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Increase of Lipid Hydroperoxides in Liver Mitochondria and Inhibition of Cytochrome Oxidase by Carbon Tetrachloride Intoxication in Rats

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The cellular localization of lipid hydroperoxides was determined for the first time in mitochondria, microsomes and cytosol of rat liver using a specific method involving chemical derivatization and HPLC. Mitochondria contained the highest level of hydroperoxides. After 6 h of intragastric administration of carbon tetrachloride (CCl_4) to rats (2 ml/kg body weight), the concentration of lipid hydroperoxides increased significantly in liver mitochondria and cytochrome oxidase activity was inhibited to 35% of the control rats. The mitochondrial content of haem a decreased to 60% of the control at 12 h of CCl_4 administration. *In vitro* reaction of mitochondria with CCl_4 caused inactivation of cytochrome oxidase. These observations suggested that cytochrome oxidase and haem a in mitochondria were targets of CCl_4.

Keywords: Carbon tetrachloride, hydroperoxide, mitochondria, lipid peroxidation, cytochrome oxidase, haem a, necrosis

Abbreviations: CCl_4, carbon tetrachloride; GOT, glutamate-oxaloacetate transaminase; LDH, lactate dehydrogenase

INTRODUCTION

Carbon tetrachloride (CCl_4) has long been known as a typical hepatotoxin to cause hepatocellular necrosis.[1] As a result of extensive studies, the initial event in the rat given CCl_4 has been assumed to be lipid peroxidation of the endoplasmic reticulum of the liver cell initiated by trichloromethyl radical generated by the reaction between the chemical and cytochrome P450.[1,2] However, it is still unknown how radical reactions cause following reactions such as disturbance in hepatocellular calcium homeostasis[3] resulting in necrosis. Recently protein kinase C,[4] glycerol-3-phosphate acyl transferase,[5] regucalcin[6] and glucose 6-phosphatase[7] were found to be targets of the poison. In this paper we made a new approach to radical reactions in the liver after the administration of the drug by evaluating the localization of lipid hydroperoxides which were suggested to be a mediator of radical

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reactions and an effective indicator of oxidative stress[8] based on their elevation in the tissues of vitamin C-[9] or vitamin E-deficient[10] rats. Furthermore, we investigated effects of CCl_4 on cytochrome oxidase in mitochondria where lipid hydroperoxides were found to accumulate.

MATERIALS AND METHODS

Materials

1-Naphthyldiphenylphosphine and its oxide were prepared according to the literature.[8] Cumene hydroperoxide was purchased from Nacalai Tesque Co. (Kyoto, Japan). All other reagents were of analytical grade and were purchased from Wako Pure Chem. Co. (Osaka, Japan).

Animals and Treatments

Guidelines of the Prime Minister's Office of Japan (No. 6 of 27 March 1980) for the care and use of laboratory animals were followed. Male Wistar rats (strain Slc) were obtained from Japan SLC Co. (Hamamatsu, Shizuoka, Japan) and fed a commercial diet (type MF, Oriental Yeast Co., Tokyo, Japan) prior to the experiments. The animals were housed in a room with a temperature of $24 \pm 2°C$, and a 12 h/12 h light dark cycle. Eight-week-old rats were fasted for 12 h and administered a mixture of CCl_4 and corn oil (1 : 1, 4 ml/kg body weight) through an intragastric tube. The control rats received corn oil alone (2 ml/kg body weight).

Cell Fractionation

The rats were anaesthetized with diethyl ether and killed by collecting the blood from the vena cava inferior into a heparinized syringe. The liver was perfused with a chilled isotonic saline through the portal vein and removed. The liver was homogenized in 4 volumes of the ice-cold fractionation buffer (0.25 M sucrose containing 50 mM Tris-HCl buffer containing 1 mM EDTA, pH 7.4)[11,12] using a Potter-Elvehjem glass-Teflon homogenizer. Mitochondria, microsome and cytosolic fractions were prepared from tissue homogenate by differential centrifugation essentially as described by literature.[11,12] The purities of the subcellular fraction were ascertained by measuring marker enzyme activities.

Enzyme Assays

Cytochrome oxidase (EC 1.9.3.1) activity was determined based on the initial rate of ferrocytochrome c oxidation.[13] Activity of NADPH-cytochrome c reductase (EC 1.6.2.4) was determined based on the reduction of ferricytochrome c in the presence of NADPH.[14] Lactate dehydrogenase (LDH: EC 1.1.1.27.) activity was measured on the basis of the rate of oxidation of NADH.[15] The activity of plasma glutamate-oxaloacetate transaminase (GOT: EC 2.6.1.1) was determined using the diagnostic kits (GOT-UV Test Wako, Wako Pure Chemicals Co., Osaka) and expressed as Karmen Units.[16]

Determination of Lipid Hydroperoxides

Mitochondria were suspended in the fractionation buffer and 2 ml of the mitochondrial solution was subjected to the determination of lipid hydroperoxides. Microsomes were suspended in 2/5-fold tissue volume of the fractionation buffer and 2 ml of the solution was used for the quantitation of hydroperoxides. Cytosol fraction (2 ml) was used to measure the level of lipid hydroperoxides. The level of hydroperoxides in each fraction was determined according to the method.[8]

Extraction and Determination of Haem a and Haem b

Haem a and haem b in the mitochondrial fraction were extracted and determined as described.[17]

In Vitro Inactivation of Cytochrome Oxidase and Haems by CCl_4

Mitochondria were separated from ca. 7 g of rat liver and suspended in the fractionation buffer (ca. 7 ml). From this solution, two aliquots of 1.5 ml were taken. To one aliquot, 18.7 µl of CCl_4 was added and to the other 18.7 µl of water was added as the control. These mixtures were incubated at 37°C. At 30 min and 1 h, the activity of cytochrome oxidase was determined.

Mitochondria and microsome fractions were separated from ca. 7 g of rat liver and these fractions were combined in the fractionation buffer to a final volume of 10 ml. From the mixture, four samples of 1.5 ml were taken. To the one solution was added 18.7 µl of water as the control and to the second solution, NADPH (final 0.3 mM) was added as the second control. To the third solution, 18.7 µl of CCl_4 was added and to the fourth solution, 18.7 µl of CCl_4 and 15 µl of NADPH solution (final 0.3 mM) were added. These solutions were incubated at 37°C. At 30 min and 1 h, the activity of cytochrome oxidase was determined.

Mitochondria were incubated with water (control) and CCl_4 (the same concentration as above). After 1 h at 37°C, the content of haem a and haem b of these reaction mixtures was determined as described.[17]

Protein Assay and Statistics

Protein concentrations were determined by the method of Lowry *et al.*[18] using bovine serum albumin as the standard. Data were expressed as mean ± SD and analyzed by ANOVA using StatView software (Abacus Concepts, Berkeley, CA). Differences between group means were analyzed using Bonferroni/Dunn procedure (Dunn's procedure as a multiple comparison procedure). Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Change of Plasma GOT

The plasma GOT level at 6 h after administration of CCl_4 was 1530 ± 540 (Karmen Units) which was significantly higher than that of the control (88.1 ± 18.1). After 12 h of the administration, GOT level increased to 6920 ± 2670, which was also significantly higher than the control (77.5 ± 18.4). These results indicated that the liver damage was already considerable 6 h after administration of CCl_4.

The Level of Lipid Hydroperoxides in the Liver

The level of lipid hydroperoxides in liver homogenate of rats after 6 h of the administration of CCl_4 was measured utilizing a specific and sensitive method developed by us[8] involving quantitative chemical conversion of hydroperoxides into 1-naphthyldiphenylphosphine oxide and HPLC and found to be 225.9 ± 64.5 pmol/mg protein, which was not significantly different from that of the control rats (269.8 ± 81.7). At 12 h after administration of CCl_4, the level of lipid hydroperoxides in liver homogenate was 377.3 ± 51.2 pmol/mg protein, which was also not significantly different from that of the control (253.0 ± 184.9). These results indicated that lipid hydroperoxides did not significantly accumulate in the liver as a whole by CCl_4 intoxication. This observation was consistent with the literature[21,22] which described that thiobarbituric acid reactive substances (TBARS) did not increase in rat liver at 15 min to 25 h after CCl_4 poisoning.

As total lipid hydroperoxides of the liver did not change, localization of hydroperoxides in the liver cell was studied. The liver was fractionated into mitochondria, microsome and cytosol fractions. Since the distribution (Table I) of activities of marker enzymes in each fraction was similar to the literature,[11] it was concluded that the

TABLE I Distribution of marker enzyme activities in subcellular fractions of the liver of the control and CCl_4-intoxicated rats at 6 and 12 h of the treatment

Subcellular fractions	Cytochrome oxidase nmol/mg protein/min	NADPH-cyt c reductase nmol/mg protein/min	LDH nmol/mg protein/min
Control (6 h)			
Mitochondria	332.8 ± 46.5	65.5 ± 3.4	1548 ± 97
Microsome	49.1 ± 7.0	195.0 ± 3.2	1200 ± 100
Cytosol	1.4 ± 0.4	7.8 ± 2.9	9407 ± 819
CCl_4-treated (6 h)			
Mitochondria	98.4 ± 25.2**	78.6 ± 14.1	1727 ± 169
Microsome	33.0 ± 12.6	156.2 ± 18.5*	1828 ± 234
Cytosol	0 ± 0	7.2 ± 2.2	9298 ± 2293
Control (12 h)			
Mitochondria	243.1 ± 19.0	53.8 ± 19.8	1426 ± 302
Microsome	37.7 ± 3.0	210.0 ± 16.5	1573 ± 305
Cytosol	0.5 ± 0.8	8.3 ± 5.8	7751 ± 1107
CCl_4-treated (12 h)			
Mitochondria	102.0 ± 30.9**	61.0 ± 10.6	1462 ± 194
Microsome	56.6 ± 9.9	90.3 ● 34.4**	1814 ± 275
Cytosol	0.4 ± 0.4	4.3 ± 1.8	8299 ± 785

Rats were administered orally CCl_4 or corn oil (control). After 6 and 12 h, fractionation of the liver was made and activities of marker enzymes of each fraction was determined as described in the text. Values are means ± SD of 3 or 4 rats. In each fraction, only the marker enzyme activity was compared between two groups. Asterisks indicate significant difference from the corresponding control (ANOVA Bonferroni/Dunn procedure, $^*P < 0.05$ and $^{**}P < 0.01$).

TABLE II The level of lipid hydroperoxides in subcelluar fractions of the liver of the control and CCl_4-intoxicated rats at 6 and 12 h of the treatment

	Lipid hydroperoxides (pmol/mg protein)		
	Mitochondria	Microsome	Cytosol
Control (6 h)	939.1 ± 25.7	300.1 ± 57.4	52.7 ± 46.3
CCl_4-treated (6 h)	1126.0 ± 31.1**	345.3 ± 22.6	23.0 ± 24.6
Control (12 h)	825.4 ± 168.0	251.5 ± 57.7	147.3 ± 63.1
CCl_4-treated (12 h)	864.0 ± 266.0	367.1 ± 117.1	49.7 ± 28.4

Rats were administered orally CCl_4 or corn oil (control). After 6 and 12 h, fractionation of the liver was made and the level of lipid hydroperoxides of each fraction was determined as described in the text. Values are means ± SD of 3 or 4 rats. Asterisks indicate significant difference from the corresponding control (ANOVA Bonferroni/Dunn procedure, $^*P < 0.05$ and $^{**}P < 0.01$).

fractionation was satisfactory. The level of lipid hydroperoxides in each fraction was determined for the first time (Table II). Among these fractions, mitochondria contained the highest level of hydroperoxides in the control rat liver.

Hydroperoxides in liver mitochondria increased significantly after 6 h of the administration of CCl_4 and returned to the normal level at 12 h (Table II). These results demonstrated that lipid peroxidation was transiently enhanced in mitochondria.

The level of lipid hydroperoxides in microsome as well as cytosol fractions did not significantly increase by CCl_4 administration (Table II). In an early work,[19] it was reported that levels of lipid hydroperoxides in liver mitochondria and microsomes did not increase on the basis of iodometry after CCl_4 poisoning. They also described that

lipid hydroperoxides increased based on absorption of conjugated dienes at 234 nm.[19] However the former method seemed to have a problem in sensitivity and the latter seems to be low in specificity.

On the other hand, the present result that the concentration of lipid hydroperoxides increased only in mitochondria does not exclude the possibility that lipid peroxidation in microsomes was enhanced by CCl_4, because the level of lipid hydroperoxides is determined by the balance between their formation and decomposition. If hydroperoxides were rapidly decomposed in microsomes by enzymes, including cytochrome P450, they would not accumulate.

It was reported that hydroperoxides of phosphatidylcholine and phosphatidylethanolamine as determined based on absorption at 234 nm increased in rat liver intoxicated with CCl_4.[22] Later Miyazawa *et al.*[23] reported that the liver content of phosphatidylcholine hydroperoxide determined by separation of total lipids with HPLC and detection with chemiluminescent reaction increased after 6 h of CCl_4 poisoning at the dose of 1 ml/kg body weight. These reports supported the accumulation of lipid hydroperoxides by CCl_4 intoxication.

Change in Activity of Cytochrome Oxidase and Decrease of Haem a and Haem b

The activity of cytochrome oxidase in rat liver mitochondria after 6 h of the treatment with CCl_4 was decreased to 35% of the control value (Table I). The activity of the enzyme remained at a similar level after 12 h (Table I). These results indicated that cytochrome oxidase was one of targets of CCl_4. This result may explain an early observation[24] that CCl_4 caused damage in rat liver mitochondrial oxidation of octanoate and uncoupling from phosphorylation. Inactivation of cytochrome oxidase may damage energy production in the liver. Recently much attention is paid to the relationship among cell death (apoptosis and necrosis) and cellular ATP.[27,28] CCl_4 is known to cause centrizonal necrosis[1] and we suggested that the extent of necrosis caused by the present dose amounted to 70% of the total liver based on the regenerative response of DNA synthesis.[20] Since the total activity of cytochrome oxidase was depressed to 35% of the control value by the treatment with CCl_4 as described above, the enzyme activity in the necrotic area was suggested to be nearly zero, resulting in ATP depletion, which would cause the disorder of calcium homeostasis[25,26] and necrosis.[27,28]

To elucidate the mechanism of the inhibition, the mitochondrial content of haem a was determined. After 12 h of administration of CCl_4, liver mitochondria were isolated. The content of haem a was 42.0 ± 7.8 pmol/mg protein, which was significantly lower than that of the control rats (71.0 ± 6.6), i.e., haem a decreased to 60% of the control. Since the activity decreased to 42% of the control at 12 h, about 70% of the inhibition may be attributed to the decrease, possibly destruction of haem a.

A significant decrease of haem b in mitochondria from 354 ± 52 to 200 ± 67 pmol/mg protein was also observed. Destruction of haem b of cytochrome P450 by CCl_4[29–31] and degradation of total haem b in liver slices by bromotrichloromethane[32] are well documented.

In Vitro Inactivation of Cytochrome Oxidase by CCl_4

Isolated rat liver mitochondria were diluted with the fractionation buffer to the original liver weight and reacted at 37°C with CCl_4 at 130 mM, which was adopted assuming that CCl_4 was concentrated in the liver by 6 times of the average concentration of the total body (2 ml/kg body weight). In Figure 1, results are shown. Each point is a mean of three independent runs. The activity of cytochrome oxidase was significantly inhibited to 35% of that of the initial at 30 min and to 32% at 1 h. These inhibition rates were comparable to the *in vivo* experiment. However, the content of haem a decreased from 79.3 ± 7.1 to 77.8 ± 8.5 pmol/mg

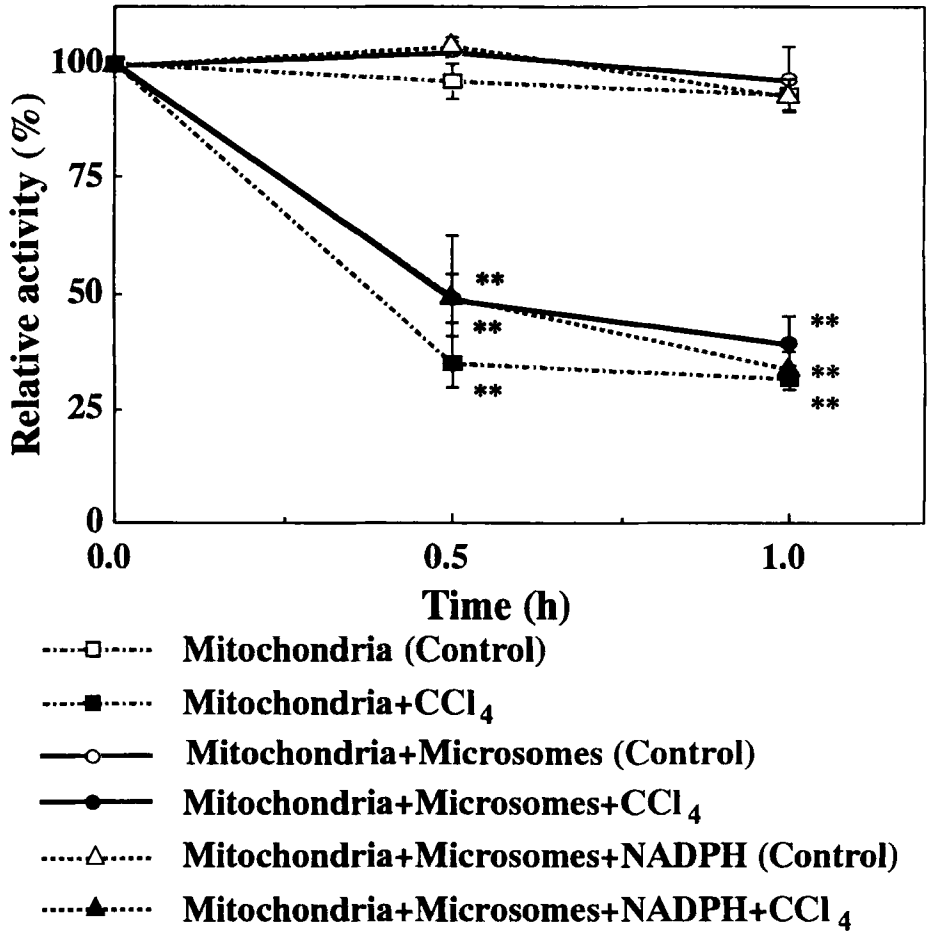

FIGURE 1 *In vitro* inactivation of cytochrome oxidase by CCl_4 and effect of microsomes. Rat liver mitochondria were incubated in the presence or absence (control) of CCl_4 and the activity change of cytochrome oxidase was determined. Similar reactions were made in the presence of microsomal fraction and microsomal fraction and NADPH. The initial activity of the enzyme in the absence of CCl_4 was defined as 100% for each run. Each point was mean ± SD of three independent runs. The bars are the standard deviations. Asterisks indicate significant difference from the corresponding control (ANOVA Bonferroni/Dunn procedure, $^{*}P < 0.05$ and $^{**}P < 0.01$).

protein by the reaction with CCl_4 for 1 h and the decrease was not significant. Haem b also did not decrease significantly in the reaction. This observation suggests that the destruction of haem a observed in the rat liver intoxicated with CCl_4 is a secondary reaction followed by an initial inactivation of the protein part of cytochrome oxidase.

When mitochondria were reacted with CCl_4 in the presence of microsomes, a significant difference was not observed in the extent of the inhibition of cytochrome oxidase compared to the reaction without microsomes (Figure 1). A significant effect of microsomes was not observed in the presence of NADPH (Figure 1) at 0.3 mM which was its concentration in rat liver.[33] In the reaction with microsomes, the total mitochondrial fraction separated from the liver was mixed with the total microsomal fraction from the same liver to mimic the relative ratio of the two organelles in the liver. These results suggested that mitochondria reacted with CCl_4 by themselves, resulting in the inhibition of the enzyme and that radical reactions caused in microsomes did not appreciably affect the inhibition reaction.

This idea is supported by a paper[34] which reported that isolated rat liver mitochondria produced trichloromethyl radicals which were detected by ESR spectra.

These observations support the idea that CCl_4 causes radical reactions in mitochondria and inhibits cytochrome oxidase, which is a target of the chemical. This was in agreement with the report[35,36] which described that the content of cytochrome oxidase increased in the cirrhotic liver of rats treated chronically with CCl_4, because this result could be explained on the ground that the induction of the enzyme was a compensatory reaction of the liver against the inactivation of the enzyme by CCl_4.

Reaction of Hydroperoxide with Cytochrome Oxidase

To investigate the role of lipid hydroperoxide in the inhibition of cytochrome oxidase, rat liver mitochondria were incubated with 1 mM concentration of cumene hydroperoxide at 37°C. After 30 min, the activity of the enzyme was significantly decreased to $80.1 \pm 5.5\%$ of the control. At 1 h, activity was $78.6 \pm 4.0\%$ of the control. When 5 mM of cumene hydroperoxide was used, the activity was inhibited to 52.7 ± 2.0 and $39.6 \pm 2.1\%$ of the initial value at 30 min and 1 h, respectively. Since the content of lipid hydroperoxides in mitochondria after 6 h of CCl_4 intoxication was about 1000 pmol/mg protein (Table II) and 1 kg (assumed to be 1 l) of mitochondria contained about 180 g of protein,[37] the concentration of lipid hydroperoxides in mitochondria was calculated to be about 180 μM. Based on the value and the inhibition by 1 mM cumene hydroperoxide, the contribution of hydroperoxide in the inhibition of cytochrome oxidase seemed to be only partial.

Inhibition of NADPH-cytochrome c Reductase

Activity of NADPH-cytochrome c reductase, a marker enzyme of microsomes, was also inhibited by CCl_4 administration (Table I). The extent of inhibition at 12 h was significantly higher than that at 6 h different from cytochrome oxidase. This enzyme belongs to the electron transport system of microsomes and therefore CCl_4 may cause inactivation of it, as for cytochrome P450 and perhaps cytochrome oxidase.

Effect of CCl_4 on LDH

Activity of LDH was not influenced by CCl_4 administration (Table I).

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