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Hepatoprotective effects of *Platycodon grandiflorum* on acetaminophen-induced liver damage in mice

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Abstract

The protective effects of an aqueous extract from the roots of *Platycodon grandiflorum* A. DC (Campanulaceae), Changkil (CK), on acetaminophen (APAP)-induced hepatotoxicities and the possible protective mechanisms involved were investigated in mice. Pretreatment with CK prior to the administration of APAP significantly prevented the increase in serum alanine aminotransferase and aspartate aminotransferase activity and hepatic lipid peroxidation in a dose-dependent manner. APAP-induced hepatotoxicity was also essentially prevented as evidenced by liver histopathology. Hepatic glutathione levels and glutathione-S-transferase activities were not affected by treatment with CK alone, but pretreatment with CK protected the APAP-induced depletion of hepatic glutathione levels. The effects of CK on cytochrome P450 (P450) 1A2 and 2E1, the major isozymes involved in APAP bioactivation, were investigated. In microsomal incubations, CK effectively inhibited P450 lA2-dependent methoxyresorufin O-deethylase activities and the P450 2E1-dependent *p*-nitrophenol and aniline hydroxylase. The results suggest that the protective effects of CK against the APAP-induced hepatotoxicity may, at least in part, be due to its ability to block P450-mediated APAP bioactivation.

Keywords: *Platycodon grandiflorum*; Acetaminophen; Hepatotoxicity; Cytochrome P450

1. Introduction

Acetaminophen (APAP; paracetamol) is widely used as an antipyretic and analgesic, and that in high doses can result in extensive liver injury [1]. There is considerable species variation with respect to susceptibility to APAP-induced hepatic toxicity; the mouse is among the more susceptible species and is frequently used in studies [2]. At normal dosage levels, the major elimination pathways of APAP are conjugation with glucuronide and sulfate [3]. The biotransformation and mechanism of APAP hepatotoxicity have been reviewed [1,3]. Studies suggest that APAP requires metabolic activation in order to exert its toxic effects. This toxicity is believed to be mediated via excessive cytochrome P450-generated production of an electrophilic intermediate, *N*-acetyl *p*-benzoquinone imine (NAPQI) [4], which under

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normal dose conditions, is efficiently detoxified by conjugation with glutathione (GSH) [5–7]. However, in an overdose with APAP the glucuronidation and sulfation routes become saturated and more extensive bioactivation of APAP occurs, leading to rapid depletion of hepatic GSH levels. Subsequently, covalent binding of NAPQI to cellular macromolecules, membrane lipid peroxidation, and the disturbance of intracellular calcium balance take place with resultant cell damage or cell death [1,3,7]. Several P450 enzymes are known to play an important role in APAP bioactivation to NAPQI. P450 2E1 and lA2 have been suggested to be primary enzymes for APAP bioactivation in liver microsomes [8–10]. Some studies demonstrated that APAP-induced hepatotoxicity can be modulated by substances that influence P450 activity [5,6].

Herbs have become attractive as food that confer a health benefit (physiologically functional foods) and as a source of material for the development of drugs. Herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases, and there is relatively little knowledge with respect to their modes of action. Natural compounds that reduce enzymes related to bioactivation of chemicals could be considered as good candidates for protection against chemical-induced toxicities. P450 1A1/2 and P450 2E1 are well recognized for their role in the activation of many chemicals to toxic and carcinogenic agents [11–13].

Platycodi Radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae), commonly known as Doraji (Chinese drug, 'Jiegeng', and Japanese name, 'Kikyo') is used as a traditional oriental medicine and the its biological significance has been reviewed [14]. 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 [14,15]. Some studies on its chemical [14,16,17] and immunopharmacological effects have been performed [18]. Recently, it has been observed that the root of *P. grandiflorum* (22 years old) helped prevent hypercholesterolemia and hyperlipidemia [19]. In previous studies, we reported that Changkil (CK), aqueous extract from the root of *P. grandiflorum* cultivated for more than 20 years (S.H. Lee, 1991, Patent on the method of cultivating the perennial balloonflower, Patent No. 045971, Korea), enhanced some of the functions of macrophages, such as, their proliferation, spreading ability, phagocytosis, cytostatic activity, NO secretion, and the gene expression of TNFα, IL-1β, and IL-6 [20,21]. However, the protective effects of *P. grandiflorum* on liver injury have not been well investigated.

The present study was undertaken to evaluate the protective effect of CK on APAP-induced hepatotoxicity and to elucidate the mechanism(s) underlying these protective effects in mice. The parameters analyzed included the serum levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activity, hepatic GSH content, and the histopathology of liver damage. The inhibition of P450 by CK and the mechanisms of its protective action were also investigated. Our results indicate that CK pretreatment significantly protected against APAP-induced hepatotoxicities. Our results suggest that this protection might be due to the blocking of the bioactivation of APAP by the inhibition of P450 1A2 and P450 2E1.

2. Materials and methods

2.1. Chemicals

Acetaminophen (APAP), diagnostic kits for serum ALT and AST, thiobarbituric acid, dithionitrobenzoic acid, phenylmethoxysulfonyl fluoride, and reduced GSH were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were the highest grade of commercially available.

2.2. Plant material

CK is the aqueous extract from the roots of *Platycodon grandiflorum* (22 years old), and 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 mixed was allowed to cool to room temperature, filtered, and lyophilized. The yield of lyophilized residue corresponded to 33.5% of the original dry root weight, and the pale-yellow extract powder was dissolved directly in distilled water. The

composition of the root of *P. grandiflorum* was determined by a previous study [19].

2.3. Animals and treatment

Male ICR mice (25–30 g) were obtained from KFDA (Seoul, Korea). 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 l2 h dark/light cycle and acclimatized for at least 1 week before use. CK in saline was administered intragastrically (i.g.) at 10–100 mg/kg once daily for 3 consecutive days. Groups of control animals were given the respective vehicles. Three hours after the final treatment, mice were treated with APAP (400 mg/kg, i.p., dissolved in saline). Food was removed 16 h prior to APAP treatment. Twenty-four hours after the administration of APAP, mice were anesthetized with CO_2, blood was removed by cardiac puncture to determine the serum ALT and AST activities, and the animals were cervical dislocation. After bleeding, the livers were weighed and a thin slice was preserved in a buffered formalin solution for histological sections. The remaining livers were frozen quickly in dry ice/methanol and stored at −70°C for analyses of GSH content and lipid peroxidation.

2.4. 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, malondialehyde (MDA) [22].

2.5. 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 a colorimetric method using Ellman's reagent as described by Sedlak and Lindsay [23]. Briefly, a weighed portion of the tissue was homogenized in 0.02 M EDTA. Protein was precipitated with 5% trichloroacetic acid, and the supernatant mixed with 2 vol. of Tris buffer (0.4 M, pH 8.9), containing 0.2 M EDTA. Color was developed by adding dithionitrobenzoic acid prepared in methanol, and absorbance was measured at 412 nm. The GSH level was quantified using a standard curve prepared by plotting with different concentrations of reduced GSH.

2.6. 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 APAP-induced necrosis was evaluated based on morphological changes in liver sections stained with hematoxylin and eosin (H&E) using standard techniques.

2.7. Hepatic cytosol isolation and glutathione-S-transferase assay

Mice were treated with CK or vehicle once daily for 3 consecutive days. Twenty-four hours after the last treatment, animals were sacrificed by cervical dislocation. The liver was 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 [24]. All preparations were stored at −70°C until use. The cytosolic fractions were used to assay glutathione-S-transferase activity. Cytosolic glutathione-S-transferase activity was determined using 1-chloro-2,4-dinitrobenzene as a substrate [25].

2.8. Hepatic microsomes isolation and P450s monooxygenase assays

P450 inducers were administered for 3 consecutive days at the following doses: 3-methylcholanthrene (20 mg/kg, P450 1A1/2 inducer) or pyridine (100 mg/kg, P450 2E1 inducer). Mice were sacrificed by decapitation 24 h after treatment. The livers were then quickly

Table 1
Dose-dependent effects of the pretreatment of mice with CK on acetaminophen-induced hepatotoxicity[a]

Treatment	Serum ALT (U/liter)	Serum AST (U/liter)	Hepatic lipid peroxidation (MDA, nmol/g wt.)
Control	35 ± 4^{b}	26 ± 3	3.1 ± 0.4
CK (100 mg/kg)	33 ± 4^{b}	23 ± 4	3.2 ± 0.4
APAP	$2175 \pm 221^{c,d}$	$1472 \pm 151^{c,d}$	$8.6 \pm 0.9^{c,d}$
CK (10 mg/kg) + APAP	$1613 \pm 172^{b,c,d}$	$1021 \pm 103^{b,c,d}$	$6.1 \pm 0.7^{b,c,d}$
CK (50 mg/kg) + APAP	$627 \pm 75^{b,c,d}$	$524 \pm 61^{b,c,d}$	$4.1 \pm 0.4^{b,c,d}$
CK (100 mg/kg) + APAP	$61 \pm 6^{b,c,d}$	$41 \pm 5^{b,c,d}$	$3.6 \pm 0.4^{b,c}$

[a] Mice were pretreated with CK (10, 50 or 100 mg/kg, i.g.) once daily for 3 consecutive days. Control mice were given saline. Three hours after the final treatment, mice were treated with acetaminophen (APAP, 400 mg/kg, i.p.). 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.
[b] Significantly different from the APAP.
[c] Significantly different from the control.
[d] Significantly different from the CK.

removed, weighed, and perfused with ice-cold 0.15 M KCI solution. Hepatic microsomes were prepared as described previously [24], rapidly frozen, and stored at −80°C until used. The microsomal fractions were used to determine P450s monooxygenase activities in vitro. Methoxyresorufin O-dealkylase activity was determined by a modified fluorometric method [26]. The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was determined spectrophotometrically as described previously [27]. Aniline hydroxylase activity was determined by measuring the *p*-aminophenol formation [27]. All assays were run in duplicate. For the inhibition study, CK was dissolved in saline and added to the incubation mixture.

2.9. Statistical analysis

All experiments were repeated at least three times. Means ± SD were calculated for each group and Dunnet's *t*-test was used to calculate statistical significance. Differences were considered statistically significant when $P < 0.01$.

3. Results

3.1. Effect of CK on APAP-induced hepatotoxicity

The effects of pretreatment with CK on the APAP-induced elevation of serum ALT and AST activities are shown in Table 1. Pretreatment with CK (100 mg/kg, i.g., 3 days) resulted in no change in serum ALT and AST activity when compared to the control. A single dose of APAP (400 mg/kg) caused hepatotoxicity in mice as indicated by the increase of ALT and AST serum levels after APAP administration (Table 1). CK pretreatment prevented the APAP-induced elevation of ALT and AST serum levels in a dose-dependent manner. Low doses of CK (10 mg/kg) partially prevented the elevation of ALT and AST serum levels. Medium or higher doses of CK (50 or 100 mg/kg) almost completely prevented hepatotoxicity. Consistent with the serum levels of ALT and AST, pretreatment of CK significantly decreased APAP-induced hepatic lipid peroxidation in a dose-dependent manner (Table 1).

The histopathologic changes produced by APAP are illustrated in Fig. 1. Histological evaluation of APAP-induced damage in the APAP treatment group (Fig. 1B) showed that 50–60% of the liver was damaged, with large damaged zones of degenerating and necrotic hepatocytes in the centrilobular region. Fragmentation and dissolution of hepatocytes were frequent. When CK (10 mg/kg, Fig. 2C) was given to mice prior to the APAP treatment, the area of liver damage was reduced to 40–50% compare with APAP treatment group. With increased of dose (50 mg/kg, Fig. 2D), the area of liver damage was reduced to 80–90% compare with APAP treatment group and the liver morphology at the higher dose of CK group (100 mg/kg, Fig. 2E) was similar to that of the normal

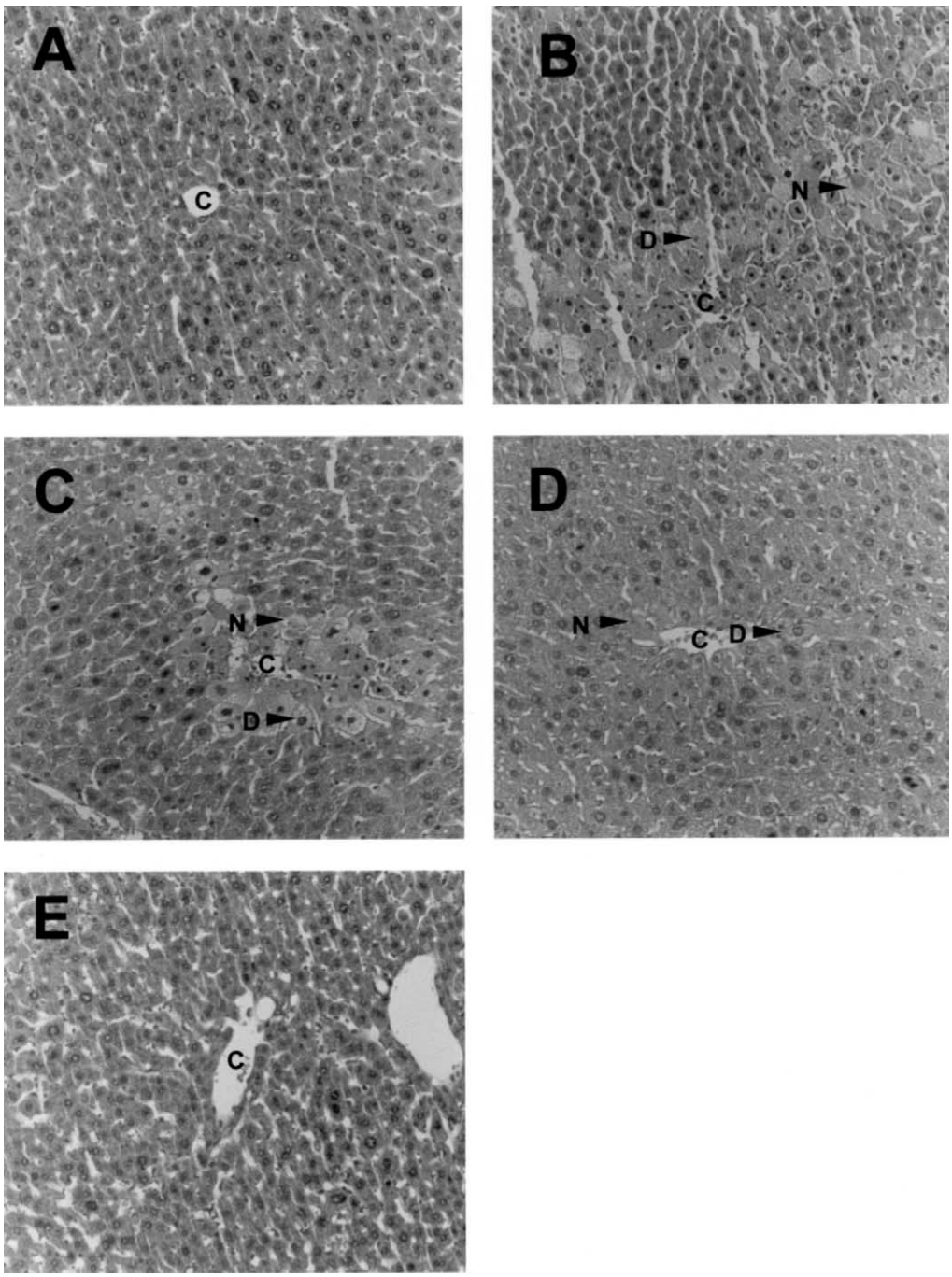

Fig. 1. Effects of CK pretreatment on acetaminophen-induced liver damage in mice. Mice were pretreated with CK (10, 50 or 100 mg/kg, i.g.) once daily for 3 consecutive days. Control mice were given saline. Three hours after the final treatment, mice were treated with acetaminophen (APAP, 400 mg/kg, i.p.). The mice were killed 24 h after the APAP administration. (A) Liver from mouse treated with saline; (B) liver from mouse treated with APAP; (C) liver from mouse treated with CK (10 mg/kg) plus APAP; (D) liver from mouse treated with CK (50 mg/kg) plus APAP; (E) liver from mouse treated with CK (100 mg/kg) plus APAP. C, central vein; N, necrosis; D, degeneration. × 100.

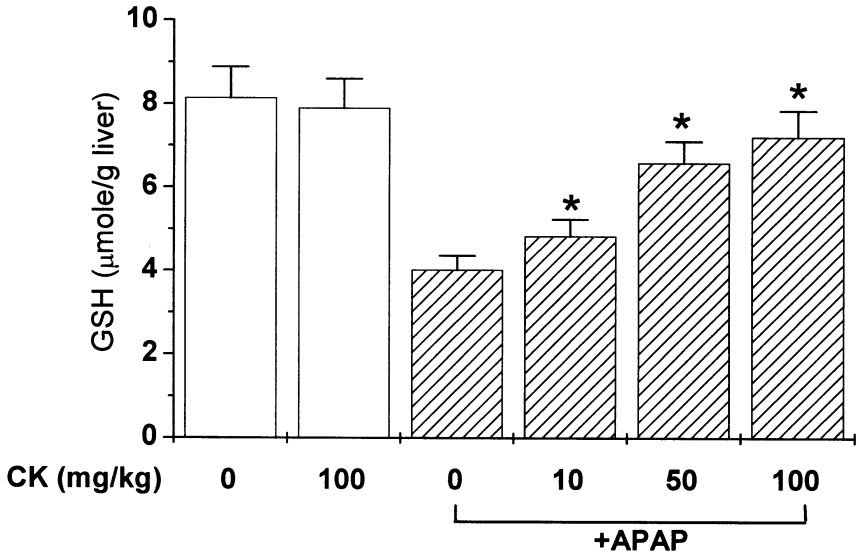

Fig. 2. Protective effect of CK on acetaminophen-induced depletion of hepatic cellular glutathione. Mice were pretreated with CK (10, 50 or 100 mg/kg, i.g.) once daily for 3 consecutive days. Control mice were given saline. Three hours after the final treatment, mice were treated with acetaminophen (APAP, 400 mg/kg, i.p.). The mice were killed 24 h after the APAP administration. Hepatic cellular glutathione (GSH) contents were measured as described in Section 2. Each bar represents the mean ± SD for five mice. *Significantly different from the APAP.

controls (Fig. 2A). CK (100 mg/kg) treatment alone did not cause a significant change in the liver histology (data not shown).

3.2. Effect of CK on APAP-induced liver GSH depletion

APAP was given to two groups of mice, with and without CK pretreatment, and hepatic GSH levels were determined 24 h after the APAP administration. CK, by itself, did not affect hepatic GSH levels (Table 2 and Fig. 2). Whereas administrations of APAP alone significantly depleted GSH level, pretreatment with CK significantly protected the GSH depletion produced by APAP (Fig. 2). A dose-dependent protective effect on the depletion of GSH level was observed on increasing CK pretreatment from 10 to 100 mg/kg. However, there was no significant difference in the activity of cytosolic glutathione-S-transferase between controls and mice given CK treatment (Table 2).

3.3. Effects of CK on APAP bioactivation-related P450 enzyme activities in vitro

In order to determine the relative effectiveness of CK in inhibiting different P450-catalyzed reactions, different substrates were used in the microsomal incubations. In incubations with mouse liver microsomes, CK showed a dose-dependent inhibition of P450 1A2-specific methoxyresorufin O-deethylase with an IC_{50} value of 7.2 mg/ml and P450 2E1-specific aniline hydroxylase and *p*-nitrophenol hydroxylase with IC_{50} values of 3.4 and 2.8 mg/ml, respectively.

4. Discussion

Liver injuries induced by APAP are commonly

Table 2
Effects of CK treatment on hepatic glutathione levels and glutathione-S-transferase activity[a]

Treatment	Glutathione content (μmol/g liver wt.)	Glutathione-S-transferase (nmol/min/mg protein)
Control	6.87 ± 0.72	831 ± 86
CK	7.06 ± 0.74	818 ± 88
P value	NS	NS

[a] Mice were treated with CK (100 mg/kg, i.g.) once daily for 3 consecutive days. Control mice were given saline. Each value represents the mean ± SD of five mice. NS; not significantly different from the control at $P < 0.01$.

Table 3
Effects of CK on hepatic microsomal P450 monooxygenase activities in vitro[a]

Assay	Control	CK (mg/ml)		
		2	5	10
MROD	5.89 ± 0.60[b]	5.34 ± 0.55	3.47 ± 0.35*	1.62 ± 0.17*
PNPH	4.78 ± 0.48[b]	2.97 ± 0.31*	1.92 ± 0.21*	1.44 ± 0.15*
AH	12.62 ± 1.23[b]	7.62 ± 0.81*	5.21 ± 0.53*	4.43 ± 0.42*

[a] Values are presented as the mean ± SD for three independent experiments, performed in triplicate. MROD, methoxyresorufin O-dealkylation; PNPH, *p*-nitrophenol hydroxylation; AH, aniline 4-hydroxylation. *Results significantly different from the control.
[b] nmol/mg protein/min.

used for the screening of hepatoprotective drugs [2]. The rise in serum levels of lactate dehydrogenase, AST and ALT has been attributed to the damaged structural integrity of the liver, because these are normally located in the cytoplasm and are released into the circulation after cellular damage [1]. Our results provide strong evidence that CK significantly inhibits the acute liver toxicity induced by high doses of APAP in mice, as shown by a reduction of serum liver enzyme activities and hepatic lipid peroxidation, as well as the preservation of the liver histopathology. These protective effects were dose-dependent. These results demonstrate that CK pretreatment at dosages from 10 to 100 mg/kg significantly decreases APAP-induced hepatotoxicity.

It is generally accepted that the initiating events in APAP-induced hepatic injury involve the formation of the toxic metabolite NAPQI by cytochrome P450-dependent enzyme systems and GSH depletion [1,3–5]. P450 2E1 and 1A2 are the major enzymes involved in the activation of APAP in animals or humans [8,9,10,28]. In mice, similar P450 enzymes are probably also involved in APAP activation [10]. Therefore, the suppression of P450 could result in the decreased formation of the reactive metabolites, and thus a decreased tissue injury. Our results support this hypothesis, in that there is a good correlation between both the decrease in P450 1A2 and P450 2E1 enzyme activities in microsomal P450-dependent monooxygenase studies in vitro and the protection against APAP-induced hepatotoxicity in mice. The hydroxylations of *p*-nitrophenol and aniline, and the dealkylation of methoxyresorufin, used in the present study, have been extensively used to probe the activity of P450 2E1 and P450 1A2, respectively [26,29]. The identities of the CK components that inhibit P450 1A2 and P450 2E1 are not known and need to be further studied. These observations suggest that the suppression of P450 1A2 and P450 2E1 by CK in mice plays an important role in the CK-induced hepatoprotection against APAP. This idea is consistent with the observation that P450 2E1 inhibitors, such as the dially sulfide, a naturally occurring component of garlic, effectively inhibited APAP-induced hepatotoxicity [30]. Several previous studies have demonstrated that APAP-induced hepatotoxicity can be modulated by substances that influence P450 activities [6,7,31]. Compounds or drugs that induce levels of specific P450s, such as ethanol and isoniazid, which induce P450 2E1, potentiate the hepatic toxicity of APAP [32,33]. On the other hand, compounds that inhibit P450 enzymes, such as disulfiram, 4-methylpyrazole, cimetidine, and α-hederin protect against APAP-induced toxicity [29,33–35]. Induction or inhibition of different enzymes that catalyze APAP biotransformation may subsequently influence the metabolic activation or detoxification of APAP.

In contrast to the toxic activation of APAP via the P450s pathway, the detoxification pathway is GSH conjugation of NAPQI, a reactive toxic metabolite of APAP. Previous studies on the mechanisms of APAP-induced hepatotoxicity have shown that GSH plays a key role in the detoxification of its reactive toxic metabolites, and that liver necrosis begins when GSH stores are markedly depleted [1,3,5–7]. Our results show that pretreatment with CK significantly reduces APAP-induced hepatic GSH depletion (Fig. 2). This result is probably due to the decreased bioactivation of APAP by pretreatment with CK (Table 3), which results in the decreased formation of NAPQI.

GSH is largely mediated through the activity of glutathione-S-transferase, and form adducts with the toxic metabolites of APAP. Therefore, it is believed that it contributes to the detoxification of this compound [1,3,5–7]. However, CK pretreatment, by itself, did not affect the hepatic GSH levels (Table 2 and Fig. 2), and CK did not significantly change cytosolic glutathione-S-transferase activity (Table 2). These results show that the mechanism of the protective effects of CK against APAP-induced hepatotoxicity may not be related to increased levels of cellular GSH content or increased glutathione-S-transferase activity. However, the possibility exists that CK is involved in the direct scavenging of the reactive NAPQI and reduced GSH depletion, or in the enhancement of other pathways involved in APAP deactivation, such as conjugation with glucuronide and sulfate. In fact multiple mechanisms might be involved in the hepatoprotective effect of CK. Further studies are necessary to explain the protective effects of CK on APAP-induced hepatotoxicity.

P450 1A2 and P450 2E1 is active in the metabolism of polyaromatic hydrocarbons and heterocyclic amines, and small organic molecules, such as APAP, carbon tetrachloride, and nitrosamines, respectively [11–13]. In addition, P450 1A2 and P450 2E1 play a critical role in the metabolism of many carcinogens, which require metabolic activation to exert their carcinogenic effect [11–13,29,36]. Thus, suppression of P450 1A2 and P450 2E1 by CK not only plays an important role in the protection against the hepatotoxicity of APAP, but also may play a role in modulating the toxicity of other xenobiotics like as a chemopreventive agents.

In summary, the present study demonstrates that CK has dose-dependent protective effect against APAP-induced hepatotoxicity, and we believe it likely that this protective effect is probably mediated by its inhibitory effect on APAP bioactivation, which is mainly catalyzed by P450 1A2 and P450 2E1.

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