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Japanese Journal of Veterinary Research, 47(3-4), 151-154

2000-02-29

10.14943/jjvr.47.3-4.151

http://hdl.handle.net/2115/2789

bulletin (article)
Application of FT-IR and ESR spectroscopic techniques to the study of CCl₄-induced peroxidation in rat liver microsomes

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(Accepted for publication : Jan. 7, 2000)

Abstract

FT-IR and ESR were used for on the investigation of the CCl₄-induced peroxidation of rat liver microsomes in combination with biochemical methods. Lipid peroxidation was assayed by TBA reagent in the presence of CCl₄ and NADPH. The CCl₃⁻ 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 CCl₄ 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

CCl₄ is not only a well-known hepatotoxicant that produces peroxidation and dephosphorylation of cell membranes in the liver but also an inducer of lipid peroxidation in liver microsomes in an in vitro biochemical system. In both systems, CCl₃⁻ is accepted to be formed during the cytochrome P450-mediated biotransformation of CCl₄ in microsomes¹,²,³,⁸,¹⁰).

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 CCl₃⁻ radical in rat liver microsomes.

Microsomes were prepared from the liver of 20-week-old female SD rats⁹). 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
reaction was started by adding 5 μl of ethanol solution containing 5 mM or 10 mM CCl₄ and followed by incubation at 37°C for 15 min.

The lipid peroxides were determined by measuring the TBARS (thiobarbituric acid-reactive substance) as described by Buege and Aust. The sample incubated with CCl₄, as shown in Table 1, gave significantly high values of the TBARS compared with the control sample incubated without CCl₄, confirming that the lipid peroxidation of microsomes occurred following metabolic activation of CCl₄ by cytochrome P450 which requires NADPH.

The radical formation was spectroscopically studied by ESR using the spin trapping reagent of N-tert-butyl-a-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 CHCl₃/CH₃OH (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 PBN-CCl₃ adduct in accordance with the literature. This assignment was also confirmed by the PBN-CCl₃ adduct photochemically formed by UV light (Figure 1D).

The IR spectroscopic data were obtained for the lipids extracted with CHCl₃/CH₃OH (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

<table>
<thead>
<tr>
<th>Group</th>
<th>TBARS (μmol/mg protein/15 min)</th>
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<tbody>
<tr>
<td>Control</td>
<td>0.81±0.20</td>
</tr>
<tr>
<td>CCl₄ 5 mM</td>
<td>5.46±0.03*</td>
</tr>
<tr>
<td>CCl₄ 10 mM</td>
<td>6.05±0.01*</td>
</tr>
</tbody>
</table>

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).
Infrared spectroscopy was used to study the impact of carbon tetrachloride (CCl4) on lipid peroxidation in rat liver microsomes. The spectra were analyzed in the range of 3050 to 2975 cm⁻¹, focusing on the C-H bond vibrations around 3012 cm⁻¹. In control enzymatic reactions without CCl4 (A), there was a peak at 3012 cm⁻¹. However, in enzymatic reactions with 5 mM (B) and 10 mM (C) CCl4, this peak decreased, indicating the abstraction of H⁺ from the C-C-H group by CCl₃ radicals. The 3012 cm⁻¹ band is seen to decrease in its intensity in Figures 2B and 2C, suggesting that the CCl₃ radicals abstract H⁺ from the -C=C-H group 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-C)), 1057 (ν (P-O-C)) and 970 cm⁻¹ (ν (C-N+·C))⁷. There appears to be no significant effect of CCl₄, 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 CCl₄ (unpublished observation).

In conclusion, we spectroscopically confirmed that the CCl₄-induced lipid peroxidation in rat liver microsomes proceeds through the reaction of lipids with the CCl₄-derived radicals. It was also demonstrated that the FT-IR spectroscopic technique is useful for observing the damage in the cell membranes.

Acknowledgments

S. Yoon is supported by research fellowships from the Japan Society for the Promotion of Science for Young Scientists. The data and results in this paper are from a thesis to be submitted in partial fulfillment for the degree of Doctor of Philosophy in the Graduate School of Veterinary Medicine, Hokkaido University.

References


