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Protective effect of L-theanine on carbon tetrachloride-induced acute liver injury in mice

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ABSTRACT

We studied effects of L-theanine, a unique amino acid in tea, on carbon tetrachloride (CCl_4)-induced liver injury in mice. The mice were pre-treated orally with L-theanine (50, 100 or 200 mg/kg) once daily for seven days before CCl_4 (10 ml/kg of 0.2% CCl_4 solution in olive oil) injection. L-theanine dose-dependently suppressed the increase of serum activity of ALT and AST and bilirubin level as well as liver histopathological changes induced by CCl_4 in mice. L-theanine significantly prevented CCl_4-induced production of lipid peroxidation and decrease of hepatic GSH content and antioxidant enzymes activities. Our further studies demonstrated that L-theanine inhibited metabolic activation of CCl_4 through down-regulating cytochrome P450 2E1 (CYP2E1). As a consequence, L-theanine inhibited oxidative stress-mediated inflammatory response which included the increase of TNF-α and IL-1β in sera, and expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) in livers. CCl_4-induced activation of apoptotic related proteins including caspase-3 and PARP in mouse livers was also prevented by L-theanine treatment. In summary, L-theanine protects mice against CCl_4-induced acute liver injury through inhibiting metabolic activation of CCl_4 and preventing CCl_4-induced reduction of anti-oxidant capacity in mouse livers to relieve inflammatory response and hepatocyte apoptosis.

1. Introduction

Acute hepatic injury induced by hepatotoxins has been recognized as one of the most important pharmacovigilance concerns and the leading cause for drug withdrawal on safety grounds [1]. Because of its unique metabolism and relationship to the gastrointestinal tract, the liver is a crucial target of the toxicity of drugs, xenobiotics, and oxidative stress [2]. Carbon tetrachloride (CCl_4) is a well-known hepatotoxin that is widely used to induce toxic liver injury in a range of laboratory animals. The toxicity of CCl_4 results from its reductive dehalogenation by cytochrome P450 (CYP450) into the highly reactive free radical trichloromethyl radical ($CCl_3^{\cdot}$). The CCl_3 radical alkylates cellular proteins and other macromolecules with a simultaneous attack on polyunsaturated fatty acids to produce lipid peroxides, leading to liver damage [3]. In CCl_4 induced hepatic injury, free radicals probably activate Kupffer cells which mediate the hepatic inflammation process by producing tumor necrosis factor-α (TNF-α) and other pro-inflammatory cytokines. An early rise of TNF-α level induces expression of inflammatory mediators including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) through activating NF-κB [4]. Death of hepatocytes and other hepatic cell types is a characteristic feature in drug/toxicant-induced liver injury [5]. CCl_4 induced the excessive ROS generation depletes the endogenous antioxidant enzymes and triggers hepatocyte apoptosis through activation of the caspases cascade, such as caspase-3, -8, and -9 [6].

L-theanine (L-glutamate-c-ethylamide), a water-soluble amino acid found specifically in green tea (*Camellia sinensis*), comprises 1–2% of dry weight of tea leaves. L-theanine has demonstrated antioxidative properties and neuroprotective effects against ischemia and Parkinson-related neurotoxicants [7]. L-theanine also attenuates β-amyloid-induced cognitive dysfunction and neurotoxicity through reducing oxidative damage and preventing signaling of ERK/p38 kinase and NF-κB pathways [7]. L-theanine decreases adriamycin (ADR) and doxorubicin induced side toxicity through normalizing the increase of lipid peroxide level and reduction of glutathione peroxidase activity and GSH level [8,9]. Our previous study shows that L-theanine prevents alcoholic liver injury through enhancing the antioxidant capability of hepatocytes

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[10]. However it is unclear if L-theanine can prevent CCl_4 or other toxic chemical-induced liver injury. S-adenosyl-L- methionine (SAM) is a precursor for polyamines, and in liver, it is also a precursor for reduced glutathione (GSH) [11]. SAM can protect against liver injury induced by many hepatotoxins [12–14].

In the present study, we studied the protective effects of L-theanine on mice against CCl_4-induced acute liver injury and analyzed the possible mechanism. SAM was used as a positive control in our study.

2. Materials and methods

2.1. Chemicals

L-theanine was purchased from Agro Biotech (Jiangsu, China). Carbon tetrachloride (CCl_4) was purchased from Jiangsu Qiangsheng Chemical Co. (Shanghai, China). Ademetionine 1,4-Butanedisulfonate (AMB) was from Hospira S.P.A. (Italy). All other chemicals were of the highest quality available commercially.

2.2. Animals

Male ICR mice (6–7 weeks) weighing 18–22 g were purchased from Laboratory Animal Center, Yangzhou University (Yangzhou, China). Laboratory animal handling and experimental procedures were performed in accordance with the requirements of Provisions and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by Science and Technology Department of Jiangsu Province.

2.3. Experimental design

Mice were randomly divided into eight experimental groups of 10 animals each. Group I served as normal control was orally administered with vehicle only (sterile distilled water) during the experiment. Mice in Group II received orally L-theanine (200 mg/kg/day) only during the experiment. Mice in Group III were orally given sterile distilled water for 7 days. Mice in Groups IV, V and VI were orally administered with three different doses of L-theanine (50, 100 or 200 mg/kg/day) respectively for 7 days. Mice in Group VII served as the positive control were orally administered AMB (300 mg/kg) daily for 7 days. On the 7th day, Mice in group III, IV, V, VI and VII received an intraperitoneal injection of CCl_4 (10 ml/kg body weight of 0.2% CCl_4 solution in olive oil) two hours after the last administration of vehicle, L-theanine, or AMB. Mice in Group VIII were orally given sterile distilled water for 7 days before CCl_4 intoxication and then were treated with L-theanine (200 mg/kg) once orally 30 min after CCl_4 treatment serving as a post administration group.

2.4. Measurement of serum ALT, AST and bilirubin

At 24 h after CCl_4 treatment, the blood samples were collected by retroorbital bleeding. Collected blood was centrifuged (3500 r/min, 10 min, 4 °C) and serum was obtained. Activities of both alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and bilirubin level in serum were measured spectrophotometrically under an Elx 800 Universal Microplate Reader (BIO-TEK, INC) according to the instructions supplied with the commercial assay kits (Jiancheng Biotechnology, China).

2.5. Detection of lipid peroxidation products, GSH/GSSG, and activities of antioxidant enzymes in liver

Livers were homogenized and the homogenates were centrifuged at 3500 r/min for 10 min at 4 °C, and the supernatants were subjected to further measurement. Lipid peroxidation was determined by measuring the thiobarbituric acid-reactive substances (TBARS) in the homogenate and expressed as MDA (malondialdehyde) concentration. The concentrations of GSH (glutathione), GSSG (oxidized glutathione) and activities of GR (glutathione reductase), SOD (superoxide dismutase) and CAT (catalase) were determined according to the instructions supplied with the commercial assay kits (Jiancheng Biotechnology, China).

2.6. Assessment of serum TNF-α and IL-1β levels

The blood samples were collected from retro-orbital sinus 18 h or 24 h after CCl_4 treatment. Serum IL-1β and TNF-α levels were determined under an Elx 800 Universal Microplate Reader (BIO-TEK, INC) according to the protocols of the ELISA kits (ExCell Biology, China).

2.7. Histological examinations

For the histopathological studies, mice were sacrificed 24 h after CCl_4 injection and the livers were removed and stored in the same fixation for 24 h at 4 °C. The fixed tissues were embedded in paraffin, sectioned, deparaffinized, and rehydrated using the standard techniques. The extent of CCl_4-induced hepatic damage was evaluated by assessing the morphological changes in the liver sections stained with hematoxylin and eosin (Jiancheng Biotechnology, China).

2.8. Immunohistochemistry of COX-2 and iNOS

Liver sections were immunostained for COX-2 and iNOS according to the instructions of the streptomyces avidin–biotin complex (SABC) kit (Boster Biotechnology, China). Briefly, sections were incubated with rabbit polyclonal antibody against COX-2 or iNOS (Bioworld Technology, USA) overnight at 4 °C and then were treated with biotinylated anti-rabbit IgG developed with DAB solution and counterstained with hematoxylin. The protein expression of COX-2 and iNOS was measured semi quantitatively by detecting total COX-2 and iNOS positive area under the Nikon E2000 microscope with Image-Pro Plus image analysis software.

2.9. Western blot

For protein preparation, liver tissues were homogenized in RIPA lysis buffer. The lysate was sonicated for 1 min and centrifuged at 15,000*g* for 20 min at 4 °C. Then protein was mixed with loading buffer, boiled for 5 min and subjected to electrophoresis on 10% SDS - polyacrylamide gels and transferred onto nitrocellulose membranes. After blocking the nitro cellulose paper in non-fat dry milk (5%) in TBS (10 mM Tris and 150 mM NaCl) for 1 h, membranes were incubated for 18 h with active Caspase-3, Cleaved PARP (Bioworld Technology, USA) and CYP2E1 (Proteintech Group, USA) primary antibodies. Then membranes were incubated with secondary antibodies for 1 h. Before and after incubation with secondary antibodies, membranes were washed with TBS and TBST (TBS containing 0.1% Tween-20). Immunoreactivity was visualized by the Li-COR Odyssey Infrared Imaging System.

2.10. Statistical analysis

Data was presented as meanSD. A one-way repeated measure analysis of variance (ANOVA) was used to compare the results between two groups and a Student's *t*-test was used to determine the significance of the difference between experimental groups. Differences with $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Effect of L-theanine on CCl_4-induced hepatic injury

The serum activities of both ALT and AST and level of bilirubin are biochemical markers for the early acute hepatic damage [15]. Serum activities of ALT and AST significantly increased and bilirubin level was elevated 24 h after CCl_4 treatment, indicating CCl_4-induced injury of hepatic cells (Fig. 1A–C). L-theanine treatment dose-dependently prevented the elevation of aminotransferase activities and bilirubin level induced by CCl_4, whereas, L-theanine itself did not alter serum ALT, AST and bilirubin (Fig. 1A–C). AMB, at dose of 300 mg/kg, significantly also normalized the change of ALT and AST activities as well as bilirubin content in the mouse serum induced by CCl_4. These results suggested that L-theanine could protect mice against CCl_4-induced liver injury.

Histopathological studies also provided important evidence supporting the results from biochemical analysis. Liver sections from control and L-theanine alone treated mice showed normal liver architecture (Fig. 1D). CCl_4 induced an apparent injury in mouse livers, which includes large areas of extensive pericentral necrosis with loss of hepatic architecture and moderate increase in inflammatory cell infiltration (Fig. 1D). L-theanine pre-treated to mice dose-dependently prevented the development of histopathological changes induced by CCl_4 (Fig. 1D). AMB also inhibited the effect of CCl_4 on mouse hepatic histopathology.

3.2. Effect of L- theanine on CCl_4-induced lipid peroxidation and CYP 2E1 expression

The level of TBARS was apparently higher in the CCl_4-treated group than that in the control group, whereas, in the mice pre-treated with 100 mg/kg, 200 mg/kg L-theanine and 300 mg/kg AMB, CCl_4-induced increase of hepatic TBARS was significantly prevented (Fig. 2A). However, in L-theanine post-treated group, TBARS level was as similar as that in CCl_4 alone treated group (Fig. 2A). These data indicated that pre-treatment of L-theanine inhibited CCl_4-induced increase of lipid peroxidation.

Many hepatotoxicants including CCl_4 require metabolic activation, particularly by the liver CYP450 enzymes, to form reactive, toxic metabolites, which in turn cause liver injury in experimental animals and humans. Immunoblot analysis was thus performed to examine the effect of L-theanine on hepatic CYP2E1 protein level. As shown in Fig. 2B, CCl_4 induced an increase of CYP2E1 protein level and this increase of CYP2E1 was dose-dependently inhibited by pre-treatment of L-theanine to mice. AMB, at dose of 300 mg/kg,

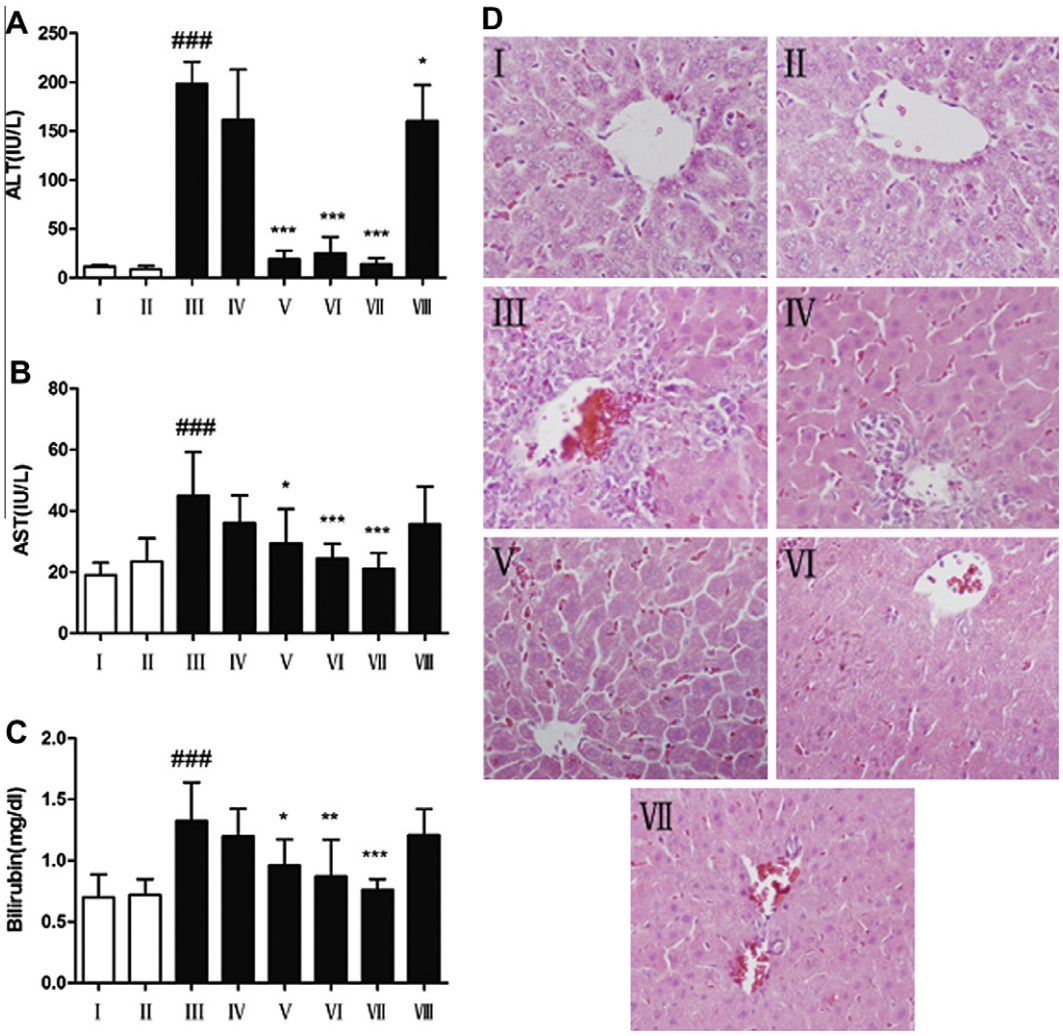

Fig. 1. Effects of L-theanine on CCl_4-induced hepatic injury. Animals were divided into following groups: (I) Normal control, (II) L-theanine(200) mg/kg alone treated group, (III) CCl_4 treated control group, (IV) L-theanine (50 mg/kg) plus CCl_4 treated group, (V) L-theanine (100 mg/kg) plus CCl_4 treated group, (VI) L-theanine (200 mg/kg) plus CCl_4 treated group, (VII) AMB (300 mg/kg) plus CCl_4 treated group, (VIII) Post-treatment of L-theanine (200 mg/kg) plus CCl_4 treated group. Serum ALT (A) and AST (B) activities and bilirubin (C) level were measured. (D) Mouse liver sections stained with HE to show histopathology of livers (200×). Each value represents the mean ± S.D. ($n = 10$). $^{###}p < 0.001$ compared with group I. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with group III.

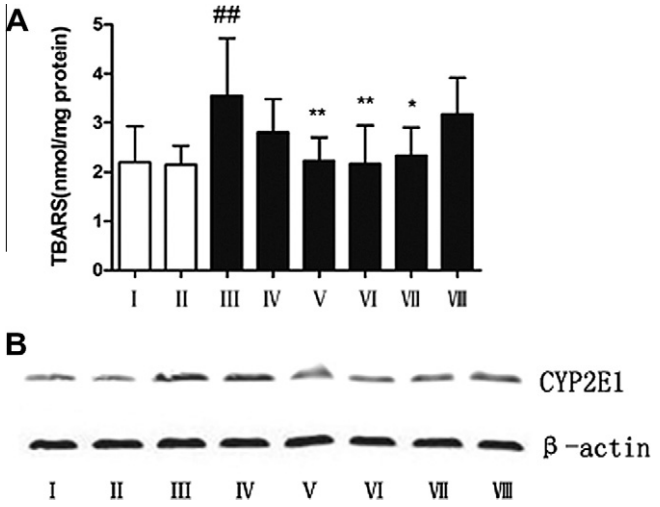

Fig. 2. Effects of L-theanine on CCl_4-induced lipid peroxidation production and CYP 2E1 expression. Animal treatment is as same as described in Fig.1. (A) The content of TBARS was determined in livers. (B) Expression of hepatic CYP2E1 was detected by western blotting. Each value represents the mean ± S.D. ($n = 10$). $^{\#\#}p < 0.01$ compared with group I. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with group III.

also suppressed CCl_4 -induced increase of CYP2E1 in mouse livers, but post-treatment of L-theanine did not alter CYP2E1 level. These results suggested that L-theanine could protect mouse livers by inhibiting metabolic activation of CCl_4.

3.3. Effect of L- theanine on CCl_4-induced dysfunction of hepatic anti-oxidation

Activated CCl_4 could lead to oxidative stress in livers. GSH, a nonenzymatic antioxidant in the detoxification pathway, presents at a high level in normal livers [16]. GR, SOD and CAT are three anti-oxidant enzymes. GR promotes the NADPH-dependent reduction of GSSG to GSH. SOD eliminated ROS derived from the peroxidative process of xenobiotics in hepatic tissues. CAT is a key component of the antioxidant defense system. Our results showed that CCl_4 significantly reduced the GSH level in mouse livers, but if the mice were pre-treated with L-theanine and AMB, hepatic GSH reduction was reversed (Fig. 3A). There was no significant difference in GSSG content among groups (Fig. 3B). As shown in Fig. 3C–E, hepatic activities of GR, SOD and CAT were conspicuously decreased in CCl_4 treated mice, whereas both L-theanine and AMB reversed such reduction of the activities of these enzymes.

3.4. Effect of L-theanine on inflammatory response and apoptosis in mouse livers

Both TNF-α and IL-1 play an important role in the pathogenesis of the experimental fulminant hepatitis model and administration

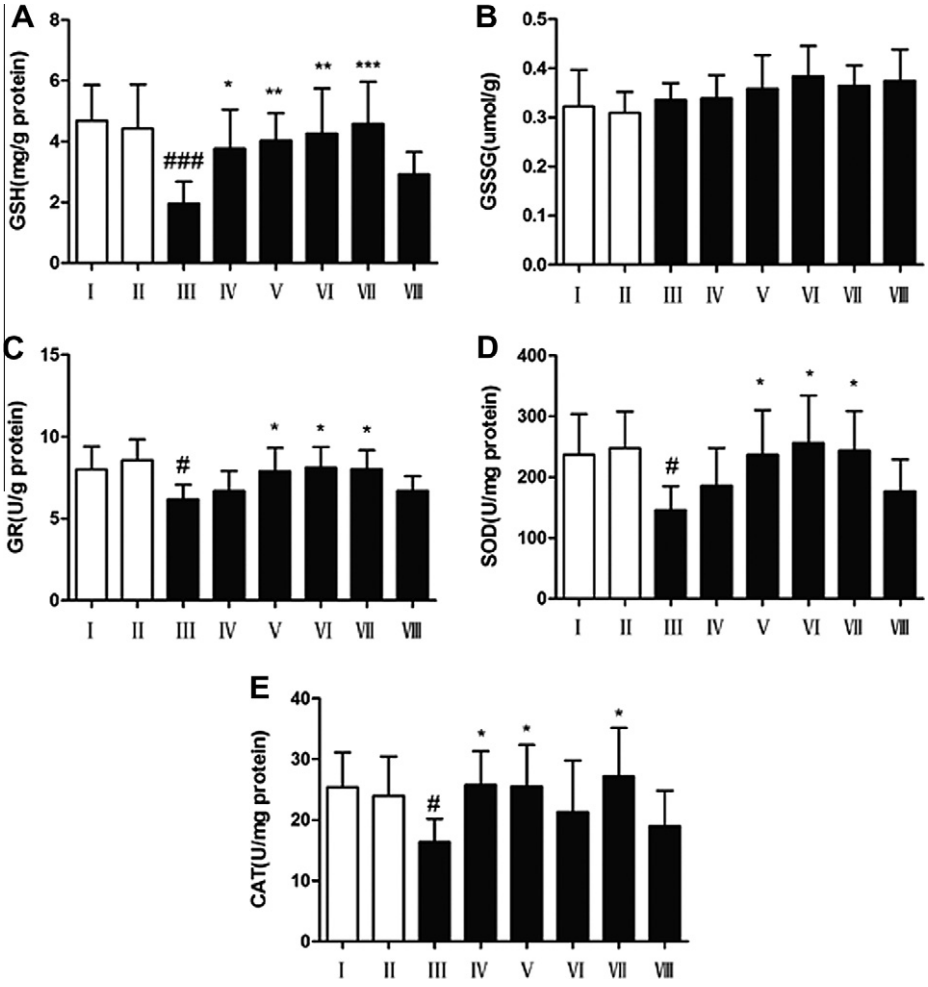

Fig. 3. Effects of L-theanine on CCl_4-induced reduction of hepatic anti-oxidant function. Animal treatment is as same as described in Fig.1. Liver GSH (A) and GSSG (B) levels and activities of antioxidant enzymes (C, D, E) were measured. Each value represents the mean ± S.D. ($n = 10$). $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ compared with group I. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with group III.

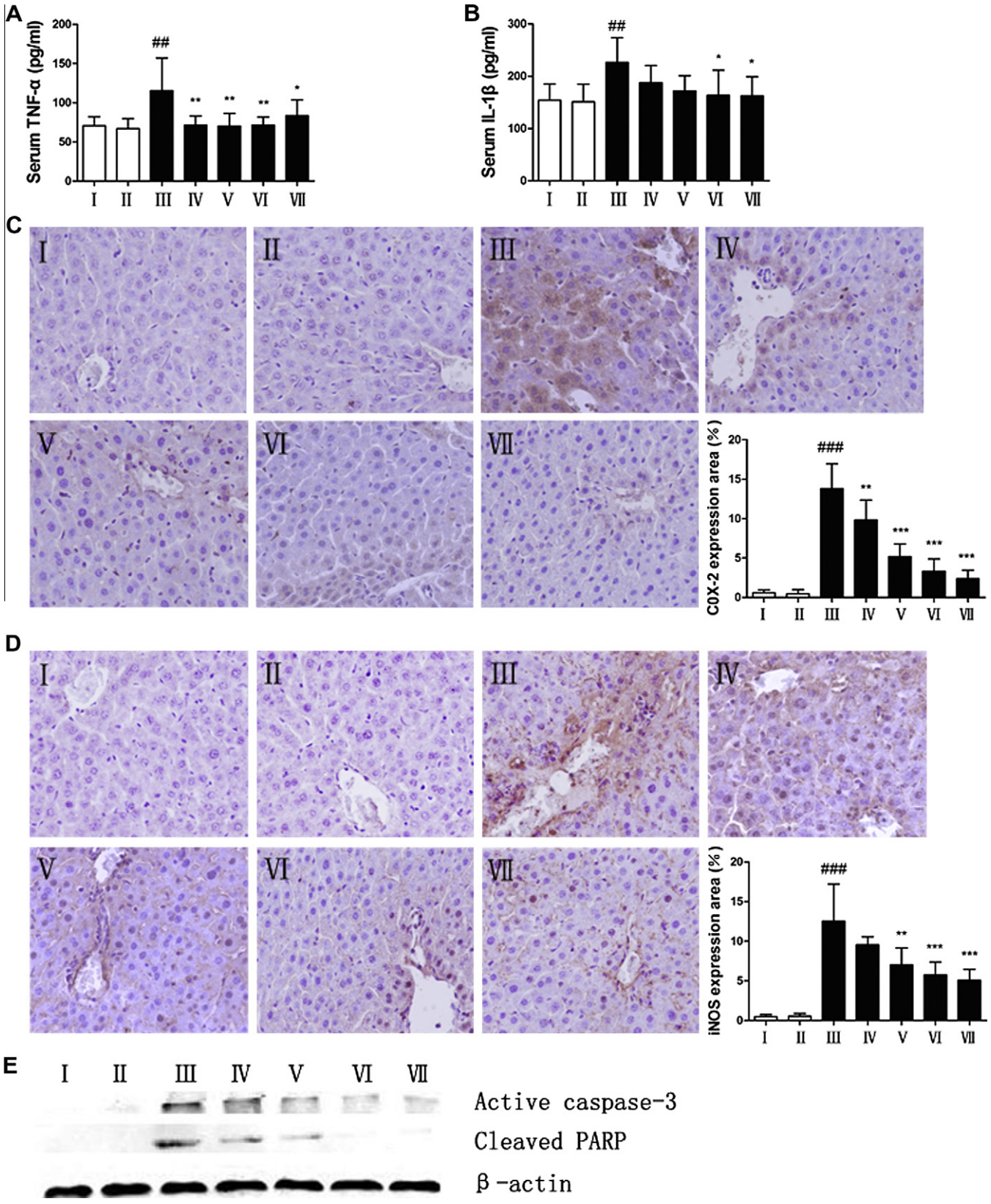

Fig. 4. Effects of L-theanine on CCl_4-induced expression of inflammatory mediators and activation of hepatocyte apoptosis. Animals were divided into eight groups described in Fig.1. Serum TNF-α (A) and IL-1β (B) levels were measured by ELISA. Hepatic COX-2 (C) and iNOS (D) were detected by immunohistochemistry (200×). The expression of COX-2 and iNOS was analyzed semi-quantitatively. (E) Hepatic active caspase-3 and cleaved PARP were detected by western blotting. Data represent the mean ± S.D. ($n = 10$). $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ compared with group I. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with group III.

of CCl_4 stimulates production of TNF-α and IL-1 in both serum and liver [17]. As expected, serum TNF-α and IL-1β levels elevated 18 h and 24 h after CCl_4 stimulation. Pre-treatment of L-theanine apparently decreased TNF-α and IL-1β level in CCl_4-intoxicated mice

(Fig. 4A and B). iNOS and COX-2 are key enzymes in production of two inflammatory factors NO and prostaglandin respectively. We observed the effects of L-theanine on hepatic COX-2 and iNOS levels by using immunohistochemical assay. As shown in Fig. 4C and D, CCl_4 injection dramatically increased COX-2 and iNOS immunopositivity in necrotic areas of the livers compared with normal control, which was attenuated by L-theanine and AMB. The semi-quantitative analysis to immunopositive staining of COX-2 and iNOS were shown in Fig. 4C and D.

It has been reported that the increased apoptosis of liver hepatocytes was observed in CCl_4-induced acute liver injury [18]. We performed immunoblot analysis to examine the effect of L-theanine on hepatic active caspase-3 and cleaved PARP proteins. As shown in Fig. 4E, active caspase-3 and cleaved PARP fragments increased in CCl_4 treated group as compared to the control group, which were attenuated by L-theanine and AMB pre-treatment. These results indicated that L-theanine prevented the signaling of apoptotic signal pathway trigged by CCl_4.

4. Discussion

CCl_4-induced hepatic injury is an experimental model widely used for hepatoprotective drug screening. In these injured livers, cell membrane integrity is broken and the enzymes (such as ALT, AST, etc.) in cell plasma leak out. In the present study, pre-treatment of L-theanine to mice significantly prevented CCl_4-induced increase of serum activity of AST and ALT and level of bilirubin. Our histological observation also provided the consistent results that suggested the protective effect of L-theanine on mice against the liver injury induced by CCl_4.

Hepatotoxicity of CCl_4 is thought to involve two phases. First, CCl_4 is metabolized mainly by microsomal CYP2E1 to form highly reactive trichloromethyl radicals, which initiates lipid peroxidation and hepatocellular necrosis [19,20]. The CYP450 system is abundantly localized in the liver [21]. The concentration of L-theanine in the liver is significantly increased 1 h after it was administration. It has been reported that, the theanine pre-administration inhibited the ethanol- enhanced CYP2E1 activity [22]. Our study demonstrated that CCl_4 increased CYP2E1, and L-theanine significantly suppressed CYP2E1 expression induced by CCl_4. Consistent with this result, the experiments showed that CCl_4 induced production of lipid peroxidation. These data indicated that L-theanine could inhibit CCl_4-induced oxide stress by preventing the activation of CCl_4. Theanine itself does not have antioxidant properties, but intake of theanine was thought to be effective against the tissue changes with GSH level reduction [22]. In the present study, we found that decrease of GSH in mouse livers induced by CCl_4 was also prevented by L-theanine. Although GSH depletion may enhance the peroxidation process, it alone is not sufficient to induce lipid peroxidation. SOD catalyzes the dismutation of the superoxide anion to H_2O_2 and O_2, whereas CAT decomposes H_2O_2 and protects the tissues from highly reactive hydroxyl radicals. GR is the primary enzyme to maintain glutathione redox status. It has been well documented that CCl_4 decreased the antioxidant capacity of mouse liver by inhibiting the activity of the antioxidant enzymes. Our study demonstrated L-theanine significantly prevented the decrease of activities of SOD, CAT and GR in mice induced by CCl_4. These findings indicated that L-theanine protected mice against CCl_4-induced oxidative stress via multiple routes.

The second phase of CCl_4 hepatotoxicity involves the inflammatory responses. Inflammatory cells including Kupffer cells activated by oxidative stress release an excess of pro-inflammatory cytokines such as TNF-α, and IL-1β [23]. In the present study, CCl_4-induced increase of the serum levels of IL-1β and TNF-α were significantly prevented by treatment of L-theanine. A previous study showed that the production of these inflammatory factors is associated with NF-κB pathway in CCl_4-induced acute liver injury [24]. NF-κB is an early response transcription factor, and nuclear translocation of NF-κB leads to gene expression of pro-inflammatory cytokines, chemokines, adhesion molecules, matrix metalloproteinases, COX-2, and iNOS [25]. The final products of iNOS and COX-2 may initiate the cascade of inflammatory response in injured liver. The release of TNF-α from activated Kupffer cells up-regulates iNOS and stimulates production of nitric oxide (NO). Overproduction of NO by iNOS may mediate CCl_4-induced acute hepatotoxicity through up-regulation of inflammatory responses [26]. COX-2 catalyses the committed step in the prostaglandin production pathway and is induced by several different stimuli including TNF-α, IL-1β and ROS [27]. The inhibition of COX-2 has been shown to exert the hepatoprotective effect in CCl_4-induced liver damage [28]. In the current study, L-theanine significantly inhibited CCl_4-induced expression of iNOS and COX-2 in mouse livers. Taken together, our results demonstrated that L-theanine attenuated CCl_4-induced inflammatory cascade in mouse livers.

CCl_4-intoxication has been suggested to cause severe apoptosis [29]. Caspase-3 is the dominant executioner of programmed cell death involved in the cleavage of many apoptosis related proteins. The cleavage of PARP by activated caspase-3 provides one of the most utilized diagnostics for detection of apoptosis in many cell types. CCl_4 promoted caspase-3 activation and PARP cleavage indicating that CCl_4-induced cell death involved caspase-3 activation. Pre-treatment of L-theanine profoundly decreased caspase-3 activation and PARP cleavage induced by CCl_4, which suggested that L-theanine decreased the apoptosis in hepatocytes trigged by CCl_4.

In summary, the present study demonstrates that L-theanine protects mice against CCl_4-induced acute liver damage. The hepatoprotective effects of L-theanine are depend on its ability to inhibit bioactivation of CCl_4 through down-regulating CYP2E1 and enhance anti-oxidant capacity which decreased by CCl_4. As a result, L-theanine inhibits CCl_4-induced hepatic damage, which includes the suppression of CCl_4-induced inflammatory response and hepatocyte apoptosis. Our results suggest that L-theanine is a potential hepatoprotective agent against chemical or drug-induced hepatotoxicity.

Acknowledgments

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