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Research section

Protective effect of *Platycodi radix* on carbon tetrachloride-induced hepatotoxicity

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Abstract

The protective effects of a *Platycodi radix* (Changkil: CK), the root of *Platycodon grandiflorum* A. DC (*Campanulaceae*) on carbon tetrachloride ($CC1_4$)-induced hepatotoxicity and the possible mechanisms involved in this protection were investigated in mice. Pretreatment with CK prior to the administration of $CC1_4$ significantly prevented the increased serum enzymatic activities of alanine and aspartate aminotransferase in a dose-dependent manner. In addition, pretreatment with CK also significantly prevented the elevation of hepatic malondialdehyde formation and the depletion of reduced glutathione content in the liver of $CC1_4$-intoxicated mice. However, hepatic reduced glutathione levels and glutathione *S*-transferase activities were not affected by treatment with CK alone. $CC1_4$-induced hepatotoxicity was also essentially prevented, as indicated by a liver histopathologic study. The effects of CK on the cytochrome P450 (P450) 2E1, the major isozyme involved in $CC1_4$ bioactivation were also investigated. Treatment of mice with CK resulted in a significant decrease of P450 2E1-dependent *p*-nitrophenol and aniline hydroxylation in a dose-dependent manner. CK showed antioxidant effects in $FeCl_2$–ascorbate-induced lipid peroxidation in mice liver homogenate and in superoxide radical scavenging activity. Our results suggest that the protective effects of CK against $CC1_4$-induced hepatotoxicity possibly involve mechanisms related to its ability to block P450-mediated $CC1_4$ bioactivation and free radical scavenging effects.

Keywords: *Platycodi radix*; Protective effects; Carbon tetrachloride; Hepatotoxicity

1. Introduction

Carbon tetrachloride ($CC1_4$), a well-known model compound for the production of chemical hepatic injury, requires biotransformation by hepatic microsomal cytochrome P450 (P450) to produce the hepatotoxic metabolite, trichloromethyl free radicals ($CC1_3$ and/or $CC1_300^-$) (Castro et al., 1974; Brattin et al., 1985). Trichloromethyl free radicals can react with sulfhydryl groups, such as glutathione (GSH) and protein thiols. The covalent binding of trichloromethyl free radicals to cell protein is considered the initial step in a chain of events, which eventually leads to membrane lipid peroxidation and finally cell necrosis (Brattin et al., 1985; Recknagel et al., 1989, 1991; Williams and Burt, 1990). Although several isoforms of P450 may metabolize $CC1_4$, attention has been focused largely on the P450 2E1 isoform, which is ethanol inducible (Koop, 1992; Raucy et al., 1993; Zangar et al., 2000). Alternations in the activity of P450 2E1 affect the susceptibility to hepatic injury from $CC1_4$ (Kim et al., 1997; Jeong and Park, 1998; Jeong, 1999). P450 2E1 is also active in the metabolism of small organic molecules including acetaminophen, aliphatic organic alcohols, nitrosamines, benzene, phenol, 4-nitrophenol and pyrazole (Guengerich et al., 1991; Koop, 1992; Lee et al., 1996). The reactive intermediates formed during the metabolism of therapeutic agents, toxicants and carcinogens by this enzyme are frequently capable of binding covalently to tissue macromolecules, which may in turn cause tissue damage (Guengerich et al., 1991; Eaton et al., 1995). Natural compounds that reduce chemical activating enzymes could be considered as good candidates for

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CK, Changkil; GSH, glutathione; MDA, malondialdehyde.

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protection against chemically induced toxicities. P450 2E1 is well recognized for its role in the activation of many chemicals to toxic and carcinogenic agents (Guengerich et al., 1991; Koop, 1992; Eaton et al., 1995; Jeong and Park, 1998; Jeong, 1999).

Herbs have recently become attractive as health-beneficial foods (physiologically functional foods) and as a source material for the development of drugs. Herbal medicines derived from plant extracts are being utilized increasingly to treat a wide variety of clinical diseases, with relatively little knowledge regarding their modes of action.

Platycodi radix, the root of *Platycodon grandiflorum* A. DC (*Campanulaceae*), commonly known as Doraji (Chinese drug, 'Jiegeng', and Japanese name, 'Kikyo') has been used as a traditional oriental medicine; moreover, the biological significance of *P. grandiflorum* has been reviewed (Lee, 1973). Extracts from the roots of *P. grandiflorum* have been reported to have wide-ranging health benefits. In Korea, the root of *P. grandiflorum* (4 years old) is used as a food and employed as a folk remedy for adult diseases such as, bronchitis, asthma and pulmonary tuberculosis, hyperlipidemia, diabetes and inflammatory diseases, and as a sedative (Takagi and Lee, 1972; Lee, 1973). Several studies on its chemical (Lee, 1973; Tada et al., 1975; Saeki et al., 1999) and immunopharmacological effects have been performed (Nagao et al., 1986), and recently it was observed that the root of *P. grandiflorum* (22 years old) help prevent hypercholesterolemia and hyperlipidemia (Kim et al., 1995). In previous studies, we reported that Changkil (CK), the aqueous extract from the root of *P. grandiflorum* cultivated for more than 20 years (Lee, S.H., 1991, Patent on the method of cultivating the perennial balloonflower, Patent No. 045971, Korea), enhanced some of the functions of macrophages, including, proliferation, spreading ability, phagocytosis, cytostatic activity and NO secretion, and induced the gene expression of TNFα, IL-1β and IL-6 (Choi et al., 2001a,b). However, scientific studies on its usefulness with respect to liver caused damage by hepatotoxicants are lacking.

The present study was undertaken to evaluate the protective effects of CK on $CC1_4$-induced hepatotoxicity and to elucidate the mechanism(s) underlying these protective effects in mice. The parameters analyzed included, serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, hepatic GSH content, lipid peroxidation, and the histopathology of liver damage. The antioxidant effects (vs $FeCl_2$–ascorbic acid induced lipid peroxidation in liver homogenate and its superoxide scavenging activity) and its inhibition of P450 2E1 activities were also investigated. Our results indicated that CK pretreatment significantly protected against $CC1_4$-induced hepatotoxicity.

2. Materials and methods

2.1. Materials

The CK is the aqueous extract from the roots of *P. grandiflorum* (22 years old), which was supplied by Jang Saeng Doraji Co. (Jinju, South Korea). The CK was prepared as follows: distilled water at 90 °C was added to powered root (5 ml/g) and the temperature maintained for 10 h. The mixture was allowed to cool to room temperature, filtered, and. The yield of freeze-dried residue corresponded to 33.5% of the original dry root weight. The pale-yellow extract powder was dissolved directly in distilled water. The composition of the root of *P. grandiflorum* has been previously published (Kim et al., 1995).

$CC1_4$, olive oil, diagnostic kits for serum ALT and AST, thiobarbituric acid, dithionitrobenzoic acid, L(−)ascorbic acid, xanthine, xanthine oxidase, phenylmethoxysulfonyl fluoride, reduced GSH, ferrous sulfate and hydrogen peroxides were obtained from Sigma Chemical Co. (St Louis, MO, USA). All other chemicals and solvents were of the highest grade commercially available.

2.2. Animals and treatment

Male ICR mice (25–30 g) were obtained from KFDA (Seoul, Korea). The animals were allowed free access to Purina Rodent Chow and tap water, maintained in a controlled environment at 21±2°C and 50±5% relatively humidity with a 12-h dark/light cycle, and acclimatized for at least l week before use. CK in saline was administered intragastrically at 10–100 mg/kg once daily for 3 consecutive days. 3 h after the final treatment, mice were treated with $CC1_4$ (20 mg/kg, intraperitoneally, dissolved in olive oil). Groups of control animals were given the respective vehicles. 24 h after the administration of $CC1_4$, mice were anesthetized with CO_2, blood was removed by cardiac puncture to determine the serum ALT and AST activities, and the animals were decapitated. After bleeding, livers were weighed and a thin slice preserved in a buffered formalin solution for obtaining histological sections. The remaining livers were frozen quickly in dry ice/methanol and stored at −70°C for GSH content and lipid peroxidation analysis.

2.3. Hepatotoxicity studies

Hepatotoxicities were assessed by quantifying the serum activities of ALT and AST, and hepatic lipid peroxidation. Serum ALT and AST activities were measured with a spectrophotometric diagnostic kit obtained from the Sigma Chemical Co. Hepatic lipid peroxidation was measured by the formation of the

thiobarbituric acid-reactive material, malondialdehyde (MDA) (Fairhurst et al., 1982).

2.4. Hepatic GSH determination

Mice were killed by cervical dislocation. Livers were excised quickly, washed in ice-cold EDTA solution (0.02 M), blotted dry, dissected to remove connective tissues and weighed. Non-protein liver GSH was estimated by colorimetric method using Ellman's reagent according to Sedlak and Lindsay (1968).

2.5. Histological examinations

Fresh liver tissues, previously trimmed to approximately 2 mm thickness, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Fixed tissues were processed routinely, and then embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The extent of $CC1_4$-induced necrosis was evaluated by assessing morphological changes in liver sections stained with hematoxylin and eosin (H&E), using standard techniques.

2.6. Microsome and cytosol isolation

Mice were treated with CK or vehicle once daily for 3 consecutive days. 24 h after the last treatment, animals were sacrificed by cervical dislocation. The livers were quickly removed, weighed and perfused with ice-cold 0.15 M KCl, and then homogenized with 4 vol (w/v) of 10 mM Tris–HCl (pH. 7.4) containing 0.15 M KCl, 0.1 mM EDTA, 1.0 mM dithiothreitol and 0.01 mM phenylmethoxysulfonyl fluoride in a Potter-Elvehjem homogenizer. Hepatic microsomal and cytosolic fractions were prepared by differential centrifugation, as described previously (Jeong and Yun, 1995). All preparations were stored at −70 °C until use. The cytosolic fractions were used to assay glutathione *S*-transferase activity. The microsomal fractions were used for P450 2E1-specific oxidative activities.

2.7. Cytosolic glutathione S-transferase assay

Cytosolic glutathione *S*-transferase activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate (Habig et al., 1974).

2.8. p-Nitrophenol and aniline hydroxylase assay

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was determined spectrophotometrically as described previously (Dicker et al., 1990). Aniline hydroxylase activity was determined by measuring *p*-aminophenol formation (Dicker et al., 1990). Microsomal protein was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

2.9. $FeC1_2$–ascorbic acid stimulated lipid peroxidation in liver homogenate

The young male ICR mice, weighing 20–25 g, were killed by decapitation and their liver tissues were quickly removed. A 2-g portion of liver tissue was sliced and then homogenized with 10 ml of 150 mM KCl–Tris–HCl buffer (pH 7.2). The protein content was determined by the method of Bradford (1976). The reaction mixture was composed of 0.25 ml liver homogenate, 0.1 ml Tris–HCI buffer (pH 7.2), 0.05 ml 0.1 mM ascorbic acid, 0.05 ml 4 mM $FeC1_2$ and 0.05 ml of various concentrations of CK. Products of lipid peroxidation were measured by the formation of the thiobarbituric acid-reactive material, MDA (Fairhurst et al., 1982). 1,1,3,3_Tetraethoxypropan was used as standard for calibration of MDA. Appropriate controls were performed to eliminate any possible interference with the thiobarbituric acid assay.

2.10. Assay of superoxide scavenging activity

Superoxide was generated by xanthine (100 μM) and xanthine oxidase (0.02 U) with or without various concentrations of added CK in 1 ml of 10 mM KH_2PO_4–KOH buffer, pH 7.4, and was detected using nitroblue tetrazolium (100 μM) and quantified spectrophotometrically at 550 nm (Ursini et al., 1994). Superoxide dismutase (100 U/ml) was used as a reference inhibitor.

2.11. Statistical analysis

All experiments were repeated at least three times. Results are reported as means ± S.D. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, a Dunnett's '*t*' test was used to compare the means of two specific groups, with $P < 0.01$ considered as significant.

3. Results

3.1. Effect of CK on $CC1_4$-induced hepatotoxicity

The effects of pretreatment with CK on the $CC1_4$-induced elevation of serum ALT and AST activities are shown in Table 1. Pretreatment with CK (100 mg/kg, ig, 3 days) resulted in no changes in serum ALT and AST activities, compared to the control. A single dose of $CC1_4$ (20 mg/kg) caused hepatotoxicity in mice, as indicated by the increase in ALT and AST serum levels after $CC1_4$ administration. CK pretreatment prevented the $CC1_4$-induced elevation ALT and AST serum levels

Table 1
Dose-dependent effects of pretreatment of mice with CK on $CC1_4$-induced hepatotoxicity

Treatment	Serum ALT (U/l)	Serum AST (U/l)
Control	42 ± 5^{c}	21 ± 4^{c}
CK (100 mg/kg)	45 ± 6^{c}	24 ± 3^{c}
$CC1_4$	$3862 \pm 396^{a,b}$	$1811 \pm 194^{a,b}$
CK (10 mg/kg) + $CC1_4$	$2126 \pm 239^{a,b,c}$	$1091 \pm 123^{a,b,c}$
CK (50 mg/kg) + $CC1_4$	$534 \pm 92^{a,b,c}$	$427 \pm 58^{a,b,c}$
CK (100 mg/kg) + $CC1_4$	$221 \pm 39^{a,b,c}$	$246 \pm 32^{a,b,c}$

Mice were pretreated with CK (10, 50 or 100 mg/kg, ig) once daily for 3 consecutive days. Control mice were given saline. 3 h after the final treatment, mice were treated with $CC1_4$ (20 mg/kg, ip). Hepatotoxicity was determined 24 h later by quantifying the serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and hepatic lipid peroxidation. Each value represents the mean ± SD of five mice.

[a] Significantly different from control.
[b] Significantly different from CK.
[c] Significantly different from $CC1_4$.

in a dose-dependent manner. Low doses of CK (10 mg/kg) partially prevented the elevation of ALT and AST serum levels, and medium or higher doses of CK (50 or 100 mg/kg) almost completely prevented hepatotoxicity.

Histopathological studies showed that $CC1_4$, compared to the control, induces degeneration in hepatocytes and hepatic cords and focal necrosis (Plate 1). By microscopic examination, the severe hepatic lesions induced by $CC1_4$ were reduced remarkably by the administration of CK, and this was in good agreement with the results of the serum aminotransferases activities and hepatic lipid peroxidation levels. Necrosis, which is a more severe form of injury, was either markedly prevented or minimized by pretreatment with CK. CK (100 mg/kg) treatment alone did not cause a change in the liver histology (data not shown).

3.2. Effects of CK on hepatic lipid peroxidation and GSH levels

$CC1_4$ was given to two groups of mice, with or without CK pretreatment, and livers were removed and homogenized 24 h after $CC1_4$ administration. In order to evaluate the effect of pretreatment with CK on $CC1_4$-induced liver lipid peroxidation, we monitored the levels of MDA, an indicator of oxidative damage, and one of the principal products of lipid peroxidation. Its concentration in liver homogenate was determined using the thiobarbituric acid method. As shown in Fig. 1, the production of MDA in the $CC1_4$-treated group increased 3.3-fold when compared with the control. Consistent with the serum levels of ALT and AST, pretreatment with CK significantly decreased $CC1_4$-induced hepatic lipid peroxidation in a dose-dependent manner. Whereas administration of $CC1_4$ alone significantly depleted GSH levels, pretreatment with CK significantly protected the GSH depletion produced by $CC1_4$ (Fig. 2). It was reported that the maximal depletion of GSH by CCl_4 occurs at 4–6 h, and by 24 h GSH levels have partially recovered, although not to the control levels (Nishida et al., 1996). A dose-dependent protective effect on the depletion of GSH level was observed. However, no significant differences in the hepatic GSH level and the activity of cytosolic glutathione *S*-transferase in the control and treatment with CK alone were observed (Table 2).

3.3. Effects of CK on $CC1_4$ bioactivation-related P450 2E1 activity

In mice, CK pretreatment showed a dose-dependent protective effect on $CC1_4$-induced hepatotoxicity, and it is known that $CC1_4$ requires P450 2E1-associated bioactivation to produce liver injury. Therefore, the effects of CK on hepatic microsomal 2E1-specific microsomal monooxygenase activities were examined as a means of investigating the protection afforded CK. As shown in Table 3, hepatic microsomal fractions from mice treated with CK significantly decreased the hydroxylation activities on the two P450 2E1-specific substrates, *p*-nitrophenol and aniline, in a dose-dependent manner.

3.4. Effects of CK on $FeC1_2$–ascorbic acid stimulated lipid peroxidation and superoxide scavenging activity

In order to determine the antioxidant effects of CK in terms of the mechanism of its hepatoprotective effect, antilipid peroxidation in liver homogenate and the superoxide scavenging activity of CK were investigated (Table 4). Consistent with the results of $CC1_4$-induced hepatic lipid peroxidation, CK showed a dose-dependent inhibition of the $FeC1_2$–ascorbic acid stimulated lipid peroxidation with an IC_{50} value of 1.94 mg/ml in liver homogenate. CK also showed superoxide scavenging activity with an IC_{50} value of 3.0 mg/ml.

4. Discussion

Liver injuries induced by $CC1_4$ are the best characterized system of xenobiotic-induced hepatotoxicity and commonly used models for the screening of the antihepatotoxic/hepatoprotective activity of drugs (Brattin et al., 1985; Recknagel et al., 1989, 1991; Williams and Burk, 1990). The rise in the serum levels of lactate dehydrogenase, AST and ALT has been attributed to the damaged structural integrity of the liver, because these are cytoplasmic in location and are released into circulation after cellular damage (Recknagel et al., 1989, 1991). The results of the present study demonstrate that pretreatment of mice with CK effectively protected mice against carbon tetrachloride-

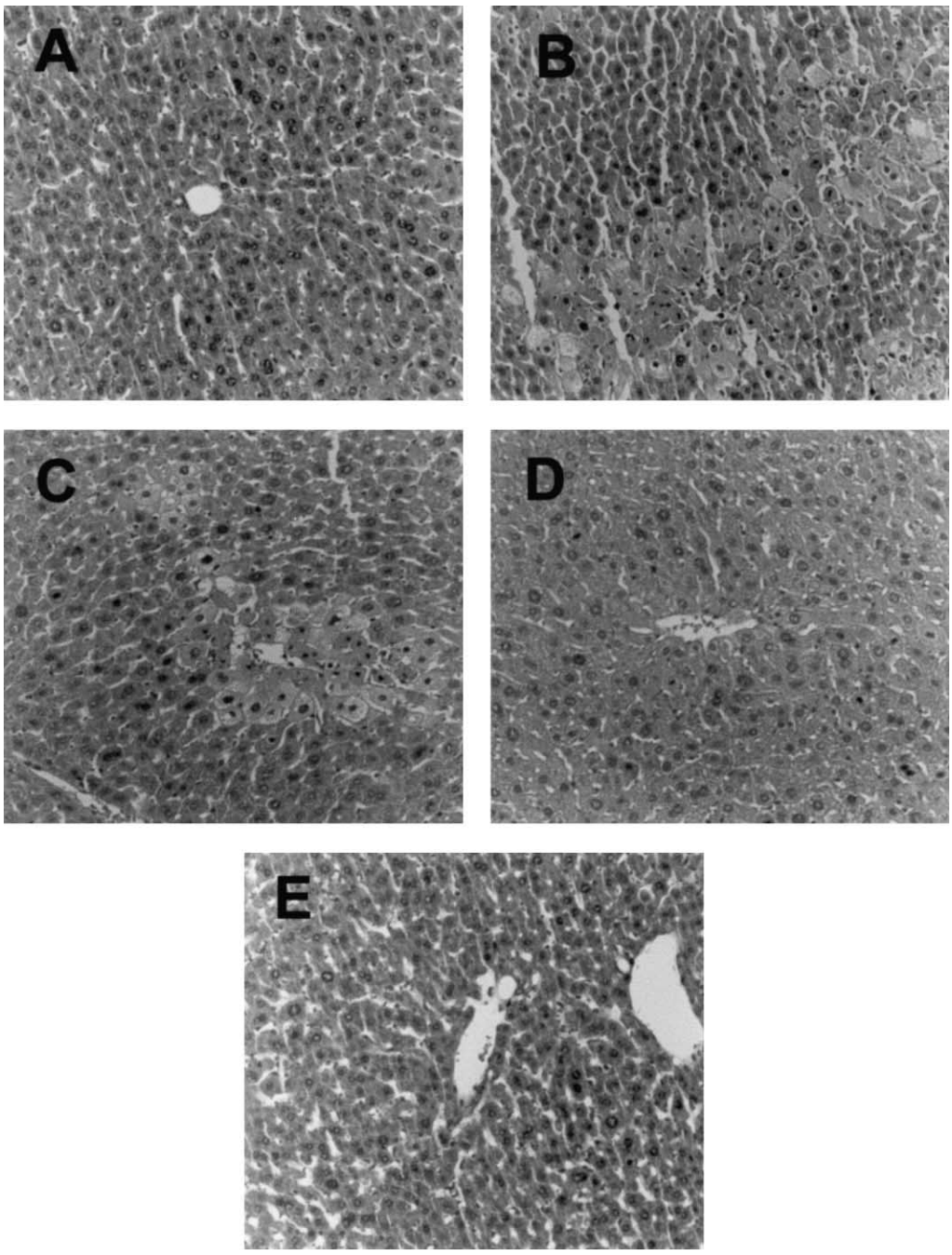

Plate 1. Effects of CK pretreatment on $CC1_4$-induced liver damage in mice. Mice were pretreated with CK (10, 50 or 100 mg/kg, ig) once daily for 3 consecutive days. Control mice were given saline. 3 h after the final treatment, mice were treated with CCl_4 (20 mg/kg, ip). Mice were sacrificed 24 h after the $CC1_4$ administration. (A) Liver from mouse treated with saline; (B) liver from mouse treated with $CC1_4$; (C) liver from mouse treated with CK (10 mg/kg) plus $CC1_4$; (D) liver from mouse treated with CK (50 mg/kg) plus $CC1_4$; (E) liver from mouse treated with CK (100 mg/kg) plus $CC1_4$. ×100.

induced hepatotoxicity, as evidenced by decreased serum aminotransferase activity, and hepatic lipid peroxidation (Table 1, Fig. 1). Moreover, these protective effects were found to be dose dependent, specifically, CK pretreatment at doses from 10 to 100 mg/kg was found to significantly decrease $CC1_4$-induced hepatotoxicity. This phenomenon was also confirmed by histological observation (Plate 1).

It is now generally accepted that the hepatotoxicity of $CC1_4$ is the result of reductive dehalogenation, which is catalyzed by P450 and forms the highly reactive trichloromethyl free radical. This then readily interacts with molecular oxygen to form the trichloromethyl peroxy radical (Brattin et al., 1985; Recknagel et al., 1989, 1991; Williams and Burk, 1990; Brent and Rumack, 1993). Both radicals are capable of binding to proteins

or lipids, or of abstracting a hydrogen atom from an unsaturated lipid, initiating lipid peroxidation and liver damage and by so doing, play a significant role in pathogenesis of diseases (Gosselin et al., 1984; Brattin et al., 1985; Recknagel et al., 1989; Halliwell and Gutteridge, 1990; Williams and Burk, 1990). Therefore, the suppression of P450 could result in reduction in the level of the reactive metabolites, and thus decreased tissue injury. The metabolic activation of $CC1_4$ is thought to be mediated through P450 2E1 (Koop, 1992; Raucy et al., 1993; Zangar et al., 2000). Our results support this hypothesis, in that there is a good correlation between the decreased P450 2E1 enzyme activity in CK-treated hepatic microsomes, and the level of protection against

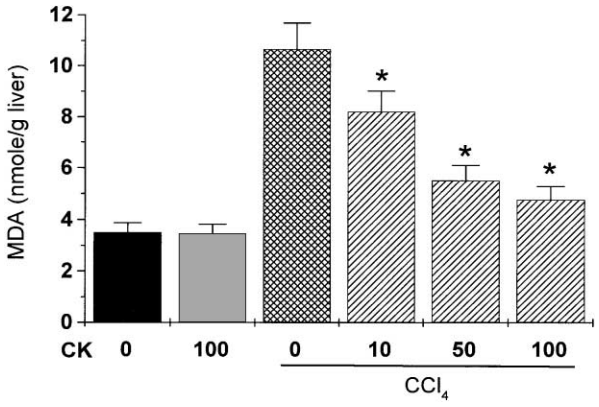

Fig. 1. Effects of CK on hepatic lipid peroxidation in $CC1_4$-intoxicated mice. Mice were pretreated with CK (10, 50 or 100 mg/kg, ig) once daily for 3 consecutive days. Control mice were given saline. 3 h after the final treatment, mice were treated with $CC1_4$ (20 mg/kg, ip). Mice were sacrificed 24 h after $CC1_4$ administration. Hepatic lipid peroxidation was measured as described in Materials and methods. Each bar represents the mean±S.D. for five mice. *Significantly different from $CC1_4$ at $P<0.01$.

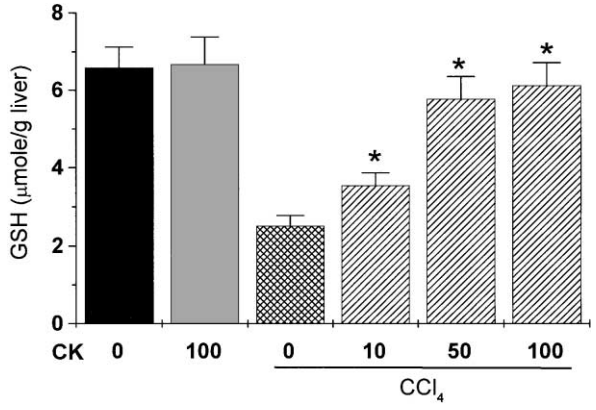

Fig. 2. Protective effect of CK on the $CC1_4$-induced depletion of hepatic cellular glutathione. Mice were pretreated with CK (10, 50 or 100 mg/kg, ig) once daily for 3 consecutive days. Control mice were given saline. 3 h after the final treatment, mice were treated with $CC1_4$ (20 mg/kg, ip). The mice were killed 24 h after $CC1_4$ administration. Hepatic cellular glutathione (GSH) contents were measured as described in Materials and methods. Each bar represents the mean±S.D. for five mice. *Significantly different from $CC1_4$ at $P<0.01$.

$CC1_4$-induced hepatotoxicity in mice (Tables 1 and 3; Plate 1; Figs 1 and 2). The hydroxylation of *p*-nitrophenol and aniline, as used in the present study, has been used extensively to probe the activity of P450 2E1 (Dicker et al., 1990; Koop, 1992; Kim et al., 1997; Jeong and Park, 1998; Jeong, 1999). The identities of the CK components that inhibit P450 2E1 are not known and need to be further studied. The observations above suggest that the inhibition of P450 2E1 by CK in mice plays an important role in the CK-induced hepatoprotection against $CC1_4$. Several previous studies have demonstrated that $CC1_4$-induced hepatotoxicity can be modulated by substances that influence the activity of P450 2E1. In particular, compounds or drugs that induce P450 2E1 potentiate the hepatic toxicity of $CC1_4$ (Day et al., 1993; Allis et al., 1996); on the other hand, compounds that inhibit P450 2E1 protect against $CC1_4$-induced toxicity (Lauriault et al., 1992; Kim et al., 1997; Jeong and Park, 1998; Jeong, 1999). The induction or inhibition of $CC1_4$ biotransformation may subsequently influence the metabolic activation or detoxification of $CC1_4$.

P450 2E1 participates in the metabolism of small organic molecules, such as $CC1_4$, acetaminophen and nitrosamines (Guengerich et al., 1991; Koop, 1992; Raucy et al., 1993; Lee et al., 1996; Zangar et al., 2000). In addition, P450 2E1 plays a critical role in the metabolism of many carcinogens, including the nitrosamines, which require metabolic activation to exert their carcinogenic effect (Yang et al., 1990; Yoo et al., 1990; Guengerich et al., 1991). Thus, the inhibition of P450 2E1 by CK not only plays an important role by protecting against the hepatotoxicity of $CC1_4$, but also may play a role in modulating the toxicity of other xenobiotics, by acting as a chemopreventive agent by decreasing metabolic activation.

In contrast to the toxic activation of $CC1_4$ via the P450 2E1 pathway, the detoxification pathway involves GSH conjugation of the trichloromethyl radical, a P450 2E1-mediated $CC1_4$ metabolite. Previous studies on the mechanism of $CC1_4$-induced hepatotoxicity have shown that GSH plays a key role in the detoxification of the reactive toxic metabolites of $CC1_4$ and that liver necrosis begins when GSH stores are markedly depleted (Recknagel et al., 1989, 1991; Williams and Burk, 1990).

Table 2
Effects of CK treatment on hepatic glutathione level and glutathione *S*-transferase activity

Treatment	Glutathione content (μmol/g liver wt.)	Glutathione *S*-transferase (nmoe/min/mg protein)
Control	6.67±0.72	784±85
CK (10 mg/kg)	6.56±0.62	801±83
CK (100 mg/kg)	6.71±0.68	795±81

Mice were treated with CK (100 mg/kg, ig) once daily for 3 consecutive days. Control mice were given saline. Each value represents the mean±S.D. of five mice.

GSH, largely mediated through the activity of glutathione *S*-transferase, forms adducts with the toxic metabolites of $CC1_4$ and may contribute to the detoxification of this compound (Recknagel et al., 1989, 1991), and it has been hypothesized that one of the principal causes of $CC1_4$-induced liver injury is lipid peroxidation by free radical derivatives of $CC1_4$ (Recknagel et al., 1989, 1991). Our results show that pretreatment with CK significantly inhibits lipid peroxidation (Fig. 1) and significantly reduces $CC1_4$-induced hepatic GSH depletion (Fig. 2). This is attributed to the decreased bioactivation of $CC1_4$ caused by the CK pretreatment (Table 3). Glutathione *S*-transferase is a soluble protein located in the cytosol, and plays an important role in the detoxification and excretion of xenobiotics (Boyer et al., 1984). Glutathione *S*-transferase functionally binds GSH and endogenous or exogenous substances. As glutathione *S*-transferase increases the solubility of hydrophobic substances, it also plays an important role in the storage and excretion of xenobiotics. Compounds that increase the activity of glutathione *S*-transferase, which metabolizes toxic compounds to non-toxic, protect the liver. However, CK pretreatment, by itself, did not affect hepatic GSH levels (Table 2). In addition, CK did not significantly change cytosolic glutathione *S*-transferase activity (Table 2). These results show that the protection afforded by CK against $CC1_4$-induced hepatotoxicity may not be related to the increase of cellular GSH content or glutathione *S*-transferase activity.

Table 3
Effects of CK on microsomal *p*-nitrophenol and aniline hydroxylation activities

Treatment	*p*-Nitrophenol hydroxylation (nmol/min/mg protein)	Aniline 4-hydroxylation (nmol/min/mg protein)
Control	2.24±0.21	0.76±0.07
OA (10 mg/kg)	1.66±0.17*	0.52±0.05*
OA (50 mg/kg)	1.14±0.14*	0.35±0.03*
OA (100 mg/kg)	0.94±0.07*	0.29±0.02*

Mice were treated with CK (OA; 10, 50 or 100 mg/kg, ig) once daily for 3 consecutive days. Control mice were given saline. Each value represents the mean±S.D. of five mice.

* Significantly different from control at $P<0.01$.

Table 4
Inhibitory effects of CK on $FeC1_2$–ascorbic acid stimulated lipid peroxidation and superoxide scavenging activity

Inhibition of lipid peroxidation[a]		Inhibition of scavenging activity[b]	
Treatment (mg/ml)	(%)	Treatment (mg/ml)	(%)
CK 0.1	13.7±1.4	CK 0.5	15.1±1.6
CK 1	46.8±4.8	CK 1	24.3±2.4
CK 5	73.5±7.2	CK 2	43.9±4.4
CK 10	92.8±9.3	CK 5	67.8±6.7

[a] Mouse liver homogenates were stimulated with $FeC1_2$–ascorbic acid in the presence or absence of CK and lipid peroxidation was measured as described in Materials and methods.

[b] Superoxide was generated by oxidation of xanthine/xanthine oxidase in the presence or absence of CK and scavenging activity was measured as described in Materials and methods. Values are presented as the mean of the percentage inhibition±S.D. for three independent experiments, performed in triplicate.

Lipid peroxidation is accepted as one of the principal causes of $CC1_4$-induced liver injury, and is mediated by the production of free radical derivatives of $CC1_4$. Thus, antioxidant activity and/or the inhibition of free radicals generation is important in terms of protecting the liver from $CC1_4$-induced damage (Campo et al., 2001). In vitro lipid peroxidation in a liver homogenate can proceed in a non-enzymatic manner. The process is induced by ascorbate in the presence of Fe^{2+}/Fe^{3+}, and it has been reported that Fe^{2+} and ascorbic acid stimulated lipid peroxidation in rat liver microsomes and mitochondria. In order to clarify the mode of action of CK, in vitro lipid peroxidation experiments were carried out. According to the results obtained, CK inhibited the $FeCl_2$–ascorbic acid-stimulated lipid peroxidation in liver homogenate (Table 4). Moreover, our experimental results show that CK exercised a powerful free radical scavenging activity on the superoxide radical generated using the xanthinexanthine oxidase system (Table 4), and may therefore act by scavenging free radicals and reactive oxygen species formed during CCl_4 metabolism. This particular aspect, which relates to antilipid peroxidation and the superoxide radical scavenging activity of compounds in CK requires further study. Active oxygen species and free radicals are known to be involved in a variety of various diseases, such as arteriosclerosis, liver disease, diabetes, inflammation, cancer and the aging process (Niki, 1995). Any compound, natural or synthetic, with antioxidant properties that might contribute towards the partial or total alleviation of this damage, may have a significant role in maintaining health when consumed as a part of the normal diet.

In conclusion, the results of this study demonstrate that CK has a potent hepatoprotective action on $CC1_4$-induced hepatic damage in mice. These results show that the hepatoprotective effects of CK may be due to its ability to block the bioactivation of $CC1_4$ by inhibiting P450 2E1, which results in the decreased formation of trichloromethyl radicals, and its antioxidant activity, in combination with its ability to scavenge free radicals and inhibit lipid peroxidation, all of which are capable of hepatocellular injury.

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