PRETREATMENT WITH TAURINE FACILITATES HEPATIC LIPID PEROXIDE FORMATION ASSOCIATED WITH CARBON TETRACHLORIDE INTOXICATION

Toshiaki NAKASHIMA, Tatsuro TAKINO and Kinya KURIYAMA*
Departments of Internal Medicine and *Pharmacology, Kyoto Prefectural University of Medicine, Kamikyo-ku, Kyoto 602, Japan
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Abstract—Pretreatment with taurine (3% aqueous solution, p.o., for 3 days) facilitated lipid peroxide formation in the rat liver associated with carbon tetrachloride (CCl₄: 2 ml/kg, b.w., i.p.) administration. The taurine pretreatment had no effect on the metabolism of ¹⁴CCl₄ in hepatic microsomes and antioxidant content in the liver, but significantly enhanced the CCl₄-induced increase in hepatic Ca²⁺ content. Ca²⁺ increased the Fe²⁺-induced NADPH-dependent lipid peroxidation in hepatic microsomes, and this Ca²⁺ effect was also enhanced by CCl₄ treatment. These results suggest that the increment of hepatic lipid peroxidation following CCl₄ administration may be, at least in part, due to the increment of Fe²⁺-induced NADPH-dependent lipid peroxidation by Ca²⁺, and the taurine pretreatment may affect the increase by increasing Ca²⁺ content in the liver.

The increased rate of lipid peroxide formation has been implicated as one of the causes of liver injury following carbon tetrachloride (CCl₄) administration. It is well known that CCl₄ is metabolized enzymatically by the mixed function oxidase system of hepatic microsomes and is cleaved into trichloromethyl radicals, which may facilitate the formation of lipid peroxides in the membrane components of hepatocytes. Treatment with CCl₄ also induces a marked increment of hepatic calcium which is suspected as one of factors mediating cellular necrosis (1).

On the other hand, taurine (2-aminoethanesulfonic acid), a sulfur amino acid, has been found in various mammalian organs and appears to increase the affinity of calcium to various membraneous structures such as the sarcoplasmic reticulum of heart (2), retina (3) and liver mitochondria (4). Considering these findings, it is probable that Ca²⁺ may take a part in hepatic lipid peroxidation following CCl₄ administration.

We have previously shown that the decrease of hepatic lipid peroxidation induced by CCl₄ is noticed if taurine is administered after occurring CCl₄-intoxication in the liver (5). In the present study, the effect of taurine pretreatment on CCl₄-induced lipid peroxide formation has been examined.

Materials and Methods

Male Wistar rats weighing 200–250 g were used in all experiments. Animals were given orally a 3% aqueous solution of taurine for three days, and subsequently received a single dose of CCl₄ (2 ml/kg, b.w., i.p.) in olive oil. The administration of taurine was continued until the sacrifice of animals. Control animals received 0.9% sodium chloride instead of the taurine solution and olive oil instead of CCl₄.

A part of each liver sample was fixed with 10% formalin solution, and it was sectioned and embedded in paraffin. Each section was
stained with hematoxylin-eosin and was examined under light microscopy.

The lipid peroxides formed in the liver were quantitated according to Ohkawa et al. (6). Briefly, tissue homogenate or microsomes were mixed with sodium dodecyl sulfate, acetate buffer (adjusted to pH 3.5 with NaOH) and aqueous solution of thiobarbituric acid. After heating at 95°C for 60 min in a water-bath, the red color formed was extracted with a n-butanol-pyridine (15:1) mixture, and the intensity of the color was estimated spectrophotometrically from the absorbance at 532 nm. Tetraethoxypropane was used as a standard for the assay, and lipid peroxide content was expressed as nmol of malonic dialdehyde (MDA) formed per mg protein.

Microsomes were prepared from 0.25M sucrose homogenates of the liver from rats fasted for 12 hr according to the procedure of Hogeboom et al. (7) as follows: The homogenate was centrifuged at 24,000×g for 10 min, and the supernatant thus obtained was further centrifuged at 105,000×g for 60 min to obtain the microsomal (P3) fraction. The microsomal pellets were suspended in 0.15 M KCl, kept in an ice-cold tube and used within 8 hr for each determination.

Aniline hydroxylation and aminopyrine demethylation were assayed spectrophotometrically by the method of Schenkman et al. (8).

The metabolism of CCl₄ was estimated by the disappearance rate of ¹⁴CCl₄ in an incubation mixture containing microsomes in vitro (9). Liver microsomes were incubated at 37°C for 5, 15 and 30 min with 0.2 M potassium phosphate buffer (pH 7.5) containing 0.8 μmoles of ¹⁴CCl₄, 200 μM NADPH and 3 mM EDTA. A part of the reaction mixture was placed in 10 ml of ice cold toluene and stored at 4°C for 3 days with occasional shaking. An aliquot of the toluene layer was mixed with toluene scintillator containing 0.5% (w/v) PPO and 0.03% (w/v) POPOP, and then the radioactivity extracted into the toluene layer was measured using a liquid scintillation spectrometer. The radioactivity in toluene was assumed to be entirely due to ¹⁴CCl₄ that was not metabolized by the microsomal mixed function oxygenase (10). The tissue blank level was determined in the absence of NADPH in the medium, and the difference in the radioactivity found between each tissue blank and experimental value (determined in the presence of NADPH) was used to calculate the disappearance rate of ¹⁴CCl₄.

It is well known that hepatic antioxidants can be divided into water-soluble and fat-soluble fractions. These antioxidants were determined by using a stable free radical, a,a-diphenyl-β-picrylhydrazyl (DPPH), according to the method of Glavind (11).

The extent of CCl₄-induced lipid peroxidation in microsomes in vitro was estimated by measuring MDA formed in the incubation media (12). Briefly, liver microsomes (2 mg protein) were preincubated at 37°C for 2 min with 0.2 M potassium phosphate buffer (pH 7.5) containing 400 μM NADPH in a final volume of 2 ml. After the addition of 2 μmoles of CCl₄, it was incubated for 30 min in tightly capped test tubes. The reaction was stopped by adding 0.2 ml of 8.1% sodium dodecyl sulfate and 1.5 ml of 20% acetic acid (pH 3.5) in an ice cold water bath. Under the same experimental conditions, the tissue blank level of MDA formation was measured in the absence of NADPH and was subtracted from each experimental value. The Fe²⁺-induced increase of NADPH-dependent MDA formation was determined by the same reaction mixture at 5 min after the addition of 0.4 mM FeSO₄.

EDTA treatment of microsomes (see Fig. 5) was employed as follows: The microsomal (P₃) fraction was washed once with 0.15 M
KCI - 1 mM EDTA (pH 7.5) and then with 0.15 M KCl. The microsomes thus obtained were used to study lipid peroxidation in vitro. The effect of EGTA and EDTA on the lipid peroxide level in the liver was also examined at 12 and 24 hr after CCl₄ administration. The liver homogenates obtained from CCl₄-intoxicated rats were preincubated at 37°C for 2 min and then incubated for 2 min with 3 mM EGTA or 3 mM EDTA. The formation of MDA in the mixture was determined as described above.

Effect of Ca²⁺ on Fe²⁺-induced increase of NADPH-dependent lipid peroxidation was examined in hepatic microsomes from untreated and CCl₄-intoxicated rats. In the case of CCl₄-intoxicated rats, the microsomes were obtained 3 hr after CCl₄ administration. Microsomes were preincubated in the reaction mixture containing CaCl₂ at 37°C for 2 min and then incubated for 2 min in the presence of 0.4 M FeSO₄. The lipid peroxide formed was then assayed as described previously. In addition, effect of Ca²⁺ on NADPH-induced microsomal lipid peroxidation was also estimated in the microsomes obtained from untreated and CCl₄-intoxicated rats. After preincubating with 0.2 M potassium phosphate buffer (pH 7.5) at 37°C for 2 min, the sample was incubated for 2 min with or without CaCl₂ in the presence of 400 μM NADPH.

The assay procedure for hepatic calcium content was as follows: Liver tissues were homogenized with a 10-fold excess of a concentrated HNO₃-H₂SO₄ (1:1) mixture in terms of the tissue volume, and the volume of the homogenate was adjusted to 10 ml with distilled water containing 1,000 ppm CaCl₂. The calcium content in the sample was determined with an atomic absorption flame photometer (Shimadzu AA-640), and the net content was calculated after subtracting the added amount of calcium.

The non-haem (free) iron content in the microsomal fraction was measured by using the method of Henry et al. (13) with the slight modification by Wills (14). Protein was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard. Statistic significances were evaluated by the Student’s t-test (16). Differences were accepted as statistically significant for all P values <0.05.

Results

Effect of pretreatment with taurine on lipid peroxide content and morphological alterations in liver at 24 hr after CCl₄ administration: Malonic dialdehyde formation in the liver was increased about three times in CCl₄-intoxicated rats, and this increase was further augmented by taurine pretreatment.

![Fig. 1. Alteration of lipid peroxide levels in liver at 24 hr after CCl₄ administration to untreated and taurine-pretreated rats. The pretreatment with taurine for three days significantly increased taurine content in the rat liver: Control rat, 3.2±0.6, and taurine-pretreated rat, 7.5±1.4 (μ moles/g wet liver, N=5). Each column represents the mean±S.E. obtained from five to seven separate experiments. *P<0.001, compared with each control value. #P<0.001, compared with the value obtained from CCl₄-treated rats.](image-url)
Light microscopic observation on the liver stained by hematoxylin-eosin revealed that there existed centrilobular hemorrhage and necrosis in CCl₄ administered rats. On the other hand, taurine pretreatment accentuated the CCl₄-induced damage, as evidenced by marked necrosis with ballooning of hepatocytes in perilobular areas (Fig. 2). These results indicate that pretreatment with taurine significantly enhances the CCl₄-induced increment of lipid peroxide formation as well as liver injury in CCl₄-intoxicated rats.

**Effect of pretreatment with taurine on demethylation of aminopyrine, hydroxylation of aniline and metabolism of ¹⁴C CCl₄ by liver microsomes:** It is well known that the induction of hepatic mixed-function oxidase by phenobarbital enhances the hepatotoxicity of CCl₄ due to metabolic activation of CCl₄ to its hepatotoxic products, which is catalyzed by cytochrome P-450 in hepatic microsomes. To examine the effect of taurine pretreatment on the activity of mixed-function oxidase, the demethylation of aminopyrine and the hydroxylation of aniline in liver microsomes from taurine-pretreated rats were also investigated. Pretreatment of rats with taurine had no effect on these activities in hepatic microsomes (Table 1). Similarly, the metabolic degradation of ¹⁴C CCl₄ by liver microsomes was not affected by taurine-pretreatment (Fig. 3). It can be concluded that taurine pretreatment does not act as an inducer of microsomal

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**Table 1. Effect of taurine on demethylation of aminopyrine and hydroxylation of aniline by hepatic microsomes**

|                      | Untreated | Taurine-treated |
|----------------------|-----------|-----------------|
| **Demethylation of aminopyrine (nmol/10 min/mg protein)** | 51.2±3.6  | 48.1±4.3        |
| **Hydroxylation of aniline (nmol/15 min/mg protein)**    | 4.9±0.2   | 4.8±0.4         |

Each value represents the mean±S.E. obtained from four separate experiments.
enzyme and has no capacity to accelerate the metabolism of CCl₄ in hepatic microsomes.

Effect of pretreatment with taurine on antioxidant level in liver following CCl₄ administration: Antioxidants may protect against CCl₄-induced hepatic damage by virtue of their free radical scavenger properties (17). The pretreatment with taurine, however, caused no change of hepatic antioxidant contents in both water-soluble and fat-soluble fractions (Table 2).

Effect of pretreatment with taurine on CCl₄-induced and Fe⁺⁺-induced lipid peroxidations in liver microsomes: Effect of taurine pretreatment on the time course of endogenous and CCl₄-induced lipid peroxidations in hepatic microsomes was examined. As shown in Fig. 4, CCl₄-induced lipid peroxidation was significantly increased in hepatic microsomes from taurine-pretreated rats.

Since a significant haemorrhage was observed in CCl₄-intoxicated rat liver, the effect of Fe⁺⁺, which might occur as non-haem Fe⁺⁺ in the liver, on the lipid peroxidation was also examined in hepatic microsomes from untreated and taurine-

![Figure 3](image1.png)

**Fig. 3.** Effect of taurine pretreatment on metabolism of ¹⁴CCl₄ in liver microsomes of rats. Each point represents the mean±S.E. obtained from four separate experiments.

![Figure 4](image2.png)

**Fig. 4.** Time course of CCl₄-induced lipid peroxidation in hepatic microsomes from untreated and taurine-pretreated rats. Each point represents the mean±S.E. obtained from four separate experiments. *P<0.05, **P<0.01, compared with each value obtained from the “untreated+CCl₄” group.

|                         | Water-soluble antioxidants (µequiv./g liver) | Fat-soluble antioxidants (µequiv./g liver) |
|-------------------------|---------------------------------------------|-------------------------------------------|
|                         | Untreated                                    | Taurine-treated                            |
| CCl₄ (-)                | 18.4±1.2                                    | 19.7±1.3                                  |
| CCl₄ (6 hr)             | 17.1±1.4                                    | 16.4±2.1                                  |
| CCl₄ (12 hr)            | 15.3±1.5                                    | 17.6±1.6                                  |
| CCl₄ (24 hr)            | 18.7±2.2                                    | 18.4±1.7                                  |
| Unreared                |                                            |                                           |
| Taurine-treated         |                                            |                                           |
|                         | 0.07±0.01                                   | 0.11±0.01                                 |
|                         | 0.13±0.02*                                  | 0.14±0.01*                                |
|                         | 0.15±0.02*                                  | 0.08±0.01                                 |
|                         | 0.11±0.01*                                  |                                           |
|                         | 0.13±0.01*                                  |                                           |

Each value represents the mean±S.E. obtained from four separate experiments. *P<0.05, **P<0.01, compared with the CCl₄ (-) value.
pretreated rats. As shown in Fig. 5, the enhancement of NADPH-dependent lipid peroxidation by Fe\textsuperscript{2+} was noticed in taurine-pretreated rats. Furthermore, this increasing effect of taurine pretreatment on microsomal lipid peroxidation was not detected in hepatic microsomes pretreated by the media containing EDTA.

These results suggest that the taurine pretreatment-induced enhancement of hepatic lipid peroxidation following CCl\textsubscript{4} administration may be, at least in part, due to the Fe\textsuperscript{2+}-induced increase of NADPH-dependent lipid peroxidation.

Effect of EDTA or EGTA on lipid peroxide level in liver following CCl\textsubscript{4} administration to untreated and taurine-pretreated rats: The suppressive effect of EDTA against CCl\textsubscript{4} induced lipid peroxidation in hepatic microsomes was further examined in the liver homogenates from CCl\textsubscript{4}-intoxicated rats (Fig. 6). Significant increments of lipid peroxidation in the liver were noticed at 12 and 24 hr after CCl\textsubscript{4} administration in both untreated and taurine-pretreated rats. These increments in hepatic lipid peroxidation decreased significantly by the additions of EDTA and EGTA which are well-known as calcium chelators. These results suggest that the effect of taurine on hepatic lipid peroxidation observed in these experiments may be mediated by alteration of hepatic calcium content. Furthermore, the suppressive effect of EDTA was found to be greater than that of EGTA, suggesting that not only Ca\textsuperscript{2+} but also Fe\textsuperscript{2+} may be involved in the increment of lipid peroxide found in CCl\textsubscript{4}-intoxicated liver.

Effect of Ca\textsuperscript{2+} on Fe\textsuperscript{2+}-induced increase of NADPH-dependent lipid peroxidation in liver microsomes from untreated and CCl\textsubscript{4}-treated rats: The accentuation of Fe\textsuperscript{2+}-induced increase of NADPH-dependent lipid per-
Fig. 7. Effect of Ca\(^{2+}\) on Fe\(^{2+}\)-induced increase of NADPH-dependent lipid peroxidation in hepatic microsomes from untreated and CCl\(_4\)-treated rats. Experimental details are given in the Methods. Each point represents the mean value of three separate experiments.

oxidation by Ca\(^{2+}\) was observed in hepatic microsomes in vitro and this effect of Ca\(^{2+}\) was most significant in the microsomes from CCl\(_4\)-treated rats (Fig. 7). These results strongly suggest intracellular Ca\(^{2+}\) may accentuate the increment of NADPH-dependent lipid peroxidation by Fe\(^{2+}\) in hepatic microsomes following CCl\(_4\) administration.

Time course of changes in hepatic lipid peroxide and calcium and non-haem (free) iron content in hepatic microsomes following CCl\(_4\) administration: The hepatic lipid peroxide content increased in parallel with the increment of calcium at 12 and 24 hr after CCl\(_4\) administration. Taurine pretreatment further increased both lipid peroxide level and calcium content in the liver. On the other hand, the content of non-haem (free) iron in hepatic microsomes showed an increase in CCl\(_4\)-intoxicated rats. Taurine has, however, no effect on the hepatic content of non-haem iron. These results also strongly suggest that the increment of intracellular Ca\(^{2+}\) may enhance the lipid peroxidation in hepatic microsomes from CCl\(_4\)-intoxicated rats by accentuating the Fe\(^{2+}\)-induced increase of NADPH-dependent lipid peroxidation.

Discussion

The present results clearly indicate that the pretreatment with taurine enhances the toxicity of CCl\(_4\) in rat liver by increasing hepatic lipid peroxidation. Hartroft and Porta (18) have reported that the susceptibility of various tissues to lipid peroxide is the consequence of the relative amount of unsaturated fatty acids and pro-oxidant substances versus the amounts of saturated fatty acids and antioxidant substances. In the taurine pretreated rats, the antioxidant level in the liver has been found to be the same as that in control animals (Table 1).

CCl\(_4\), which acts as a pro-oxidant, is known to be metabolized to CCl\(_3\) in hepatic microsomes. The pretreatment of animals with taurine, however, had no effect on the demethylation of aminopyrine and hydroxylation of aniline in hepatic microsomes (Table 2), suggesting that taurine pretreatment has no effect on the mixed function oxidase system in hepatic microsomes as measured by the above parameters. The lack of any effect of taurine on the metabolism of CCl\(_4\) in liver microsomes (Fig. 3) further supports this conclusion.
One of the interesting findings obtained in the present study is that the stimulating effect of taurine pretreatment on hepatic lipid peroxide formation induced by CCl₄ treatment disappeared in EGTA or EDTA washed microsomes. These results indicate that divalent cations being chelated by these agents such as Ca²⁺ and Fe²⁺ have important roles in the enhancement of CCl₄ intoxication-induced lipid peroxidation in the liver by the pretreatment with taurine.

Increased calcium content in guinea-pig heart and the enhancement of ATP-dependent calcium uptake in liver mitochondria following taurine treatment (2, 4) have been reported. Considering the present results together with these previous findings, it is suggested that the accentuating effect of taurine pretreatment on hepatic lipid peroxide formation found in an animal intoxicated by CCl₄ may also be mediated by calcium ion. In fact, we have found in this study that calcium facilitates the Fe²⁺-induced increase of NADPH-dependent lipid peroxidation in hepatic microsomes, and this calcium effect is also significantly enhanced by CCl₄ treatment (Fig. 7). Furthermore, calcium content in the liver has been found to increase at 12 and 24 hr after CCl₄ treatment, and this increment of calcium in the liver is further enhanced by taurine pretreatment (Fig. 8).

The content of non-haem (free) iron in hepatic microsomes also showed an increase following CCl₄ administration, possibly due to hepatocellular haemorrhage and damage induced by this agent. These results strongly suggest that the increment of free-iron may facilitate the lipid peroxidation in the liver, and intracellular calcium enhances the increase of lipid peroxidation induced by iron in hepatic microsomes from CCl₄-intoxicated rat liver.

It has been reported that hepatic microsomes have an ability to sequester calcium (19). Recently, evidence has been also presented that early disturbance of the microsomal calcium pump activity may be a key event in the occurrence of CCl₄-induced liver injury (20). Taurine pretreatment did
not modify the calcium content in the liver of the untreated rat, but it increased significantly at 12 and 24 hr after CCl₄-treatment, suggesting that taurine may also modulate the calcium sequestration mechanism in the microsomes of the CCl₄-intoxicated rat liver. Exact mechanisms underlying this action of taurine in the liver, however, remain to be elucidated.

The present results strongly suggest that the increment of hepatic lipid peroxidation in CCl₄-intoxicated rats may be, at least in part, due to the facilitation of Fe²⁺-induced increase of lipid peroxidation by intracellular calcium, and taurine pretreatment may affect the facilitation by increasing calcium concentration in the liver.

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