Potential roles of AMP-activated protein kinase in liver regeneration in mice with acute liver injury

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Abstract. Liver regeneration post severe liver injury is crucial for the recovery of hepatic structure and function. The energy sensor AMP-activated protein kinase (AMPK) has a crucial role in the regulation of nutrition metabolism in addition to other energy-intensive physiological and pathophysiological processes. Cellular proliferation requires intensive energy and nutrition support, therefore the present study investigated whether AMPK is involved in liver regeneration post carbon tetrachloride (CCl_4)-induced acute hepatic injury. The experimental data indicated that phosphorylation level of AMPK increased 48 h post-CCl_4 exposure, which was accompanied with upregulation of proliferating cell nuclear antigen (PCNA) and recovery of alanine aminotransferase (ALT) level. Pretreatment with the AMPK inhibitor compound C had no obvious effects on ALT elevation in plasma and histological abnormalities in liver 24 h post CCl_4 exposure. However, treatment with compound C 24 h post CCl_4 exposure significantly suppressed CCl_4-induced AMPK phosphorylation, PCNA expression and ALT recovery. These data suggest that endogenous AMPK was primarily activated at the regeneration stage in mice with CCl_4-induced acute liver injury and may function as a positive regulator in liver regeneration.

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Introduction

Acute liver injury induced by drugs, poisons or infections is a common health problem worldwide, which remains one of the leading causes of death (1). To alleviate the liver damage and improve the outcomes, extensive studies have been conducted to investigate the mechanisms underlying the development of liver injury (2-5). Importantly, the liver has strong regenerative activity after injury to compensate liver cell loss (6-8). The regeneration process is crucial for the recovery of hepatic structure and function (9-12).

AMP-activated protein kinase (AMPK) is a serine/threonine kinase. It is usually regarded as a sensor of cellular energy status used to keep up a delicate balance by monitoring both the short- and long-term total body energy requirements (13). Interestingly, recent studies have found that AMPK was extensively involved in various energy-intensive physiological and pathophysiological processes, such as inflammation (14), autophagy (15-17) and proliferation (18-21). Therefore, AMPK is rapidly emerging as a novel target for pathophysiological and pharmacological research (22).

There is increasing evidence that AMPK is involved in the development of hepatic disorders. Several studies have found that AMPK plays crucial roles in the development of cholestatic liver diseases, nonalcoholic fatty liver disease and liver fibrosis (23-25). In addition, AMPK also have potential value for the pharmacological intervention of liver injury induced by carbon tetrachloride (CCl_4), endotoxin or ischemia (26-28). Although the crucial roles of AMPK in liver injury have been reported, the pathological significance of AMPK in the regeneration stage post acute liver injury was unclear.

Because liver regeneration includes a serial of highly active molecular responses, which requires intensive energy supply (9), we then suspected that AMPK might regulate the procedure of liver regeneration. In the present study, the potential role of AMPK in liver regeneration was investigated in mice with CCl_4-induced toxic liver injury (13). In this widely used animal model, the phosphorylation status of AMPK post CCl_4 exposure was detected. Then, the activity of AMPK was inhibited by a selective AMPK inhibitor,

compound C (29,30), and the degree of liver regeneration was determined.

Materials and methods

Experimental animals. The male KM mice *(Mus musculus Km)* weighing 18-22 g were purchased from the Experimental Animal Center of Chongqing Medical University (Chongqing, China). Mice were kept in a 12-h light/dark cycle with *ad libitum* water and food. All experimental protocols involving the animals were approved by the Institutional Animal Care and Use Committee of Chongqing Medical University.

Reagents. CCl_4 was obtained from Chengdu Kelong Chemical Reagent Factory (Chengdu, China). The AMPK inhibitor F6-[4-[2-(1-piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine (compound C) was purchased from Cayman Chemical (Ann Arbor, Michigan, MI, USA). The alanine aminotransferase (ALT) detection kit was purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Rabbit anti-mouse antibodies against AMPK, phosphorylated AMPK (p-AMPK, Thr^{172}) and β-actin were purchased from Cell signaling Technology (Danvers, MA, USA). Rabbit anti-mouse antibody against proliferating cell nuclear antigen (PCNA) antibody was purchased from Abcam (Cambridge, UK). The BCA protein assay kit, the horseradish peroxidase-conjugated goat anti-rabbit antibody and the enhanced chemiluminescence (ECL) reagents were obtained from Pierce Biotechnology (Rockford, IL, USA).

Experimental design. To induce acute liver injury, the mice received intraperitoneal injection of CCl_4 (1%, dissolved in olive oil, 0.8 ml/kg). To determine the phosphorylation status of hepatic AMPK, the mice were anesthetized and sacrificed at various timepoints post CCl_4 exposure (0, 24 and 48 h; n=8 for each group), the liver and plasma samples were harvested for further experiments. To investigate the potential roles of AMPK in acute liver injury, the mice were randomly divided into 4 groups (n=8 for each group): i) the control (CON) group, mice received vehicle only; ii) the compound C group, mice received the AMPK inhibitor compound C (15 mg/kg, i.p.) only; iii) the CCl_4 group, mice exposed to CCl_4; iv) the CCl_4 + compound C group, mice received compound C 0.5 h prior to CCl_4 challenge. The animals were sacrificed 24 h post CCl_4 exposure, the liver and plasma samples were harvested. To investigate the potential roles of AMPK in liver injury, the mice were randomly divided into 4 groups (n=8 for each group): i) the CON group, mice received vehicle only; ii) the compound C group, mice received the AMPK inhibitor compound C (15 mg/kg, i.p.) only; iii) the CCl_4 group, mice exposed to CCl_4; iv) the CCl_4 + compound C group, mice received compound C 24 h post CCl_4 challenge. The animals were sacrificed 48 h post CCl_4 exposure, the liver and plasma samples were harvested. The plasma samples were collected in the heparin tubes and were then centrifuged at 5,000 RPM for 15 min at 4˚C. The supernatants were collected for further examinations.

Determination of liver enzymes. The plasma samples were collected with the method described above. The levels of ALT in plasma samples were determined following the manufacturer's instructions. The enzymatic activities were calculated according to the standard curve.

Histochemistry. The liver samples were fixed in formalin, embedded in paraffin, and the liver sections were evaluated with hematoxylin and eosin staining under light microscope (Olympus Corp., Tokyo, Japan).

Immunoblot analysis. The proteins were extracted from the liver samples and the concentration of the protein samples was determined by BCA protein assay kit (Pierce Biotechnology). Protein extracts were separated on 10 or 8% SDS-PAGE gels, then transferred to nitrocellulose membrane (Millipore, Billerica, MA, USA). After incubation with the 5% (w/v) skimmed milk in Tris-buffered saline (TBST) containing 0.1% Tween-20 for 2 h at room temperature, the membranes were incubated with primary antibodies overnight at 4˚C. And then, the membranes were washed by TBST (containing 0.05% Tween-20), then incubated with secondary antibody for 1.5 h at 37˚C and then washed by TBST. Antibody binding was visualized with an ECL chemiluminescence system (Pierce Biotechnology).

Statistical analysis. Data were presented as mean ± SD and analyzed by one-way ANOVA with Turkey's post hoc test in SPSS13.0. $P<0.05$ was considered to indicate a statistically significant difference.

Results

AMPK was activated post CCl_4 challenge. The immunoblot analysis showed that CCl_4 exposure did not stimulate the phosphorylation of AMPK 24 h post-CCl_4 administration, but the phosphorylation level of AMPK was upregulated 48 h post-CCl_4 exposure (Fig. 1). The upregulation of p-AMPK 48 h post-CCl_4 exposure was accompanied with increased expression of PCNA and recovery of ALT level (Figs. 2 and 3).

Pre-insult treatment with an AMPK inhibitor had no obvious effects on liver injury. To determine the potential roles of AMPK on CCl_4-induced liver injury, the AMPK inhibitor compound C was administered before CCl_4 exposure. The experimental data shown that pretreatment with compound C had no significant effects on CCl_4-induced elevation of ALT in plasma (Fig. 4). Consistently, the histopathological examination found no obvious difference in CCl_4-challenged mice with or without compound C administration (Fig. 5).

Delayed inhibition of AMPK suppressed liver regeneration. To investigate whether AMPK is involved in liver regeneration post CCl_4 challenge, compound C was administered 24 h post CCl_4 exposure. The experimental data shown that post-treatment with compound C significantly suppressed AMPK phosphorylation 48 h post CCl_4 exposure (Fig. 6). Post-treatment with compound C also suppressed CCl_4-induced expression of PCNA 48 h post CCl_4 exposure (Fig. 7). Meanwhile, the recovery of ALT level 48 h post CCl_4 exposure was impaired by compound C administered post CCl_4 exposure (Fig. 8).

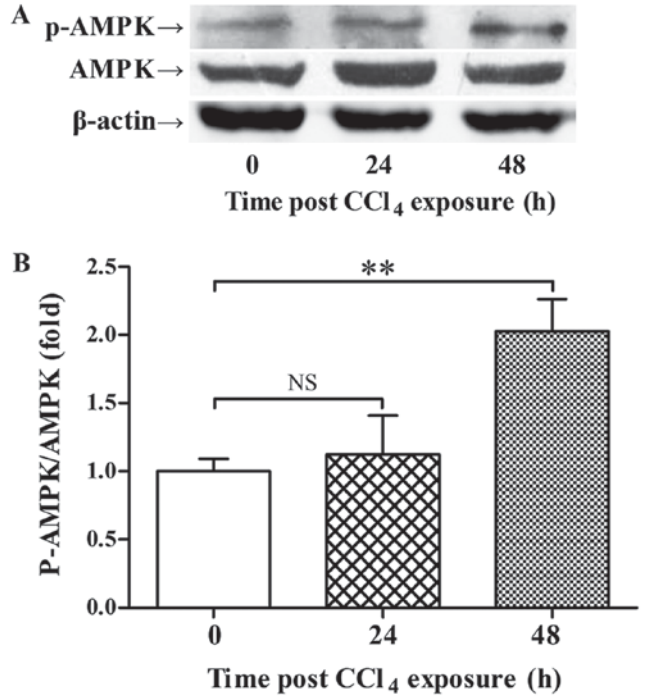

Figure 1. CCl_4-induced phosphorylation of AMPK in liver. Acute liver injury was induced by intraperitoneal injection of CCl_4. The liver samples were harvested 0, 24, 48 h post CCl_4 exposure. (A) The level of p-AMPK and total AMPK were determined by immunoblot analysis. The target proteins were indicated by arrows in the blot. (B) The bands of p-AMPK and AMPK were semi-quantified by gray scale. n=4, $^{NS}P>0.05$, $^{**}P<0.01$. AMPK, AMP-activated protein kinase; CCl4, carbon tetrachloride; p-AMPK, phosphorylated AMPK.

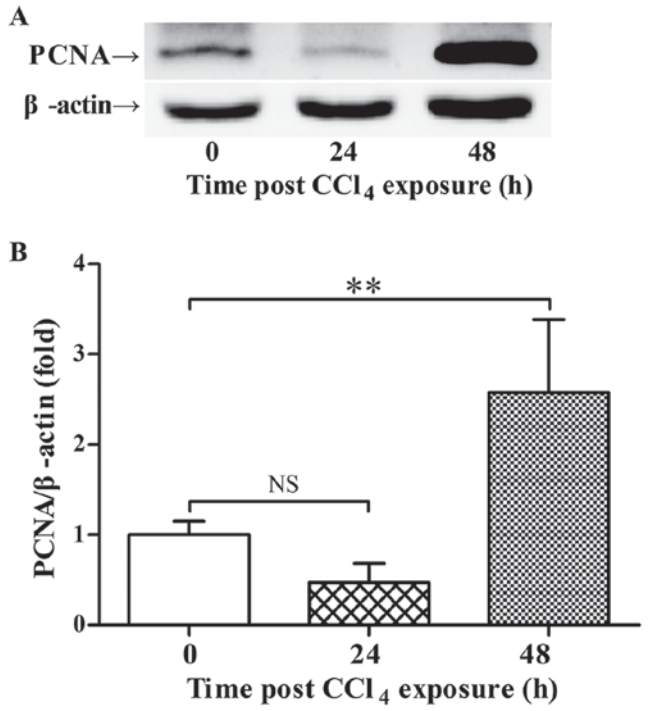

Figure 2. CCl_4-induced expression of PCNA in liver. Acute liver injury was induced by intraperitoneal injection of CCl_4. The liver samples were harvested 0, 24, 48 h post CCl_4 exposure. (A) The level of PCNA was determined by immunoblot analysis. The target proteins were indicated by arrows in the blot. (B) The bands of PCNA and β-actin were semi-quantified by gray scale. n=4, $^{NS}P>0.05$, $^{**}P<0.01$. CCl4, carbon tetrachloride; PCNA, proliferating cell nuclear antigen.

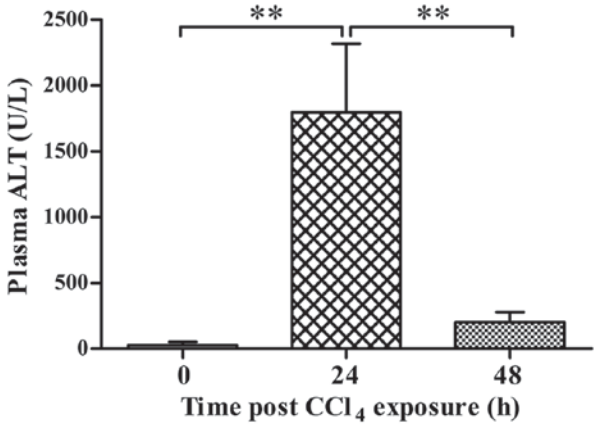

Figure 3. CCl_4-induced elevation of ALT in plasma. Acute liver injury was induced by intraperitoneal injection of CCl_4. The plasma samples were harvested 0, 24, 48 h post CCl_4 exposure and the activity of ALT was determined. n=8, $^{**}P<0.01$. CCl4, carbon tetrachloride; ALT, alanine aminotransferase.

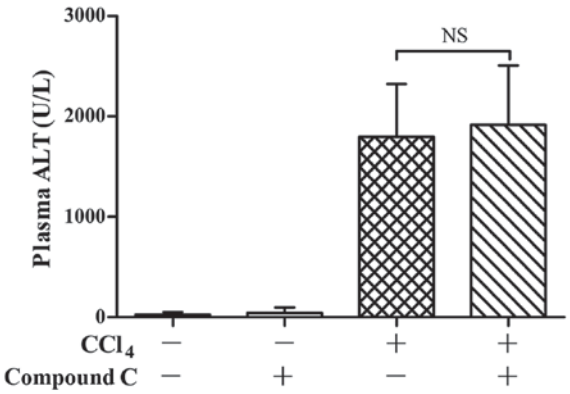

Figure 4. Pretreatment with compound C had no significant effects on CCl_4-induced elevation of ALT in plasma. Acute liver injury was induced by intraperitoneal injection of CCl_4 and the AMPK inhibitor compound C was administered 30 min before CCl_4 exposure. The plasma samples were harvested 24 h post CCl_4 exposure and the activity of ALT was determined. n=8, $^{NS}P>0.05$. AMPK, AMP-activated protein kinase; CCl4, carbon tetrachloride; ALT, alanine aminotransferase; compound C, F6-[4-[2-(1-piperidinyl) ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine.

Discussion

AMPK has been regarded as a crucial metabolic regulator which plays central roles in the maintenance of energy homeostasis (22). Our previous studies have found that AMPK provided anti-inflammatory benefits in mice with acute hepatitis induced by carbon tetrachloride or endotoxin (26,31). Several studies have found that AMPK was involved in the regulation of cellular proliferation (32-34). In the present study, we found that pharmacological inhibition of AMPK suppressed liver regeneration post CCl_4-induced acute liver injury. Consistently, similar results have been obtained in a regenerative model with partial hepatectomy (11,18,35). These data suggests that AMPK might have positive roles in liver regeneration.

CCl_4 is a representative hepatotoxin which induces severe liver injury quickly, the degree of liver damage usually peaks at 24 h after CCl_4 exposure, followed by liver regeneration and

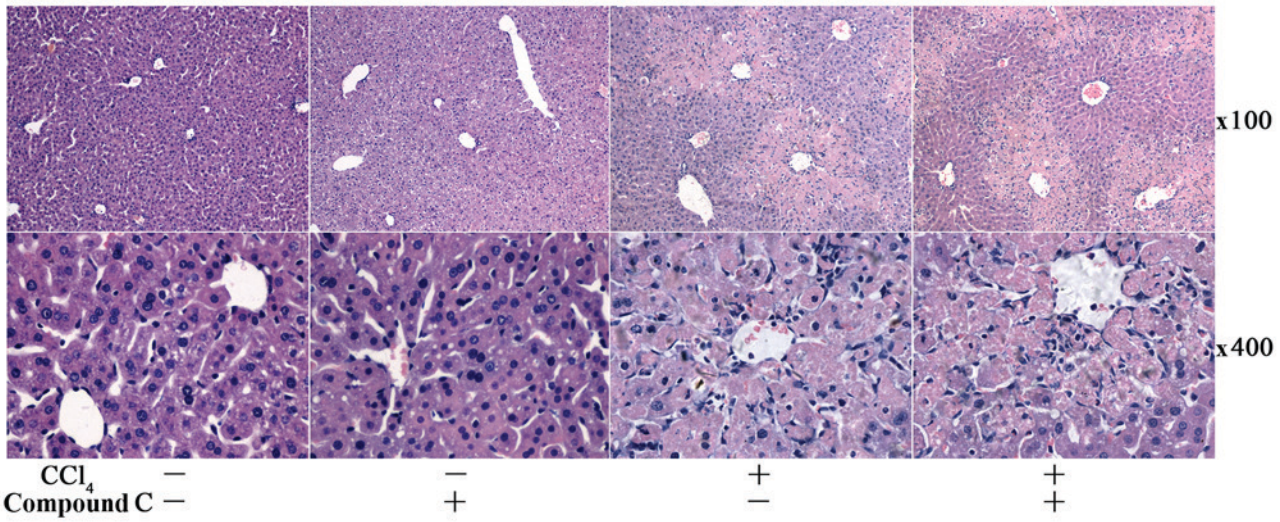

Figure 5. Pretreatment with compound C had no significant effects on CCl_4-induced histological abnormalities in liver. Acute liver injury was induced by intraperitoneal injection of CCl_4 and the AMPK inhibitor compound C was administered 30 min before CCl_4 exposure. The liver samples were harvested 24 h post CCl_4 exposure and stained with hematoxylin and eosin for morphological examination. The representative liver sections of each group were shown. AMPK, AMP-activated protein kinase; CCl4, carbon tetrachloride compound C, F6-[4-[2-(1-piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine.

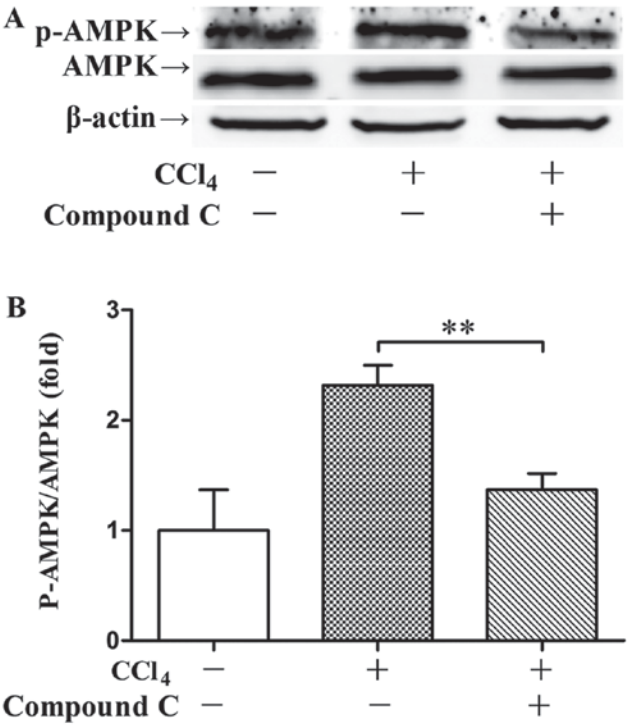

Figure 6. Post-treatment with compound C suppressed CCl_4-induced phosphorylation of AMPK in liver. Acute liver injury was induced by intraperitoneal injection of CCl_4 and the AMPK inhibitor compound C was administered 24 h post CCl_4 exposure. The liver samples were harvested 48 h post CCl_4 exposure. (A) The level of p-AMPK and total AMPK were determined by immunoblot analysis. The target proteins were indicated by arrows in the blot. (B) The bands of p-AMPK and AMPK were semi-quantified by gray scale. n=4, **P<0.01. AMPK, AMP-activated protein kinase; CCl4, carbon tetrachloride compound C, F6-[4-[2-(1-piperidinyl)ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine; p-AMPK, phosphorylated AMPK.

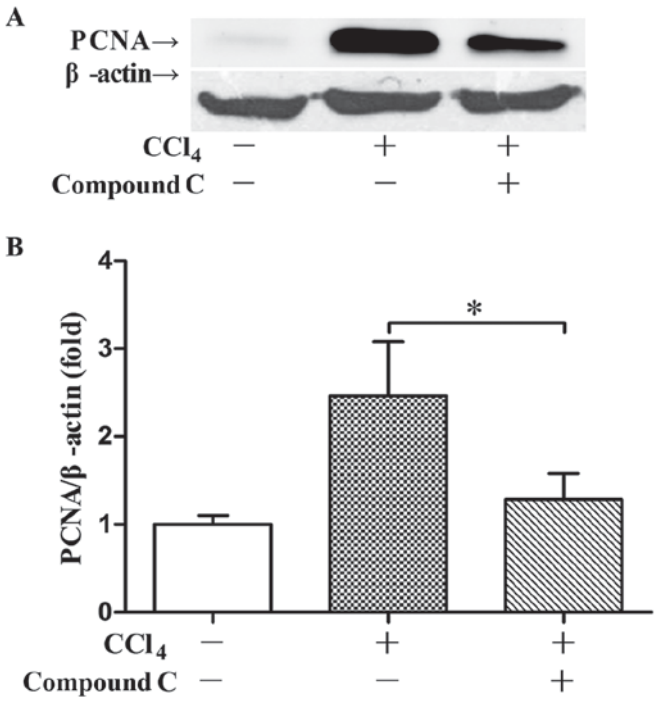

Figure 7. Post-treatment with compound C inhibited CCl_4-induced expression of PCNA in liver. Acute liver injury was induced by intraperitoneal injection of CCl_4 and the AMPK inhibitor compound C was administered 24 h post CCl_4 exposure. The liver samples were harvested 48 h post CCl_4 exposure. (A) The level of PCNA was determined by immunoblot analysis. The target proteins were indicated by arrows in the blot. (B) The bands of PCNA and β-actin were semi-quantified by gray scale. n=4, *P<0.05. AMPK, AMP-activated protein kinase; CCl4, carbon tetrachloride; PCNA, proliferating cell nuclear antigen compound C, F6-[4-[2-(1-piperidinyl) ethoxy]phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine.

recovery (13). In the present study, the immunoblot analysis indicated that the phosphorylation level of AMPK significantly increased 48 h post CCl_4 challenge. In another model with endotoxin-induced acute liver injury, we also found that endogenous AMPK was mainly phosphorylated at the late stage (36). In the present study, pretreatment with the AMPK inhibitor compound C had little effects on the elevation of ALT in plasma and the degree of histological abnormalities in liver. Therefore,

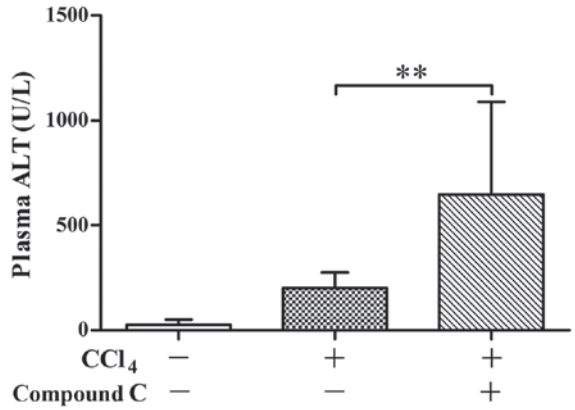

Figure 8. Post-treatment with compound C impaired the recovery of plasma ALT 48 h post CCl_4 exposure. Acute liver injury was induced by intraperitoneal injection of CCl_4 and the AMPK inhibitor compound C was administered 24 h post CCl_4 exposure. The plasma samples were harvested 48 h post CCl_4 exposure and the activity of ALT was determined. n=8, **P<0.01. AMPK, AMP-activated protein kinase; CCl4, carbon tetrachloride; ALT, alanine aminotransferase compound C, F6-[4-[2-(1-piperidinyl)ethoxy] phenyl]-3-(4-pyridinyl)-pyrazolo[1,5-a]pyrimidine.

endogenous AMPK mainly functions at the regeneration stage in CCl_4-exposed mice.

Because the phosphorylation of AMPK was associated with the expression of PCNA, a well-documented molecular marker of cellular proliferation (37), we also determined the roles of AMPK at the regeneration stage by treatment with the AMPK inhibitor 24 h post CCl_4 exposure. The results indicated that post-insult inhibition of AMPK significantly suppressed the expression PCNA, which was accompanied with delayed decline of ALT. These data suggests that CCl_4-induced activation of AMPK might act as a positive regulator in liver regeneration.

There is a considerable amount of researches have demonstrated a crucial link between AMPK and cellular proliferation (18-20). A study in cardiac fibroblasts found that AMPK suppressed cell cycle progression via modulating the expression of p21 and p27 (38). In addition, the suppressive effects of AMPK on tumor cell proliferation have been observed in neuroblastoma cells, breast cancer cells, prostate cancer cells and cervical cancer cells (39). These data suggests that activation of AMPK might prevent proliferation under some circumstance.

On the contrary, it was reported that treatment with the AMPK inhibitor compound C induced cell cycle arrest and suppressed the growth of colorectal cancer cells (40,41). The stimulatory actions of AMPK on proliferation were also confirmed by molecular approaches in prostate cancer cells and glioma cells (20,42). In addition, the *in vivo* promotive activities of AMPK on regeneration were observed in mice with mitochondrial myopathy or partial hepatectomy (19,21). Therefore, AMPK might have positive or negative effects on cellular proliferation in different pathological conditions.

Taken together, the present study found that endogenous AMPK was mainly activated at the regeneration stage in mice with CCl_4-induced acute liver injury and it might function as a positive regulator in liver regeneration. Although the molecular mechanisms underlying the stimulatory activities of AMPK on liver regeneration remain to be further investigated, the present study suggests that AMPK might play crucial roles in the recovery of liver structure and function after severe liver damage.

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