The Protective Effects of PMC against Chronic Carbon Tetrachloride-Induced Hepatotoxicity in Vivo

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Received June 14, 2001; accepted August 28, 2001

In this study, PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane), a derivative of \( \alpha \)-tocopherol, dose-dependently (1—10 mg/kg) ameliorated the increase in plasma aspartate aminotransferase (GOT) and alanine aminotransferase (GPT) levels caused by chronic repeated carbon tetrachloride (CCL\(_4\)) intoxication in mice. Moreover, PMC significantly improved the CCL\(_4\)-induced increase of hepatic glutathione peroxidase, reductase, and superoxide dismutase activities. PMC also restored the decrement in the glutathione content of hepatic tissues in CCL\(_4\)-intoxicated mice. Furthermore, it also dose-dependently inhibited the formation of lipid peroxidative products during carbon tetrachloride treatment. Histopathological changes of hepatic lesions induced by carbon tetrachloride were significantly improved by treatment with PMC in a dose-dependent manner. These results suggest that PMC exerts effective protection in chronic chemical-induced hepatic injury in vivo.

Key words 2,2,5,7,8-pentamethyl-6-hydroxychromane; antioxidant; carbon tetrachloride; hepatotoxicity

Many studies have shown that reactive oxygen species including oxygen free radicals are causative factors in the etiology of degenerative diseases, including some hepatopathies.\(^1\) Carbon tetrachloride (CCL\(_4\)) is most frequently used as a chemical inducer of experimental liver cirrhosis.\(^2\) It has been suggested that hepatic necrosis caused by carbon tetrachloride involves bioactivation by the microsomal cytochrome P450-dependent monoxygenase system, resulting in the formation of trichloromethyl free radical and reactive oxygen species that initiate lipid peroxidation and protein oxidation.\(^3\) Free radicals in both \( \textit{in vitro} \) and \( \textit{in vivo} \) models have also been shown to modify and damage proteins, carbohydrates, and DNA.\(^4\) Therefore, under such disseminated oxidative stress, biomembranes and bioactive molecules are disturbed or inactivated. Furthermore, hepatic microsomes, mitochondria, and the nuclei of hepatocytes are also impaired by lipid peroxide, with hepatocytes ultimately being destroyed.\(^5\)

According to \( \textit{in vitro} \) and \( \textit{in vivo} \) studies, several classical antioxidants have been shown to protect hepatocytes against lipid peroxidation or inflammation, therefore preventing the occurrence of hepatic necrosis.\(^6,7\) \( \alpha \)-Tocopherol is well known for its antioxidant properties in biomembranes where it acts to prevent lipid peroxidation.\(^8\) It also protects against carbon tetrachloride-induced hepatotoxicity.\(^9,10\) The \( \alpha \)-tocopherol analogue, PMC (2,2,5,7,8-pentamethyl-6-hydroxychromane), in which the phytyl chain is replaced by a methyl group, is more hydrophilic than other \( \alpha \)-tocopherol derivatives, and has potent radical scavenging activity and potent inhibition of nuclear factor-\( \kappa \)B activity.\(^11,12\) The antiperoxidative potency of PMC was approximately 18-times than that of \( \alpha \)-tocopherol.\(^12\) Recently, we reported that PMC is a potentially effective antioxidant and antiplatelet agent through the inhibition of cyclooxygenase.\(^13\) Therefore, we suggested that PMC may be effective against diseases in which reactive oxygen species play a role as potent causative factors.

In the present study, we examined and compared the relative inhibitory activities of PMC with silymarin in chronic carbon tetrachloride-induced liver injury in mice. We also evaluated the role of oxidative stress in this model of liver injury.

MATERIALS AND METHODS

Materials PMC was obtained from Wako Pure Chemical Ind. (Osaka, Japan). Carbon tetrachloride, silymarin, hydrogen peroxide, sodium azide, 5,5'-dithiobis (2-nitro benzoic acid) (DTNB), 2-thiobarbituric acid and other reagents in the study were obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Diagnostic kits for assaying alanine aminotransferase (GPT) and aspartate aminotransferase (GOT) were also purchased from Sigma.

Animals Male ICR mice, 5 weeks old, weighing 20—25 g used in this study were obtained from the Laboratory Animal Center of National Taiwan University. All the animal experiments and care were performed according to the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, D.C., 1996). The animals were maintained under a 12-h light/dark cycle in a temperature- and humidity-controlled room. Mice were fed with a laboratory pellet chow and given water \textit{ad libitum}.

Chronic CCL\(_4\)-Induced Liver Injury in Vivo Mice were divided into six groups: (1) normal control, (2) vehicle (corn oil) plus CCL\(_4\) treatment, (3) PMC (1 mg/kg) plus CCL\(_4\) treatment, (4) PMC (5 mg/kg) plus CCL\(_4\) treatment, (5) PMC (10 mg/kg) plus CCL\(_4\) treatment and (6) silymarin (50 mg/kg) plus CCL\(_4\) treatment. Mice were treated with sublethal doses of carbon tetrachloride (40% CCL\(_4\)/olive oil, 0.1 ml/10 g body weight per day, s.c. twice per week) for 8 weeks to induce chronic hepatic liver injury. Groups of mice were treated with PMC (1, 5, 10 mg/kg per day, respectively, p.o., 4 d per week) or vehicle solution (corn oil) for 8 weeks. Control mice were treated with normal saline for 8 weeks. Blood samples at 200 \( \mu \)l with heparin (10 U/ml) were collected from the tail vein at the end of the 1st, 3rd, 6th, and 8th
weeks. Blood and livers were immediately obtained after the animals were sacrificed. Livers were weighed and utilized for the following biological analysis. Liver homogenates (10%, w/v) were obtained in 50 mM phosphate buffer (pH 7.0) and stored at −80°C within 2 weeks before experiments of analyses.

**Determination of Plasma Transaminase Activities**

GPT and GOT activities in the plasma were measured by using GPT and GOT EIA kits (Sigma) according to the manufacturer’s instructions.

**Glutathione Reductase Assay**

Liver glutathione reductase (GSH Rd) was assayed by a reaction mixture containing 0.99 ml of 100 mM potassium phosphate buffer (pH 7.0), 1.1 mM MgCl₂, 5 mM oxidized glutathione (GSSG), and 0.1 mM NADPH. Ten microliters of liver homogenate was added to trigger the NADPH conversion reaction. Changes in absorbance were monitored by a continuous-recording spectrophotometer (V-530, Jasco) at 340 nm for 5 min at 25°C. The specific enzyme activity of GSH Rd was expressed as nmol NADPH/min per mg protein.

**Glutathione Peroxidase Assay**

Glutathione peroxidase (GSH Px) activity was expressed as nanomoles of NADPH oxidized to NADP per minute per milligram of protein, with a molar extinction coefficient for NADPH at 340 nm of 6.22×10⁴ (Paglia and Valentine, 1967). Liver GSH Px was assayed in a 1-ml crystal cuvette containing 0.8 ml of 100 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM NaN₃, 0.2 mM NADPH, 1 U/ml GSH reductase, and 1 mM GSH. Five microliters of the liver homogenate and buffer was added to make a total volume of 0.9 ml. The reaction was started by the addition of 100 μl of 2.5 mM H₂O₂, and the conversion of NADPH to NADP was monitored by a continuous-recording spectrophotometer at 340 nm for 3 min. The specific enzyme activity of GSH Px was expressed as nmol protein.

**Determination of Superoxide Dismutase Activity**

According to the method of Geller and Winge, a part of the liver tissue was homogenized (10%, w/v) in a solution (0.32 mM sucrose, 1 mM EDTA, and 10 mM Tris–HCl, pH 7.4) using a polytron homogenizer, after which it was centrifuged at 10000 rpm in a centrifuge (Beckman, Microfuge) at 4°C for 30 min. Supernatant was assayed for superoxide dismutase (SOD) activity by following the inhibition of nitroblue tetrazolium (NBT) reduction. SOD was assayed by a reaction mixture containing 985 μl of 100 mM phosphate buffer (pH 7.4), 0.3 mM K₂H₂EDTA, 0.5 mM NBT, and 0.1 mM xanthine. The mixture was preincubated for 3 min at 25°C, and 10 μl of 0.02 U/ml xanthine oxidase was added to generate superoxide and to induce NBT reduction. Changes in absorbance at 560 nm were recorded at 20-s intervals for 5 min. SOD activity was determined from a standard curve of percentage inhibition of NBT reduction with standard SOD activity. Data were expressed as SOD units/mg protein as compared with the standard.

**Catalase Assays**

Liver homogenates (10%, w/v) were centrifuged (10000 rpm) at 4°C for 10 min. Supernatant (5 μl) was added to a crystal cuvette containing 0.995 ml of 30 mM H₂O₂ solution prepared in potassium phosphate buffer. Changes in absorbance were read at 240 nm for 1 min. Commercially available catalase was used as a standard. Using the reaction time interval (Δt) of absorbance (A₁ and A₂), the following equation was generated to calculate the rate constant (K): K=(2.3/Δt) log(A₁/A₂). The specific activity of the enzyme was expressed as K/mg protein.

**Determination of Glutathione Content**

GSH was determined by titration with DTNB as described previously. Proteins of 0.4-ml liver homogenates were precipitated by the addition of 0.4 ml of a metaphosphoric acid solution (1.67 g metaphosphoric acid, 0.20 g EDTA, and 30.0 g NaCl in 100 ml H₂O). After 40 min, the protein precipitate was separated from the remaining solution by centrifugation at 5000 rpm in a microcentrifuge (Beckman, Microfuge) at 4°C for 5 min. Forty hundred microliters of the supernatant was combined with 0.4 ml of 300 mM Na₂HPO₄, and the absorbance at 412 nm was read against a blank consisting of 0.4 ml supernatant plus 0.4 ml H₂O. Then, 100 μl DTNB (0.02%, w/v; 20 mg DTNB in 100 ml of 1% sodium citrate) was added to the blank and sample, and absorbance of the sample was read against the blank at 412 nm. The glutathione content was determined using a calibration curve prepared with an authentic sample. An aliquot of lyase was used for determination of the protein content. GSH values were expressed as μmol/mg protein.

**Measurement of Malonaldehyde**

The content of malonaldehyde (MDA) formation in liver homogenate was determined using the thiobituric acid (TBA) method with some modification. Briefly, 250 μl of liver homogenate (10%, w/v) was first mixed with the same volume of 50 mM potassium phosphate buffer. Then 20 μl of iced-cold trichloroacetic acid (TCA) solution (4% [w/v] in 0.3 M HCl) and 200 μl of TBA-reactive substance reagent (0.5% [w/v] TBA in 50% [v/v] acetic acid) were also added. Tube contents were vortexed briefly. After boiling for 15 min, samples were cooled, extracted with n-1-butanol, and centrifuged by microcentrifuge for 10 min at 10000 rpm. The butanol layer containing the thiobarbituric acid-reactive substances (TBARS) was read at 532 nm. The results were expressed as absorbance (at 532 nm)/mg protein.

**Determination of Protein Contents**

Protein contents of liver homogenates were determined by the Bio-Rad method using bovine serum albumin as a standard.

**Liver Histology**

Mice were sacrificed at the end of the 8th week, and livers were freshly and quickly obtained. Liver slices were made from a part of the left and central lobes, and immediately fixed in 10% buffered formalin phosphate solution, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The reticulin silver stain and Masson trichrome stain were also performed.

**Statistical Analysis**

The experimental results were expressed as the mean±S.E.M. and are accompanied by the number of observations. Data were assessed by the method of analysis of variance (ANOVA). If this analysis indicated significant differences among the group means, then each group was compared by the Newman-Keuls method. A p value of <0.05 was considered statistically significant.

**RESULTS**

**Serum Transaminases**

Blood was collected at indicated time points for GPT and GOT determinations after CCl₄ administration. Both plasma GPT and GOT levels had markedly increased to their maximum values (U/l) at
At the end of the third week, and then decreased to 410±82 and 508±109 by the eighth week (Figs. 1A, B). In the normal group, serum GPT levels were 67±9 and 37±5, and GOT levels were 195±24 and 100±36 at the end of the third and eighth weeks, respectively. On the other hand, PMC treatment (1, 5, 10 mg/kg) produced dose-dependent reductions of GPT and GOT levels at indicated time points. PMC (10 mg/kg) markedly reduced the activities of GPT and GOT by about 84 and 87% at the eighth week for continuous treatment with CCl₄ as compared with the vehicle group (Fig. 1). Silymarin (50 mg/kg) also significantly decreased the GPT and GOT levels to about 69 and 93% as compared with the vehicle groups (Fig. 1).

**Glutathione Peroxidase and Reductase Activities**

Figure 2 shows the changes of hepatic glutathione peroxidase and reductase activities at 8 weeks in CCl₄-intoxicated mice. Activities of GSH peroxidase and reductase had increased about 1.6- and 3.4-fold, respectively, at 8 weeks after CCl₄ administration as compared with the control group. On the other hand, PMC reduced both enzyme activities in a dose-dependent manner (1, 5, 10 mg/kg). However, PMC (5 mg/kg) slightly inhibited the elevated GSH peroxidase activity induced by CCl₄, but it did not reach statistical significance. PMC at a higher dose (10 mg/kg) markedly attenuated the activity of GSH peroxidase (Fig. 2A). On the other hand, PMC (1—10 mg/kg) also markedly inhibited the CCl₄-induced increase in GSH reductase activity. This enzyme activity was completely restored to the normal level by treatment with PMC at 5 mg/kg (Fig. 2B). As a positive hepatoprotective control, silymarin (50 mg/kg) also significantly restored both GSH-related enzyme activities to the normal level under the same conditions (Fig. 2).

**Superoxide Dismutase Activity in Liver Tissues**

Figure 3 shows the SOD activity of liver tissues. SOD activity in liver tissues was increased about 1.6- and 3.4-fold, respectively, at 8 weeks after CCl₄ administration as compared with the control group. On the other hand, PMC (1, 5, 10 mg/kg) markedly reduced the SOD activity by about 84 and 87% at the eighth week for continuous treatment with CCl₄ as compared with the vehicle group (Fig. 3). Silymarin (50 mg/kg) also significantly decreased the SOD activity to about 69 and 93% as compared with the vehicle groups (Fig. 3).
CCL₄-intoxicated mice compared with the control group liver tissues was reduced by approximately a half at 8 weeks in CCL₄-intoxicated mice at the end of the eighth week. Catalase activities were determined as described in Materials and Methods. Results are presented as the mean±S.E.M. (n=5). *p<0.01 compared with the normal control (without CCL₄ treatment). #p<0.01 compared with the vehicle group (corn oil).

Fig. 5. Effects of PMC on Hepatic Glutathione Levels in CCL₄-Intoxicated Mice
CCL₄-intoxicated mice were treated with vehicle solution (corn oil), various doses of PMC (1, 5, 10 mg/kg), and silymarin (50 mg/ml) for 8 weeks. Homogenates of liver tissues were obtained from CCL₄-intoxicated mice at the end of the eighth week. Glutathione contents were determined by the DTNB conjugation method as described in Materials and Methods. Results are presented as the mean±S.E.M. (n=5). *p<0.05 compared with the normal control (without CCL₄ treatment). #p<0.05 compared with the vehicle group (corn oil).

Glutathione Content in Liver Tissues Figure 5 shows the changes of glutathione levels of liver tissues in CCL₄-intoxicated mice. Hepatic glutathione levels (nmol/mg protein) significantly decreased from 47.2±4.0 of the control group to 23.6±4.8 in CCL₄-intoxicated mice at 8 weeks. PMC at doses of 5 and 10 mg/kg significantly increased the glutathione level of hepatic tissues. In addition, PMC and silymarin both almost completely restored the enzymatic activity to the normal level at the respective doses of 10 and 50 mg/kg (Fig. 4).

In Vivo Lipid Peroxidation The hepatotoxicity of carbon tetrachloride is thought to be due to lipid peroxidation. TBARS increased about 2.5-fold at 8 weeks in CCL₄-intoxicated mice compared with the control group (Fig. 6). PMC (1, 5, 10 mg/kg) dose-dependently reduced the formation of TBARS in liver tissues in CCL₄-intoxicated mice (Fig. 6). TBARS levels of liver tissues were almost completely restored to normal levels (0.41±0.04 vs. 0.30±0.03) at a dose of 10 mg/kg of PMC in CCL₄-intoxicated mice (Fig. 6). Similarly, silymarin (50 mg/kg) markedly reduced TBARS formation, which approximately returned to the normal level at 8 weeks in CCL₄-intoxicated mice (Fig. 6).

Hepatopathology Histopathological changes of necrotic, ballooning, and lipid-laden hepatocytes of liver sections were assessed at 8 weeks after CCL₄ administration (Fig. 7). Typical intense centrilobular necrosis of hepatocytotoxicity was observed at 8 weeks in CCL₄-intoxicated mice (Fig. 7B), at which time there was also a drastic increase in plasma transaminase activities (Fig. 1). Marked macro- and microvesicular fatty changes of hepatocytes around the central vein and parenchymal disarrangement were found. Ballooning degeneration and infiltration of inflammatory cells (such as hemosiderin-laden macrophages) were also found (Fig. 7B), as compared to normal liver sections (Fig. 7A). Moreover, bridging necrosis (mainly C–C, focal C–P) with frame collapse was also observed by reticulum staining (data not shown). Interestingly, the hepatohistological changes induced...
by CCl₄ were markedly improved by treatment with PMC in a dose-dependent manner (Figs. 7C, D, E). PMC administration (10 mg/kg) markedly decreased macro- and microvesicular changes, and they were rarely found in CCl₄-intoxicated mice treated with PMC. Lipid-laden cells or lipid droplets were also rarely observed. In addition, ballooning-degenerated hepatocytes or inflammatory cells were markedly reduced (Fig. 7E). Furthermore, silymarin (50 mg/kg) also markedly reduced the alterations of necrotic, ballooned, and lipid-laden cells (Fig. 7F).

**DISCUSSION**

It is well known that free radicals derived from oxygen and other chemicals are an important factor related to injury of the liver. Another mechanism of cellular injury is local action of cytotoxic cytokines such as TNFα. The expression of inflammatory cytokines such as TNFα occurs through NF-κB activation during the actual period of hepatocellular injury in acute CCl₄-mediated liver damage. An early mediator of hepatocyte injury and activation of Kupffer cells is probably ROS. Thus, cells then manifest increased

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**Fig. 7. Effects of PMC on Hepatic Morphological Analysis in CCl₄-Intoxicated Mice**

CCl₄-intoxicated mice were treated with vehicle solution (corn oil), various doses of PMC (1, 5, 10 mg/kg), and silymarin (50 mg/ml) for 8 weeks. Livers were sectioned and stained with hematoxylin-eosin by standard techniques. Figure (A) is from a representative mouse with no treatment; Fig. (B) is from a representative mouse treated with CCl₄ alone, the hepatocytes were shown either with necrotic cells (thick arrow), abundant microvesicular fat (thin arrow) or macrovesicular fat (droplet, double arrows); Figs. (C), (D), (E) and (F) are from representative mice treated with 1, 5, and 10 mg/kg of PMC or silymarin (50 mg/kg) plus CCl₄ treatment, respectively.
NF-κB activation and produce a number of other factors that have been implicated in the induction of hepatocyte necrosis, including proteolysis enzymes, ROS, and several cytokines (e.g., IL-1, IL-2, and TNFα). It was reported that NF-κB activity is increased by metabolites of CCl₄, the agent that enhances hepatic cell injury and necrosis. Therefore, reduction of NF-κB activity may decrease cell necrosis, perhaps through affecting cytokine expression. Moreover, it was reported that antioxidants may protect against CCl₄-induced hepatotoxicity through anti-peroxidation, NF-κB inactivation, or induction of defense enzyme expression.

In this study, the activities of GOT and GPT were rapidly elevated at 3—4 weeks after CCl₄ administration, and then gradually decreased at 6—8 weeks. However, at the end of 8 weeks, hepatic necrosis seemed to be attenuated in CCl₄-intoxicated mice. This phenomenon may be explained by redox adaptation or autoprotection. The detailed mechanisms of this adaptation or autoprotection may occur through induction of genes of some defense enzyme systems or by CYP2E1 inactivation. However, the detailed mechanisms are not fully understood and remain to be further resolved. In this study, we found that PMC markedly prevented hepatic necrosis in a dose-dependent manner. PMC (10 mg/kg) exerts more potent hepatoprotective activity than silymarin (50 mg/kg). The hepatoprotective activity of PMC was also supported by histological examinations.

Some endogenous protective factors such as glutathione peroxidase, catalase, and superoxide dismutase are activated in the defense against oxidative cell injury by means of their being free-radical scavengers. The most important hepatic enzymes for the detoxication of lipid peroxide or ROS are glutathione peroxidase, glutathione reductase, and glutathione. Under oxidative stress, glutathione is largely consumed by the glutathione-related enzymes, thereby resulting in induction of some intoxication. In the present study, chronic sublethal doses of CCl₄ enhanced the activities of glutathione-related enzymes, and decreased the glutathione content, whereas PMC markedly reversed these effects. It is conceivable that the effect of PMC may be due initially to a reduction in hepatic peroxidative activities followed by inhibition of the activities of glutathione-related enzymes, thereby leading to restoration of the glutathione content in CCl₄-induced hepatotoxicity.

Superoxide dismutase and catalase are the major enzymes which catalyze ROS in most cells. Both enzymes play an important role in the elimination of ROS derived from the redox process of xenobiotics in liver tissues. It was suggested that catalase and SOD are easily inactivated by lipid peroxides or ROS. In this study, catalase and SOD were appreciably elevated by administration of PMC, suggesting that it can restore SOD enzymes and/or activate enzyme activity in CCl₄-damaged liver tissue. This result is consistent with a previous report which showed a significant restoration of SOD activity by treatment with an antioxidant in CCl₄-intoxicated animals.

In the present study, PMC, a derivative of α-tocopherol, was highly effective at prevention of CCl₄-induced hepatotoxicity. Its protective effects against various aspects of CCl₄-induced hepatotoxicity are attributed to its ability to scavenge free radicals involved in CCl₄-mediated lipid peroxidation. It was reported that inhibition of cytochrome P450 (CYP2E1) can also account for some hepatoprotectant actions. However, determining whether PMC is a cytochrome P450 inhibitor still requires further investigation. It is conceivable that PMC exerts its hepatoprotective activity by, at least partly, scavenging the free radical formation or by inhibition of both NF-κB activation and inflammatory eicosanoid formation. The inhibitory effects of dietary PMC may be able to prevent hepatic injury caused by CCl₄. Therefore, PMC may be useful as a hepatoprotective agent against chronic chemical-induced hepatotoxicity in vivo.

Acknowledgements This work was financially supported by a research grant of the National Science Council of Taiwan (NSC 89-2320-B-038-058).

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