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# EVIDENCE FOR CARBON TETRACHLORIDE-INDUCED LIPID PEROXIDATION IN MOUSE LIVER*

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**Abstract**—The involvement of lipid peroxidation in the mechanism of carbon tetrachloride-induced hepatotoxicity has been a point of controversy. Previous investigators have reported an absence of lipid peroxidative degradation products in mice after exposure to carbon tetrachloride and have used this evidence against the hypothesis that lipid peroxidation is an integral part of the events that cause tissue damage. We have compared the extent of lipid peroxidation caused by carbon tetrachloride between Sprague–Dawley rats and three strains of mice (A/J, BALB/cJ, and C57B1/6J) in *in vitro* and *in vivo* systems. Hepatic microsomes isolated from fasted mice of each strain produced more malondialdehyde (a degradation product of lipid peroxidation) per mg microsomal protein than those isolated from fasted rats at all times of incubation with $CCl_4$. *In vivo* lipid peroxidation was estimated by the lipid conjugated diene content in hepatic microsomes from the rat and three strains of mice. Increased conjugated diene formation was observed in microsomal lipids of these animals after intraperitoneal injection of $CCl_4$ (1 ml/kg as a 20% solution in corn oil) when compared to animals given only corn oil, but no differences were found in the amount of conjugated dienes between mice and rats. Our observations show that the $CCl_4$-treated mouse undergoes hepatic lipid peroxidation at least as well as the rat, and indicate that lipid peroxidation cannot be excluded as a mechanism of carbon tetrachloride hepatotoxicity as has been claimed on the basis of its ineffectiveness in the mouse.

The mechanism of carbon tetrachloride ($CCl_4$) hepatic toxicity has been investigated for many years, but the mechanism of liver injury and lethality is still unknown [1]. In the forefront of the hypotheses to account for this toxicity has been the role of lipid peroxidation. $CCl_4$ has been shown to increase indicators of lipid peroxidation such as conjugated diene formation [2, 3], and ethane [4, 5] and pentane [6, 7] evolution (*in vivo*), and production of malondialdehyde [8–10] (*in vitro*). Most of the studies have been done using the rat. Previous studies [11, 12] have shown that conjugated dienes are not present in hepatic microsomes of mice 3 or 6 hr after $CCl_4$ administration and suggested that lipid peroxidation is not important in $CCl_4$-mediated liver damage. Because the mouse is quite susceptible to liver injury by $CCl_4$, these studies have been cited as evidence that lipid peroxidation is not involved in the injury process. In this report we provide evidence that hepatic lipid peroxidation does occur in mice as a consequence of $CCl_4$ exposure as well as in rats when livers are taken 30 min after $CCl_4$ administration or when $CCl_4$ is added to isolated microsomal preparations. Thus, the mouse is not an exception to the hypothesis that lipid peroxidation contributes to $CCl_4$-induced hepatic damage.

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## MATERIALS AND METHODS

The three strains of male mice, A/J, BALB/cJ, and C57B1/6J (15–20 g), were obtained from Jackson Laboratories (Bar Harbor, ME). The male rats were Sprague–Dawley-derived, bred and maintained at the University of Oklahoma Health Sciences Center Animal Resources and Facilities. All animals were fed a standard laboratory chow diet and were allowed water *ad lib*. For each group of experiments, three livers from each mouse strain were pooled and one rat liver was used.

All chemicals were obtained from commercial sources. Methanol, chloroform, cyclohexane and carbon tetrachloride were distilled before use.

For the experiments involving malondialdehyde formation *in vitro*, microsomes were prepared as described by Rikans *et al.* [13]. Livers from animals fasted 12–16 hr were homogenized with a Teflon–glass homogenizer in 4 vol. of a 0.05 M Tris–HCl buffer solution (pH 7.5) containing 1.15% KCl. The microsomes were separated from the homogenate by differential centrifugation at 12,000 *g* for 15 min and the resulting supernatant solution at 105,000 *g* for 90 min. The pellets thus obtained were washed once with the homogenizing solution and repelleted by centrifuging at 105,000 *g* for 50 min. The microsomes were resuspended in 0.1 M Tris–HCl (pH 7.5) to a protein concentration of 7–15 mg/ml. All preparations were kept in melting ice between centrifugations.

Malondialdehyde was measured by the thiobarbituric acid assay [14]. Lipid peroxidation was induced either by the addition of $CCl_4$ (20 $\mu$l) or ADP–Fe (0.4 mM ADP, 0.12 $\mu$M $FeCl_3$). The incu-

bation volume was 1 ml. This solution contained 0.1 M Tris–HCl (pH 7.5), and an NADPH-generating system (glucose-6-phosphate, 56 mM; NADP, 3 mM; and glucose-6-phosphate dehydrogenase, 0.5 Sigma Units). The incubation temperature was 25°. The reaction was initiated by adding microsomes (0.7 to 1.5 mg protein). Blank tubes contained no ADP–Fe or $CCl_4$. Blanks were incubated for the designated times and were used to correct for lipid peroxidation attributable to NADPH oxidation. Microsomes were incubated with the ADP–Fe for 0 or 10 min, and with $CCl_4$ for 0, 2.5, 5, 7.5, or 10 min. The reactions were stopped by adding 0.5 ml of 35% trichloroacetic acid. The chromophore was developed by adding 1 ml of a 0.5% thiobarbituric acid solution and heating the mixture in a boiling water bath for 15 min. The tubes were capped with marbles during this procedure to prevent evaporation. Finally, 1.0 ml of trichloroacetic acid (70%) was added to complete the color development. The solution was cleared of opaque materials by adding 1 ml $CHCl_3$, mixing, and then centrifuging at 1000 *g* for 15 min. The aqueous layer was measured at 532 nm with a Gilford Stasar II spectrophotometer. Malondialdehyde concentration was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ [15].

Protein was determined by a modification [16] of the method of Lowry *et al.* The content of cytochrome P-450 was assayed by the method of Omura and Sato [17].

Animals used for the assay of conjugated dienes in liver lipids were fasted for 12–16 hr and then injected with $CCl_4$ (1 ml $CCl_4$/kg) as a 1:5 mixture of $CCl_4$ in corn oil (Mazola) 30 min before livers were taken.

Microsomes used for the determination of conjugated dienes were prepared as described by Rao and Recknagel [2]. Livers were homogenized in 4 vol. of a 0.3 M sucrose–3 mM EDTA solution. The microsomes were isolated by differential centrifugation of the homogenate at 12,000 *g* for 15 min and of the resultant supernatant solution at 105,000 *g* for 90 min. Lipids were extracted by the method of Folch *et al.* [18] as modified by Rao and Recknagel [2]. For each 3 g of wet weight liver, 5 ml $CH_3OH$ was added to the microsomal pellet for resuspension. Chloroform, at twice the volume of methanol, was then added to the mixture. The microsomes were shaken in the solvent mixture for 20 min with a mechanical shaker. The $CHCl_3$:$CH_3OH$ suspension was filtered and the filter was rinsed with 15 ml of $CHCl_3$:$CH_3OH$ (2:1). Water (10 ml) was shaken with the filtrate. The resulting emulsion was centrifuged at 1000 *g* for 20 min. The aqueous layer was aspirated and the $CHCl_3$ organic layer evaporated under $N_2$ made $O_2$ free by bubbling it through a 1% solution of pyrogallic acid. The lipid residues were suspended in a small volume of $CHCl_3$, were transferred to tared screw cap tubes, and the solvent was again removed with $O_2$-free $N_2$. Two ml absolute ethanol was added to remove remaining traces of water, and this solution was evaporated under $O_2$-free $N_2$. The tubes were then reweighed to determine total lipids and the lipids were resuspended to 5–10 mg/ml in $CHCl_3$. Aliquots of lipid (5–10 mg) were transferred to tared tubes and the chloroform was removed with $O_2$-free $N_2$. These tubes were reweighed and the lipids were resuspended in cyclohexane to a dilution of 1 mg lipid/ml. Conjugated dienes were measured by scanning the cyclohexane-suspended lipids between 400 and 220 nm in a Pye Unicam SP8-100 ultraviolet spectrophotometer. The reading at 400 nm was used as the zero absorbance for both control and $CCl_4$-treated microsomes. The differences in conjugated diene content between microsomal lipids from $CCl_4$-treated and control animals are reported as $\Delta E^{1\%}_{1cm}$ lipids which is the difference in absorbance between the two samples at 233 nm in a 1% solution through a 1 cm light path [2].

Statistical treatment was by Student's *t*-test (two-tailed).

## RESULTS

The microsomal cytochrome P-450 values for the rat and three strains of mice are shown in Table 1. These values as well as protein content were used to normalize the malondialdehyde data. Microsomal protein/g wet weight liver was 23.9, 16.8, 20.7 and 18.0 for the rat and A/J, BALB/cJ and C57B1/6J mice respectively. Malondialdehyde formation (which includes all thiobarbituric acid reacting substances produced during lipid peroxidation) has been widely accepted as an *in vitro* indicator of lipid peroxidation [14]. In Table 2, malondialdehyde formation is measured in the microsomal system incubated with ADP–Fe. The ADP–Fe incubations were used to give an indication of the relative capacity of the microsomal preparation to undergo lipid peroxidation. The data in Table 2 show that no difference existed in the peroxidative capacity between mice and rats under the conditions of these experiments in relation to mg microsomal protein, and that peroxidation was not less than that of the rat when related to nmoles cytochrome P-450. Preliminary studies showed that formation of malondialdehyde in the NADPH–ADP–Fe–microsomal system was maximal by 7.5 min and did not change with further incubation. The data are normalized to either mg microsomal protein or nmoles microsomal cytochrome P-450. When normalized to mg microsomal protein, the amounts of malondialdehyde formed

Table 1. Liver microsomal cytochrome P-450 content in rats and in three strains of mice*

| Animal | Cytochrome P-450 (nmoles/mg microsomal protein) |
|---|---|
| Rat | 1.10 ± 0.16 |
| A/J | 0.57 ± 0.10† |
| BALB/cJ | 1.16 ± 0.14 |
| C57B1/6J | 0.66 ± 0.02† |

* Washed microsomes were suspended in 0.1 M Tris–HCl buffer (pH 7.5). Na dithionite was added and CO was bubbled through the solution for 1 min. A difference spectrum between the CO bubbled microsomes and microsomes not treated with CO was scanned. Cytochrome P-450 was calculated from the absorption maxima at 450 nm using an extinction coefficient of 91 $mM^{-1}\ cm^{-1}$. Each value is the mean ± S.E.M. for four microsomal preparations.

† Different from the rat ($P < 0.05$).

Table 2. Malondialdehyde (MDA) formation after the addition of ADP–ferric ion to microsomes from livers of rats and three strains of mice*

| Animal | MDA (nmoles/mg microsomal protein) | MDA (nmoles/nmole cytochrome P-450) |
|---|---|---|
| Rat | 22.3 ± 2.1 | 21.5 ± 2.0 |
| A/J | 24.7 ± 0.7 | 46.4 ± 6.4† |
| BALB/cJ | 23.3 ± 0.5 | 20.9 ± 2.5 |
| C57B1/6J | 25.1 ± 0.9 | 37.8 ± 1.7† |

* Incubation mixtures containing washed microsomes in 0.1 M Tris–HCl (pH 7.5) with an NADPH-generating system were mixed with and without ADP–Fe for 10 min. NADPH-induced malondialdehyde formation was subtracted from that caused by ADP-Fe. The data are corrected to either mg microsomal protein or nmole cytochrome P-450. Malondialdehyde concentration was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Each value is the mean ± S.E.M. for four microsomal preparations.

† Different from the rat ($P < 0.05$).

among the three strains of mice and the rat were the same ($P > 0.05$). However, when these data were corrected to nmoles cytochrome P-450, the values for the rat and BALB/cJ were similar, and less than those of the A/J and C57B1/6J ($P < 0.05$).

In Table 3a, the development of malondialdehyde is followed over time after adding $CCl_4$ and the data are normalized to mg microsomal protein. No difference was found in the amount of malondialdehyde formed at a particular time between the different mouse strains ($P > 0.05$). However, each mouse strain produced more malondialdehyde at any particular time point than the rat, except for the C57B1/6J mice at 7.5 min and the A/J at 10 min ($P < 0.05$). Table 3b presents the same data but corrected to nmoles cytochrome P-450. No difference in the amount of malondialdehyde formed could be shown between the three strains of mice after 7.5 and 10 min ($P > 0.05$). However, the A/J strain was statistically higher than the BALB/cJ and C57B1/6J mice at 2.5 and 5 min ($P < 0.05$). The rat produced considerably less malondialdehyde than any of the mouse strains at each of the time points investigated. The amount of malondialdehyde formed was higher for the A/J and C57B1/6J mice when related to the amount of cytochrome P-450 as compared to being expressed with respect to mg microsomal protein. This increase was not observed with the rat or BALB/cJ mouse.

Table 3. Production of malondialdehyde (MDA) *in vitro* by hepatic microsomes from rats and three strains of mice over time after addition of $CCl_4$*

| Time | Rat | A/J | BALB/cJ | C57B1/6J |
|---|---|---|---|---|
| | (a) MDA (nmoles/mg microsomal protein) | | | |
| 2.5 | 0.5 ± 0.1 | 1.4 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.2 |
| 5.0 | 0.8 ± 0.2 | 2.8 ± 0.2 | 2.7 ± 0.5 | 2.3 ± 0.3 |
| 7.5 | 1.8 ± 0.5 | 4.0 ± 0.8 | 4.4 ± 0.7 | 2.8 ± 0.6† |
| 10.0 | 2.6 ± 0.4 | 4.1 ± 0.8† | 5.6 ± 1.1 | 4.2 ± 0.7 |
| | (b) MDA (nmoles/nmole cytochrome P-450) | | | |
| 2.5 | 0.5 ± 0.2 | 2.8 ± 0.4‡ | 1.0 ± 0.2 | 1.5 ± 0.2 |
| 5.0 | 0.8 ± 0.2 | 5.4 ± 0.5‡ | 2.5 ± 0.6 | 3.5 ± 0.4 |
| 7.5 | 1.8 ± 0.6 | 7.4 ± 1.6 | 4.1 ± 0.8 | 4.2 ± 0.9 |
| 10.0 | 2.5 ± 0.5 | 7.5 ± 1.7 | 5.0 ± 1.1 | 6.4 ± 1.1 |

* Incubation mixtures were prepared as described in Table 2 except that 20 μl $CCl_4$ was substituted for the ADP–Fe. Incubations were run at 25° for the times indicated. NADPH-induced malondialdehyde formation was subtracted from that induced by $CCl_4$. The data are corrected to either mg microsomal protein or nmole cytochrome P-450. Malondialdehyde concentration was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. Each mouse strain produced more malondialdehyde at any particular time point than the rat, unless marked † or ‡. Each value is the mean ± S.E.M. for four microsomal preparations.

† Not significantly different ($P < 0.05$) from rat at same time.

‡ Statistically different ($P < 0.05$) from BALB/cJ and C57B1/6J at same time.

Conjugated diene formation in hepatic microsomes after administering (i.p.) $CCl_4$ (1:5 $CCl_4$: corn oil; 1 ml $CCl_4$/kg) was used to evaluate *in vivo* lipid peroxidation. Table 5 presents these data. These

Table 4. Rates of malondialdehyde formation calculated from the averages of the $CCl_4$ time curve data*

| Animal | Rate† | Rate‡ |
|---|---|---|
| Rat | 0.26 ± 0.05 | 0.25 ± 0.06 |
| A/J | 0.44 ± 0.08 | 0.83 ± 0.16‖ § |
| BALB/cJ | 0.58 ± 0.11 | 0.53 ± 0.11 |
| C57B1/6J | 0.41 ± 0.07 | 0.62 ± 0.11§ |

* Rates were calculated as the averages of the regression lines from the values given in Table 3.

† nmoles malondialdehyde · (mg microsomal protein)$^{-1}$ min$^{-1}$.

‡ nmoles malondialdehyde · (nmole cytochrome P-450)$^{-1}$ · min$^{-1}$.

§ Each of the values for the mice is different from rat ($P < 0.05$).

‖ Different from BALB/cJ ($P < 0.05$).

Table 5. Conjugated dienes formed in liver microsomes of rats and three strains of mice 30 min after $CCl_4$ administration*

| Animal | ΔABS† |
|---|---|
| Rat | 1.65 ± 0.14 |
| A/J | 1.21 ± 0.19 |
| BALB/cJ | 2.08 ± 0.14 |
| C57B1/6J | 1.70 ± 0.27 |

* All animals were given 1 ml of $CCl_4$/kg body weight (i.p.) as a 20% solution (v/v) in corn oil. Livers from $CCl_4$-injected animals were compared to those from oil-injected animals. The data for mice represent seven to nine different liver samples with three livers pooled for each sample. The data for rats represent six different livers with two different oil-injected control samples. Each value is the mean ± S.E.M. expressed as $\Delta E^{1\%}_{1\,cm}$ values.

† ABS is the absorption difference between a 1% solution of microsomal lipids from $CCl_4$ and from control animals at 233 nm through a 1 cm light path.

results do not show statistically significant differences between the lipid diene content of similarly corrected microsomal lipids from the different groups of animals ($P > 0.05$).

## DISCUSSION

The mechanism by which $CCl_4$ exerts its toxic effects on the liver has been an active area of investigation and has given rise to some inconsistencies [19]. A prooxidant effect on hepatic microsomal lipids by the trichloromethyl free radical metabolite of $CCl_4$ has been proposed [20]. The cytochrome P-450 enzyme system has been implicated as the activating system for the formation of a toxic metabolite because induction studies with phenobarbital [3, 21, 22] potentiated $CCl_4$ toxicity and lipid peroxidation, and the cytochrome P-450 inhibitors SKF-525A [3] and allylisopropylacetamide [23] protect against $CCl_4$-induced damage. Furthermore, Poyer *et al.* [24] have obtained unequivocal evidence for the formation of the trichloromethyl radical both *in vivo* and in preparations of hepatic microsomes incubated with $CCl_4$ and an NADPH-generating system.

However, other workers [11, 12] argue against lipid peroxidation as the mechanism by which the trichloromethyl free radical causes liver damage since they were unable to observe conjugated dienes in mouse microsomal lipids after the animals had been administered $CCl_4$. Our investigations have re-examined the lipid peroxidative effects of $CCl_4$ on the mouse and rat liver in *in vitro* and *in vivo* systems, and have compared the *in vitro* effects with the content of cytochrome P-450.

By following the formation of malondialdehyde in *in vitro* preparations, we have demonstrated that microsomes from A/J, BALB/cJ, and C57B1/6J mice do undergo lipid peroxidation as a consequence of metabolizing $CCl_4$ (Table 3). When compared with the amount of malondialdehyde produced from rats, lipid peroxidation occurred to a greater extent in microsomes from the three mouse strains. Two possible explanations for this difference between species are: (1) more unsaturated fatty acid substrate in mouse microsomes, and (2) a more efficient $CCl_4$-activating system in the mouse. The mouse microsomes do not appear to have more peroxidizable lipid substrate since the amount of lipid peroxidation induced by ADP–Fe was not different between the mice and the rat when normalized to mg microsomal protein. However, the differences observed when the data are normalized to nmoles cytochrome P-450 could be a consequence of either the activity or amount of the $CCl_4$-activating system. As can be seen in Table 1, the amount of cytochrome P-450 is greatest in the rat and the BALB/cJ mice followed by the A/J strain and finally the C57B1/6J mice, but the rate of malondialdehyde production is essentially the inverse of this order. Thus, the difference in malondialdehyde formed per nmole cytochrome P-450 between the mice and rat after $CCl_4$ administration (Table 3) might be explained as an increased activity of the $CCl_4$-activating system of the mouse.

*In vivo* lipid peroxidation as measured by lipid conjugated dienes indicates that lipid peroxidation does occur in the mouse after administering $CCl_4$ intraperitoneally. However, no differences in the amount of lipid peroxidation between the three mouse strains and the rat were observed using this index of lipid peroxidation (Table 5). Diaz-Gomez *et al.* [11] and de Toranzo *et al.* [12] used A/J, GXF, CF1, and Swiss strain mice in their studies of conjugated diene formation. However, they measured conjugated dienes 3 and 6 hr after administering $CCl_4$. Table 5 demonstrates that the microsomal lipids of mice treated with $CCl_4$ contained significantly more conjugated dienes than control mice at 30 min. Thus, it appears that lipid conjugated dienes formed as a result of $CCl_4$ treatment disappear from liver microsomes of mice but not of rats within 3 hr of such treatment. Earlier work by us [25] is consistent with this explanation.

In summary, we have presented evidence that lipid peroxidation occurs *in vivo* in the liver endoplasmic reticulum of mice after administering $CCl_4$ and that it occurs to the same extent that it does in rats. The *in vitro* studies on the formation of malondialdehyde have shown that lipid peroxidation also occurs in hepatic microsomes of mice as well as rats, and that mice produce more thiobarbituric acid-reacting products than the rat. The differences between the mice and rat may be due to an increased $CCl_4$-activating ability of the mouse cytochrome P-450 system. These results are in accord with the hypothesis that lipid peroxidation is involved in the mechanism of $CCl_4$ poisoning.

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