CARBON TETRACHLORIDE-INDUCED LOSS OF MICROSONAL GLUCOSE 6-PHOSPHATASE AND CYTOCHROME P-450 IN VITRO

Yasusuke MASUDA
Department of Toxicology, Niigata College of Pharmacy, Kamishin'ei-cho, Niigata 950-21, Japan
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Abstract—Rat liver microsomes were incubated in the presence of NADPH and CCl₄ under various conditions, and losses of glucose 6-phosphatase (G-6-Pase) and cytochrome P-450 were examined in terms of lipid peroxidation and CCl₄ metabolism. Loss of G-6-Pase activity correlated well with the enhancement of lipid peroxidation. Loss of cytochrome P-450 was also dependent on the lipid peroxidation, under aerobic conditions. However, the cytochrome was destroyed under anaerobic conditions in which lipid peroxidation and loss of G-6-Pase were greatly suppressed. This anaerobic loss of cytochrome P-450 may be linked with the metabolism of CCl₄ by this hemoprotein, as evidenced by the observation that CCl₄ metabolism occurred only under anaerobic conditions and was inhibited by carbon monoxide accompanied by the suppression of the loss of cytochrome P-450.

A number of studies on the mechanisms of hepatotoxicity of CCl₄ emphasize the importance of endoplasmic reticulum (ER) as an early site of liver injury. According to the current hypothesis, CCl₄ is first activated by the drug metabolizing enzyme system of ER to the reactive free radical (•CCl₃), which then triggers peroxidative breakdown of lipids constituting ER membranes accompanying various biochemical and structural alterations such as loss of drug metabolizing enzyme activities, cytochrome P-450 and glucose 6-phosphatase (G-6-Pase), and detachment of ribosomes from rough ER in an early phase of CCl₄ toxicity (1–3).

Whether or not these early microsomal impairments can all be ascribed to lipid peroxidation induced by CCl₄ remains controversial (4–6). Direct attack of the reactive metabolites of CCl₄ to ER membranes is also considered to be destructive, as determined from irreversible incorporation of CCl₄ metabolites into the microsomal components (7–9). Recknagel (2) and Glende et al. (10) stressed the importance of the lipid peroxidative process but did not rule out participation of the irreversible binding process.

One such example is the loss of cytochrome P-450; Glende et al. (10) and Reiner et al. (11) emphasized the peroxidative damage, while Castro et al. (12) suggested the direct attack by trichloromethyl free radicals. In addition, this hemoprotein probably plays an essential role in the metabolic activation of CCl₄ (13, 14). Loss of G-6-Pase activity in the peroxidative process was also questioned by Cawthorne et al. (4) and Cignoli and Castro (5).
Although both of these proposed mechanisms appear to be involved in the onset and development of CCl₄ hepatotoxicity, no definite conclusion has been reached. In the present study, losses of microsomal cytochrome P-450 and G-6-Pase were comparatively examined in vitro in relation to lipid peroxidation and CCl₄ metabolism and the findings are reported herein.

MATERIALS AND METHODS

Chemicals: Sources of the reagents are as follows: NADPH and isocitric dehydrogenase, P-L Biochemicals, Inc.; CCl₄ and CHCl₃, Wako Pure Chemicals, spectral grade; malonaldehyde Bis (diethylacetal), Tokyo Kasei Kogyo Co. Ltd. SKF-525 A (2-diethylaminoethyl-2,2-diphenylvalerate HCl) was kindly provided by Smith Kleine & French Laboratories. All other chemicals were of the highest grade commercially available.

Preparation of rat liver microsomes: Healthy male Wistar rats weighing 180–220 g were used. After exsanguination, the liver was well perfused in situ with cold 0.15 M KCl solution through a portal vein. The 20% liver homogenate in 0.15 M KCl-10 mM EDTA (pH 7.5) was prepared and centrifuged at 12,000×g for 15 min. The supernatant was centrifuged at 100,000×g for 60 min to precipitate the microsomes which were then floated off from the glycogen fraction at the bottom and washed once with 0.1 M potassium phosphate buffer (pH 7.5). The final microsomal precipitates were suspended in 0.15 M KCl-20 mM Tris-HCl buffer (pH 7.5) so as to bring the protein concentration to 40 mg/ml and were then kept on ice under nitrogen and used within 24 hr. The microsomal suspension thus prepared was well preserved against peroxidative damage during the experimental period. Protein concentration was determined by the method of Lowry et al. (15).

Experiments for lipid peroxidation: Complete incubation mixtures regularly containing microsomes (2 mg protein/ml), CCl₄ (20.8 µmoles (2.0 µl)) and NADPH-generating system (200 µM NADPH, 2.5 mM nicotinamide, 5 mM MgCl₂, 10 mM sodium isocitrate and 0.4 U of isocitrate dehydrogenase) in a final volume of 10 ml of 0.1 M phosphate buffer (pH 7.5) were prepared in a 25 ml-flask. CCl₄ was then carefully added at the bottom of the preparation, using a microsyringe, and the flask was tightly capped with a silicon rubber cap. In anaerobic conditions, the phosphate buffer was bubbled with N₂ gas through a fine mesh for about 1 hr prior to use and after all ingredients had been added, the flask was lightly flashed with N₂ for about 30 sec and capped immediately. Carbon monoxide (CO) treatment was performed by bubbling CO for 1 min through a capillary into the incubation medium containing microsomes before adding CCl₄. All reaction mixtures were prepared quickly in ice and finally the capped flasks were mixed vigorously using a Vortex-mixer. After incubation at 37°C for 30 min, the reaction mixture was cooled in ice and 1.0 ml, in duplicate, was assessed for malondialdehyde (MDA) content, using the thio-barbituric acid method (16). The remainder was centrifuged at 100,000×g for 60 min, the sediment being suspended in 1.6 ml of 0.15 M KCl-20 mM Tris-HCl (pH 7.5) and assayed for protein concentration (15), cytochrome P-450 content (17) and G-6-Pase activity (18). Cytochrome P-450 difference spectrum was measured in 0.1 M phosphate buffer (pH 7.5) containing 20% glycerol. In samples treated with CO, some CO still appeared to bind to microsomes even after precipitation of microsomes from the incubation mixture, as the dithionite difference spectrum gave a peak at 450 nm without CO bubbling and such was not observed in the case of untreated microsomes. Therefore,
this portion calculated by using extinction coefficient of 104 mM$^{-1}$ cm$^{-1}$ (19) was added for correction in CO-pretreated samples.

**Measurement of CCl$_4$ metabolism:** Preparation of the regular incubation mixtures (total 10 ml) was essentially the same as that for lipid peroxidation experiments; in order to visualize distinct metabolism, 4 mg of microsomal protein/ml and 5.2 µmoles of CCl$_4$ were added. Incubation was carried out at 37°C for 1 hr.

Extraction of CCl$_4$ and CHCl$_3$ from the reaction mixture and gaseous phase was as follows: at the end of incubation, the flasks were immersed in salted ice to freeze the contents, 5.0 ml of toluene was injected through the caps and the preparation shaken vigorously for 2 hr. The entire contents were then subjected to steam distillation, and CCl$_4$ and CHCl$_3$ in the toluene layer were determined by the method described below. Recoveries of CCl$_4$ (5.2 µmoles) and CHCl$_3$ (6.2 µmoles (0.5 µl)) from the buffer solution incubated at 37°C for 1 hr were 82.9±2.1% (S.D.), n=8, and 83.8±1.7% (S.D.), n=8, respectively, with no significant difference from the recoveries in the presence of microsomes alone. The results were corrected for these values.

**Simultaneous determination of CCl$_4$ and CHCl$_3$:** A colorimetric method for the simultaneous determination of CCl$_4$ and CHCl$_3$ in a solution was developed by application of the methods described by Freimuth (20) and Recknagel and Litteria (21). Details of the present method are given in brief. Toluene solution (0.5 ml) containing CCl$_4$ or CHCl$_3$, or both was added to 5.0 ml of pyridine, in duplicate, and exactly 0.075 ml of acetone was added to one of them. Color development was greatly affected by the amount of acetone: with 0.075 ml of acetone, color intensity by CCl$_4$ was about 10 times higher than that in the absence of acetone, whereas color intensity by CHCl$_3$ decreased to about one half (Fig. 1). Finally, 1.5 ml of 35% (W/W) KOH solution was added to both tubes. Blanks without CCl$_4$ and CHCl$_3$ were made in parallel. The test tubes were capped, well shaken, heated at 70°C for 20 min in a water bath, and then cooled in ice. Three ml of the colored phase was transferred to a test tube containing 0.85 ml of 0.01 N NaOH solution and the optical density was read at 530 nm. Standard curves for CCl$_4$ and CHCl$_3$ in the presence or absence of acetone were straight and the color development by CCl$_4$ plus CHCl$_3$ was completely additive (Fig. 1). Therefore, contents of CCl$_4$ (x µmoles) and CHCl$_3$ (y µmoles) in the sample may be calculated from the following equations:

$$x = \frac{b'c-bc'}{ab'-a'b} \cdot y = \frac{a'c-ac'}{a'b-ab'}$$

![Fig. 1. Standard curves of CCl$_4$, CHCl$_3$ and CCl$_4$ plus CHCl$_3$ in the presence and absence of acetone by means of the modified Fujiwara chromogen method (21). Details are given in Methods.](image)
where,

\( a \) and \( a' \): O.D./\mu \text{mole} of CCl\(_4\) in the absence and presence of acetone,

\( b \) and \( b' \): O.D./\mu \text{mole} of CHCl\(_3\) in the absence and presence of acetone,

\( c \) and \( c' \): O.D. of the sample in the absence and presence of acetone, respectively.

With this method, no color development was observed with CH\(_2\)Cl\(_2\) or CCl\(_3\)CCl\(_3\), while CBrCl\(_3\) reacted to the same degree as CHCl\(_3\).

This colorimetric method was applied to the determination of CCl\(_4\) metabolism described in the previous paragraph without further identification of metabolites. As, according to gas chromatographic analysis\(^{(10)}\), metabolites of CCl\(_4\) in the microsomal system consist of a covalently bound fraction to microsomes, free CHCl\(_3\) and some unidentified fraction.

**RESULTS**

**Relationship between lipid peroxidation and loss of microsomal enzymes:** In this series of experiments, relationships between microsomal MDA production and losses of G-6-Pase and cytochrome P-450 were examined in parallel under various conditions.

As shown in Table 1, under aerobic conditions, microsomes produced only negligible amounts of MDA in the presence of CCl\(_4\) alone (minus NADPH-generating system in Table 1). Microsomes plus NADPH-generating system (minus CCl\(_4\) in Table 1) produced MDA accompanying the loss of G-6-Pase and cytochrome P-450. Further stimulation of MDA production was observed in the presence of both the NADPH-generating system and CCl\(_4\) (complete system) accompanied by an enhanced loss of both enzymes.

Anaerobic incubation of microsomes suppressed considerably the MDA production in the NADPH-generating system with a slight loss of these enzymes. In the complete system, MDA production was also markedly suppressed, as compared to conditions of aerobic incubation with a good preservation of G-6-Pase activity, but the loss of cytochrome P-450 was little prevented. MDA production observed under the present anaerobic conditions may be due to the oxygen mainly dissolved in original microsomal suspensions and in other additives.

On the other hand, when microsomes were peroxidized by activator (Fe\(^{+++}\)-pyrophosphate) and NADPH under aerobic conditions,
various types of lipid peroxidation inhibitors such as SKF-525 A, menadione or N,N-diphenyl-p-phenylenediamine (DPPD) protected cytochrome P-450 or G-6-Pase or both against the peroxidative damages (Table 2).

From these observations shown in Tables 1 and 2, under aerobic conditions, CCl₄-induced loss of G-6-Pase and cytochrome P-450 may be coupled with lipid peroxidation, such as was suggested earlier (10, 22). Under anaerobic conditions, however, different mechanisms may be involved in the CCl₄-induced loss of this hemoprotein.

In addition, as shown in Table 3, the lipid peroxidation inhibitors also suppressed CCl₄-induced MDA production and preserved G-6-Pase activity to the level of the minus CCl₄ system, under aerobic conditions, while the extent of the preservation of cytochrome P-450 content was less. Thus, even under aerobic conditions, the nature of the CCl₄-induced losses of both enzymes seems to be different.

### Table 2. Effect of lipid peroxidation inhibitors on peroxidative loss of microsomal enzymes induced by activator

| Conditions       | MDA (nmol/mg prot.) | G-6-Pase (µmolPi/mg prot./hr) | Cytochrome P-450 (nmol/mg prot.) |
|------------------|---------------------|-------------------------------|---------------------------------|
| Whole system     | 29.1±0.5            | 13.2±0.1                      | 0.61±0.00                       |
| minus activator  | 0.9±0.1             | 20.0±0.2                      | 0.98±0.02                       |
| plus SKF-525 A   | 8.9±0.2             | 13.7±0.3                      | 0.93±0.01                       |
| (2×10⁻⁴ M)       |                     |                               |                                 |
| plus Menadione   | 1.5±0.0             | 19.9±0.2                      | 0.91±0.03                       |
| (10⁻⁶ M)         |                     |                               |                                 |
| plus DPPD        | 1.4±0.0             | 20.2±0.1                      | 0.95±0.01                       |
| (10⁻⁶ M)         |                     |                               |                                 |

Microsomes (20 mg/10 ml) were peroxidized in the presence of 400 µM NADPH and activator (20 µM Fe⁺⁺⁺=200 µM pyrophosphate) (whole system) under aerobic condition and processed as described in Methods. Each value represents the mean and deviation of duplicate experiments.

### Table 3. Effect of various lipid peroxidation inhibitors on CCl₄-induced lipid peroxidation and loss of microsomal enzymes under aerobic condition

| Conditions       | MDA (nmol/mg prot.) | G-6-Pase (µmolPi/mg prot./hr) | Cytochrome P-450 (nmol/mg prot.) |
|------------------|---------------------|-------------------------------|---------------------------------|
| Complete system  | 44.0±0.2            | 3.8±0.1                       | 0.34±0.02                       |
| minus CCl₄       | 4.4±0.3             | 11.7±0.4                      | 0.85±0.01                       |
| minus NADPH-GS*  | 0.9±0.1             | 19.8±0.2                      | 0.91±0.02                       |
| plus SKF-525 A   | 3.2±0.1             | 12.4±0.2                      | 0.56±0.05                       |
| (2×10⁻⁴ M)       |                     |                               |                                 |
| plus Menadione   | 4.9±0.2             | 11.3±0.4                      | 0.53±0.01                       |
| (10⁻⁶ M)         |                     |                               |                                 |
| plus DPPD        | 2.7±0.1             | 14.5±0.1                      | 0.58±0.02                       |
| (10⁻⁶ M)         |                     |                               |                                 |

Experimental details are given in Methods. Each value represents the mean and deviation of duplicate experiments *NADPH-generating system.
Table 4. Effect of carbon monoxide on microsomal lipid peroxidation and loss of enzymes induced by CCl₄ under anaerobic conditions

| Conditions              | MDA (nmoles/mg prot.) | G-6-Pase (µmoles Pi/mg prot./hr) | Cytochrome P-450 (nmoles/mg prot.) |
|-------------------------|-----------------------|----------------------------------|-----------------------------------|
| Complete system         | 7.1±0.3               | 17.4±0.2                         | 0.46±0.00                         |
| minus CCl₄              | 1.4±0.1               | 19.2±0.3                         | 0.72±0.01                         |
| minus NADPH-GS*         | 0.7±0.0               | 20.0±0.3                         | 1.07±0.00                         |
| plus CO                 | 16.8±1.6              | 10.6±0.1                         | 0.68±0.03                         |
| plus CO and DTC (10⁻⁶ M)| 2.9±0.1               | 14.0±0.1                         | 0.89±0.04                         |
| plus CO and DPPD (10⁻⁶ M)| 2.3±0.1           | 17.1±0.5                         | 0.90±0.05                         |

CO treatment of either microsomes alone or minus NADPH-generating system did not affect the MDA production, G-6-Pase activity and cytochrome P-450 content. In the minus CCl₄ system CO produced about 30% increase in MDA production and a slight increase in cytochrome P-450 content with no change in G-6-Pase activity. Experimental details are given in Methods. Each value represents the mean and deviation of duplicate experiments. *NADPH-generating system.

Nature of the loss of cytochrome P-450 under anaerobic conditions was further examined by CO treatment. CO reportedly inhibits microsomal CCl₄ metabolism, under anaerobic conditions (9) and such was confirmed in the experiment described in the next section. Therefore, if the breakdown of cytochrome P-450 is coupled with the metabolism of CCl₄, then it is expected that CO will inhibit the loss of cytochrome P-450, under anaerobic conditions. This is supported by the following findings (Table 4), i.e., CO treatment increased MDA production appreciably and such was accompanied by an increased loss of G-6-Pase activity, while, on the contrary, the loss of cytochrome P-450 was partially protected and the addition of lipid peroxidation inhibitors such as DPPD and diethyldithiocarbamate (DTC) further protected the hemoprotein from the breakdown.

Metabolism of CCl₄: Other investigators studied metabolism of CCl₄ by liver microsomes in vitro by using gas chromatographic analysis (10) or isotopically-labelled CCl₄ (9, 13). In subsequent experiments, a colorimetric method based on the Fujiwara chromogen formation (20, 21) was applied. The incubation conditions for the metabolism of CCl₄ were similar to those for lipid peroxidation.

In agreement with other observations (9, 10, 13), CCl₄ was metabolized to CHCl₃ only under anaerobic conditions in the presence of NADPH, and the extent of the metabolism was dependent on the concentration of microsomes (Fig. 2A). Production of CHCl₃ by 4 mg protein of microsomes per ml was maximum at the concentration of 5–10 µmoles of CCl₄ in a total volume of 10 ml (Fig. 2B). Time courses of CCl₄ disappearance and CHCl₃ production are shown in Fig. 3. Non-linearity of both of these time-courses may be partly due to a simultaneous and progressive loss of cytochrome P-450. At the end of 60 min of incubation, approx. 70% of the added CCl₄ (5.2 µmoles) were metabolized, in which about 1.2 µmoles were determined as CHCl₃. The remainder would be consumed for covalent binding to microsomal components, as determined from the data reported by Glende et al. (10).
To obtain a clear profile of the metabolism, this experimental condition was slightly different from that used for the previous lipid peroxidation experiments, where the regular microsomal concentration was 2 mg protein/ml and the amount of CCl₄ added was 20.8 μmoles per 10 ml of the incubation medium. It may be expected from these series of experiments, however, that there was a considerable metabolism of CCl₄ under the anaerobic conditions described in the previous section.

CHCl₃ (6.2 μmoles) was not metabolized under the same condition, irrespective of aerobic or anaerobic conditions (data not shown). As shown in Table 5, CO inhibited the production of CHCl₃ under anaerobic conditions, as reported by Uehleke et al. (9). SKF-525 A itself did not affect CHCl₃ production but did prevent the inhibition of CHCl₃ production by CO.

**DISCUSSION**

In agreement with the findings of others (10), CCl₄ stimulated microsomal MDA production in the presence of NADPH, under aerobic conditions accompanying a loss of G-6-Pase activity. Anaerobic conditions or addition of various lipid peroxidation inhibitors protected against the loss of G-6-Pase activity as well as against increased MDA production. From these and other data, the loss of G-6-Pase activity appears to be coupled with the lipid peroxidation process.

Microsomal cytochrome P-450 was also sensitive to lipid peroxidation, under aerobic conditions in which MDA production was
greatly enhanced by either CCl₄ or an activator (Fe³⁺-pyrophosphate) as reported previously (22). The various lipid peroxidation inhibitors prevented the loss of cytochrome P-450 induced by the activator, though such was less effective on the CCl₄-induced loss.

Unlike the case of G-6-Pase, however, under anaerobic conditions, prevention of the loss of cytochrome P-450 was not observed despite a marked suppression of CCl₄-induced MDA production. This may indicate that cytochrome P-450 may be lost through different mechanisms, under aerobic and anaerobic conditions, i.e., lipid peroxidation-dependent and -independent processes. The lipid peroxidation-independent loss of cytochrome P-450 may be associated with the metabolism of CCl₄, because marked metabolism of CCl₄, as measured by disappearance of CCl₄ and production of CHCl₃, occurred only under anaerobic conditions, and furthermore, CO inhibited the metabolism of CCl₄ accompanying partial prevention of the loss of cytochrome P-450.

Glende et al. (10) observed no loss of cytochrome P-450 under conditions of anaerobic incubation of microsomes in the presence of EDTA where a great enhancement of CCl₄ metabolism did occur, as evidenced by increased CHCl₃ production and irreversible binding of CCl₄ metabolites to microsomal proteins. On the other hand, recent observations by Yamazoe et al. (23) are in line with the findings in the present work. The latter authors attributed the difference of the results to the washing procedure; they determined cytochrome P-450 after washing the incubated microsomes, while the former assayed cytochrome P-450 directly in the incubation medium. Since CCl₄ shows an absorption peak at 454 nm when added to reduced microsomes under anaerobic conditions (24), it may be necessary to separate microsomes from the incubation medium. In the present experiments, cytochrome P-450 was determined after precipitation of microsomes from the incubation mixture which contained no EDTA, and CCl₄ alone had a minimal effect on the cytochrome P-450 content.

Several investigators (9-11) reported that CCl₄ was covalently bound to microsomal proteins under anaerobic conditions in the presence of NADPH. In the present work, CCl₄ binding was not determined, but approx. two thirds of the CCl₄ metabolized was not recovered as CHCl₃. This unidentified fraction may be bound to microsomes, as CHCl₃ was not further metabolized, under the same conditions.

### Table 5. Effect of carbon monoxide on microsomal CCl₄ metabolism

| Conditions                  | CHCl₃ produced (μmoles/flask) |
|-----------------------------|-------------------------------|
| (Under air)                 |                               |
| Complete system plus CO     | 0.037±0.020 (4)               |
| (Under N₂)                  |                               |
| Complete system plus CO     | 1.154±0.052 (4)               |
| plus SKF-525 A (2×10⁻³ M)   | 0.253±0.020 (4)               |
| plus CO and SKF-525 A (2×10⁻³ M) | 1.029±0.010 (2) |

Experimental details are given in Methods. Each value represents the mean±S.D. of 2-4 experiments.
anaerobic conditions. Uehleke et al. (9) and Sipes et al. (13) suggested that CCl₄ is metabolized to trichloromethyl radicals on the microsomal cytochrome P-450-dependent reductive pathway. Possibly, a large amount of the active radical metabolites produced by this reductive pathway in the absence of oxygen may destroy the nearby hemoprotein specifically and directly, by still unknown mechanisms. Under aerobic conditions no detectable metabolism occurred. In the presence of oxygen, however, a minimal extent of metabolism may be sufficient to provoke propagative lipid peroxidation in microsomal lipids for which cytochrome P-450 is also destroyed.

From these in vitro studies, it appears that, in vivo, loss of G-6-Pase activity is primarily due to lipid peroxidation induced by CCl₄, but in the case of cytochrome P-450 the additive mechanisms noted above may also be concerned. This is not inconsistent with our previous studies in vivo (25), in which diethylthiocarbamate—a lipid peroxidation inhibitor—protected against the CCl₄-induced loss of G-6-Pase but not the loss of cytochrome P-450 in phenobarbital-pretreated rats.

Finally, it is well known that necrosis and fat accumulation after CCl₄ administration occurs to a great extent around the central veins in the liver lobules. This can partially be attributed to a lowered oxygen tension due to decreased blood flow (26) and uneven distribution of drug metabolizing enzyme activity (27), i.e., higher activity around central veins. In addition, propagative lipid peroxidation processes require much oxygen, thus resulting in a deficiency in local oxygen in ER membranes. Under such conditions, the loss of cytochrome P-450 linked to CCl₄ metabolism might be partially involved. Detection of CHCl₃ in the expired air of the dogs exposed to CCl₄ vapor (28) may be also supporting evidence for the occurrence of the reductive metabolism of CCl₄ in vivo.

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