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FULL PAPER

Accumulation of diacylglycerol induced by CCl_4-derived radicals in rat liver membrane and its inhibition with radical trapping reagent - FT-IR spectroscopic and HPLC chromatographic observations-

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Abstract

We have investigated the accumulation of diacylglycerol (DAG) induced by carbon tetrachloride (CCl_4)-derived radicals in the liver of female Sprague-Dawley (SD) rats after intraperitoneally injecting CCl_4. DAG is an intracellular activator of protein kinase C (PKC) which regulates cell proliferation and differentiation. The electron spin resonance (ESR) study gave the signal of the PBN-CCl_3 adduct in the liver of the rats which were pretreated with PBN, confirming that CCl_4 was metabolized into CCl_3· radicals with cytochrome P450 enzyme and indicating that PBN could trap them. The blood biochemical assay supported the trapping of the CCl_3· radicals; the pretreatment of rats with PBN inhibited the increase in the GOT and GPT values upon exposure to CCl_4. The Fourier transform-infrared (FT-IR) study indicated in comparison with the model compounds that the CCl_4-injected rats accumulated DAG in addition to phosphatidylcholine, phosphatidylethanolamine and triglyceride (TG) in the lipid membrane fraction of the liver homogenate. DAG was found to be ca. 10-15 % of the membrane phospholipids by weight. However, DAG was not found in the lipid of the liver microsomes, suggesting that it is formed only in the cell membrane of liver. Also, neither DAG nor TG was found in the lipid membrane of the rats that were pretreated with PBN followed by an injection of CCl_4. The formation of DAG was confirmed by an HPLC study. The activation of PKC was observed in liver homogenate in the rats that were injected with CCl_4. On the basis of the above findings, it was concluded that the CCl_4-derived radicals stimulate PKC through the accumulation of DAG in the liver membrane of the rats. Furthermore, it was shown that PBN has a protective and therapeutic effect against CCl_4-induced damage.

Key words: carbon tetrachloride, diacylglycerol, infrared spectroscopy, PKC, radicals

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Introduction

Carbon tetrachloride (CCl_4) is a well-known hepatotoxicant, which produces hepatitis in rats or mice. The CCl_4-derived radicals are known to be formed during cytochrome P450-mediated biotransformation followed by the lipid peroxidation of the cell membrane in the liver[2,6,14]. Thus, CCl_4 induces typical radical-derived hepatitis in rats. Free radicals have been reported to stimulate an intracellular signal transducer through activation of its ligands. Diacylglycerol (DAG) is an intracellular activator of protein kinase C (PKC) in the cell membrane, which plays an important role in the intracellular signal transduction controlling cell proliferation and differentiation. Recently, PKC has been reported to be activated in the rats that were exposed to CCl_4[15]. Our goal is to elucidate if the CCl_4-derived radicals result not only in the lipid peroxidation of the membrane but also in the stimulation of the intracellular signal transduction. We especially focused on the accumulation of DAG in the liver of female SD rats intraperitoneally injected CCl_4. We used an FT-IR spectroscopic technique along with HPLC. IR spectroscopy has been expanding its application in biology, and its usefulness is expected in molecular diagnosis. Recently, we have reported that Long-Evans Cinnamon (LEC) rats accumulate DAG in the liver, and it was suggested that the accumulated DAG plays an important role in the development of hepatomas through the activation of the proto-oncogene of c-fos[9,18].

Materials and Methods

Animal treatments

Twenty-week old female SD rats were purchased from Nihon SLC Co. (Hamamatsu, Japan). The rats were housed for 7 days before the experiment in an environmentally regulated room with a 12 hr light-dark cycle, and fed with commercial diet and water *ad libitum*. The 0.8 ml/kg b.w. of CCl_4 was intraperitoneally injected. When detecting CCl_4-derived radicals with ESR or inhibiting toxic effect due to the radicals by radical trapping, 300 mg/kg b.w. of PBN were intraperitoneally injected 30 min before the injection of CCl_4. The rats were then killed by decapitation at 3, 12 or 24 hr later, followed by removing the liver. A homogenate was prepared from three volumes of 1.15 % KCl solution. Furthermore, the liver microsomes were prepared according to the method of Omura and Sato[11]. The homogenate and microsomes were stored at -80℃ until assayed. The serum was collected for blood chemical assay using a conventional blood chemical analyzer (COBAS READY, Roche Japan).

Chemicals

N-*tert*-butyl-α-phenylnitrone (PBN) (Aldrich Chem. Co., U.S.A.) and CCl_4 (Wako, Japan) were used. Phosphatidylcholine (PC) from an egg yolk and phosphatidylethanolamine (PE) from an egg were obtained from Avanti Polar Lipids Inc. (Arabaster, USA). Triglyceride (TG) from pig liver and 1,2-diacylglyceride (DAG) from pig liver lecithin were also obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, USA). These chemicals were used as the model compounds without further purification. Pyridine and 3,5-dinitrobenzoylchloride were obtained from Wako (Tokyo, Japan). All other chemicals used were of analytical grade.

FT-IR and ESR measurements

FT-IR or ESR spectroscopy was used to detect the phospholipids and their metabolites or CCl_4-derived radicals in the extracts from the liver homogenate and microsomes, respectively. IR samples were prepared by extracting neutral lipids and phospholipids from both the homogenate and microsomes with $CHCl_3/CH_3OH$ (2:1, v/v) under a nitrogen en-

vironment according to the modified method of Folch *et al.*[4]. The IR sample of the model compound was prepared by mixing PC, PE, TG and DAG in $CHCl_3$. A 200 μl aliquot of the liver extract or the model compound in $CHCl_3/CH_3OH$ or $CHCl_3$ was directly applied onto a disposable polyethylene IR card (3M, USA) and dried in air. FT-IR spectra were recorded using a JASCO FT-IR 420 spectrometer that was connected to an IBM compatible microcomputer. Each sample was scanned 50 times through the frequency range of 400-4000 cm^{-1} with resolution of 2 cm^{-1}. Baseline correction or background subtraction was performed using the JASCO's spectrum manager.

ESR samples were prepared by extracting PBN-adducts from the liver homogenate with hexane. The extracts were concentrated under vacuum. The spectra were recorded with a Varian E-4 ESR spectrometer in an airtight ESR cell at room temperature.

HPLC measurements

The formation of DAG was detected by an HPLC measurement according to Eaton *et al.*[3]. Twenty mg of the liver sample were homogenized in 8 ml of CH_3OH:water (1:1), followed by vortexing and centrifuging at 750 x g for 5 min after adding 8 ml of CH_3OH. The lipids extracted into the $CHCl_3$ were dried under nitrogen. Neutral lipids were removed from the mixture of phospholipids and DAG by dissolving it into 4 ml of $CHCl_3$ and applying it to Silica cartridges (Waters, USA). DAG was analyzed by HPLC at 254 nm after derivatizing it with 3,5-dinitrobenzoyl chloride[8] and dissolving it into the mobile phase (1 ml) of hexane, cyclohexane, diethyl ether and ethanol (49:49:2:0.1, v/v). A Waters μ Porasil column (10 μm, 3.9 x 300 mm) was used, and the mobile phase was gradiated to that of cyclohexane, diethyl ether and ethanol (85:15:0.1, v/v) over 30 min at a flow rate of 1 ml/min.

Protein kinase C assay

PKC activity in the liver homogenate was assayed using the Biotrak protein kinase C enzyme assay system (Amersham Pharmacia Biotech, Sweden). The system is based upon the PKC catalyzed transfer of the γ-phosphate group of adenosine-5`-triphosphate to a peptide which is specific for PKC. The incorporation of phosphorous-32 is quantitatively measured using a scintillation beta counter (TRI-CARB 2100TR, Packard, U.S.A.).

TBA assay of microsomal lipid peroxides

The Microsomal lipid peroxides were determined by measuring the TBARS as described by Buege and Aust[1].

Statistics

The data were expressed as mean values and standard deviations. Student's *t* test was used to determine the statistical significance of differences at the $P<0.01$ level of confidence.

Results

1. CCl_4-treated rats

The results of the blood biochemical assay in the rats killed at 3, 12 or 24 hr post-injection of CCl_4 are shown in Table 1. All of the rats exposed to CCl_4 had the significantly high value of GOT (glutamic oxaloacetic transaminase), GPT (glutamic pyruvic transaminase) and LDH (lactate dehydrogenase) activities compared with those of the control group, indicating that the amount of CCl_4 injected was sufficient to study its toxic effect.

1.1 Liver homogenate

Figure 1a depicts the IR spectra obtained with the lipid extracts of the liver homogenate from the control or CCl_4-injected SD rats in the range of 1300-900 cm^{-1}. This particular range is quite sensitive to the metabolism of phospholipids and the accumulation of the neutral lipids. Phospholipids give the band of phosphate head groups at 1248 (ν (P = O)), 1091(ν (P-O-)), 1058 cm^{-1} (ν (P-O-C)) and the choline group at 969 cm^{-1} (ν (C-N + -C))[7].

Table 1. Biochemical parameters in serum of female Sprague-Dawley rats

	GOT (IU/L)	GPT (IU/L)	LDH (IU/L)	ALB (g/dL)	TBIL (mg/dL)	TP (g/dL)
Control group	160.0±38.9	39.8±6.7	612.3±50.5	3.8±0.3	0.4±0.6	7.1±0.5
CCl_4-injected group						
3hr post-injection	>1,000¶	191.3±5.3*	1625.1±120.1*	3.3±0.3	1.0±0.3	6.5±0.2
12hr post-injection	>1,000¶	545.2±3.6*	2325.2±95.1*	3.5±0.4	1.0±0.2	6.9±0.4
24hr post-injection	>1,000¶	606.5±51.4*	3474.2±371.5*	3.6±0.2	1.0±0.5	6.8±0.3

Each value represents mean±S.D. from 4 rats.
Abbreviations: LDH : lactate dehydrogenase, ALB: albumin, TBIL: total bilirubin, TP: total protein
*Significantly different from the control group ($P<0.01$).
¶ over Cobas Ready detection limit.

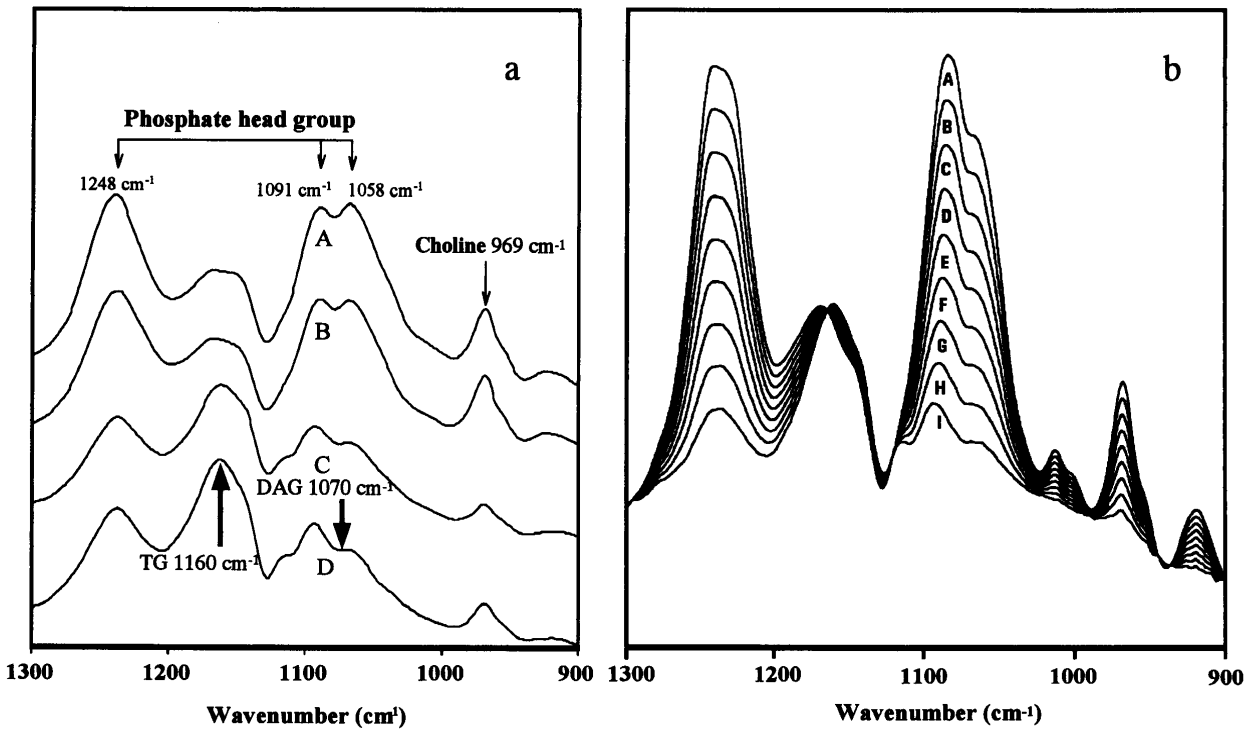

Fig. 1 Infrared spectra of lipid extracts from liver homogenate (a) and a mixture of model compounds of phosphatidylcholine (PC), phosphatidylethanolamine (PE), triglyceride (TG) and 1, 2-diacylglycerol (DAG) (b) between 1300-900 cm^{-1}. (a) A) Control SD rat, B) SD rat injected i.p. with 0.8 ml/kg b.w. CCl_4 and killed at 3 hr post-injection, C) SD rat injected i.p. with 0.8 ml/kg b.w. CCl_4 and killed at 12 hr post-injection, D) SD rat injected i.p. with 0.8 ml/kg b.w. CCl_4 and killed at 24 hr post-injection, (b) A) PC(60): PE (30): TG (8): DAG (2), B) PC (53): PE (27): TG (16): DAG (4), C) PC (47): PE (23): TG (24): DAG (6), D) PC (40): PE (20): TG (32): DAG (8), E) PC (33): PE (17): TG (40): DAG (10), F) PC (27): PE (13): TG (48): DAG (12), G) PC (20): PE (10): TG (56): DAG (14), H) PC (13): PE (7): TG (64): DAG (16), I) PC (7): PE (3): TG (72): DAG (18). Number in parenthesis show percentage of each model compound in weight.

TG and DAG give the respective bands around 1160 and 1070 cm^{-1}. Spectrum A is from the control rats, and based upon the literature[16], can be roughly analyzed as being composed of a 2:1 mixture of PC and PE by weight. The rats injected with CCl_4 showed very different spectra as depicted in B, C and D. The accumulation of TG and DAG is recognized with increasing exposure period. It has been reported that CCl_4 damages the ribosomes in the

hepatocyte to inhibit the synthesis of the TG carrier protein[12].

To analyze these spectra, we carried out IR spectroscopic observations using a mixture of PC, PE, TG and DAG model compounds. TG and DAG were added to the mixture of PC and PE (2:1) at their various ratios. The series of spectra was depicted in Figure 1b, and it was estimated that DAG amounted to ca. 10-15% of the phospholipids contained in the homogenate from the rats killed at 12 and 24 hr post-injection of CCl_4.

This was confirmed by the HPLC assays of DAG. As is listed in Table 2, the CCl_4-injected rats gave significantly higher values of 2.37 and 2.45 mg/g wet liver weight at 12 and 24 hr post-injection of CCl_4, respectively. These values agreed with the IR spectroscopic results, because the rat liver usually contains about 20 mg/g wet liver weight of phospholipids.

Table 2. Quantity of 1,2-diacylglycerol in SD rat liver

Group	DAG (mg/g wet liver weight)
Control group	0.35±0.02
CCl_4-injected group	
3hr post-injection	0.48±0.04
12hr post-injection	2.37±0.05*
24hr post-injection	2.45±0.07*

Each value represents mean±S.D. from 3 rats.
Each sample was measured in duplicate.
DAG: 1,2-diacylglycerol
*Significantly different from the control group ($P<0.01$).

Table 3. PKC activity in SD rat liver

Group	Mean cpm
Control group	4443±26
CCl_4-injected group	
3hr post-injection	7515±59*
12hr post-injection	17955±87*
24hr post-injection	14586±158*

Each value represents mean±S.D. from 3 rats.
Each sample was measured in duplicate.
PKC: protein kinase C ; cpm : count per minute
*Significantly different from the control group ($P<0.01$).

In Table 3, the results of the PKC activities are tabulated. It is clear that the PKC activity increases with the accumulation of DAG, even though the value is slightly decreased in the sample from the rats killed at 24 hr post-injection.

1.2 Liver microsomes

Figure 2a shows the IR spectra obtained with the lipid extracts of microsomes from the control or CCl_4-injected SD rats in the range of 1300-900 cm^{-1}. Few changes were observed between the spectra, indicating that CCl_4 does not affect the phosphate head and choline group of the phospholipids in the microsomes. As shown in Figure 2b, however, the absorption intensity in the band of -C-H of -C=C-H was observed to decrease around 3012 cm^{-1}, indicating that the peroxidation proceeded in the phospholipids of the microsomes. These spectra were normalized by using the peak of C=O in the phospholipid at 1760 cm^{-1} as the internal standard. The peroxidation was confirmed by observing the significantly high values of the TBARS (Table 4).

2. CCl_4 treatment of the rats pretreated with PBN

Figure 3 shows the results of the blood biochemical assay in the rats, which were pretreated with PBN and were killed at 3 hr post-injection of CCl_4, together with those in rats treated by other methods. The pretreatment of rats with PBN inhibited the increase in the values of GOT and GPT upon exposure to CCl_4, suggesting that the CCl_4-derived radicals actually injure the liver of rats.

Figure 4 shows the IR spectra from the samples of those which were used in the blood biochemical assay. The pretreatment of rats with PBN inhibited the development of the

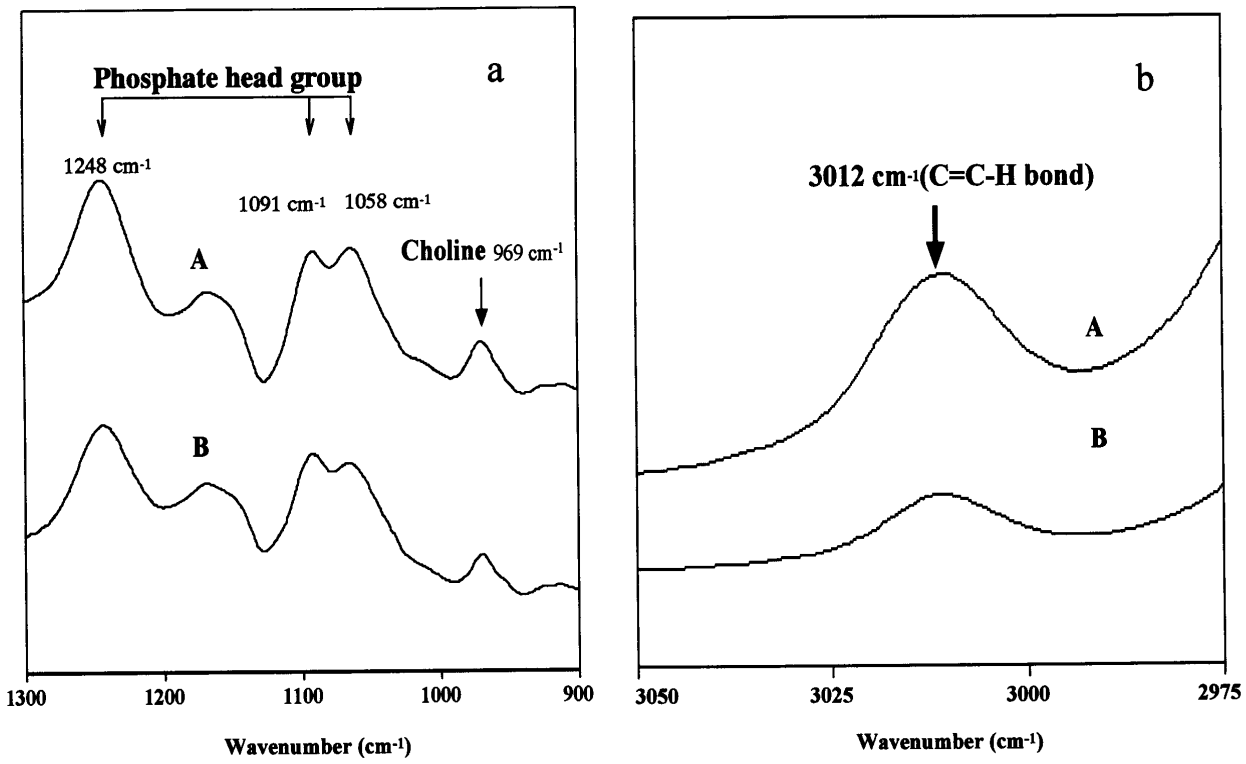

Fig.2 Infrared spectra of lipid extracts from liver microsomes between 1300 and 900 cm^{-1} (a) and around 3012 cm^{-1} (b). A) Control SD rat, B) SD rat injected i.p. with 0.8 ml/kg b.w. CCl_4 and killed at 24 hr post-injection.

Table 4. TBARS of liver microsomes from SD rat injected with carbon tetrachloride

Group	TBARS (μmol/mg)
Control	0.65±0.01
CCl_4-injected group	
24hr post-injection	12.05±0.05*

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

absorption bands due to TG and DAG at 1160 and 1070 cm^{-1}, respectively.

We carried out an ESR study of the CCl_4-derived radicals in the sample prepared from the rats pretreated with PBN and injected with CCl_4. The ESR spectrum with the respective parameters, g=2.02, AN=14.3 G and AH=1.4 G, was obtained from the sample, and can be assigned to a PBN-CCl_3 adduct with the reference to the spectrum of the PBN-CCl_3 adduct formed by UV irradiation in the PBN-hexane solution (10 mg/ml) containing CCl_4 (100 μl) by UV irradiation for 30 min under anaerobic conditions (Figure 5). The parameters found in the experiment agreed with these reported in the literature[5].

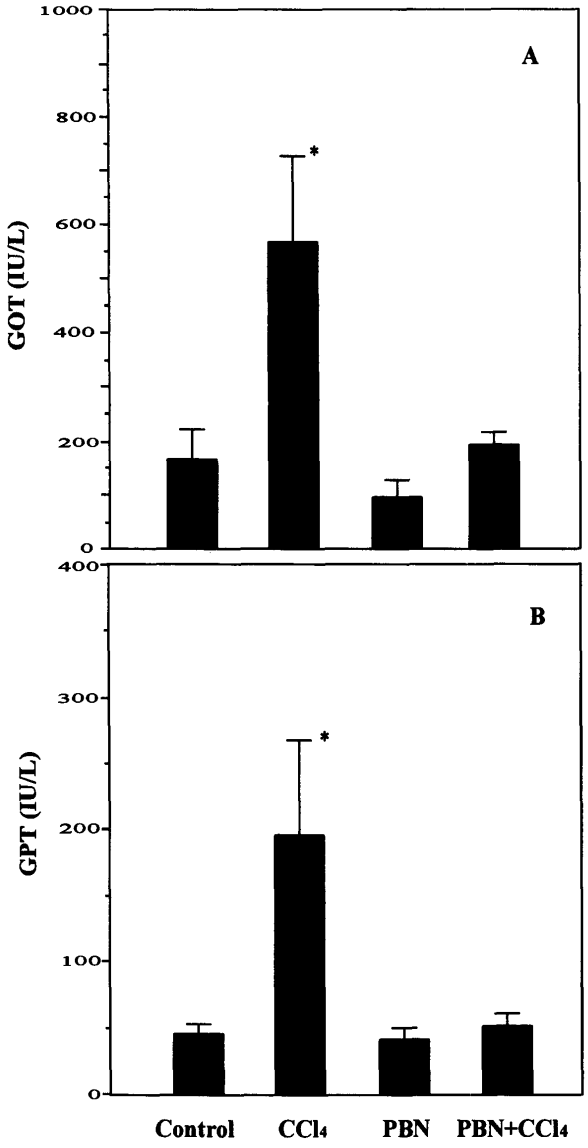

Fig 3. Activities of GOT (A) and GPT (B) in serum of SD rats. "Control", "CCl_4", "PBN" and "PBN + CCl_4" represent GOT (A) and GPT (B) values in control SD rats, SD rats injected with 0.8 ml/kg b.w. CCl_4, SD rats treated with 300 mg/kg b.w. PBN and SD rats pretreated with 300 mg/kg b.w. PBN and then injected with 0.8 ml/kg b.w. CCl_4, respectively. All rats were killed at 3 hr post-injection of CCl_4. Each datum is mean ± S.D. of four determinants.
* Significantly different ($P < 0.01$) when compared to other groups.

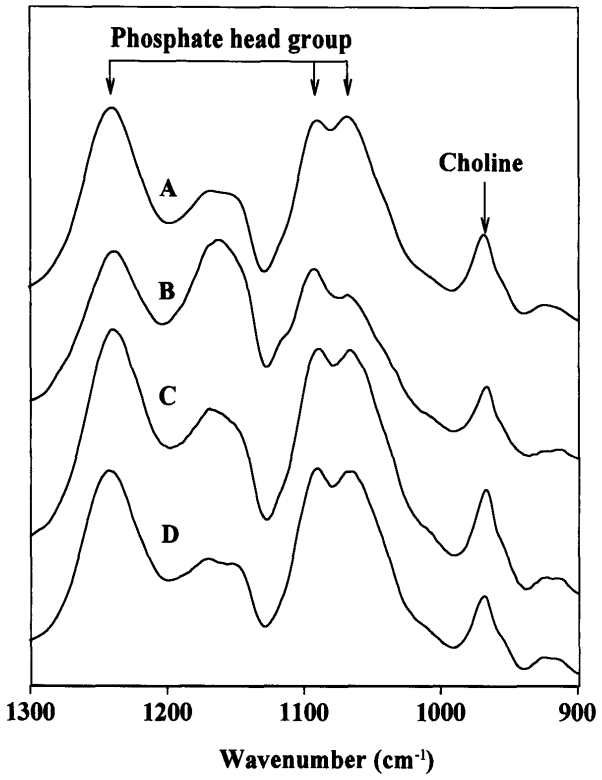

Fig 4. Infrared spectra of lipid extracts from liver homogenate between 1300 and 900 cm^{-1}. A) Control SD rat, B) SD rats injected with 0.8 ml/kg b.w. CCl_4 C) SD rats treated with 300 mg/kg b.w. PBN, D) SD rats pretreated with 300 mg/kg b.w. PBN and injected with 0.8 ml/kg b.w. CCl_4. All rats were killed at 3 hr post-injection of CCl_4.

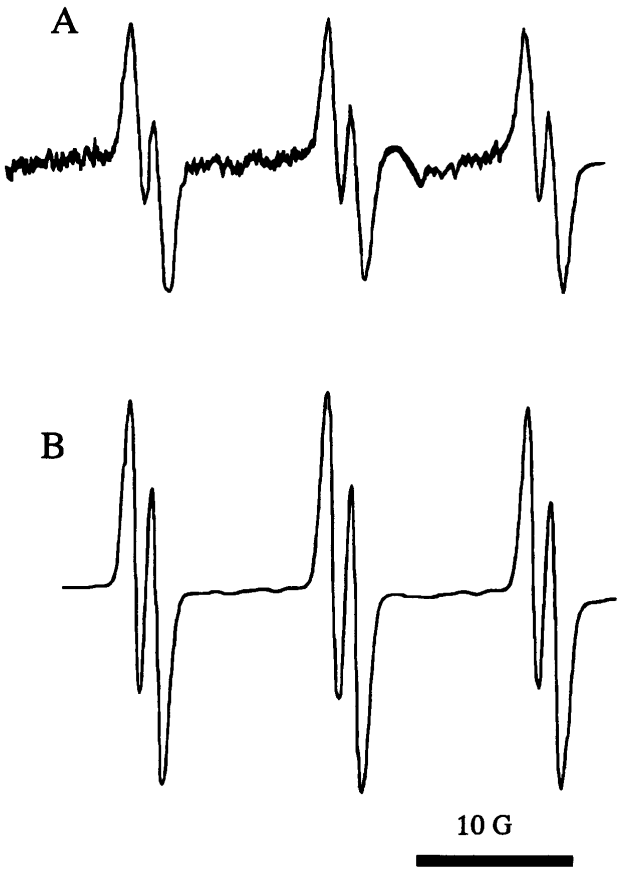

Fig 5. ESR spectra of PBN-CCl_3 adduct formed in SD rats liver (A) and in UV-photochemical reaction (B). SD rats were pretreated with 300 mg/kg b.w. PBN, injected with 0.8 ml/kg b.w. CCl_4 at 30 min later and killed at 3 hr post-injection of CCl_4. Photoformed PBN-CCl_3 was prepared in PBN-hexane solution (10 mg/ml) containing CCl_4 (100 μl) by UV irradiation (Receiver gain : A) 6.3×10^3, B) 3.2×10^2)

Discussion

We have investigated the accumulation of DAG induced by the CCl_4-derived radicals in the liver of female SD rats. First, we confirmed the *in vivo* formation of radicals from CCl_4. When injecting CCl_4 into the rats, which were pretreated with PBN, the ESR signal of the PBN-CCl_3 adduct was observed with the respective parameters of $g = 2.02$, $A_N = 14.3$ G and $A_H = 1.4$ G, confirming that CCl_4 was first metabolized into the $CCl_3\cdot$ radicals by the cytochrome P450 enzyme. These radicals will be followed by a nonenzymatic change in its form by oxygenation. Consequently, it is difficult to determine what type of radical is hepatotoxicant. However, even with exposure to CCl_4, the blood biochemical assay showed that the PBN pretreatment of the rats resulted in the low GOT and GPT values. Thus, it is evident that CCl_4 forms hepatotoxic radicals in the rat liver. Also, the above results suggest that PBN has a protective and therapeutic effect on the radical-induced hepatitis. In fact, Yamashita *et al.* reported that PBN has a therapeutic value for the treatment of LEC rat hepatitis[17].

Second, we have showed by using IR and HPLC techniques that DAG was accumulated in the liver cell membrane and not in the microsomes. It was found to be ca. 10-15 % of the membrane phospholipids by weight. It should be noted that the PBN pretreatment inhibited the accumulation of DAG. Therefore, this DAG accumulation is significantly related to the formation of the radicals. DAG activates PKC in the plasma membrane by making covalent bond between them[10]. We found high PKC enzymatic activity in the liver of rats injected with CCl_4. PKC plays an important role in the intracellular signal transduction controlling cell proliferation and differentiation[10,13]. Thus, the accumulation of DAG must play an

important role in the cell regeneration in the liver damaged by CCl_4[13,15].

It was clarified in the IR spectrum that a high amount of TG had accumulated in the lipid phase extracted from the liver homogenates of rats with CCl_4-induced hepatitis. The IR spectroscopic observation of TG may be useful for the simple clinical diagnosis of hepatitis.

In this study we have succeeded in the spectroscopic measurement of the lipid properties. Also, we have confirmed these ideas by routine biochemical methods. Although further study is required, the present work can form the basis for the elucidation of hepatitis induced by various radical-forming chemicals.

Acknowledgments

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