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FULL PAPER

Protective effect of *Pleurotus cornucopiae* mushroom extract on carbon tetrachloride-induced hepatotoxicity

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Abstract

Pleurotus cornucopiae **(PC) mushrooms are found in the field and commonly known in Japan as** ***Tamogidake*** **mushrooms. The present study investigated the protective effects of an aqueous extract of PC on carbon tetrachloride (CCl_4)-induced hepatotoxicity and the possible mechanism involved in this protection including cytochrome P450 (CYP) 2E1. Wistar rats were pretreated with aqueous extracts of PC (0, 100, 200, and 400 mg/kg) orally for 8 days prior to the intraperitoneal administration of a single dose of CCl_4 (0.5 ml/kg) or corn oil. Pretreatment with PC mushroom extract significantly prevented the increased serum enzyme activities of alanine and aspartate aminotransferases in a dose-dependent manner, and suppressed the expression of CYP2E1. PC mushroom extract also protected hepatocytes from the damage effects of CCl_4 as remarked by histological and electromicroscopical findings. It was concluded that repeated daily doses of aqueous extracts of PC mushroom reduced the toxic effects exerted by CCl_4 on the liver.**

Key words: CCl_4, CYP2E1, cytochrome P450, mushroom, *Pleurotus cornucopiae*

Introduction

Herbs have recently attracted attention as health-beneficial foods (physiologically functional foods) and as source materials for drug development. Herbal medicines derived from plant

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extracts are being increasingly utilized to treat a wide variety of clinical diseases despite relatively little knowledge regarding their modes of action[8]. Pleurotus species are commonly called oyster mushrooms. There are about 40 species of this mushroom distributed worldwide both in temperate and tropical zones of the world. The production amount of oyster mushrooms now ranks second among the cultivated mushrooms in the world[14]. In Asia, including Japan and far eastern Russia, a form of *Pleurotus cornucopiae* (PC) with a brilliant yellow pileus and also known as *P. citrinopileatus* Singer is commonly sold in markets.

In a previous study, we found that the extract of PC suppressed cytochrome P450 (CYP) 1A1- and CYP1A2-dependent activities[2]. CYPs are drug-metabolizing enzymes that contribute to the elimination of xenobiotics and are also involved in the metabolic activation of procarcinogens. We showed that administration of PC extract prevented DNA damage due to benzo[a]pyrene, which is metabolically activated by CYP1A1/2 reactions. In addition, we performed the Comet assay using these PC extracts. PC mushroom extract also showed a marked dose-dependent, protective effect against oxidative DNA damage caused by H_2O_2. Thus, we concluded that the antigenotoxic effects of PC mushroom extract are due to the concerted effects of the inhibition of metabolic activation by down-regulation of CYP1A and the inhibition of reactive oxygen species-induced DNA damage. These results led us to investigate the protective effects of PC extract on other metabolic activations of xenobiotics by CYP isoforms in the liver.

Carbon tetrachloride ($CC1_4$) is well-known to cause chemical hepatic injury. Liver injury induced by $CC1_4$ is the best characterized system of xenobiotic-induced hepatotoxicity and is a commonly used model for the screening of antihepatotoxic/hepatoprotective activities of drug[11]. It is now generally accepted that the hepatotoxicity of $CC1_4$ is the result of reductive dehalogenation, which is catalyzed by CYP2E1, and which forms the highly reactive trichloromethyl free radical[15,16]. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical. Both trichloromethyl and its peroxy radical are capable of binding to proteins and lipids, or of abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage, and thus playing a significant role in the pathogenesis of diseases[3,11,15]. Trichloromethyl free radicals can react with sulfhydryl groups such as glutathione and protein thiols. The covalent binding of trichloromethyl free radicals to cell proteins is considered the initial step in a chain of events that eventually leads to membrane lipid peroxidation, and finally, cell necrosis[1,12].

The purpose of this study was to evaluate the protective effect of PC mushroom extract on CCl_4-induced hepatotoxicity via CYP2E1 and to examine the mechanism(s) of this protection.

Materials and Methods

Chemicals: CCl_4 was obtained from Wako, Japan, and other chemicals and solvents were of analytical grade. Solutions of CCl_4 were prepared in corn oil (1:1 v/v; Sigma Chemical Co., St. Louis, MO, USA) before experiments.

Animals: Seven-week-old male Wistar rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan) were housed in cages at $24 \pm 1°C$ with a 12-h light and 12-h dark cycle and given laboratory feed and water ad libitum. The study was carried out under the guidelines for experiments using experimental animals at Hokkaido University (No. 16089).

Aqueous extract (tea-like preparation): Fresh PC mushroom was minced and dried under vacuum at room temperature (25°C) for 2 hr. Distilled water (40 ml/g) was added with agitation for one hour and then the homogenate was placed in a water bath at 60°C for 15 min[2]. The resultant

homogenate was filtered under suction, and the filtrate was centrifuged at 10,000 × g for 20 min to remove any mushroom debris. The supernatant was sterilized by filtration through two Millipore filters (0.22 μm) and then stored at 4°C. We used it within 3 days from preparation.

Treatments: The protocol of this study was summarized in Table 1. Animals were assigned to one of eight groups of 4 rats each. The first group served as a control, and they were intubated orally with water at dose of 1 ml/kg for 8 days. On the 9th day, control group was received a single i.p. dose of corn oil. The second and third groups received crude PC mushroom extract which corresponding to 100 mg of PC/kg body weight using a gavage technique once a day daily for 8 days. The fourth and fifth groups received 200 mg/kg of crude PC mushroom extract by gavage once a day daily for 8 days. The sixth and seventh groups received 400 mg/kg of crude PC mushroom extract by gavage once a day daily for 8 days. The last CCl_4 group was intubated orally with water for 8 days and on the 9th day received a single intraperitoneal (i.p.) dose of CCl_4 (0.5 ml/kg) dissolved in corn oil. On the 9th day of the experiment, rats of groups 3, 5 and 7 received a single i.p. dose of CCl_4 (0.5 ml/kg) dissolved in corn oil. One day after administrations of CCl_4, the rats were anesthetized with carbon dioxide, and liver and blood samples (serum) were collected for histological and biochemical analyses.

Sampling for histological and electron microscopic analysis: Livers were removed and weighed, and part of the right lobe was sliced, fixed in 10% buffered formaldehyde solution, and used for histological examination. Liver tissue was embedded in paraffin and sectioned. A series of 5 μm sections was cut and stained with hematoxylin and eosin. Lipid vacuoles recorded in CCl_4 treated

Table 1. Protocol of animal treatments

Treatments	PC 0 mg/kg	PC 100 mg/kg	PC 200 mg/kg	PC 400 mg/kg
corn oil	Group 1	Group 2	Group 4	Group 6
CCl_4	Group 8	Group 3	Group 5	Group 7

Rats are orally administrated with water (control) or PC extracts for 8 days. At the 9th day, we intraperitoneally injected corn oil (control) or 0.5 ml/kg CCl_4 to each group of rats.

Table 2. Effects of PC extract on liver tissue injury by CCl_4

Group	Lipid vacuoles / field (mean of 5 independent fields)	Hemorrhage	Leukocyte infiltration	Special notes
CCl_4	107	Perfused hemorrhage between hepatocytes due to destruction of endothelial lining	Massive infiltration with leukocytes	Hepatocytes with lost nuclei
PC 100 + CCl_4	45	Slight hemorrhage between hepatocytes	Some leukocytes	Edema around blood vesselswith damaged hepatocytes
PC 200 + CCl_4	35.2	No	No	Dilatation of sinusoidal capillaries
PC 400 + CCl_4	23.3	Some hemorrhage among hepatocytes	No	

and PC co-treated rats were counted per microscopical field and we took the mean of 5 different fields as summarized in Table 2. For electron microscopic analysis, small pieces of liver tissues were fixed with 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 hr and post fixed with 1% osmium tetroxide (OsO_4) in 0.1 M phosphate buffer (pH 7.3) for 2 hr. Then, the tissues were dehydrated in ethanol and embedded in Quetol 812 (Nissin EM, Tokyo, Japan), then, ultrathin sections were doubly stained with uranium acetate and lead citrate and photographed using a transmission electron microscope (JEM 1210, JEOL, Tokyo, Japan).

Biochemical analysis: A conventional blood chemical analyzer (COBAS Ready; Roche Diagnostic Systems, Basel, Switzerland) was used to quantify the alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities.

Microsome preparation: The liver was homogenized in 3 volumes of 1.15% potassium chloride and centrifuged at 9,000 × g for 20 min. The supernatant fraction was centrifuged at 105,000 × g for 70 min and the precipitates were used as microsomal fraction[10]. The washed microsomes were then suspended in 0.1 M potassium phosphate buffer, pH 7.4[8]. Microsomal protein concentrations were determined by the method of Lowry[9] using bovine serum albumin (Sigma Chemical Co.) as a standard.

Western immunoblotting of CYP2E1 protein: The effects of PC mushroom extract treatments and CCl_4 administration on expression levels of CYP isoforms 2E1 was studied using Western immunoblot analysis. Liver microsomes (5 mg protein) were electrophoresed (Mini-Protean II, Bio-Rad Laboratories, Richmond, CA, USA) through 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) slab gel[13]. Resolved proteins were transferred to nitrocellulose Trans-Blot membranes (Bio-Rad Laboratories). The blots were stained with Ponceau S (Sigma Chemical Co.) to confirm that the protein concentration was approximately the same in all lanes. Membrane filters were soaked in phosphate buffer solution, pH 7.5 (PBS), to remove the Ponceau S and incubated overnight in PBS containing 5% dried skimmed milk and 0.1% Tween-20 to block excess protein binding sites. The membranes were then incubated with goat polyclonal anti-rat CYP2E1 antibody (Gentest Co., Woburn, MA), detected with horseradish peroxidase-labeled rabbit anti-goat IgG and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Ltd, Buckinghamshire, UK), and visualized. The CYP2E1 content was quantified by densitometric scanning using NIH image software 1.61.

RNA extraction: Total RNA was isolated from 50 mg of liver using Trizol reagent (Life Technologies Inc, Grand Island, NY, USA). Briefly, liver tissue samples were homogenized in 1 ml of Trizol, and then 0.3 ml of chloroform was added to the sample. The mixtures were then shaken for 30 sec, followed by centrifugation at 4°C and 15,000 × g for 20 min. The supernatant layers were transferred to a new set of tubes, and an equal volume of isopropanol was added to the samples, shaken for 15 sec, and centrifuged at 4°C and 15,000 × g for 15 min. The RNA pellets were washed with 70% ethanol. RNA was dissolved in diethylpyrocarbonate-treated (DEPC) water. The integrity of the prepared RNA was checked by electrophoresis, and then the optic density (OD) was measured on a spectrophotometer. The OD of all RNA samples was 1.7 to 1.9 based on the 260/280 ratio.

Semi-quantitative RT-PCR: A mixture of 5 μg total RNA and 0.5 ng oligo dT primer in a total volume of 24 μl sterilized ultra-pure water was incubated at 70°C for 10 min, then removed from the thermal cycler and adjusted to 40 μl with a mixture of 8 μl (5X) RT-buffer, 2 μl 10 mM dNTP, 2 μl DEPC water, and 2 μl of reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan). It was incubated in the thermal cycler at 30°C for 10 min,

42°C for 1 hr, and 90°C for 10 min. For PCR, 1 μl aliquots of the synthesized cDNA were added to 20 μl of a mixture containing sterilized ultra-pure water, 2 μl of PCR buffer, 2 μl of dNTP (2.5 mM), 0.3 μl of sense and anti-sense primers (10 μM), and 0.1 μl of Taq polymerase (Takara Shuzo Co. Ltd., Shiga, Japan). Specific CYP2E1 primers were designed according to Gonzalez *et al.*[4]: 5'-GAAAAAGCCAAGGAACACC-3' (sense) and 5'-GCAGACAGGAGCAGAAACA-3' (antisense). For semi-quantitative RT-PCR assays, a co-amplification approach was used with glyceraldehyde-3-phosphate dehydrogenase (G3PDH) as the control gene. Rat liver and PCR conditions were the same as described above, except that 10 pmol of G3PDH primers (sense: 5'-TGAAGGTCGGT GTGAACGGATTTGGC-3', and antisense: 5'- CAT GTAGGCCATGAGGTCCACCAC-3') was used. Amplification was initiated by 1 cycle of denaturation at 95°C for 1 min followed by denaturation at 94°C for 1 min, and annealing at the proper temperature for 1 min, then extension at 72°C for 1 min for the proper number of cycles for each gene using a DNA thermal cycler (BioRad Laboratories). The samples were finally incubated for 7 min at 72°C after the last cycle of amplification. The amplified PCR products were separated by electrophoresis on 1–1.5% agarose gel. Bands of cDNA were stained with ethidium bromide and visualized by ultraviolet illumination. The CYP2E1 and G3PDH concentrations were densitometrically scanned using NIH image software 1.61. G3PDH mRNA levels were used for the correction of CYP2E1 mRNA expression as endogenous genes; the ratio between CYP2E1 and G3PDH was determined by densitometry.

Statistical analyses: The results are expressed as mean values ± SD. Multiple comparisons were performed using the Student's *t*-test followed by adjusting the p-values using the Bonferroni correction. $p < 0.05$ was considered statistically significant.

Results

Biochemistry

A single dose of CCl_4 (0.5 ml/kg i.p.) caused hepatotoxicity in Wistar rats, as indicated by increased ALT (Fig. 1A) and AST (Fig. 1B) serum levels compared to those of controls and those with PC extract pretreatment at each dose. Pretreatment with an aqueous extract of PC mushroom drastically prevented the CCl_4-induced elevation of ALT and AST serum levels in a dose-dependent manner (Fig. 1).

Histology

Fig. 2 shows the results of light microscopy analyses. Perfused hemorrhage was observed covering hepatocytes 24 hr post CCl_4 (0.5 ml/kg) injection, and fat globules of different sizes were observed all over the livers of CCl_4-treated group. Some leukocyte infiltration was also observed in the livers of CCl_4-treated rats. We summarized the CCl_4-injection results in Table 2, and photomicrographs are shown in Fig. 2. On the other hand,

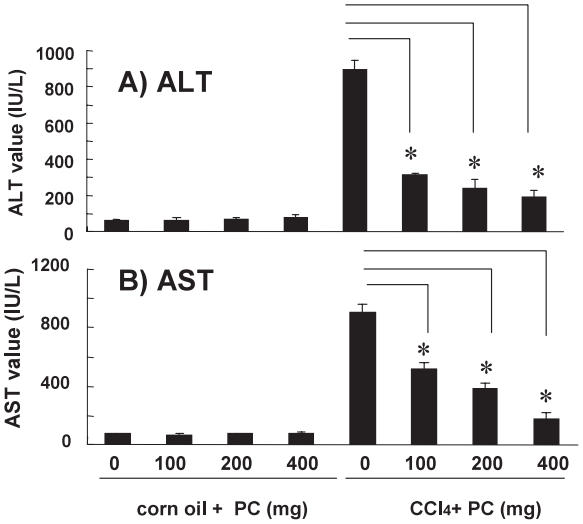

Fig. 1. Effect of PC extracts on serum ALT and AST after administration of CCl_4. ALT (A) and AST (B) levels after treatments with vehicle or CCl_4 with/without PC extract pretreatment were determined. The results are expressed as mean values ± SD. ALT and AST activities in all CCl_4-treated samples showed significant elevations compared to non-CCl_4-treated samples at the same PC dose (*$p < 0.05$).

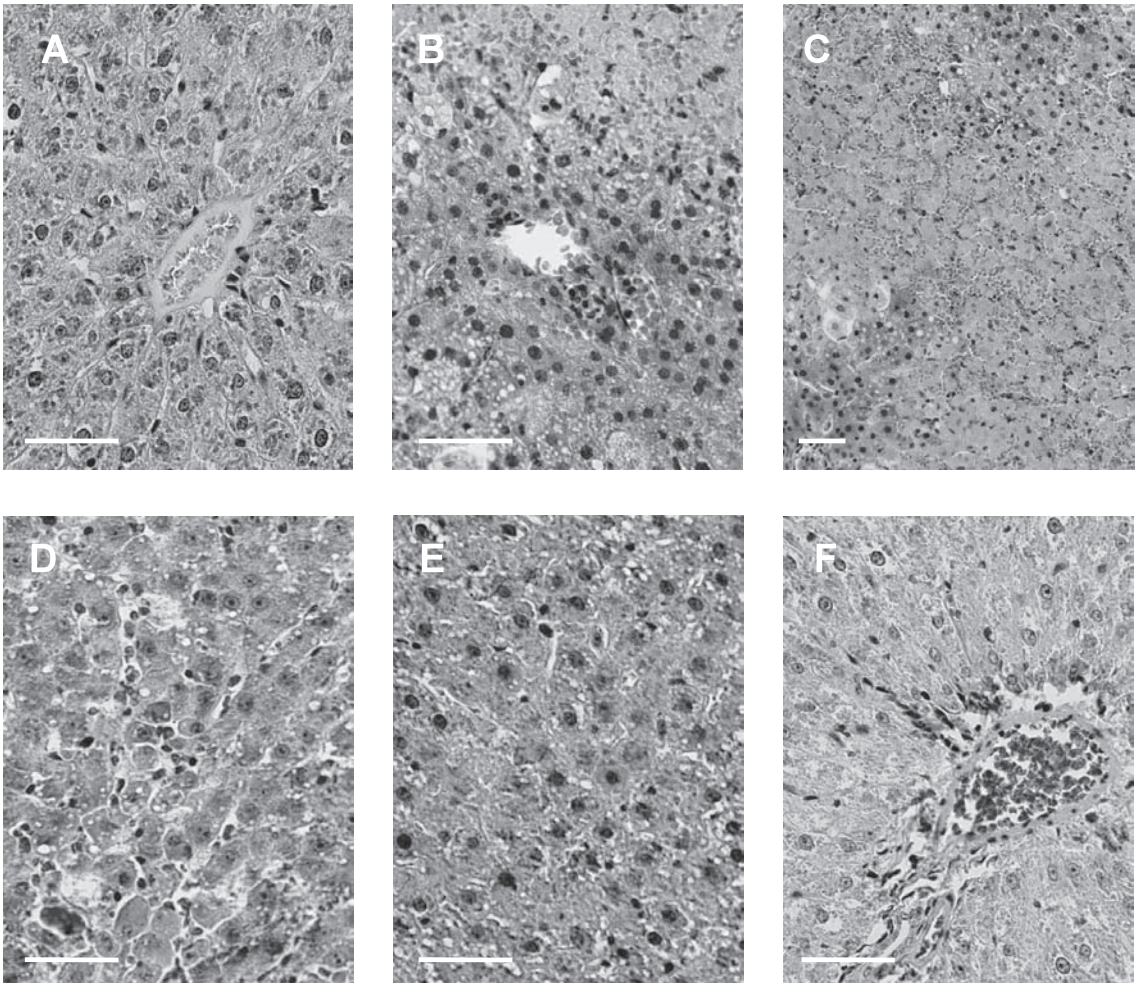

Fig. 2. Histological microscopic analyses after CCl_4 and PC extract treatments. Livers were histologically examined. A) Control; B and C) CCl_4-treated sample; D) treated with 100 mg/kg PC and CCl_4; E) treated with 200 mg/kg PC and CCl_4; F) treated with 400 mg/kg PC and CCl_4. Scale bar = 50 μm.

pretreatment of rats with aqueous extract of PC mushroom prior to injection of CCl_4 ameliorated the hepatic injury in a dose-dependent manner, leading to a lower percentage of fat droplets and absence of marked hemorrhage. Rats that received only aqueous extract of PC mushroom had apparently normal livers.

Electron microscopy

Fig. 3 shows the results of electron microscopy analyses. The cytoplasm of hepatocytes of CCl_4-treated rats showed many large lipid droplets, some fusing into larger ones, accumulated around the nucleus. Hepatocytes appeared dark colored with condensed lipid droplets as a step toward degenerative changes. Among the lipid droplets there are many mitochondria (massive in size compared to controls) with cross association with rough endoplasmic reticulum. The mitochondria have begun to change from spherical to elongated to increase the surface area as a method of cell defense. The cytoplasm was darkened with smaller nuclei. The percentage of fat globules was lower and proportional to the dose in groups pre-treated with aqueous PC mushroom extract prior to CCl_4 injection. In addition, the cytoplasmic and mitochondrial changes were not observed in PC extract-treated rats.

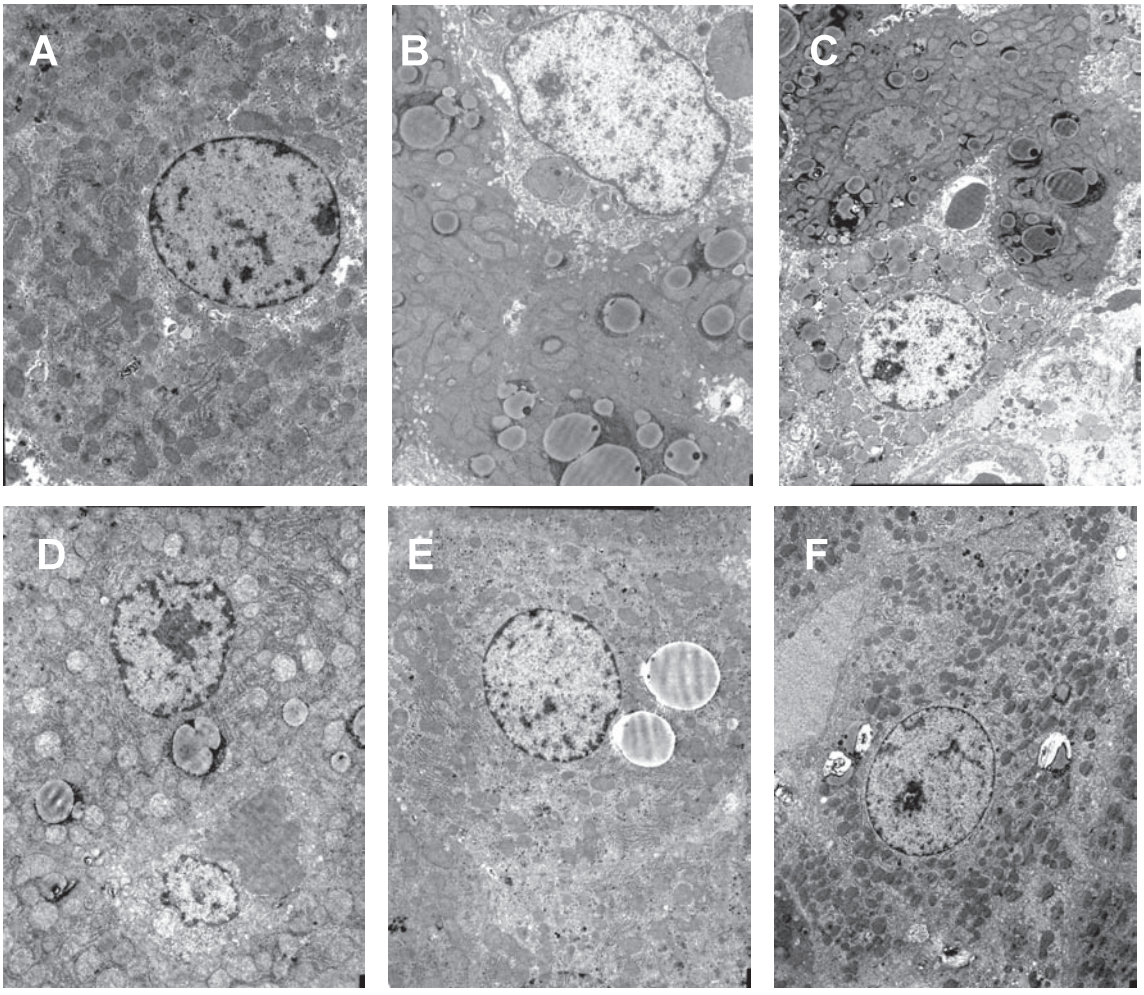

Fig. 3. Electron microscopic analyses after CCl_4 and PC extract treatments. Ultra-thin sections were photographed using a transmission electron microscope. A) Control (× 6,000); B and C) CCl_4-treated (B: × 6,000, C: × 4,000); D) treated with 100 mg/kg PC and CCl_4 (D: × 6,000); E) treated with 200 mg/kg PC and CCl_4 (× 6,000); F) treated with 400 mg/kg PC and CCl_4 (× 4,000).

CYP2E1 expression

It is known that CCl_4 requires CYP2E1-associated bioactivation to produce liver injury. Therefore, the inhibitory activities of aqueous extract of PC mushroom on hepatic microsomal CYP2E1-specific microsomal monooxygenase activities were confirmed by immunoblot analysis. The hepatic microsomes from control, CCl_4-treated, and PC-treated rats were resolved by SDS-PAGE and immunoblotted with anti-CYP2E1 antibody. A CYP2E1 protein immunoblot is shown in Fig. 4A. In CCl_4 non-treated rats, CYP2E1 expressions were suppressed by treatment with aqueous extract of PC mushrooms in a dose-dependent manner. In addition, aqueous extract of PC mushroom significantly reduced mRNA expression of CYP2E1 gene in a dose-dependent manner in comparison to control rats ($p < 0.05$, Fig. 4B). However, in the CCl_4 and PC co-treated rats, the expressions of CYP2E1 apoprotein and mRNA were lower than those of non-treated control rats, but higher than those of CCl_4-alone treated rats.

Discussion

The results of the present study demon-

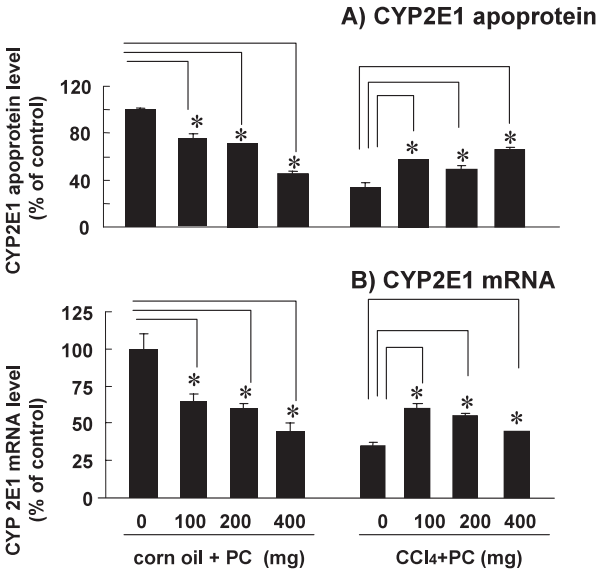

Fig. 4. Effects of PC mushroom extract and CCl_4 administration on expression levels of CYP2E1. CYP2E1 apoprotein and mRNA expressions were detected and estimated. A) CYP2E1 apoprotein expression levels. B) Semi-quantitative CYP2E1 mRNA expression in liver of CCl_4- and PC extract-treated animals. The values of CYP2E expression were corrected to G3PDH expression levels. The results are expressed as mean values ± SD. *$p < 0.05$ was considered statistically significant compared to control group.

strated that pretreatment of rats with aqueous extract of PC mushroom effectively protected them against CCl_4-induced hepatotoxicity, as evidenced by a dose-dependent decrease in serum enzymatic activities of alanine and aspartate aminotransferase (Fig. 1). Increased serum levels of aminotransferases have been attributed to damage to the structural integrity of the liver because these enzymes are cytoplasmic in location and released into the circulation after CCl_4-induced cellular damage, which is manifested histologically by fat vacuoles, massive hemorrhage, and increased numbers of mitochondria, which take an elongated shape to increase the surface area, with some degenerative changes in blood vessels (Table 2, Fig. 2 and 3).

The constituent(s) responsible for the hepatoprotection afforded by the edible PC mushroom have not been identified. Analysis of PC mushroom components revealed that it contained (1–3) β-D-glucans, ergosterol, mannitol, phenolic compounds, linoleic acid, peptides, and carbohydrates[5]. Recently, oleanolic acid, a triterpenoid saponin found in medicinal plants, was shown to be effective at inhibiting CCl_4-induced liver injury, and this protective effect is associated with inhibition of the CCl_4-induced biotransformation by the reduced CYP2E1 protein level[6].

CYP2E1 is involved in the biotransformation of several organic chemicals, including ethanol, acetone, pyridine, nitrosamines, CCl_4, and many others. The metabolic activation of CCl_4 is thought to be mediated through CYP2E1[15,16]. The regulation of CYP2E1 by some of these chemicals is highly complex and occurs at transcriptional, post-transcriptional, translational, and post-translational levels[4]. In the current study, the expressions of both mRNA and apoprotein CYP2E1 were reduced after treatment with the PC extract. The suppression of CYP2E1 may reduce the levels of reactive metabolites, and thus decreased tissue injury[3]. Actually, compounds that induce CYP2E1 potentiate the hepatic toxicity of CCl_4, and conversely, compounds that inhibit CYP2E1 protect against CCl_4-induced toxicity[7,8]. Our results partially support this hypothesis because a good correlation was found in the immunoblot results: PC extract treatment reduced the expression of CYP2E1 (Fig. 4) in a dose-dependent manner, and the decreased levels of CYP2E1 due to PC extract treatment were consistent with the biochemical and histological results. However, the co-treatments of CCl_4 and PC extract did not show the strong inhibition of CYP2E1 expressions compared to those of rats treated with CCl_4 alone, due to the drastic suppression and degradation of CYP2E1 in this group. The slight elevation of CYP2E1 expression in CCl_4 and PC extract co-treated group compared to that of CCl_4 treated animals suggested to be recovering process of cell injury[8].

Jayakumar *et al.*[5] reported that the antioxidative effects of PC extract against CCl_4 in kidney, heart and brain. In a previous study, we also reported the scavenging of reactive oxygen

species in hepatocytes by PC extract[2]. Further study is needed to elucidate exactly which part in the PC mushroom is responsible for the hepato-protection. However, in addition to the radical scavenging ability of PC components reported in previous studies, our current results suggest that the hepato-protection obtained by ingestion of aqueous extract of PC mushroom may be partially due to the blocking of bioactivation of CCl_4 by the inhibition of CYP2E1.

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