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Protective effects of saponins from the root of *Platycodon grandiflorum* against fatty liver in chronic ethanol feeding via the activation of AMP-dependent protein kinase

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ABSTRACT

Fatty liver and steatosis induced by alcohol is the earliest and most common response of the liver to alcohol and may be a precursor of more severe forms of liver injury. However, the mechanism of liver injury and deposition of fatty liver due to alcohol is complex. The protective effects of saponins from the root of *Platycodon grandiflorum* (Changkil saponins: CKS) against ethanol-induced liver injury in an enteral alcohol feeding model was investigated. Male Sprague–Dawley rats were given control diets or ethanol-containing diets enterally for 4 weeks. Treatment with CKS for 2 weeks significantly prevented the alcohol-induced increase in serum alanine aminotransferase and aspartate aminotransferase activities or decrease in serum albumin levels. Alcohol elevated the hepatic triglyceride content and induced cytochrome P450 2E1 (CYP2E1) expression. CKS treatment reduced CYP2E1 expression and hepatic triglyceride accumulation and prevented alcoholic liver steatosis. Chronic alcohol feeding decreased AMP-activated protein kinase-alpha (AMPKα) phosphorylation, which was restored by CKS treatment. Recovery of AMPKα phosphorylation by CKS was also followed by an increase in acetyl-CoA carboxylase phosphorylation. Our study suggests that CKS is a promising agent for preventing or treating human alcoholic fatty liver disease.

1. Introduction

Chronic ethanol-induced liver disease is linked with oxidative stress. Fatty liver disease is the earliest and most common response of the liver to heavy alcohol consumption (Wang et al., 2009). Excess consumption of alcohol affects the immune system and alters cytokine production, in turn increasing the level of hepatic triglycerides and leading to steatosis. An individual with alcoholic fatty liver often goes onto experience increased fibrosis, cirrhosis, and liver failure (Naveau et al., 1997).

Alcohol consumption results in the generation of ROS through many pathways. Cytochrome P450 2E1 (CYP2E1) is involved in ethanol-mediated generation of oxidative stress and plays an important role in the pathogenesis of ethanol-induced liver injury (Stewart et al., 2001). Induction of CYP2E1 by ethanol is a key pathway, and pathological changes in ethanol-induced liver injury are associated with CYP2E1 levels. Furthermore, CYP2E1 expression levels are induced during ethanol-dependent metabolic changes during early liver injuries such as steatosis and steatohepatitis (Bondy, 1992; Tsukamoto et al., 1995). CYP2E1 is well recognized for its role in activating many chemicals to toxic and carcinogenic agents (Guengerich et al., 1991; Koop, 1992; Eaton et al., 1995).

In animals, chronic alcohol feeding causes fat accumulation, which leads to formation of steatosis in the liver. Accumulation of fat in alcohol-fed animals may result from increased hepatic triglyceride (TG) content, inhibition of fatty acid oxidation and excessive oxidative stress and may lead to fibrosis (Sorensen et al., 1984). AMP-activated protein kinase (AMPK) is a multi-subunit protein kinase that acts as a primary regulator of lipid metabolism through direct phosphorylation of its substrates and indirect control over gene transcription (Yan et al., 2007; Xu et al., 2003). Chronic alcohol exposure decreases AMPK activity in cultured rat hepatocytes and in alcohol-fed mice and rats (You et al., 2004; Ajmo et al., 2008; Tomita et al., 2005). Hepatocytes isolated from alcohol-fed rats confirm the impaired AMPK activity (Garcia-Villafranca et al., 2008). The alcohol-mediated inhibition of AMPK is also associated with enhanced acetyl-Coenzyme A carboxylase (ACC) activity and development of liver steatosis in these animals (Ki et al., 2007); ACC is generally regarded as the rate-limiting

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enzyme in fatty acid biosynthesis in liver and other tissues (You et al., 2005).

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

Platycodi Radix is the root of *Platycodon grandiflorum* A. DC (Campanulaceae) (4 years old). It is used as a food additive and in traditional oriental medicines as a sedative and to treat adult diseases (e.g. bronchitis, asthma and pulmonary tuberculosis) and inflammatory diseases (Lee, 1973). In our previous studies, saponins isolated from the root of *P. grandiflorum* (CKS) showed protective effects against acute ethanol, acetaminophen- and CCl_4-induced hepatotoxicity in mice and inhibit the progress of hepatic fibrosis in rats (Khanal et al., 2009; Lee et al., 2001, 2008, 2004b). In addition, the saponins isolated from the root of *P. grandiflorum* reportedly have potent antioxidant effects, such as superoxide radical scavenging activity, and inhibit reactive oxygen species (ROS) production by tert-butyl hydroperoxide in hepatocytes and in liver (Lee et al., 2004a). The biological significance of CKS has previously been reviewed (Lee, 1973). Previous studies found that Changkil (CK), which is the aqueous extract of the root of *P. grandiflorum* plants, prevents hypercholesterolemia and hyperlipidemia (Kim et al., 1995). Although we already reported that CKS prevents chemical-induced hepatotoxicity and that CKS has *in vivo* and *in vitro* antioxidant effects, the physiological functions and the protective mechanisms of CKS in chronic ethanol-induced liver injury are not clear. In the present study, we investigated the protective effect of CKS against chronic ethanol-induced alcoholic steatosis via modulation of AMPK and explore the mechanisms underlying its protective effects in rat.

2. Materials and methods

2.1. Chemicals

Ethanol was obtained from Merck (KGaA Darmstadt, Germany). Dithionitrobenzoic acid, L-(−)-ascorbic acid, phenylmethoxysulfonyl fluoride, Bradford solution, and diagnostic kits to measure serum alanine aminotransferase (ALT), serum aspartate aminotransferase (AST), and serum albumin levels were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Lieber–DeCarli liquid diet was purchased from Dyets, Inc. (Bethlehem, PA). Antibodies against CYP2E1 and β-actin were purchased from Abcam (UK) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Antibodies that specifically recognize AMPKα phospho-AMPKα, ACC and phospho-ACC were obtained from Cell Signaling (Beverly, MA). All other chemicals were of the highest commercial grade available.

2.2. Preparation of CKS

CK refers to the aqueous extract from the 20-year-old roots of *P. grandiflorum*, which was supplied by Jang Saeng Doraji Co., Jinju, South Korea. The CKS were prepared as described elsewhere and their compositions were previously published (Kim et al., 1995; Tada et al., 1975). The composition of the root of CKS were deapioplatycoside E, platycoside E, deapioplatycodin D3, platycodin D3, polygalacin D2, platycodin D2, deapioplatycodin D and platycodin D (Kim et al., 2005). Briefly, CK was subjected to column chromatography over amberlite XAD-2, Diaion MCl Gel HP20 or Kogel BG4600. After removing the saccharides and amino acids with water, the column was eluted with methanol to obtain the CKS, which is the saponin fraction of CK (Tada et al., 1975).

2.3. Animals and treatments

Male Sprague–Dawley (SD) rats, weighing 190–200 g, were obtained from DAE HAN BIOLINK CO., Ltd. (Chungbuk, Korea). The use of animals complied with the guidelines established by the Animal Care Committee of Chosun University. Animals were acclimated to temperature (22 ± 2 °C) and humidity (55 ± 5%)-controlled rooms with 12 h light/dark cycle for 1 week prior to use. The body weight and conditions of the animals were monitored at least twice a week. The rats were individually housed and fed for 4 weeks (in groups of four each) an equal amount of the control diet (47% of calories as carbohydrates) or the alcohol Lieber–DeCarli liquid diet, containing 36% of calories as ethanol, 11% as carbohydrates, and 18% as protein (Dyets Inc., Bethlehem, PA) (Lieber and DeCarli, 1994). Food consumption was limited to the average intake of the ethanol groups fed *ad libitum*. The diet was kept refrigerated. Ethanol was mixed into the diet just before the supply. After 2 weeks, the rats were treated daily for 2 weeks with CKS [0.5, 1 or 2 (mg/kg/day) $n = 4$] dissolved in saline. Control animals ($n = 4$) received saline only.

2.4. Serum biochemistry

ALT, AST, and albumin levels were measured to assess liver damage. The ALT and AST activities or albumin levels were measured using spectrophotometric diagnostic kits (Sigma Chemical Co.) as previously described (Lee et al., 2001). Briefly, samples were centrifuged at 10,000*g* for 10 min immediately after collection. The serum was stored in the −80 °C freezer before analysis. ALT and AST activities or albumin levels in the serum were evaluated using an ELISA reader (Varioskan, Thermo Electron Co.).

2.5. Histological examinations

Fresh liver tissues were immersed in neutral buffered formalin for 24 h. Fixed tissues were processed routinely, then embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The extent of ethanol-induced steatosis was evaluated by assessing morphological changes in liver sections stained with hematoxylin and eosin to assess steatosis using the method described by Nanji et al. (1989). The number of cells containing fat was estimated and the tissues were graded as follows: steatosis <5%, 1; 25–50%, 2; 50–75%, 3; >75%, 4.

2.6. Hepatic TG content determination

For determination of total triglyceride content, 100 mg of liver was homogenized in 1 ml of 50 mM NaCl. A total of 4 ml of chloroform:methanol (2:1) was added to each sample. Samples were then centrifuged and the organic layer was transferred to another test tube and dried under nitrogen. The resulting pellet was dissolved in phosphate-buffered saline containing 1% Triton X-100, and the TG content was determined using an enzymatic reagent kit (Sigma Chemical Co.).

2.7. Immunoblot analysis

Livers were removed quickly, weighed, perfused with ice-cold 0.15 M KCl, and then homogenized in a Potter–Elvehjem homogenizer with 4 vol. (w/v) of 10 mM Tris–HCl (pH 7.4) containing 0.15 M KCl, 0.1 mM EDTA, 1 mM dithiothreitol and 0.01 mM phenylmethoxysulfonyl fluoride. Protein concentrations in the liver homogenates were determined using the Bradford method with bovine serum albumin as the standard (Bradford, 1976). The liver homogenates were resolved by 8–10% SDS–polyacrylamide gel electrophoresis followed by electroblotting onto polyvinylidene difluoride membranes and development using the ECL chemiluminescence system (Amersham, Buckinghamshire, UK). A representative blot is shown in each figure and one sample per treatment is included in the blot.

2.8. Statistical analysis

All experiments were performed in duplicate. Mean ± S.E.M. was calculated for each group and the Tukey–Kramer test was used to calculate statistical significance at the $p < 0.05$ level.

3. Results

3.1. Effect of CKS on blood biochemistry

After 4 weeks on the chronic alcohol diet, ALT and AST activities were increased as shown in Table 1. ALT and AST activities elevated

Table 1
Effects of CKS on blood biochemistry.

Treatment ($n = 4$)	ALT (IU/L)	AST (IU/L)	Albumin (g/dL)
Control	9.8 ± 0.2	23.7 ± 1.8	4.49 ± 0.2
Ethanol	24.4 ± 2.7$^{\#}$	44.8 ± 4.1$^{\#}$	3.4 ± 0.4$^{\#}$
Ethanol + CKS 0.5	14.3 ± 1.9*	35.5 ± 2.4*	3.52 ± 0.2
Ethanol + CKS 1	13.1 ± 0.3*	32.7 ± 3.6*	3.77 ± 0.2
Ethanol + CKS 2	11.9 ± 2.1*	26.4 ± 1.9*	4.01 ± 0.6*

Rats were pair-fed control or alcohol-containing diet for 4 weeks. After 2 weeks on the diets, the animals were treated daily with CKS 0.5, 1 or 2 mg/kg for another 2 weeks. ALT and AST activities or albumin levels were determined in the rat plasma. Values represent mean ± S.E.M. from 4 animals. Significantly different from control at $^{\#}p < 0.05$. Significantly different from ethanol at $^{*}p < 0.05$.

by alcohol administration tended to decrease after treatment with CKS. CKS treatment (2 mg/kg) improved ALT and AST activity to a level similar to that in control. Chronic alcohol administration to rats significantly decreased the albumin level, which was marginally recovered by CKS (Table 1).

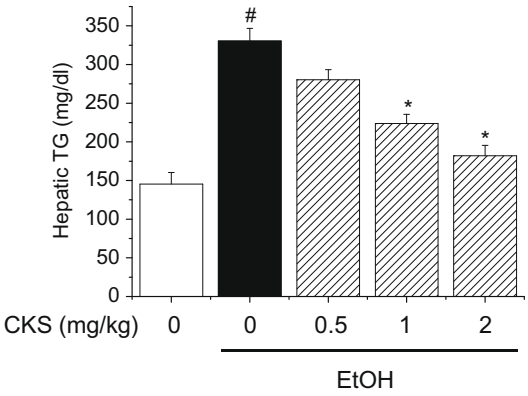

Fig. 1. Effects of CKS on hepatic TG content. Rats were pair-fed control or alcohol-containing diet for 4 weeks. After 2 weeks on the diets, the animals were treated daily with CKS 0.5, 1 or 2 mg/kg for another 2 weeks. Hepatic TG contents were measured as described in Section 2. Values represent mean ± S.E.M. from 4 animals. Significantly different from control at $^{\#}p < 0.05$. Significantly different from ethanol at $^{*}p < 0.05$.

3.2. Effects of CKS on hepatic TG contents

Chronic alcohol-fed rats exhibited significant increases in hepatic TG contents compared to control. TG measurements indicated that livers of alcohol-fed rat also accumulated large amounts of triglycerides. Based on the elevated hepatic triglycerides and the histological scoring, all alcohol-fed rats developed steatosis. However, this development was attenuated by CKS (Fig. 1). These results indicate that CKS feeding might prevent alcoholic steatosis.

3.3. Histopathological changes

To determine the effects of CKS on alcoholic steatosis, we histopathologically assessed the amount of fat deposited in the liver. Where we did not see any pathological changes, liver histology was normal (Fig. 2A). Chronic alcohol feeding for 4 weeks resulted in fat deposition, and faint microvesicular and macrovesicular fat droplets were observed (Fig. 2B), which caused degenerative morphological changes in the liver. These ethanol-induced hepatic pathological changes were significantly inhibited in CKS-treated rats (Fig. 2C).

3.4. Effects of CKS on CYP2E1 protein expression

We examined the effects of CKS on CYP2E1 protein expression. Liver homogenates from CKS-treated rat were resolved by

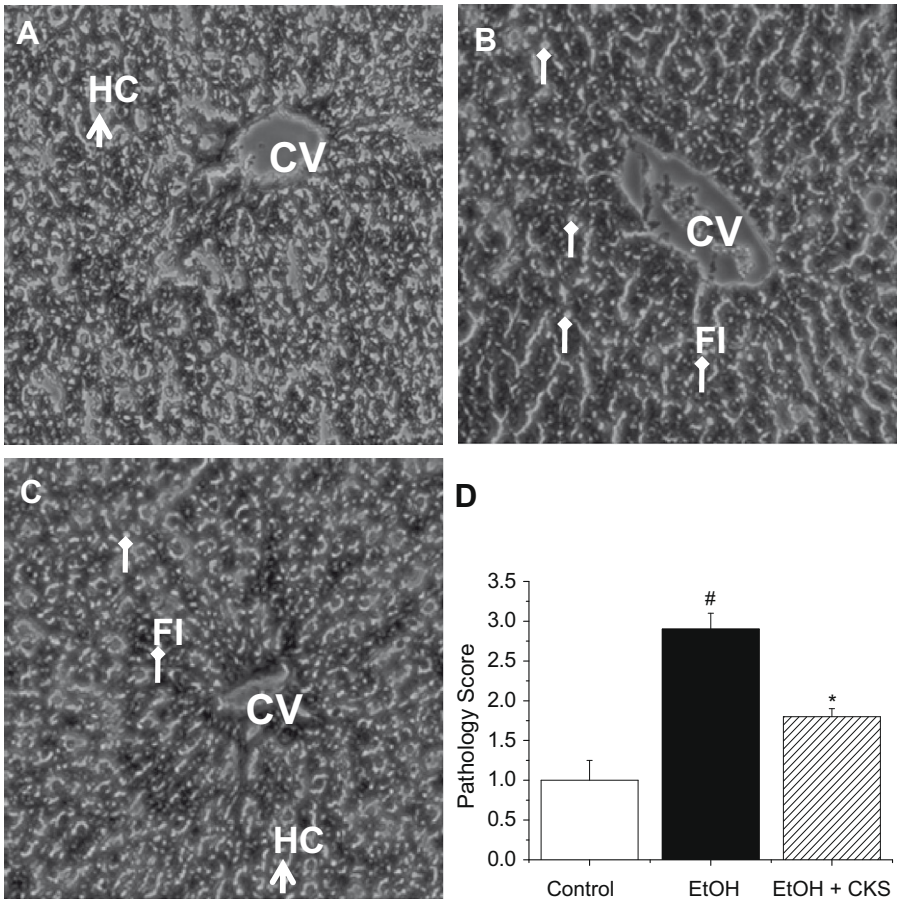

Fig. 2. Effects of CKS on histopathological changes in the liver. Rats were pair-fed control or alcohol-containing diet for 4 weeks. After 2 weeks on the diets, the animals were treated daily with CKS 2 mg/kg for another 2 weeks. Livers were harvested, fixed in formalin, and stained with hematoxylin-eosin for evaluation of pathological changes due to feeding of control diet (A), ethanol diet (B), or ethanol diet plus CKS (2 mg/kg) (C). Photomicrographs show views of the liver sections: central vein (CV), hepatocytes (HC), and fat infiltration (FI). Magnification ×200. (D) Pathology was scored as described in Section 2. Significantly different from control at $^{\#}p < 0.05$. Significantly different from ethanol at $^{*}p < 0.05$.

SDS–PAGE and immunoblotted with anti-CYP2E1 antibodies. As shown in Fig. 3, alcohol administration increased the CYP2E1 expression. CKS intake for 2 weeks did not affect the expression of CYP2E1 (data not shown). But the elevation of CYP2E1 expression in alcoholic liver was significantly inhibited by CKS. These results suggest that the suppression of CYP2E1 by CKS in rat is an important aspect of the hepatoprotective effect of CKS against ethanol.

3.5. Effect of CKS on phosphorylation of AMPK

AMPK regulates lipid metabolism in the liver (Winder and Hardie, 1999). The inhibition of AMPK by alcohol feeding was also associated with decreased phosphorylation of the target proteins, ACC. Chronic alcohol feeding for 4 weeks notably decreased the levels of phosphorylated AMPK in liver homogenates (Fig. 4). Treatment of alcohol-fed rats with CKS for 2 weeks resulted in the recovery of AMPK phosphorylation. Our results demonstrate that chronic alcohol feeding decreased AMPK protein expression, which was recovered after 2 weeks of CKS treatment.

3.6. Effect of CKS on phosphorylation of ACC

We next measured the levels of phosphorylated ACC, a rate-limiting enzyme involved in fatty acid biosynthesis in the liver. Alcohol administration inhibited ACC phosphorylation in the liver, while CKS treatments for 2 weeks improved this effect of alcohol (Fig. 5). Our results demonstrate that chronic alcohol feeding decreased phosphorylated ACC protein expression, which was recovered after 2 weeks of CKS treatment.

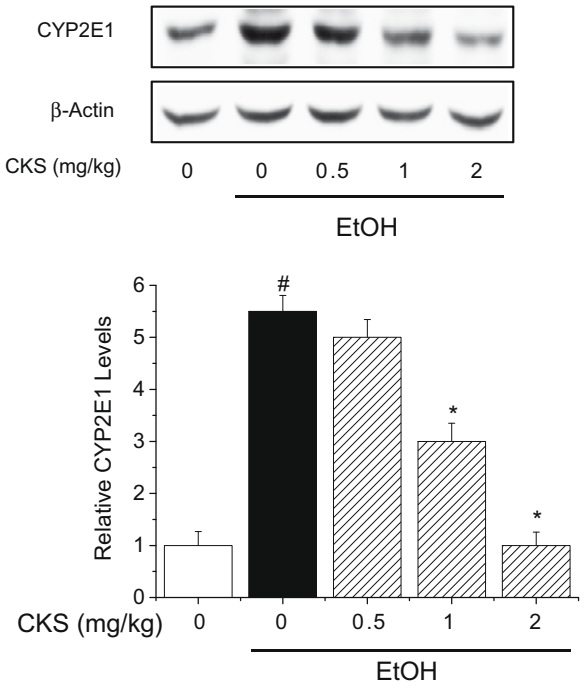

Fig. 3. Effects of CKS on CYP2E1 protein expression. Rats were pair-fed control or alcohol-containing diet for 4 weeks. After 2 weeks on the diets, the animals were treated daily with CKS 0.5, 1 or 2 mg/kg for another 2 weeks. CYP2E1 protein expressions were determined by immunoblot of each liver protein sample with specific antibodies. β-Actin protein was used as a loading control. Densitometry ratios of CYP2E1 protein expressions were normalized to β-actin. Significantly different from control at $^{\#}p < 0.05$. Significantly different from ethanol at $^{*}p < 0.05$.

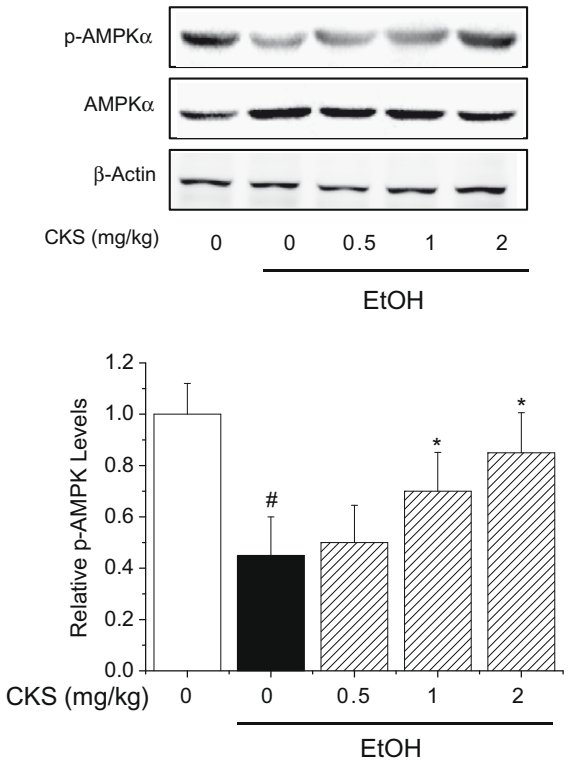

Fig. 4. Effect of CKS on phosphorylation of AMPK. Rats were pair-fed control or alcohol-containing diet for 4 weeks. After 2 weeks on the diets, the animals were treated daily with CKS 0.5, 1 or 2 mg/kg for another 2 weeks. Phosphorylated AMPK was immunoblotted in liver homogenates prepared from four animals per treatment. A representative blot is shown in the figure; one sample per treatment was included in the blot. β-Actin protein was used as a loading control. Densitometry ratios of phospho-AMPK were normalized to β-actin. Significantly different from control at $^{\#}p < 0.05$. Significantly different from ethanol at $^{*}p < 0.05$.

4. Discussion

Liver damage due to ingestion of alcohol is a well known phenomenon in animals. It is well-established that ethanol inhibits the mitochondrial electron transport chain, resulting in increased ROS production (Song et al., 2008). Hepatic injury also results in the leakage of cellular enzymes into plasma (Baldi et al., 1993). Chronic ingestion of alcohol leads to a sequence of hepatic pathologies associated with alcoholic liver disease (ALD), ranging from alcoholic steatosis to alcoholic steatohepatitis, cirrhosis and liver failure. Steatosis or the accumulation of fat in the liver is the initial pathology that is common to all aspects of alcoholic liver disease (You et al., 2005). As we have already reported that, CKS protect against acute ethanol-induced hepatotoxicity in mice (Khanal et al., 2009). Acute alcoholic fatty liver has been employed as a model by numerous investigators and continues to be used, despite the original recognition of the fact that most of this acute effect may be nonspecifically due to the stress generated by alcoholic administration (Baraona and Lieber, 1979). Gene expression changes in the liver after acute drinking differs from the changes observe in chronic ethanol feeding. The changes in gene expression after chronic ethanol feeding sensitize the liver to alcohol-induced liver damage, which is not seen after acute drinking (Bardag-Gorce et al., 2009). Due to lacking of some result in acute drinking we further performed chronic ethanol. In the current experiments, we used CKS in an *in vivo* animal model of chronic alcohol feeding. Our results showed that supplementation with CKS attenuated chronic ethanol-induced liver injury.

Increased activities of serum enzymes such as ALT and AST and decreased levels of albumin have been observed in alcohol-treated

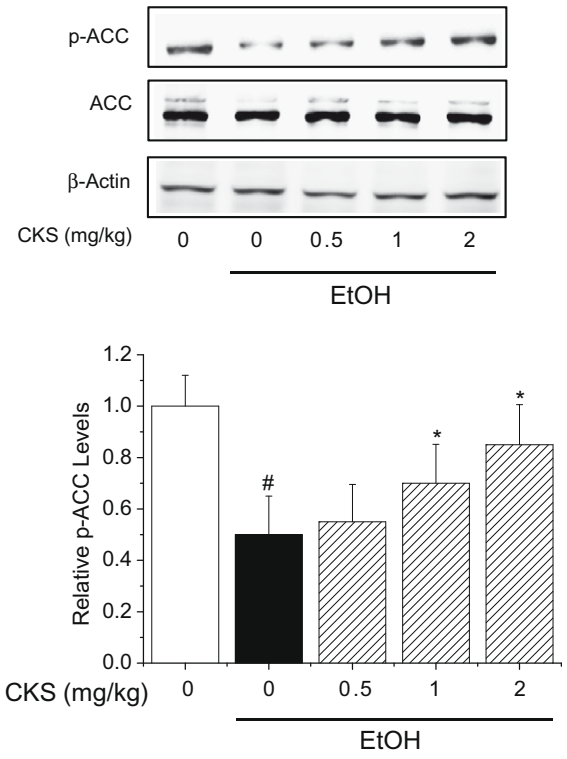

Fig. 5. Effects of CKS on phosphorylation of ACC. Rats were pair-fed control or alcohol-containing diet for 4 weeks. After 2 weeks on the diets, the animals were treated daily with CKS 0.5, 1 or 2 mg/kg for another 2 weeks. The levels of phosphorylated ACC were determined in the liver homogenates prepared from four animals per treatment. A representative blot is shown in the figure; one sample per treatment was included in the blot. β-Actin protein was used as a loading control. Densitometry ratios of phospho-AMPK were normalized to β-actin. Significantly different from control at $^{\#}p < 0.05$. Significantly different from ethanol at $^{*}p < 0.05$.

mice or rats, indicating increased permeability, damage, steatosis and inflammation (Goldberg and Watts, 1965; Ki et al., 2007). Ingestion of alcohol in animals affects the immune system and alters cytokine production (Dey and Cederbaum, 2006). Our results show that CKS treatment significantly inhibits the ethanol-induced ALT and AST activities. At the same time, CKS increases albumin levels. In addition, our results show that CKS dramatically prevents the ethanol-induced elevation of inflammatory infiltrates based on histopathologic analysis. We have already reported that CKS exerts a suppressive effect on inflammatory cytokines (Kim et al., 2006). These anti-inflammatory effects of CKS may play an important role in protecting against ethanol-induced hepatotoxicity.

CYP2E1 plays a critical role in the metabolism of many carcinogens, including nitrosamines, which require metabolic activation to exert their carcinogenic effect (Yang et al., 1990; Yoo et al., 1990; Guengerich et al., 1991). CYP2E1 can generate ROS during its catalytic circle, and CYP2E1 levels are increased by chronic alcohol feeding. Furthermore, CYP2E1 has been suggested to be a major contributor to ethanol-induced oxidative stress and to ethanol-induced liver injury. Also, CYP2E1 catalyzes the metabolic conversion of small organic molecules to reactive intermediates that are frequently capable of covalently binding to tissue macromolecules and thereby cause liver injuries. Initial suggestions of a role for CYP2E1 in alcoholic liver injury came from studies in the intragastric model of alcohol feeding in which prominent CYP2E1 induction occurs (Lu and Cederbaum, 2008). A major mechanism of CYP2E1 induction by ethanol is post-transcriptional, which protects CYP2E1 from rapid degradation via proteolytic pathways (Roberts et al., 1995). Given the importance of CYP2E1 in the bioactivation of toxicants, the selective inhibition of CYP2E1 may present protective effects against toxic injuries and possibly fat accumulation. In this study, CKS supplementation depressed the ethanol-induced elevation of CYP2E1 and may therefore have a role in the inhibition of hepatic TG accumulation and liver injury. The mechanism of inhibition of ethanol-induced CYP2E1 elevation by CKS warrants further studies.

AMPK is the primary protein kinase responsible for the inactivation of ACC, which results in increased fatty acid oxidation in liver (Chen et al., 2007) and suggest that alcohol inhibits AMPK activity. Phosphorylation of the AMPK catalytic subunit is required for its activation (Crute et al., 1998). Downstream substrates are then phosphorylated by AMPK to reduce ATP-consuming anabolic pathways including cholesterol, fatty acid and TG synthesis but increase ATP-generating catabolic pathways such as lipolysis and fatty acid oxidation (Zang et al., 2004). In the present study, we found that AMPK phosphorylation was decreased after 4 weeks of alcohol consumption and that the inhibition of AMPK by alcohol accompanied a decrease in ACC phosphorylation. We also found that CKS treatment enhanced hepatic AMPK phosphorylation in rats fed an alcohol-containing diet; this led to an increase in ACC phosphorylation. Our data indicate that CKS inhibits alcohol-dependent TG accumulation in hepatocytes presumably through AMPK-dependent fatty acid oxidation. The favorable effect of CKS against ethanol-induced fat infiltration in the liver may be mediated at least in part by AMPK activation.

In summary, CKS prevents alcoholic liver injury, decreasing steatosis and cellular damage caused by alcohol. The present results indicate that CKS supplementation may antagonize the development of oxidative liver injury induced by chronic alcohol feeding. CKS supplementation depressed the ethanol-induced elevation of CYP2E1 protein expression and led to recovery of p-AMPK and p-ACC. The hepatoprotective effects of CKS may be related to its ability to block the bio-activation of ethanol by inhibiting CYP2E1 activity, as well as its ability to scavenge free radicals. Taken together, these results suggest that CKS may be a good candidate for development as a therapeutic agent for alcoholic fatty liver.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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