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NOTE

Application of FT-IR and ESR spectroscopic techniques to the study of CCl_4-induced peroxidation in rat liver microsomes

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Abstract

FT-IR and ESR were used for on the investigation of the CCl_4-induced peroxidation of rat liver microsomes in combination with biochemical methods. Lipid peroxidation was assayed by TBA reagent in the presence of CCl_4 and NADPH. The CCl_3· radical was detected by ESR spectroscopy with a spin trapping reagent of PBN. The FT-IR spectroscopy revealed that absorption band of -C-H in -C=C-H decreased in intensity at 3012 cm^{-1}, but the absorption bands of the phosphate head and choline in the phospholipids did not significantly change between 1300 and 900 cm^{-1}. These findings were interpreted to be due to the removal of H· from -C=C-H by radicals as the first step of lipid peroxidation, and to the absence of dephosphorylation of phospholipids in the microsomal membrane. This is the first IR spectroscopic evidence indicating the nature of damage to a microsomal membrane caused by CCl_4 treatment. The spectroscopies used here demonstrated that they are useful tools to observe the damage to microsomal membranes.

Key words: lipid peroxidation, liver microsomes, spectroscopy

CCl_4 is not only a well-known hepatotoxicant that produces peroxidation and dephosphorylation of cell membranes in the liver but in also an inducer of lipid peroxidation in liver microsomes in an *in vitro* biochemical system. In both systems, CCl_3· is accepted to be formed during the cytochrome P450-mediated biotransformation of CCl_4 in microsomes[1,3-5,8,10].

Fourier transform infrared (FT-IR) spectroscopy is expanding its application in toxicological science. We applied FT-IR and electron spin resonance (ESR) spectroscopic techniques to study the biochemical events induced by the CCl_3· radical in rat liver microsomes.

Microsomes were prepared from the liver of 20-week-old female SD rats[9]. The reaction mixture of 1.0 ml volume contained 1 mg microsomal protein, 10 μl ethanol, while the NADPH-generating system consisted of 0.5 mM NADP, 5 mM glucose-6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase and 0.1 M potassium phosphate buffer (pH 7.4). The

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reaction was started by adding 5 μl of ethanol solution containing 5 mM or 10 mM CCl_4[11] and followed by incubation at 37℃ for 15 min.

The lipid peroxides were determined by measuring the TBARS (thiobarbituric acid-reactive substance) as described by Buege and Aust[2]. The sample incubated with CCl_4, as shown in Table 1, gave significantly high values of the TBARS compared with the control sample incubated without CCl_4, confirming that the lipid peroxidation of microsomes occurred following metabolic activation of CCl_4 by cytochrome P450 which requires NADPH.

The radical formation was spectroscopically studied by ESR using the spin trapping reagent of N-*tert*-butyl-α-phenylnitrone (PBN). PBN was added to the incubation mixture to reach to the final concentration of 10 mM. The spin adducts were extracted with 0.5 ml of cold $CHCl_3/CH_3OH$ (2:1, v/v) solution after stopping the reaction. The organic phase was dried and resuspended with 0.7 ml of hexane. After evacuating the air, the ESR spectrum was observed at room temperature using a Varian E-4 spectrometer. In Figures 1B and 1C are shown the obtained ESR spectra in comparison with that of the control sample (Figure 1A). The parameters of $g=2.02$, $AN=14.3$ G and $AH=1.4$ G were attributed to the signal of the

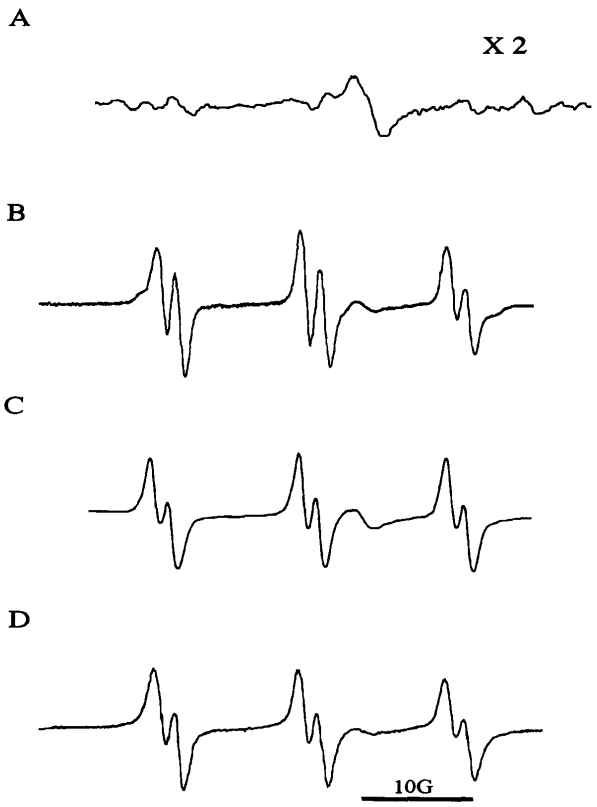

Fig 1. ESR spectra of PBN-adduct obtained in enzymatic reaction of CCl_4 in rat liver microsomes and photochemical reaction of CCl_4; A) in control enzymatic reaction without CCl_4, B) in enzymatic reaction of 5 mM CCl_4, C) in enzymatic reaction of 10 mM CCl_4, and D) in photochemical reaction of 100 μl CCl_4. PBN was 10 mM at final concentration in enzymatic reaction, and 10 mg/ml in hexane solution in photochemical reaction (Receiver gain : A) 6.3×10^3, B), C) and D) 3.2×10^3)

Table 1. TBARS in liver microsomes reacted with carbon tetrachloride

Group	TBARS (μmol/mg protein/15 min)
Control	0.81±0.20
CCl_4 5 mM	5.46±0.03*
CCl_4 10 mM	6.05±0.01*

Each value represents mean±S.D. from 3 rats.
Each sample was duplicated.
TBARS : thiobarbituric acid-reactive substances
*Significantly different from the control group ($P<0.01$).

PBN-CCl_3 adduct in accordance with the literature[6]. This assignment was also confirmed by the PBN-CCl_3 adduct photochemically formed by UV light (Figure 1D).

The IR spectroscopic data were obtained for the lipids extracted with $CHCl_3/CH_3OH$ (2:1, v/v) under a nitrogen environment and deposited on a disposable polyethylene IR card (3M, USA). The IR spectra were recorded using a JASCO FT-IR 420 spectrometer. The spectrum change was observed in the absorption band of -C-H of an isolated -C=C-H around 3012 cm^{-1} (Figure 2). These spectra

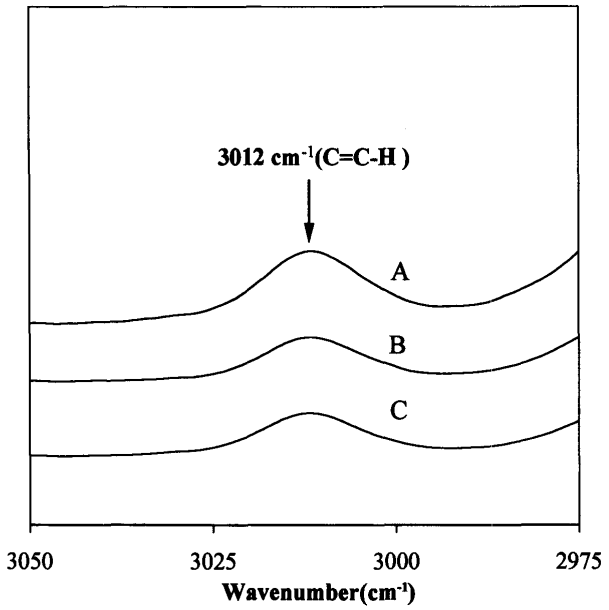

Fig 2. Infrared spectra of phospholipids in range of C-H bond in isolated -C=C-H group around 3012 cm^{-1}, A) in control enzymatic reaction without CCl_4, B) in enzymatic reaction of 5 mM CCl_4, and C) in enzymatic reaction of 10 mM CCl_4.

were normalized using the peak intensity of C=O in the phospholipid at 1760 cm^{-1} as the internal standard. The 3012 cm^{-1} band is seen to decrease in its intensity in Figures 2B and 2C. This can be interpreted as suggesting that the CCl_3· radicals abstract H· from the -C=C-H group in phospholipids as the first step in lipid peroxidation. Figure 3 shows the spectra characteristic of phospholipids in the range of the phosphate or choline group between 1300 and 900 cm^{-1}; 1249 (ν (P=O)), 1090 (ν (P-O-)), 1057 (ν (P-O-C)) and 970 cm^{-1} (ν (C-N+-C))[7]. There appears to be no significant effect of CCl_4, indicating that enzymatic or chemical dephosphorylation does not proceed in the microsomal phospholipids. On the contrary, we have previously focused on the diacylglycerol of the signal transducer in the plasma membrane of the liver in rats injured by CCl_4 (unpublished observation).

In conclusion, we spectroscopically confirmed that the CCl_4-induced lipid peroxidation in rat liver microsomes proceeds through the

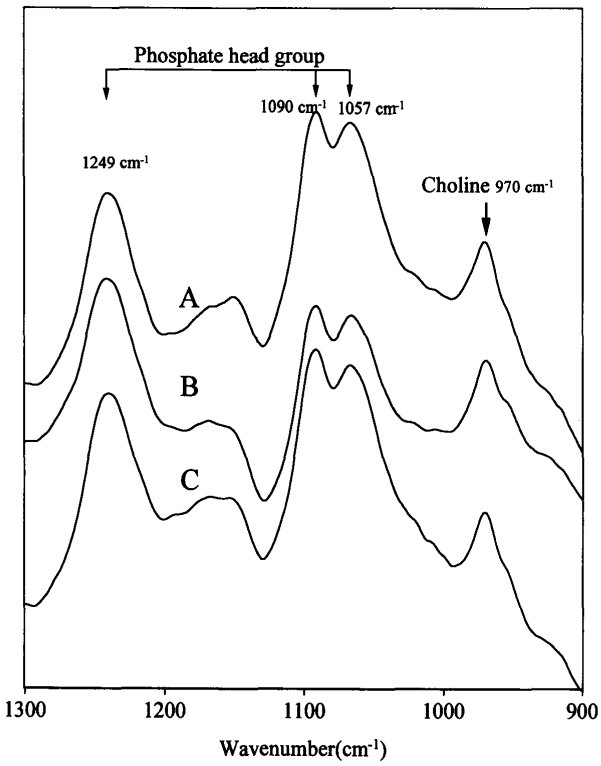

Fig 3. Infrared spectra of phospholipids in range of phosphate head and choline between 1300 and 900 cm^{-1}, A) in control enzymatic reaction without CCl_4, B) in enzymatic reaction of 5 mM CCl_4, and C) in enzymatic reaction of 10 mM CCl_4.

reaction of lipids with the CCl_4-derived radicals. It was also demonstrated that the FT-IR spectroscopic technique is useful for observing the damage in the cell membranes.

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