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Licochalcone E protects against carbon tetrachloride-induced liver toxicity by activating peroxisome proliferator-activated receptor gamma

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Abstract. The present study aimed to investigate the hepatoprotective role of Licochalcone E (LCE) and its mechanism of action in a mouse model of carbon tetrachloride (CCl_4)-induced liver toxicity. Hepatotoxicity was induced in Kunming mice via an intraperitoneal injection (IP) of CCl_4, 10 ml/kg body weight, diluted with corn oil at a 1:500 ratio. LCE was administered once a day for 7 days (IP) as pretreatment at a dose of 5 mg/kg/day. The levels of C-reactive protein (CRP) and tumor necrosis factor (TNF)-α were analyzed to determine the inflammation status. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were analyzed using ELISA assays. Liver ultrastructure was observed via optical microscopy. The mRNA and protein expression levels of peroxisome proliferator-activated receptor (PPAR)γ, and nuclear factor (NF)-κB were assayed using quantitative polymerase chain reaction and western blot analysis, respectively. Pretreatment with LCE decreased levels of ALT, AST, CRP and TNF-α, and NF-κB expression in the experimental hepatotoxicity mice model induced by CCl_4. In addition, LCE increased the expression of PPARγ and normalized the hepatic histoarchitecture. However, the effects of LCE were reversed by cotreatment with the PPARγ inhibitor GW9662. The present study suggests that LCE may be used for the treatment of hepatotoxicity, and primarily exhibits its protective role through a PPARγ/NF-κB-mediated pathway.

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Abbreviations: LCE, Licochalcone E; CRP, C-reactive protein; TNF-α, tumor necrosis factor-α; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PPARγ, peroxisome proliferator-activated receptor gamma; NF-κB, nuclear factor-k-gene binding

Key words: licochalcone E, PPARγ, NF-κB, Anti-inflammatory, hepatotoxicity

Introduction

As a vital organ of the human body, the liver is in control of the detoxification of exogenous xenobiotics, drugs, viral infections, and chronic alcoholism. Liver diseases are one of the major causes of mortality and morbidity worldwide, and drug-induced liver toxicity is a major cause of hepatic dysfunction (1). Liver damage is a widespread pathology, which in most cases involves oxidative stress and is characterized by a progressive evolution from steatosis to chronic hepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (2,3). In recent years, attention has been focused on the biotransformation of chemicals into highly reactive metabolites that initiate cellular toxicity. Carbon tetrachloride (CCl_4-)-induced hepatotoxicity in animal models has been widely used to investigate the hepatoprotective effect of natural compounds (4,5).

Hepatotoxicity injury is a complicated process that involves various mechanisms. Inflammatory damage, which is one of the most important mechanisms involved in hepatotoxicity injury (6). Several studies demonstrate that numerous inflammatory cytokines are produced in hepatotoxic liver tissues (7). In addition, oxidative damage is considered as a mechanism that contributes to the initiation and progression of hepatic damage in a variety of liver disorders (8,9). Therefore, anti-inflammatory drugs and antioxidants obtained from plants represent a logical therapeutic strategy for the treatment of liver diseases.

Several lines of evidence suggest that PPARγ/NF-κB signaling pathway may exert anti-inflammatory effects by negatively regulating the expression of proinflammatory genes induced during macrophage differentiation and activation. Study demonstrates that activation of PPARγ can inhibits the activation of NF-κB, resulting in decreased release of inflammatory factors and degree of inflammatory damage (10,11).

Licochalcone E (LCE) is a flavonoid, which belongs to the retrochalcone family, is isolated from the roots of Chinese licorice (12). Some previous studies suggest that LCE possesses several useful pharmacological properties, such as antioxidant and anti-inflammatory activities (13,14). There are several studies that show LCE-mediated anti-inflammatory properties maybe though activation of PPARγ, and inhibition of NF-κB (15,16). Therefore, in the present study, we evaluate that whether the hepatoprotective effects of LCE is related to the PPARγ/NF-κB-mediated signal pathway.

Materials and methods

Test compounds, chemicals, and reagents. Licochalcone E (purity 98≥%) was purchased from Chengdu Must Bio-Technol Co., Ltd., (Chengdu, China). GW9662 was purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland). The other chemicals and reagents were of analytical grade.

Animals and experimental groups. Kunming mice (20-30 g) were obtained from Jinan Jinfeng Experimental Animal Breeding Co., Ltd., (Jinan, China; license no. SCXK (lu) 2014-0006). All experimental procedures were approved by the Institutional Animal Care and Use Committee of the National Institute of Pharmaceutical Education and Research.

Preliminary experiments. Dosage of 1, 2.5, 5 and 10 mg/kg/day of LCE were selected to determine the optimal dose for hepatoprotection. According to the biochemical parameters (ALT and AST) and general histology survey of livers, we found that 5 mg/kg/day of LCE had the best hepatoprotective effect. Therefore, the dose of 5 mg/kg/day of LCE was selected for using in next experiments (see Fig. 1).

Animals and experimental groups. The mice were randomly divided into four groups (n=12/group): Control group (Con), CCl_4 group (CCl_4), LCE+CCl_4 group (LCE + CCl_4) and LCE + GW9662 + CCl_4 group (LCE + GW + CCl_4). In the Con group, the mice received distilled water for seven days. In the CCl_4 group, the mice received distilled water as in the previous group and intraperitoneally administered with 10 ml/kg body weight of CCl_4 diluted with corn oil at a ratio of 1:500 once on day 8. In the LCB + CCl_4 group, LCE was administered intragastrically once daily for seven days (5 mg/kg/day) followed by a single IP dose of CCl_4 (10 mg/kg body weight) on day 8. In the LCB + GW + CCl_4 group, LCE was administered intragastrically once daily for seven days (5 mg/kg/day), and then followed by a single IP dose of GW9662 (5 mg/kg body weight) and a single IP dose of CCl_4 (10 mg/kg body weight) on day 8.

Serum separation. After 24 h of CCl_4 administration, the animals were anesthetized using ether, and 1 ml of blood was collected through cardiac puncture. Blood was allowed to clot and centrifuged at 4,000 g for 10 min, the serum was separated and used for next assays.

Estimation of biochemical parameters. The levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were spectrophotometrically analyzed according to the instructions of ELISA (Nanjing Jiancheng Bio-Engineering Institute Co., Ltd., Nanjing, China).

Table I. Primer sequences.

Gene	Primer sequence (5'→3')
PPARγ	
Forward	GGAAGACCACTCGCATTCCTT
Reverse	GTAATCAGCAACCATTGGGTCA
NF-κB/p65	
Forward	ATGGCAGACGATGATCCCTAC
Reverse	CGGATCGAAATCCCCTCTGTT
GAPDH	
Forward	TGCTGGTGCTGAGTATGTCG
Reverse	TTGAGAGCAATGCCAGCC

Table II. The effect of LCE (5 mg/kg) on the normal mice liver.

Group	ALT(U/L)	AST(U/L)
Control	41.62±2.56	61.57±3.68
Mice with only LCE (5 mg/kg) administration	45.13±2.54	66.75±4.23

Assay of oxidative stress and inflammation. The serum was separated and used for assays of oxidative stress, inflammation. Superoxide dismutase (SOD) activity, malondialdehyde (MDA) level, tumor necrosis factor-α (TNF-α), C-reactive protein (CRP), and interleukin-6 (IL-6) were spectrophotometrically analyzed using ELISA (Tsz Biosciences, Greater Boston, USA) and following manufacturer's instructions.

General histology survey of livers. The mice were killed by cervical dislocation, and the livers were excised, washed in phosphate buffer, and dried using tissue paper. Hepatic tissue was fixed in 10% formalin and embedded in paraffin. The paraffin-embedded tissue was sectioned 5 μm thick, placed on slides, deparaffinized in xylene, hydrated in decreasing concentrations of ethanol, and washed in water. Conventional hematoxylin and eosin staining was done. After HE staining, the sections were observed under a light microscope.

Quantitative real-time PCR. Total RNA was isolated and purified using the RNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. One microgram of total RNA per sample was converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Primers for quantitative real-time PCR (qPCR) were designed using Primer3 software and are listed in Table I. Amplification of each target DNA was performed using SsoFast EvaGreen Supermix (Bio-Rad, USA) with 500 nM of each primer and 10 ng of cDNA per reaction. Each qPCR assay was performed in triplicate in a Bio-Rad CFX96 thermal

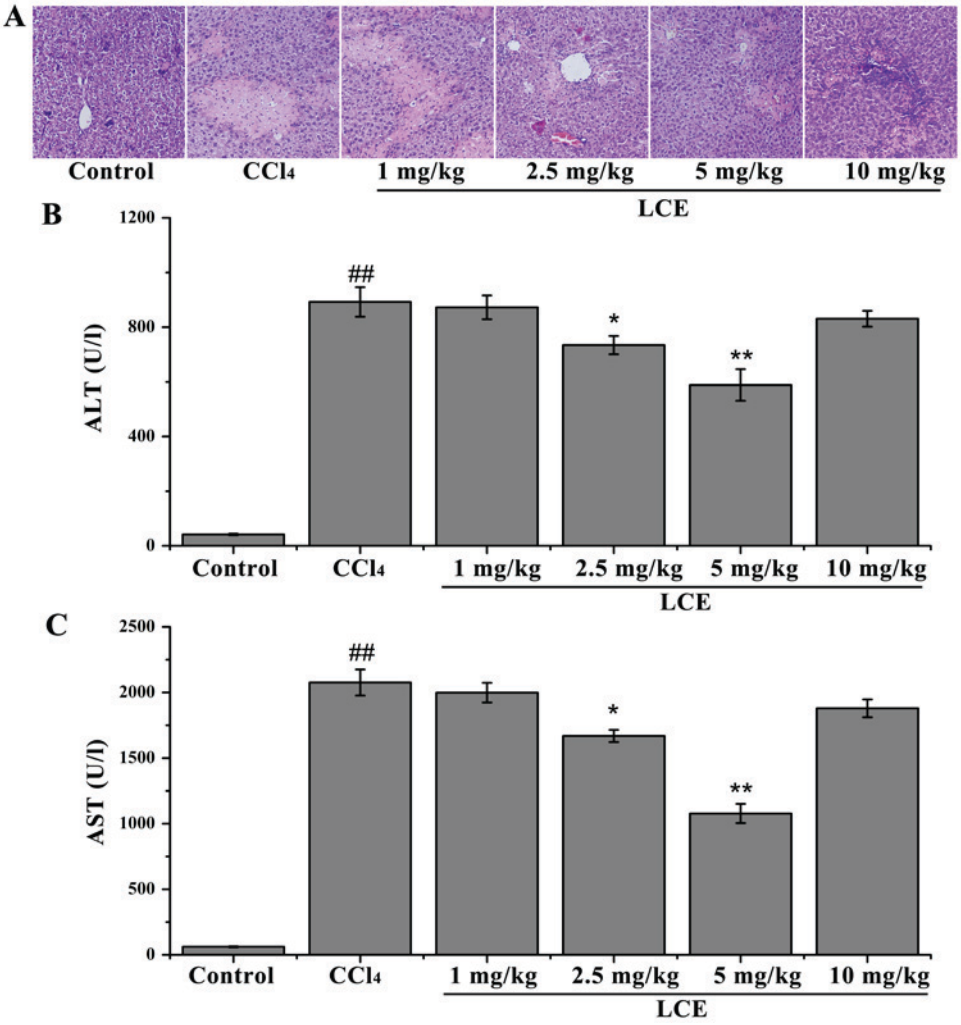

Figure 1. Different concentrations of LCE protected against CCl_4-induced histopathological damage and hepatic dysfunction. (A) Hematoxylin and eosin staining (Original magnification, x200) showed that livers in CCl_4 group exhibited massive inflammatory cells and cellular necrosis than those in Control group, and symptoms of those histopathological damage were significantly alleviated by LCE (2.5 and 5 mg/kg/day) treatment (n=6). Photographs of livers were taken 24 h postCCl_4 injection. Levels of ALT (B) and AST (C) increased obviously after CCl_4 challenge. However AST and ALT levels were significantly decreased with LCE (2.5 and 5 mg/kg/day) treatment (n=6). Taken together, preliminary experiments results that the dose of 5 mg/kg/day of LCE have a best hepatoprotective effects. Values are expressed as means ± SD (n=6/group). $^{\#\#}P<0.01$ compared with thel control group, $^{*}P<0.05$ and $^{**}P<0.01$ compared with the CCl_4 group.

cycler. Data was collected and analyzed using the Bio-Rad CFX software package. The internal control gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and target genes were amplified at equal efficiencies. The fold change in target gene expression was calculated as $2^{-\Delta\Delta CT}$.

Western blot analysis. PPARγ and NF-κB protein expression were examined using western blot analysis. The protein extracts from the liver tissue were prepared using a lysis buffer (50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, and 20% glycerol). The extracts were then subjected to centrifugation (15,000 g, 15 min at 4˚C). The supernatant fractions were assayed for protein concentration using a Bradford reagent (Bio-Rad, Richmond, CA, USA) and were used for the western blot analyses of PPARγ, NF-κB, and β-actin (Cell Signaling, Beverly, MA, USA). Horseradish peroxidase-conjugated IgG (Zymed, South San Francisco, CA, USA) was used as a secondary antibody. Finally, the bands were visualized using ECL-plus reagent, and the Bio-Rad Gel Doc 2000 imaging system and software were used to calculate the integrated absorbance (IA) of the bands. IA=area x average density. Following normalization to β-actin levels, the ratios of the IAs of PPAR-γ and NF-κB to the IA of β-actin were used to represent relative levels of activated PPAR-γ and NF-κB, respectively.

Statistical analysis. Data are presented as mean ± standard deviation from at least six independent experiments. Significance was determined by one-way analysis of variance (ANOVA) followed by bonferroni correction. $P<0.05$ was considered to indicate a statistically significant difference. The analyses were performed using the Statistical Program for Social Sciences Software (IBM SPSS, International Business Machines Corporation, Armonk City, New York, USA).

Results

The results of preliminary experiments. As shown in Fig. 1, according to the biochemical parameters (ALT and AST) and

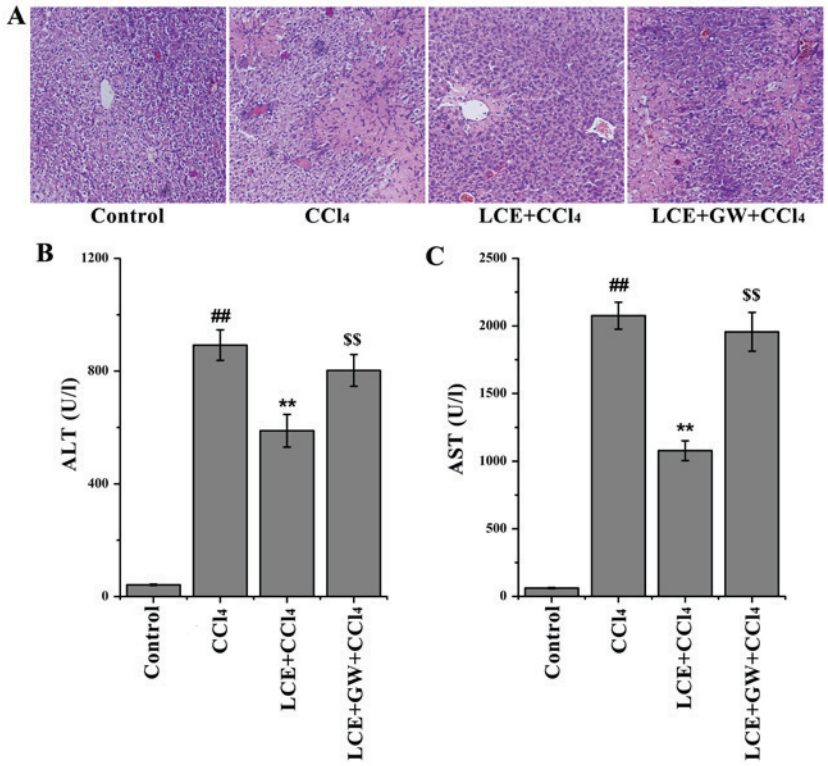

Figure 2. LCE protected against CCl_4-induced histopathological damage and hepatic dysfunction. (A) Hematoxylin and eosin staining (original magnification, x200) showed that livers in CCl_4 group and LCE+GW group exhibited massive inflammatory cells and cellular necrosis than those in Control group and LCE+CCl_4 group, and symptoms of those histopathological damage were significantly alleviated by LCE treatment. Photographs of livers were taken 24 h postCCl_4 injection. Levels of ALT (B) and AST (C) increased obviously after CCl_4 challenge. However, ALT and AST levels did not markedly increase in mice treated with LCE alone, and ALT and AST levels were significantly decreased with LCE treatment. Values are expressed as means ± SD (n=6/group). $^{\#\#}$P<0.01 compared with thel control group, **P<0.01 compared with the CCl_4 group, $^{\$\$}$P<0.01 compared with the LCE+CCl_4 group.

general histology survey of livers, we found that 5 mg/kg/day of LCE had the best hepatoprotective effect (see Fig. 1). To investigate the effect of LCE (5 mg/kg/day) on the liver of normal mice, we measured the biochemical markers in the mice with only LCE administration. Compared with control group, there were no obvious changes in the biochemical markers of mice with only LCE administration (see Table II). This result suggests that the dose of 5 mg/kg/day LCE may have almost no side effects on the liver. Therefore, the dose of 5 mg/kg/day of LCE was selected for using in next experiments.

In the present study, we found that high dose of LCE may have side effects on the liver. The HE staining assays demonstrate that a large number of inflammatory cells appeared in the high dose group (10 mg/kg LCE group). This result suggests that high doses of LCE may have proinflammatory effects. Therefore, high doses of LCE do not effectively prevent acute liver injury.

Hepatoprotective effect of LCE on carbon tetrachloride-induced liver toxicity in mice. As shown in Fig. 2A, liver sections showed normal cell morphology, with well-preserved cytoplasm, prominent nucleus, central vein (CV), and compact arrangement of hepatocytes without fatty lobulation in control group. However, significant anomalies of liver cells were observed in CCl_4-injured mice, the liver sections showed hydropic changes in centrilobular hepatocytes with cell necrosis surrounded by neutrophils. Congestion of the CV and sinusoids was observed, along with inflammatory cells infiltrating sinusoids mainly in the central zone, and symptoms of those histopathological damage were significantly alleviated by LCE treatment. However, this protective effect of LCE was reversed by co-treatment with GW9662. To investigate the hepatoprotective activities of LCE, the biochemical parameters (ALT and AST) and general histology survey of livers were measured. As shown in Fig. 2B and C, compare with control group, the intoxication of mice with CCl_4 resulted in a marked increase in the levels of liver function serum markers (ALT and AST). On the contrary, the increased levels of these liver function markers decreased nearer to normalcy because of the ameliorative effect of LCE. Importantly, the attenuation effect of LCE on serum markers (ALT and AST) was counteracted by co-treatment with GW9662.

LCE alleviated oxidative stress of hepatotoxicity injury induced by CCl_4. As shown in Fig. 3, the oxidative stress markers in liver homogenates revealed that the intoxication of mice with CCl_4 significantly decreased the activity of SOD in the serum, in comparison with those in control group. In addition, a significant increase in the levels of MDA was observed in CCl_4-intoxicated mice in contrast to the control animals. Compared with the CCl_4 group, the LCE-pretreated group exhibited significant ameliorative effect by elevating the reduced activity of SOD and by reducing the increase in the MDA levels. Meanwhile, the

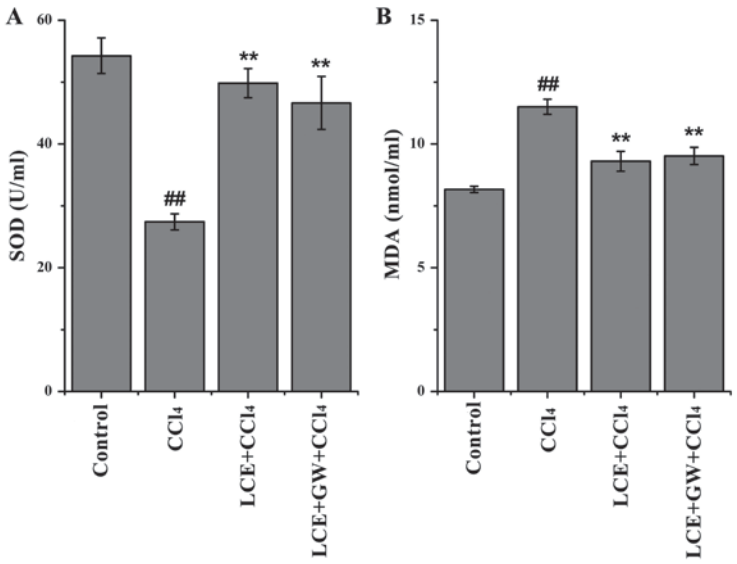

Figure 3. The effects of LCE on the levels of (A) SOD and (B) MDA in mice serum subjected to CCl_4 (values are means with their standard deviation, n=6). ##P<0.01 compared to the control group; **P<0.01 compared to the CCl_4 group.

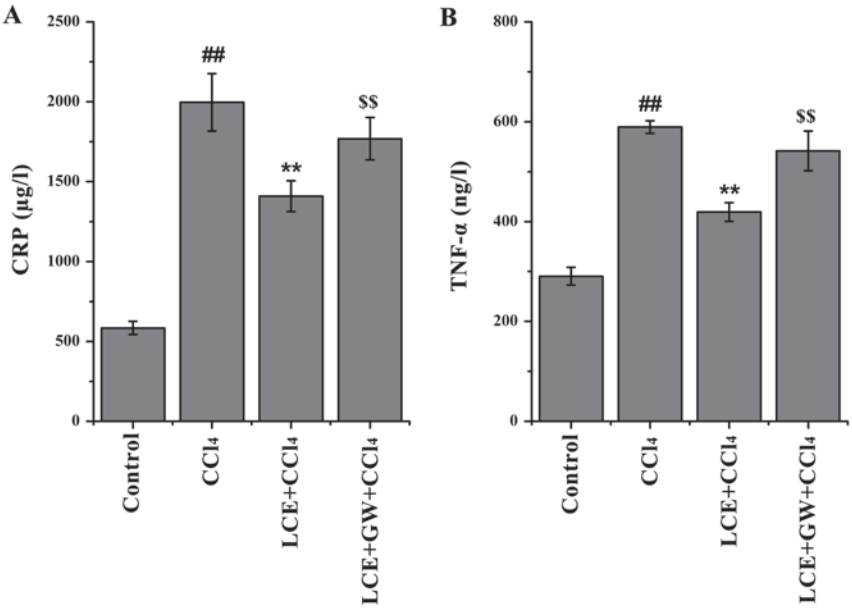

Figure 4. Effect of LCE on the levels of (A) CRP and (B) TNF-α in mice subjected to CCl_4-induced hepatotoxicity (values are presented as means ± SD, n=6). ##P<0.01 compared with the control group, **P<0.01 compared with the CCl_4 group, $$P<0.01 compared with the LCE+CCl_4 group.

effects of LCE on the levels of SOD and MDA were not counteracted by GW9662.

LCE attenuated inflammation of hepatotoxicity injury induced by CCl_4. Inflammation is an important mechanism underlying hepatotoxicity injury. The presence of inflammatory cytokines (CRP and TNF-α) associated with hepatotoxicity injury was determined in the serum to identify the possible mechanisms underlying the hepatoprotective activity of LCE. As shown in Fig. 4, compared with control group, the activity of TNF-α and CRP were significantly increased in the CCl_4 group. Compare with CCl_4 group, the activity of TNF-α and CRP in the group pretreated with LCE were significantly lower than those in the CCl_4 group. However, the effects of LCE on the levels of CRP and TNF-α were counteracted by GW9662.

Effect of LCE on PPARγ and NF-κB expression. To gain insight into the inflammatory milieu of the liver, we measured the levels of PPARγ and NF-κB mRNA using qPCR. As shown in Fig. 5, the mRNA levels of the anti-inflammatory PPARγ were significantly elevated in the CCl_4 groups compared to the control group. Compared to the CCl_4 group, the PPARγ mRNA levels were significantly higher in the LCE+CCl_4 groups. Meanwhile, there were no differences in the amount of PPARγ mRNA between the CCl_4 group and LCE+GW9662 group. The mRNA level of NF-κB increased in mice with CCl_4-induced liver injury. Interestingly, LCE

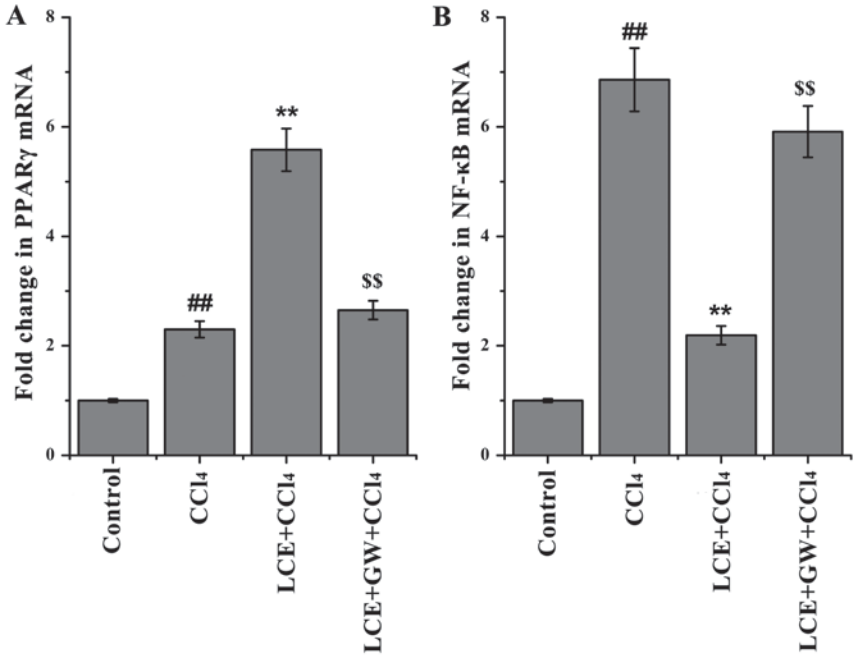

Figure 5. Effect of LCE on the mRNA levels of (A) PPAR-γ and (B) NF-κB in a CCl_4-induced liver damage mice model. GAPDH was used as an internal control. $^{\#\#}$P<0.01 compared to the control group; **P<0.01 compared to the CCl_4 group, $^{\$\$}$P<0.01 compared with the LCE+CCl_4 group.

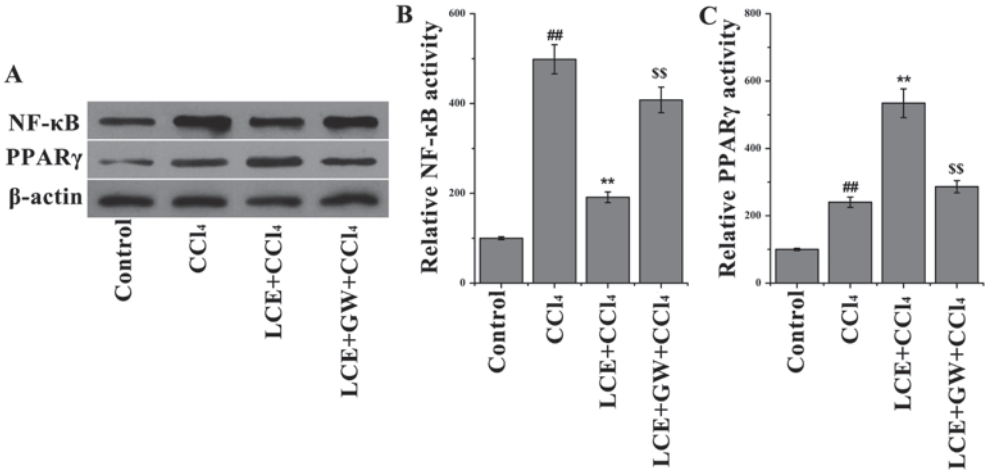

Figure 6. Effect of LCE on PPARγ and NF-κB protein levels in mice liver tissue. (A) Measurement of PPARγ and NF-κB protein levels in mice liver were analyzed by western blot. Expression of NF-κB (B) and PPARγ (C) in tissue of mice liver by western blot analysis with β-actin was used as an internal control. $^{\#\#}$P<0.01 compared with control group; **P<0.01 compared with CCl_4 group, $^{\$\$}$P<0.01 compared with the LCE+CCl_4 group.

treatment counteracted the CCl_4-related effects. Furthermore, the effects of LCE on the mRNA expression of PPARγ and NF-κB were counteracted by GW9662. There were no differences in NF-κB expression between the CCl_4 group and LCE+GW9662 group.

The effects of LCE on the protein levels of PPARγ and NF-κB in the liver. The expression of NF-κB and PPARγ proteins was measured using western blot analysis and quantified by densitometry. As shown in Fig. 6, it is found that in LCE+CCl_4 group, PPARγ level was elevated, demonstrating that LCE may be an effective PPARγ agonist. Compared with the control group, the protein level of PPARγ was significantly increased in CCl_4 group and LCE group. Compared with the CCl_4 group, PPARγ activity was much higher in the LCE+CCl_4 group. Compared with the LCE+CCl_4 group, PPARγ activity was much lower in the LCE+GW group. Interestingly the inhibitor treatments (GW9662) reversed these effects of LCE on PPARγ protein expression. Compared with the control group, NF-κB protein level increased significantly in CCl_4 group. And in contrast with the CCl_4 group, the protein level of NF-κB was much lower in LCE+CCl_4 group, suggesting that LCE treatment significantly relieved NF-κB protein level increase induced by CCl_4. Interestingly the inhibitor treatments (GW9662) reversed the hepatoprotective effects of LCE. Taken together, these results suggested that LCE may protect against CCl_4-induced liver injury by modulating

NF-κB-mediated signaling pathways, which are dependent on PPARγ activation.

Discussion

In the present study, the hepatoprotection of LCE in the mice model of CCl_4-induced liver toxicity was investigated. The results show that LCE suppressed the CCl_4-induced increase in AST, ALT, CRP, and TNF-α levels. CCl_4 is a well-known hepatotoxin, which is widely used to induce toxic liver injury and to study cellular mechanisms behind oxidative damages in laboratory animals (17). CCl_4-induced liver damage has been studied in mice and rats, and findings show a significant elevation of the serum aminotransferase (e.g., AST and ALT) levels (18,19). In the present study, a significant elevation in the levels of serum marker enzymes (e.g., AST and ALT) is observed among the animals treated with CCl_4. The administration of LCE reduces the toxic effect of CCl_4 by restoring the levels of serum marker enzymes to normalcy. CCl_4-induced hepatic lesions are characterized by coagulation necrosis and hepatocyte vacuolation, which is mainly situated in the central to middle portion of the hepatic lobules. The HE staining assays demonstrate that LCE pretreatment attenuates liver damage in mice upon CCl_4 administration. All these results demonstrate the protective effects of LCE on mice liver against CCl_4. In the present study, we found that the hepatoprotective effect of LCE was within a certain dose-range, high dose of LCE does not effectively prevent acute liver injury, this result suggests that high doses of LCE may have side effects on the liver. The HE staining assays demonstrate that a large number of inflammatory cells appeared in the high dose group (10 mg/kg LCE group), this result suggests that high doses of LCE may have proinflammatory effects.

Inflammation, which plays an important role in many disease states, is associated with enhanced expression of adhesive molecules in the vasculature, resulting in the infiltration of larger populations of neutrophils and monocytes/macrophages (20). The release of proinflammatory cytokines from these activated leukocytes can then in turn cause tissue damage (21). Previous studies have suggested that many inflammatory cytokines were released in the CCl_4-induced liver injury, several lines of evidence also suggest that PPARγ may exert anti-inflammatory effects by negatively regulating the expression of proinflammatory genes induced during macrophage differentiation and activation (22). Along these lines, inflammation plays a key role in CCl_4-induced liver injury, and the deleterious events that follow these events include an increased release of proinflammatory mediators (e.g., TNF-α and CRP) (23,24). In the present study, we found that LCE exhibits significant hepatoprotective effects against CCl_4-induced liver injury, as well as significantly increases PPARγ mRNA and protein expression. However, concurrent use of GW9662 abrogated these effects. Altogether, this data suggests that the reduction in liver tissue injury afforded by these drugs is, at least in part, due to their ability to activate PPARγ. PPARγ also has inhibitory interactions with other transcription factors, such as NF-κB (25,26). Specifically, we found that LCE treatment significantly decreased CCl_4-induced NF-κB mRNA and protein expression in the absence of, but not in the presence of GW9662. This data suggests that NF-κB is one target of the anti-inflammatory effects of PPARγ, and that one of the hepatoprotective mechanisms of PPARγ ligands is PPARγ inhibition of NF-κB. In the present study, we found that LCE exhibits significant hepatoprotective effects. LCE treatment also significantly increased PPARγ mRNA and protein expression, and decreased CRP and TNF-α production compared to the CCl_4 group. However, these hepatoprotective effects of LCE were blocked by GW9662. Our data show that the expression of NF-κB mRNA and protein in the CCl_4 group are markedly higher than that of control, but LCE treatment significantly decreased this expression. Intriguingly, when co-treatment with LCE and GW9662, NF-κB expression is significantly increased, indicating that activation of PPARγ reduces production of NF-κB and, thus, inhibits downstream expression of inflammatory genes that contribute to liver injury. Taken together, these results indicate that one of the mechanisms of LCE-mediated hepatoprotection is through its anti-inflammatory properties, including activation of PPARγ, and, thus, inhibition of NF-κB. In this study, we also found that CCl_4 also leads to an upregulation in the mRNA and protein of PPARγ expression, this may be related to the stress response.

A widely-accepted assumption is that the reactive oxygen species (ROS) are the main causes of CCl_4-induced acute liver injury. Therefore, anti-oxidative therapy is an effective means of preventing and attenuating oxidative stress-related liver diseases (27). Antioxidant enzymes, such as SOD, perform important functions in defense mechanisms against the harmful effects of ROS and free radicals in biological systems. In addition, the increased levels of MDA in the liver tissue homogenate of mice treated with CCl_4 reflect lipid peroxidation and damage to plasma membrane (10,11) as consequences of oxidative stress. In the present study, our results show that LCE treatment increases SOD activity and decreases MDA levels back to their normal control levels. Taken together, these results indicate that one of the mechanisms of LCE-mediated hepatoprotection is through its antioxidative effect. In the present study, we found that the effects of LCE on the levels of SOD and MDA were not counteracted by GW9662, this results suggested that PPARγ signaling pathway plays an important role in the regulation of inflammation, but no significant regulation in oxidative stress.

In the present study, LCE exhibited hepatoprotective activity in CCl_4-induced hepatotoxicity in mice. Apart from antioxidant action, LCE may exert an anti-inflammatory effect by activating the PPARγ signaling pathway. In the future, LCE may be developed as a drug with antioxidant and anti-inflammation properties for use in human liver diseases.

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