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Microsomal Lipid Peroxidation

II. Stimulation by Carbon Tetrachloride

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SUMMARY

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Carbon tetrachloride initiated lipid peroxidation in isolated rat liver microsomes in the absence of free metal ions. In contrast to the nonenzymatic process stimulated by ferrous iron, CCl_4-induced peroxidation showed an absolute requirement for NADPH and appeared dependent on the integrity of cytochrome *P*-450 as reflected by measurement of aminopyrine demethylase and aniline hydroxylase activities. The peroxidation subsided after only 10% of the total peroxidizable polyunsaturated fatty acids had reacted, at which point the cytochrome *P*-450 activities were 85–95% inhibited, whereas NADPH–cytochrome *P*-450 reductase activity was not significantly affected. Ferrous iron, in contrast, caused peroxidation of 100% of the available polyunsaturated fatty acids and produced decreases in cytochrome *P*-450 activities as well as a loss of susceptibility to CCl_4-induced peroxidation that approximately paralleled the extent of peroxidation. Thus, CCl_4-induced peroxidation appeared to be 10 times more potent in inhibiting cytochrome *P*-450 activities than the peroxidation caused by iron. Boiled liver microsomes or liposomes prepared from extracted liver lipid underwent extensive peroxidation in the presence of untreated microsomes when ferrous iron was the initiating species. In contrast, the CCl_4-induced peroxidative response was not affected by the addition of these exogenous forms of lipid substrate and thus the initiating species appeared to be confined to the active microsomes. No detectable peroxidation was induced by CCl_4 in microsomes from brain, kidney, or lung, and microsomal aminopyrine demethylase and aniline hydroxylase activities were more than 10-fold lower in these tissues compared to liver. These results are consistent with activation of CCl_4 by cytochrome *P*-450 to a reactive short-lived radical which initiates peroxidation in the immediate vicinity of the cytochrome and thereby inhibits enzyme activity either by destruction of essential lipids or by direct attack on the enzyme by reactive intermediates of the peroxidative process. Loss of cytochrome *P*-450 activity then results in cessation of the CCl_4-induced peroxidative response prior to more extensive reaction of membrane polyunsaturated lipids.

INTRODUCTION

Many of the structural and functional defects observed in the endoplasmic reticulum following exposure to various chemical agents are believed to be manifested through a process of lipid peroxidation. *In vitro,* this process has been observed to be promoted by iron salts, and we have presented evidence which suggests that the peroxidation-stimulating properties of NADPH, ascorbate, or superoxide anion may be attributable to their ability to promote reduction of iron to the active ferrous form (1). Carbon tetrachloride has also been reported to induce microsomal lipid peroxidation both *in vitro* (2, 3) and *in vivo* (4, 5), and this phenomenon has been the focus of numerous investigative attempts to relate the subcellular lesions observed to the large-scale organ pathology associated with CCl_4 poisoning (6).

Early studies yielded recognition of some of the conditions necessary for CCl_4 to promote lipid peroxidation in liver. Although a small amount of malondialdehyde was produced when liver homogenates were incubated

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with CCl_4 (7), it was subsequently shown that liver mitochondria were capable of metabolizing MDA[1] (8), and thus isolated microsomal fractions were henceforth employed. Glende and Recknagel (2) characterized the requirement for NADPH, and Recknagel (6) as well as Slater and Sawyer (3) presented evidence for the involvement of the microsomal NADPH–cytochrome *P*-450 reductase in the peroxidation process. In these early experiments, however, "endogenous" MDA formation (i.e., in the presence of NADPH but without CCl_4) was relatively high and the stimulatory effect of CCl_4 was not very dramatic. More recently, Masuda and Murano (9) reported conditions which yielded a very low background rate of MDA formation, against which a 3- to 10-fold stimulation of lipid peroxidation by CCl_4 was observed.

In the present paper we have attempted to further characterize microsomal lipid peroxidation as promoted by CCl_4 with regard to enzyme and cofactor requirements, rate and substrate limitations, functional (i.e., enzyme) disturbances, and ubiquity of effects in different tissues. Our findings have revealed major qualitative and quantitative differences between CCl_4-induced peroxidation and the ferrous iron-stimulated peroxidation described in the previous paper (1). In particular, CCl_4-induced peroxidation appears to (i) lack any requirement for free metal ions; (ii) occur in liver, but not in kidney, lung, or brain microsomes; and (iii) be self-limiting in magnitude.

EXPERIMENTAL PROCEDURES

Reagents. Taurocholic acid, butylated hydroxytoluene, α-tocopherol, metyrapone, *p*-hydroxymercuribenzoic acid, epinephrine, and xanthine oxidase were obtained from the Sigma Chemical Company. Aminopyrine, ammonium acetate, and acetylacetone were purchased from Aldrich Chemical Company, Metuchen, New Jersey, and acetaldehyde was a product of Eastman Chemical, Rochester, New York. All other reagents were obtained as described in the preceding paper (1).

Methods. The preparation of microsomes[2] and liposomes, protein determinations, assay of NADPH–cytochrome *c* reductase activity, and measurement of MDA formation as an index of lipid peroxidation were described in the previous paper (1).

Quantitation of lipid peroxidation. Lipid peroxidation was expressed in terms of percentage peroxidation, which is simply the percentage of maximum MDA which would be produced by complete peroxidation of the peroxidizable polyunsaturated fatty acids present in the microsomes. Maximum MDA formation was routinely determined by incubating liver microsomes with a sufficient amount of $FeSO_4$ and NADPH to completely peroxidize microsomal lipids and give the maximum MDA production possible from each preparation, which varied between 70 and 110 nmol MDA/mg microsomal protein. The amount of MDA produced during the experimental liver microsomal incubations was then expressed in terms of the percentage of this maximum MDA value, which has been shown to be equivalent to the percentage depletion of peroxidizable lipid substrate (1) and is referred to as percentage peroxidation. Peroxidation in brain, kidney, and lung microsomes was similarly quantitated in terms of the percentage of the maximum possible MDA formation, which was calculated by multiplying the value for the yield of MDA per mole of peroxidizable PUFA in liver microsomes by the peroxidizable PUFA content of the other fractions, as determined by phospholipid content (10) and fatty acid analyses by gas–liquid chromatography (1).

Assay of cytochrome P-450 activity. Aminopyrine demethylase (APDM) activity was measured by a procedure similar to that described by Orrenius (11) and involved the incubation of 200–400 μg of microsomal protein with 40 mM Tris–maleate, pH 7.4, 300 μM NADPH, 5 mM $MgCl_2$, and 8 mM aminopyrine (total volume 1.0 ml) for 30 min at 37°C. Formaldehyde was determined by the method of Nash (12). The procedure for measuring aniline hydroxylase (AH) activity was derived from Imai (13) and included the incubation of 300–500 μg of microsomal protein with 80 mM Tris–maleate, pH 7.4, 300 μM NADPH, 5 mM $MgCl_2$, and 8 mM aniline (total volume 1.0 ml) for 20 min at 37°C, following which *p*-aminophenol formation was determined. When APDM and AH activities were measured following peroxidation of the microsomes, a NADPH-generating system was used which consisted of 3 mM glucose-6-phosphate, 0.05 unit of glucose-6-phosphate dehydrogenase, and 300 μM NADPH. Termination of NADPH/Fe^{2+}- or CCl_4-induced peroxidation prior to measurement of APDM or AH activities was accomplished simply by adding either aminopyrine or aniline, respectively, which have potent antioxidants properties but have no effect on the reaction between MDA and thiobarbituric acid. In experiments where CCl_4-induced peroxidation was determined following peroxidation by NADPH/Fe^{2+}, the addition of EDTA, as specified in the figure legends, prevented subsequent iron-induced peroxidation without affecting MDA formation by CCl_4.

Specific activities are expressed in nanomoles per minute per milligram microsomal protein. All enzyme assays were linearly proportional to time and added protein over the ranges used.

RESULTS

Incubation of rat liver microsomes for 30 min in the presence of 200 μM NADPH produced less than 1% peroxidation of peroxidizable membrane lipids. The addition of CCl_4 to this system increased the percentage peroxidation to 10%. The addition of EDTA did not affect this value, indicating that the CCl_4-induced peroxidation does not require reduced iron or any chelatable free metal ion. In the presence of added iron up to 1 μM, the CCl_4 component was also observed as an increase of 10% peroxidation over the 30% induced by iron alone. At higher iron concentrations which induced over 50% per-

[1] Abbreviations used: MDA, malondialdehyde; APDM, aminopyrine demethylase; AH, aniline hydroxylase; PUFA, polyunsaturated fatty acid; reductase, NADPH–cytochrome *P*-450 reductase; pHMB, *p*-hydroxymercuribenzoic acid.

[2] The use of pentobarbital to sacrifice animals was found to have no effect on CCl_4-induced lipid peroxidation, NADPH–cytochrome *c* reductase activity, or aminopyrine demethylase and aniline hydroxylase activities.

oxidation, the CCl_4 component was diminished, most probably due to depletion of lipid substrate and an inhibitory effect of peroxidation on CCl_4 activation as described later.

Peroxidation by CCl_4 was found to be absolutely dependent on the addition of NADPH, in contrast to Fe^{2+}, which was shown to directly initiate peroxidation in the absence of NADPH (1). These findings attest to a requirement for microsomal metabolism of CCl_4 to peroxidation-initiating free radicals, as previously postulated (3, 6). Neither ascorbate nor NADH would substitute for NADPH, which is consistent with suggestions that the mixed-function oxidase system is the site of CCl_4 metabolic activation (3, 6, 14, 15).

Peroxidation by CCl_4 exhibited a maximal rate at 200 μM NADPH, or with a NADPH-generating system, but was observed to subside after 30 min, at which point 10% of the membrane polyunsaturated fatty acids had been peroxidized, as determined by MDA formation and fatty acid analysis by gas–liquid chromatography. Therefore, unlike iron-stimulated peroxidation, which continues until all of the available PUFAs have reacted (1), peroxidation by CCl_4 appears to be highly limited. At saturating NADPH concentrations, maximal CCl_4-induced peroxidation was proportional to the amount of microsomal protein over the range of 0.05 to 2.5 mg.

Attempts to increase the extent of CCl_4-induced peroxidation by varying the manner in which the haloalkane was added to the incubation system were not successful. The techniques employed included delivery of CCl_4 to the aqueous microsomal suspensions either directly, as an ethanol solution, or as a sonicated suspension in sodium taurocholate, as well as via gaseous diffusion, accomplished by mixing CCl_4 with mineral oil and placing a drop on the inside of the Teflon-coated caps of the culture tubes. In general, these methods were equally effective in terms of the extent of peroxidation produced and the particular procedure chosen was largely a matter of convenience.

Figure 1 shows the effect on lipid peroxidation obtained by varying the amount of CCl_4 in the microsomal incubations. Similar curves were obtained either by varying the concentration of CCl_4 in a fixed volume of ethanol or by adding increasing volumes of a sodium taurocholate solution containing 0.5% CCl_4. Although stimulation by CCl_4 was maximal at 0.5 μl CCl_4/mg protein with ethanol as the solvent and 2.0 μl CCl_4/mg protein when taurocholate was used, the maximum level of peroxidation in both cases did not exceed 11% peroxidation.

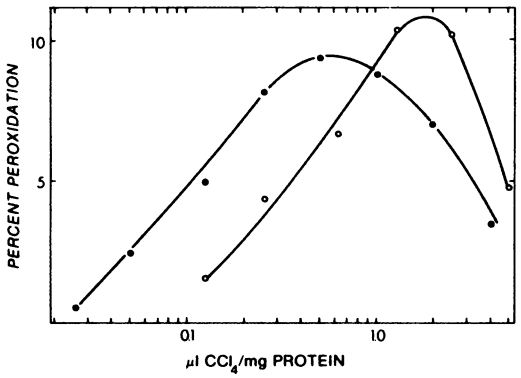

FIG. 1. *Dependence of microsomal lipid peroxidation on the concentration of CCl_4*

Microsomes (196 μg) were incubated for 20 min with 50 μM EDTA, 200 μM NADPH, and CCl_4, added either as increasing concentrations in 10 μl of ethanol (●) or as increasing volumes of a 0.5% aqueous solution of CCl_4 in 4.65 mM sodium taurocholate (○). The total volume was 0.5 ml. Each point represents the average of two experiments.

Further attempts to augment peroxidation by CCl_4 involved additions of various combinations of ADP- or EDTA-chelated iron. Studies by Pederson *et al.* (16) have suggested that these complexes might function as initiators or intermediary electron transport components in NADPH/iron-dependent microsomal lipid peroxidation, although we did not observe any enhancement of CCl_4-induced peroxidation by these agents. The influence of pH on CCl_4-induced peroxidation was also investigated. As was the case for ferrous iron-stimulated peroxidation, CCl_4 activity did not show any remarkable sensitivity to pH when varied one unit in either direction from the maximal at pH 7.4 (data not shown). Thus, lack of extensive peroxidation by CCl_4 does not appear to be attributable to less than optimal CCl_4 concentration, absence of ADP- or EDTA–iron complexes, or suboptimal pH.

Alternatively, the termination of CCl_4-induced peroxidation prior to exhaustion of membrane PUFA may be due to a loss of the capacity of microsomes to activate CCl_4 metabolically. A number of investigators have demonstrated that liver microsomal cytochrome *P*-450 activity is markedly inhibited following *in vitro* incubation with (9, 17, 18) or *in vivo* administration of (19, 20) CCl_4. Figure 2 compares the time course of CCl_4-induced peroxidation with the aminopyrine demethylase, aniline hydroxylase, and NADPH–cytochrome *P*-450 reductase (i.e., NADPH–cytochrome *c* reductase) activities of liver microsomes. Control values were obtained by incubating microsomes with CCl_4 in the absence of NADPH, which produced no lipid peroxidation and resulted in only 13 and 14% losses of APDM and AH activities, respectively, over the time course of the experiment.[3] In the presence of NADPH, however, increasing peroxidation by CCl_4 with time was accompanied by a parallel loss of both APDM and AH activities, and the peroxidative activity of CCl_4 subsided at the point where cytochrome *P*-450 was almost totally inhibited. In contrast, CCl_4-stimulated peroxidation was associated with only a slight effect on NADPH–cytochrome *P*-450 reductase activity. Thus, NADPH-dependent activation of CCl_4 to peroxidation-initiating free radicals appears to depend on the integrity of cytochrome *P*-450, and loss of the metabolic activity of this enzyme during CCl_4-induced peroxidation may account for the cessation of the peroxidative response after reaction of only 10% of the membrane PUFA.

Inhibition of cytochrome *P*-450 during CCl_4-induced peroxidation may occur through (i) direct attack of CCl_4 free radical metabolites on the cytochrome, (ii) direct

[3] With higher amounts of CCl_4 the direct effect of CCl_4 (i.e., in the absence of NADPH) on cytochrome *P*-450 activities was more pronounced; no direct effect of CCl_4 on NADPH–cytochrome *P*-450 reductase activity was observed.

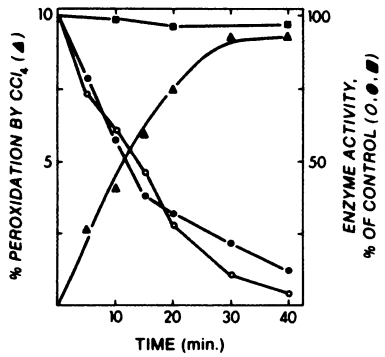

FIG. 2. *Correlation between the rate of CCl_4-induced lipid peroxidation* (▲) *and NADPH-cytochrome c reductase* (■), *aminopyrine demethylase* (●), *and aniline hydroxylase* (○) *activities*

For APDM and AH experiments, microsomes (300–500 μg protein) were first incubated in the presence of 100 μM EDTA, a NADPH-generating system (see Methods), and 0.5–0.8 μl CCl_4/mg protein (in ethanol) for the indicated lengths of time, following which an aliquot was removed for MDA determination and a mixture of the enzyme assay reagents (see Methods) was immediately added to the remaining suspension. Reductase experiments were conducted similarly except that only 10–20 μg of protein was used. The presence of aniline or aminopyrine was sufficient to terminate further peroxidation in the cytochrome *P*-450 assays, whereas 0.01% butylated hydroxytoluene was added prior to measurement of reductase activity. APDM and AH activities in the absence of CCl_4 were 10.9 ± 0.7 (mean ± standard deviation) and 0.99 ± 0.09 nmol product/min/mg protein, respectively, whereas APDM and AH activities following incubation with CCl_4 in the absence of NADPH (i.e., control activities) were 9.5 ± 0.6 and 0.85 ± 0.06 nmol product/min/mg, respectively after 40 min. Control activity of the reductase was 262 ± 4 nmol cytochrome *c* reduced/min/mg protein. Each point represents the mean value from two (reductase) or four (APDM and AH) experiments.

effect of toxic products generated during peroxidative decomposition of membrane lipids, or (iii) indirect inactivation stemming from peroxidation of lipids required for enzyme activity (21). Consistent with the second and third hypotheses, cytochrome *P*-450 has been shown to be inhibited by peroxidation elicited by a number of different agents, including NADPH/iron, ascorbate, or ionizing radiation (22, 23). Figure 3 shows that increasing peroxidation by NADPH/iron produced losses in APDM and AH activities which closely approximated the extent of peroxidation such that at 100% peroxidation, these activities were nearly totally inhibited. NADPH/iron-stimulated peroxidation was also accompanied by an inhibition of the peroxidation-inducing activity of CCl_4 which roughly paralleled the loss of cytochrome *P*-450 activities such that after 80% peroxidation by NADPH/iron, the peroxidative response to CCl_4 was completely abolished.[4] In contrast, microsomal reductase activity was less than 10% inhibited even after 100% peroxidation by NADPH/iron. Results similar to those of Fig. 3 were also obtained when microsomal peroxidation was induced by ascorbate/iron (data not shown).

The results to this point strongly support the involvement of cytochrome *P*-450 in the initiation of CCl_4-induced lipid peroxidation, since susceptibility to CCl_4-induced peroxidation is strongly correlated with the activity of cytochrome *P*-450, whereas there is no association with NADPH-cytochrome *P*-450 reductase activity. These data also demonstrate the lability of cytochrome *P*-450 to lipid peroxidation initiated by three different systems, including CCl_4, which supports the hypothesis that the lack of extensive peroxidation by CCl_4 is due to a peroxidation-induced loss of the requisite metabolic activity of cytochrome *P*-450. However, a comparison of Figs. 2 and 3 reveals that maximal inhibition of APDM and AH activities occurs at a 10-fold lower level of lipid peroxidation when CCl_4 is the initiating agent rather than $FeSO_4$. This difference may be accounted for by the relative reactivities of the initiating species. Whereas Fe^{2+} is a relatively stable ion which may freely diffuse throughout the microsomal suspensions, free radical metabolites of CCl_4 may be considerably less stable and would probably react only in the immediate vicinity of their formation. Peroxidation of membrane lipids which are closely associated with the cytochrome *P*-450 could lead to a relatively rapid inactivation of the enzyme and

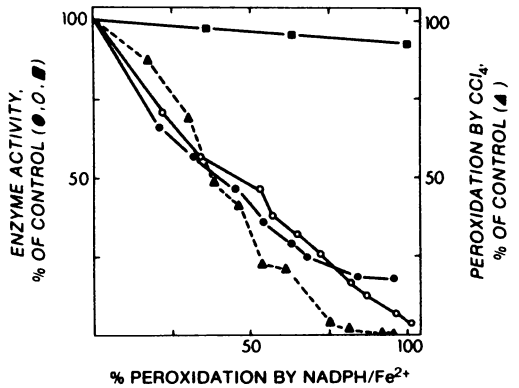

FIG. 3. *Effect of NADPH/Fe^{2+}-induced peroxidation on NADPH-cytochrome c reductase* (■), *aminopyrine demethylase* (●), *and aniline hydroxylase* (○) *activities and on the peroxidative response to CCl_4* (▲- - -▲)

For APDM and AH experiments, microsomes (300–500 μg) were incubated for 30 min with 0–20 μM $FeSO_4$ and a NADPH-generating system (see Methods), following which aliquots were removed for MDA determination and enzyme assay reagents were added as in Fig. 2. For CCl_4 experiments, an analogous procedure was employed where NADPH/Fe^{2+}-induced peroxidation was terminated by the addition of 100 μM EDTA. The remaining microsomal suspension was then incubated for 30 min with 2.1 μl CCl_4/mg protein (added as an aqueous solution in sodium taurocholate). Reductase experiments were similar to APDM and AH determinations except that 10–20 μg of protein was used. Control incubations were performed in the absence of iron (i.e., with 100 μM EDTA, which had no direct effects on the enzymes) for 30 min and yielded activities of 9.3 ± 1.1 (mean ± standard deviation), 0.93 ± 0.3, and 231 ± 33 nmol product/min/mg protein for APDM, AH, and reductase, respectively. Control CCl_4-induced peroxidation obtained in this manner was 7.61 ± 0.7% peroxidation. Each point represents the mean value from three or four experiments.

[4] Since 20% of the peroxidizable PUFA remained in the Fe^{2+}-peroxidized membranes that had been rendered insensitive to CCl_4, the lack of a CCl_4 effect in these preparations cannot be attributed to a complete lack of peroxidizable lipid. Inhibition of cytochrome *P*-450 activity toward CCl_4 by the partial peroxidation of membrane lipids seems a more likely explanation. The relatively low APDM and AH activities remaining in the membranes which were apparently rendered incapable of CCl_4 activation are consistent with a small portion of these activities residing in a form(s) of cytochrome *P*-450 which cannot metabolize CCl_4. Multiple forms of cytochrome *P*-450 with differing substrate specificities have been reported (24).

a termination of peroxidation prior to extensive loss of microsomal PUFA. Support for this hypothesis was obtained by investigating the effects of the addition of exogenous lipid on CCl_4-induced microsomal peroxidation, the results of which are shown in Table 1. Peroxidation of liposomes derived from extracted liver lipid was elicited by the addition of ferrous iron alone, as has been previously observed (1), whereas CCl_4 alone was incapable of directly initiating peroxidation in liposomes. When liposomes were added to liver microsomal suspensions containing NADPH and $FeSO_4$, peroxidation of the liposomal lipid occurred, as reflected by MDA formation in excess of that obtained with microsomes alone. Liposomes in the presence of microsomes were peroxidized to a fivefold greater extent than in the presence of Fe^{2+} alone, which is consistent with the previously suggested role of the microsomal reductase in catalyzing the reduction of iron, thereby generating higher levels of the active ferrous form (1). Apparently this process, which promotes high rates of peroxidation in microsomes, will also enhance peroxidation of exogenously added lipid.

In contrast, the addition of liposomes to a CCl_4-stimulated liver microsomal system (no iron) does not result in any additional MDA formation, which indicates that the peroxidation induced by CCl_4 is confined to the microsomes. Similarly, the addition of boiled liver microsomes to otherwise untreated microsomes did not result in any enhancement of peroxidation, which also demonstrates that the extent of CCl_4-induced peroxidation is independent of the amount of peroxidizable lipid substrate added to the incubations. Thus, it appears that CCl_4 may induce relatively focal peroxidative damage, most likely involving those lipids closely associated with the cytochrome *P*-450 site of CCl_4 activation, which would account for the greater potency of CCl_4 in inhibiting the enzyme.

TABLE 1

Peroxidation of exogenous lipid by liver microsomes plus iron or CCl_4

Liver microsomes (117 μg) were incubated for 20 min in the presence of 200 μM NADPH and either 1.0 μM $FeSO_4$ or CCl_4 (2.5 μl/mg protein) in sodium taurocholate, with and without liposomes derived from 100 μg of liver microsomes (1) or with and without boiled liver microsomes (98 μg) as indicated. The direct effect of Fe^{2+} or CCl_4 on liposomes was also tested. Peroxidation of the liposomal lipid was quantitated by subtracting the amount of MDA formed in the absence of liposomes and dividing by the number of milligrams of microsomal protein from which the liposomal lipid was prepared (assuming 85% of the lipid in the original microsomes was recovered as liposomal lipid). Percentage peroxidation values are then obtained in the same manner as with microsomal peroxidation. Each number represents the mean value from two to four experiments. (ND indicates no detectable MDA.)

Condition	MDA	Percentage peroxidation	
		Microsomal lipid	"Exogenous" lipid
	nmol/20 min		
Liver liposomes			
Plus $FeSO_4$	0.33	—	4.6
Plus CCl_4	ND	—	ND
Liver microsomes			
Plus $FeSO_4$	1.69	31.4	—
Plus $FeSO_4$, plus liposomes	3.52	31.4	26.5
Plus CCl_4	0.77	8.6	—
Plus CCl_4, plus liposomes	0.75	8.4	ND
Plus CCl_4, plus boiled liver microsomes	0.74	8.3	ND

The effects of various inhibitors on iron- and CCl_4-induced microsomal peroxidation and on microsomal reductase and cytochrome *P*-450 activities were investigated. As previously reported (1), cytochrome *c* inhibits NADPH/Fe^{2+}-induced peroxidation, possibly by competing with iron for electrons transferred from the reductase enzyme but also apparently by directly oxidizing Fe^{2+}, as indicated by an inhibitory effect of cytochrome *c* on the peroxidation stimulated by iron alone. This same amount of cytochrome *c* (200 μM) also caused a 90% reduction in peroxidation initiated by CCl_4, which is again probably due to the ability of cytochrome *c* to compete for electrons from the reductase, in this case, with the cytochrome *P*-450, which was reflected by an 80–90% inhibition of cytochrome *P*-450 activities in the presence of cytochrome *c*.

Inhibition of CCl_4-stimulated peroxidation as well as Fe^{2+}/NADPH- and Fe^{2+}-induced peroxidation was produced by the addition of the free radical scavengers butylated hydroxytoluene and α-tocopherol. These agents did not effectively inhibit the cytochrome *P*-450 or reductase activities and thus probably suppress peroxidation by reacting with free radicals essential to initiation or propagation of the peroxidation process.

Superoxide dismutase or catalase had no effect on CCl_4-induced peroxidation or on the resulting loss of cytochrome *P*-450 enzyme activities.[5] Thus the peroxidative or enzymatic changes in microsomes produced by CCl_4 do not appear to involve superoxide or hydrogen peroxide.

The cytochrome *P*-450 substrates, aminopyrine and aniline, suppressed over 80% of the CCl_4-induced lipid peroxidation. Similar effects were previously observed and were interpreted to represent competitive inhibition of cytochrome *P*-450-mediated metabolism of CCl_4 (9). However, we found that these substrates also drastically inhibited peroxidation initiated by NADPH/Fe^{2+} or even Fe^{2+} alone, and thus their effects on CCl_4-induced peroxidation may also be attributed to their antioxidant properties.

In contrast, the competitive inhibitor of cytochrome *P*-450, metyrapone, at 40 mM, had no effect on Fe^{2+}-stimulated peroxidation, although peroxidation induced by NADPH/Fe^{2+} and CCl_4 was 60 and 93% inhibited, respectively. The suppression of CCl_4-induced peroxidation is probably due to a reduction in the extent of metabolic activation of CCl_4, since metabolism of aniline and aminopyrine was highly inhibited by metyrapone. Inhibition of NADPH/Fe^{2+}-dependent peroxidation may

[5] Experiments in which a xanthine oxidase system was employed to generate superoxide, as quantitated by conversion of epinephrine to adrenochrome (1), have verified the integrity of superoxide dismutase activity in the presence of CCl_4, EDTA, and other conditions employed to obtain CCl_4-induced peroxidation (data not shown). Thus, the lack of effect of superoxide dismutase cannot be attributed to its inactivation under these conditions.

be due to an increase in microsomal electron flow from the reductase to cytochrome *P*-450, away from the reduction of iron.

The addition of pHMB (200 nmol/mg protein) produced a 90% loss of cytochrome *P*-450 activities and caused a similar reduction in the extent of peroxidation induced by CCl_4, although it had little or no effect on NADPH/Fe^{2+}- or Fe^{2+}-stimulated peroxidation or on the microsomal reductase. Thus, the effects of all the inhibitors studies are consistent with the hypothesis that cytochrome *P*-450 is the site of conversion of CCl_4 to peroxidation-initiating free radicals.

Further experiments were conducted to test whether CCl_4 could elicit peroxidation in microsomes from other organs, particularly those which appear to be damaged by CCl_4. Both kidney and brain microsomes have been shown to undergo iron-stimulated lipid peroxidation resulting in nearly total depletion of membrane PUFAs, whereas lung microsomes appear to be only slightly peroxidizable by $FeSO_4$ due to a relatively high content of vitamin E (25). However, no peroxidation was produced by CCl_4 in brain, kidney, or lung microsomes (data not shown), although histopathological changes induced by CCl_4 have been observed in kidney (26) and in lung (27). The lack of stimulation of peroxidation by CCl_4 in these organs may be due to a low capacity to activate CCl_4 metabolically to peroxidation-initiating radicals. Consistent with this, we observed that ADPM and AH activities, which were well correlated with the peroxidation-inducing capacity of CCl_4 in liver microsomes (Fig. 3), were 20- and 50-fold lower in kidney and 28- and 11-fold lower in lung microsomes, respectively, than in liver microsomes (data not shown), and were not detectable in brain microsomes. It therefore appears that the histopathological alterations in lung or kidney occurring during CCl_4 poisoning are not a consequence of metabolism of CCl_4 within those tissues, but may be due to some other disturbances such as restricted blood flow (28); or they may be secondary to hepatic dysfunction.

DISCUSSION

Unlike NADPH, ascorbate, or xanthine oxidase-generated superoxide anions which apparently promote lipid peroxidation by maintaining iron in the reduced state, carbon tetrachloride appears to initiate microsomal peroxidation by a mechanism which is independent of exogenous iron, an observation which has not been previously documented. In fact, Benedetti *et al.* (18) observed very little MDA formation by CCl_4 in the presence of EDTA, and Masuda and Murano (9) reported that EDTA partially inhibited CCl_4-induced peroxidation, although the inhibition was not complete even at 10 mM EDTA, consistent with an iron-independent component of CCl_4-induced peroxidation. The reasons for these discrepancies are not fully understood but may be attributable to greater metal ion contamination of the microsomal preparations used in the previous studies, as indicated by the level of endogenous peroxidative activity (i.e., the absence of CCl_4) occurring during their incubations. In contrast, peroxidation in our EDTA-washed microsomes is absolutely dependent on the addition of iron or CCl_4. Thus, in microsomes highly contaminated with iron, a certain portion of the iron may be trapped or protein bound and therefore inactive during the control incubations (no CCl_4 or no NADPH) but may be released during CCl_4-induced peroxidation and thereby generate an EDTA-sensitive component of the CCl_4-iniated peroxidative response. However, we believe that the peroxidative influence of metal ions released during CCl_4-induced peroxidation is probably not of physiological significance since, *in vivo*, free metal ions would most likely be rapidly scavenged and inactivated by endogenous chelators like citrate or histidine, or proteins like ferritin, which are absent in a purified microsomal preparation.

An alternative explanation for the apparently EDTA-inhibitable component of CCl_4-induced peroxidation observed by other investigators is that the presence of contaminating metal ions during the colorimetric determination of TBA can lead to additional chromogen formation. It has recently been shown that lipid hydroperoxides present in peroxidized microsomes can react with ferrous iron during the boiling step to yield TBA-reactive products (29). In the studies by both Benedetti *et al.* (18) and Musada and Murano (9), acid-insoluble factors were not removed prior to boiling and would consequently be available to react with contaminating ferrous ions. EDTA may prevent this reaction and would thus appear to partially inhibit CCl_4-induced peroxidation. Since acid-insoluble factors were removed by centrifugation in the present studies, this effect of EDTA would not be observed.

The limited extent of CCl_4-induced microsomal peroxidation observed here might be considered inconsistent with lipid peroxidation as a mechanism for the extensive histological damage produced by CCl_4 *in vivo*. It must be considered, however, that even a small loss of microsomal membrane constituents *in vivo* could disrupt membrane fluidity or alter permeability (30) to an extent which impairs microsomal function. In addition, recent evidence indicates that lipid peroxidation results in the formation of certain products having hemolytic (31) or hormone-like (32) properties which could account for cellular damage in regions remote from the center of peroxidative activity.

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