Green tea infusion protects against alcoholic liver injury by attenuating inflammation and regulating the PI3K/Akt/eNOS pathway in C57BL/6 mice†

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Alcohol intake is a major risk factor for the pathogenesis of alcoholic liver diseases. Accumulating evidence suggests that green tea protects against alcoholic liver injury; however, the underlying mechanisms remain unclear. The present study investigated the role of endothelial nitric oxide synthase (eNOS) in the protective effects of green tea against alcohol-induced liver injury and inflammation. Ethanol was intragastrically administered to male C57BL/6 mice once a day, and the mice were allowed free access to green tea infusion or water for two weeks. We assessed the plasma levels of alanine aminotransferase and aspartate aminotransferase, hepatic contents of thiobarbituric acid reactive substances, malondialdehyde and triglyceride and hepatic mRNA expression of pro-inflammatory cytokines (interleukin-1β, tumor necrosis factor-α, and interleukin-6). Our results showed that compared with water alone, green tea infusion markedly reduced liver damage, hepatic oxidative stress, hepatic lipid accumulation and inflammatory response. Green tea infusion also significantly reduced hepatic nuclear factor-κB expression and its downstream inflammatory mediators (inducible nitric oxide synthase and cyclooxygenase-2) mRNA levels in ethanol-treated mice. Additionally, green tea infusion significantly activated hepatic phosphorylated phosphatidylinositol 3-kinase (PI3K) and phosphorylated protein kinase B (Akt), which are associated with the upregulation of phosphorylated eNOS expression and the increase of plasma nitric oxide levels in ethanol-treated mice. Furthermore, the protective effects of green tea infusion were considerably inhibited by the eNOS inhibitor N \textsuperscript{G}-nitro-L-arginine methyl ester in ethanol-treated mice. In conclusion, our study demonstrated that the protective effects of green tea infusion on alcohol-induced liver injury and inflammation involve the modulation of the PI3K/AKT/eNOS pathway.

Introduction

Alcoholic liver diseases present major health problems worldwide. Alcohol abuse causes various alcoholic liver diseases including steatosis, steatohepatitis and cirrhosis, which contribute to high morbidity and mortality.\(^1\) The liver is the primary target for ethanol toxicity because it is the main organ for ethanol metabolism. In the liver, ethanol is metabolized to the highly toxic metabolite acetaldehyde that interacts with cellular macromolecules (lipids and proteins) and engenders liver injury by activating the ubiquitous proinflammatory transcription factor nuclear factor-κB (NF-κB), resulting in the expression of inflammatory mediators such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).  

The liver is composed of true hepatic parenchymal cells (hepatocytes), vascular endothelial cells, stellate cells, resident hepatic macrophages (Kupffer cells), bile duct cells, and fat-storing cells. Nitric oxide (NO) can be produced in all of these cells and can participate in a variety of metabolic processes including second messenger cell signaling.\(^3\) NO, an endogenous vasodilatory gas, maintains an antiproliferative and anti-apoptotic environment and plays a crucial role in the antagonism of stellate cell contraction, inhibition of platelet adhesion, and neutralization of oxygen free radicals in the liver.\(^4\) Nitric oxide (NO) is produced by at least two isofoms of nitric oxide synthase in the liver, namely endothelial nitric oxide synthase (eNOS) and iNOS. eNOS is a Ca\(^{2+}\)- and calmodulin (CaM)-dependent constitutive isoform and plays a crucial role in vasorelaxation,
whereas iNOS is not a constitutive enzyme, and its expression may be induced by stimuli such as lipopolysaccharides (LPS) or proinflammatory cytokines. 

During liver injury, hepatic stellate cells contract and are activated to become proliferative and fibrogenic myofibroblast-like cells, thus leading to a reduction in the level of NO and an induction of hepatic cell damage. NO dysregulation has been implicated in the pathogenesis of alcoholic liver diseases.

eNOS catalyzes the production of NO, a key regulator of blood pressure, vascular remodeling, and angiogenesis. The activity and expression of eNOS are regulated by the phosphorylation states of phosphatidylinositol 3-kinase (PI3K) and protein kinase B (Akt). Akt is a survival kinase and a main downstream target of PI3K. Active Akt phosphorylates its substrates including Forkhead box protein transcription factors, Bcl-2-associated death promoter, and eNOS. The decreased expression of eNOS in chronic liver disease reduces hepatic perfusion and accelerates fibrosis. eNOS deficiency and overexpression have been demonstrated to exacerbate and protect against liver injury, respectively. In particular, reduced eNOS activity in the liver of rats was reported after chronic alcohol exposure, which is correlated with liver damage, particularly the inflammatory activation of NF-κB. Conversely, eNOS affects the expression level of NF-κB and ultimately downregulates many pathophysiological processes including steatosis, oxidative stress, inflammation, and hepatic apoptosis. Specifically, eNOS is part of a negative feedback loop for preventing injury and inflammation in hepatocytes. Therefore, potential therapeutic interventions for activating eNOS expression will be promising strategies for protection against liver injury.

Green tea is one of the most commonly consumed beverages worldwide, and it has been considered a herbal medicine for over 4000 years in China. It contains caffeine, theanine, and polyphenolic compounds known as catechins. Green tea exhibits multiple biological activities such as anti-inflammatory, anti-oxidant, anti-hypertensive, anti-diabetic, and anti-obesity activities. In particular, increasing bodies of evidence from animal studies have demonstrated that green tea protects against alcohol-induced liver injury and inflammation, whose mechanisms refer to the alleviation of oxidative stress, inhibition of inflammatory response and attenuation of lipid peroxidation. Recently, it was reported that green tea is able to activate eNOS expression and enhance NO production in endothelial cells, here we hypothesize that the eNOS pathway is involved in the protective effects of green tea against alcohol-induced liver injury and inflammation. In the current study, we identified the liver injury-shielding efficacy of green tea via the activation of the PI3K/Akt/eNOS signal pathway.

Materials and methods

Reagents

Commercial kits for the measurement of alanine aminotransferase (ALT), aspartate aminotransferase (AST), thiobarbituric acid reactive substances (TBARS), malondialdehyde (MDA), and triglyceride (TG) and NO levels were obtained from Jiancheng Bioengineering Institute (Jiangsu, China). ELISA kits for the measurement of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, and IL-6 levels were purchased from BD Biosciences (CA, USA). N⁶O⁵-Nitro-L-arginine methyl ester (L-NAME) was purchased from Selleck Chemicals (TX, USA). The anti-β-actin antibody was purchased from Sigma (MO, USA). Anti-mouse PI3K, anti-p-PI3K (Tyr199), anti-Akt, anti-p-Akt (Ser473), anti-NF-κB p65, and anti-p-NF-κB p65 antibodies were purchased from Cell Signaling (MA, USA). Anti-mouse eNOS and p-eNOS (Ser1177) antibodies were purchased from Santa Cruz (TX, USA). The anti-mouse COX-2 antibody was purchased from Novusbio and anti-mouse iNOS was purchased from Millipore (MA, USA). Other reagents were of the highest grade available.

Tea infusion

Green tea (#43 Longjing) was manufactured in the Tea Processing Practice Base of Anhui Agricultural University. A concentrated infusion (1 : 50, w/v in water) was prepared by immersing 2 g of dried tea leaves in 100 mL of hot water (100 °C) for 5 min; the resultant beverage was cooled to room temperature (RT) in a cylinder. A tea infusion (1 : 100, w/v in water) was prepared by adding 50 mL of the concentrated infusion (1 : 50) to 50 mL of pure water (RT) in a cylinder. The tea infusion was prepared fresh and handled daily.

Animal models and treatments

Healthy male C57BL/6 mice (8 weeks, 20–22 g) were obtained from Shanghai SLAC Laboratory Animal Co. Ltd (Shanghai, China). The mice were housed in a controlled environment at 22 ± 2 °C with 40%–60% humidity and a 12 h light–dark cycle. The mice had free access to water or tea infusion and a regular diet. All experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals (Ministry of Science and Technology of the People's Republic of China), and all animal protocols were reviewed and approved by the ethics committee of Anhui Agricultural University.

Experiment 1 was designed to investigate the protective effects of replacing drinking water with tea infusion on alcohol-induced liver injury and inflammation. The mice were randomly divided into the following four experimental groups: (i) control, (ii) ethanol, (iii) ethanol + 1% tea infusion, and (iv) ethanol + 2% tea infusion (n = 6 per group). The mice in each group were allowed free access to tea infusion or water for 14 consecutive days. In the tea infusion and ethanol groups, ethanol (50% v/v, 6 g kg⁻¹) was administered to the mice intragastrically once a day over a 14-day period.

Experiment 2 was designed to investigate the effects of eNOS inhibition on alcohol-induced liver injury and inflammation. The mice were randomly divided into the following five experimental groups: (i) control, (ii) ethanol, (iii) ethanol + 2% tea infusion, (iv) ethanol + 2% tea infusion + eNOS inhibitor, and (v) eNOS inhibitor (n = 6 per group). The mice in each
group were allowed free access to tea infusion or water for 14 consecutive days. In the three ethanol groups, ethanol (50% v/v, 6 g kg⁻¹) was administered intragastrically to the mice once a day for 14 consecutive days. The mice in the two eNOS inhibitor groups were injected intraperitoneally with L-NAME (10 mg kg⁻¹) once a day for 14 consecutive days.

At the end of each experiment, the mice were anesthetized and sacrificed by cervical dislocation. Plasma was obtained through the centrifugation of blood samples at 3000 rpm for 10 min and stored at −80 °C until analysis. The samples of the livers were excised, sectioned transversely or longitudinally, and fixed in 10% (v/v) neutral-buffered formalin, and the remaining tissue was rinsed in ice-cold phosphate-buffered saline (PBS) and then stored at −80 °C.

**Plasma biochemical parameters**

The plasma levels of ALT and AST were measured using commercial kits. NO production in plasma was measured as nitrite–nitrate and was performed according to the protocols provided by the manufacturer, using the Griess Reagent. The plasma levels of IL-1β, TNF-α, and IL-6 were measured using the corresponding ELISA kits described previously according to the manufacturer’s protocol.

**Histopathological analysis**

The histopathological changes of the liver were observed by haematoxylin and eosin (H&E) staining. In brief, the fixed tissues were dehydrated in graded ethanol and embedded in paraffin. Paraffin sections were stained with H&E using a standard protocol. The stained specimens were examined and recorded by using a light microscope with a camera (Olympus, Tokyo, Japan) by two pathologists in a blinded fashion.

**Determination of hepatic TBARS, MDA and TG levels**

Liver tissue was homogenized in ice-cold 150 mM pH 7.2 PBS containing 1 mM disodium EDTA to prepare a 10% liver homogenate. The homogenate was centrifuged at 15 000g for 15 min at 4 °C to remove cell debris and nuclei, and the resulting supernatant was analyzed to determine the TBARS, MDA, and TG levels using the corresponding commercial assay kits. Protein levels were determined by using the Bradford assay with bovine serum albumin as a standard. Values were normalized to hepatic total protein.

**RNA isolation and real-time PCR**

Total RNA was extracted from the different groups of liver tissue using a TRIzol reagent (Takara Biotechnology, Dalian, China). Total RNA was converted to cDNA following the instructions provided in the PrimeScript™ RT Reagent Kit (Takara Biotechnology). Real-time PCR was performed using a Quant qPCR kit (TIANGEN Biotech, Beijing, China) and with a CFX System (Bio-Rad). The aforementioned experimental procedures were performed according to the guidelines for minimum information for publication of quantitative real-time PCR experiments. The gene-specific primers listed in Table 1 were designed using the available gene sequences (National Center for Biotechnology Information, US National Library of Medicine, http://www.ncbi.nlm.nih.gov/nuccore/).

**Western blot analysis**

Whole liver tissue lysates were extracted with commercial lysis buffer (Beyotime Biotechnology, Beijing, China) for western blot analysis. The protein content of the lysate was determined using a protein assay kit (Beyotime Biotechnology) following the manufacturer’s recommended protocol. The proteins were loaded onto 10%–15% SDS polyacrylamide gels, transferred to PVDF membranes (Bio-Rad), and blocked with 5% nonfat milk powder in PBS with 0.1% Tween. The membranes were probed overnight with a monoclonal primary antibody at 4 °C. The membranes were incubated with the secondary antibody, which had been diluted in tris buffered saline with Tween-20 (TBS-T) by 5000-fold to 10 000-fold, for 1 h at RT. The blots were visualized using an enhanced chemiluminescence kit (Bio-Rad) according to the manufacturer’s recommended instructions and were detected using the ChemiDoc XRS+ detection system (ECL; Bio-Rad). The Quantity One Image Analyzer software program (Bio-Rad) was used for quantitative densitometric analysis. The ratio of the targeted band to β-actin was considered the relative intensity of the targeted band.

**Statistical analysis**

In this paper, data are presented as means ± standard errors of the mean (SEM). Differences between groups were examined through one-way ANOVA, with post hoc analysis conducted using either Tukey’s or Dunnett’s test for multiple comparisons according to the result of Bartlett’s test for equal variances by using GraphPad software (Prism, San Diego, CA, USA). A two-sided P value of <0.05 was considered statistically significant.

**Results**

**Green tea prevented liver injury and inflammation in alcohol-induced liver injury**

We first determined the protective effect of green tea against acute alcohol-induced liver injury and inflammation in mice. As illustrated in Fig. 1A–E, ethanol alone caused liver damage, oxidative stress and lipid accumulation in mice, as indicated by the increase in the plasma ALT level and hepatic TBARS, MDA and TG contents. However, liver injury was significantly reduced in green tea-treated groups. Similarly, ethanol alone also caused hepatic inflammation in mice, as indicated by the increase in the mRNA levels of hepatic proinflammatory cytokines, namely IL-1β, TNF-α, and IL-6. However, the proinflammatory mRNA levels in the green tea-treated groups were significantly lower than those in the ethanol group (Fig. 1F–H). In experiment 1, treatment with ethanol did not cause noticeable hepatic histopathological alterations and did not influence daily consumption of green tea infusion in each experimental group (ESI Fig. 1†).
Green tea inhibited alcohol-induced hepatic NF-κB activation
NF-κB is activated in animal models of alcohol-induced liver injury, which plays a critical role in the inflammatory response by upregulating proinflammatory cytokine production.19,28 In this study, we observed that green tea treatment dose-dependently inhibited ethanol-induced hepatic NF-κB protein elevation (Fig. 2A). Next, we evaluated the effects of green tea on iNOS and COX-2 mRNA expression. We found that the hepatic iNOS and COX-2 transcription levels were significantly higher in the ethanol-treated group than in other groups; however, the tea infusion treatments prevented an increase in these inflammatory mediator gene expression levels (Fig. 2B and C).

Green tea-mediated protection involved activation of the hepatic eNOS pathway
eNOS plays a vital role in hepatic cellular functions such as proliferation, apoptosis, oxidation resistance, and stress resistance.29 eNOS activation has been shown to inhibit apoptosis, protect against inflammation and oxidative stress, and ameliorate liver injury.19,20 Akt binds to eNOS after phosphorylation at Ser-473, leading to eNOS activation.15 To determine whether green tea affects the PI3K/Akt/eNOS signaling pathway, the protein expression levels of PI3K and Akt were examined in the livers of mice. As shown in Fig. 3B–D, ethanol alone did not affect the expression of the eNOS protein and its regulatory proteins (PI3K and Akt) under the experimental conditions used. However, after the administration of green tea, hepatic p-PI3K and p-Akt expression significantly increased, without affecting the expression of the total circulating PI3K and Akt (Fig. 3B and C). Similar to PI3K phosphorylation at Tyr-199 and Akt phosphorylation at Ser-473, green tea enhanced hepatic eNOS phosphorylation at Ser-1177 and resulted in a major increase in the plasma NO level (Fig. 3A and D). Together, these data suggest that green tea-mediated protection against alcohol-induced liver injury and inflammation involves eNOS upregulation and an increase in NO levels.

Green tea-mediated eNOS activation plays a protective role in alcohol-induced liver injury
To confirm whether the PI3K/Akt/eNOS signaling pathway participates in green tea-mediated protection against alcohol-induced liver injury and inflammation, we blocked eNOS signaling with L-NAME, an in vivo eNOS inhibitor. As presented in Fig. 4A–E, green tea treatment significantly reduced the levels of ALT, AST, IL-1β, TNF-α, and IL-6 in the plasma compared with ethanol treatment. Consistent with the results of plasma biochemical analysis, the mRNA expression levels of the proinflammatory cytokines, namely IL-1β, TNF-α, and IL-6, in the liver induced by ethanol were also significantly inhibited by green tea treatment (Fig. 4F–H). By contrast, treatment with L-NAME significantly elevated the plasma levels of ALT, AST, IL-1β, TNF-α, and IL-6, in the liver after ethanol exposure (Fig. 4F–H). Notably, L-NAME alone did not alter the levels of the proinflammatory cytokines in either the plasma or the liver. Additionally, we found that L-NAME significantly attenuated green tea-mediated activation of hepatic NF-κB and its downstream inflammatory mediators and COX-2 inhibition in ethanol-treated mice (Fig. 5A and C). Notably, L-NAME is a nonselective inhibitor of both iNOS and eNOS. As shown in Fig. 5B and D, L-NAME treatment significantly reduced iNOS protein and mRNA expression. Subsequently, we evaluated the effect of L-NAME on the transcriptional expression of COX-2. Similarly, L-NAME reversed the green tea-mediated down-regulation of hepatic COX-2 transcription in the liver (Fig. 5E). To further investigate the molecular mechanism through which green tea inhibited alcohol-induced liver injury and inflammatory response in mice, western blot analysis was performed to study the changes in the PI3K/AKT/eNOS signaling pathway. L-NAME is responsible for inhibiting the expression of eNOS. We observed that L-NAME treatment significantly attenuated the green tea-induced expression of phosphorylated PI3K and Akt in the livers of the treated mice (Fig. 6B and C). Next, we investigated the effect of L-NAME on green tea-mediated eNOS activation at the protein level in the liver.
livers of mice. The results revealed that L-NAME reversed the upregulation of hepatic eNOS protein expression and the increase in the NO level in the plasma caused by green tea (Fig. 6A and D). All these results demonstrated the necessity of the activation of the PI3K/Akt signaling pathway for green tea-mediated eNOS activity and NO production.

**Discussion**

Alcohol-induced liver injury and inflammation have been recognized as a common occurrence in alcoholic liver diseases; therefore, insights into the molecular mechanisms underlying these conditions and appropriate therapeutic agents are relevant for the development of potential therapies. Green tea prevents the development of liver diseases by inhibiting inflammatory processes. Recent clinical studies and meta-analyses have also shown that green tea reduces the incidence of alcoholic liver diseases. However, the mechanisms through which green tea prevents the development and onset of alcoholic liver diseases is not fully understood. In this study, we found that green tea effectively rescued the alcohol-mediated reduction in eNOS activation and NO production by activating PI3K/Akt expression in mice. According to our review of the lit-

![Fig. 1 Effects of green tea on alcohol-induced hepatic injury and inflammatory response in mice. The mice in each group were allowed free access to green tea infusion (1:50 or 1:100, w/v) or water for 14 consecutive days. In three ethanol groups, ethanol (50% v/v, 6 g kg\(^{-1}\)) was administered to the mice intragastrically once a day over a 14-day period. (A) Plasma ALT activities. (B) Plasma AST activities. (C) Liver TBARS levels. (D) Liver MDA levels. (E) Liver TG levels. (F) Liver IL-1β mRNA levels. (G) Liver TNF-α mRNA levels. (H) Liver IL-6 mRNA levels. Data are presented as mean ± SEM (n = 6).](image-url)
erature, our study is the first to demonstrate that the hepatoprotective effects of green tea are mainly related to the modulation of the PI3K/Akt/eNOS pathway.

Green tea leaves contain various components including polyphenols (35%–40% of dry weight), caffeine (3%–5%), amino acids (4%–6%), carbohydrates (25%), proteins (15%), lipids (2%), and trace amounts of other substances, although the percentage of these ingredients could vary with the season, climate, horticultural practices, and age of the leaf.32 Accumulating evidence suggests that green tea protects against alcohol-induced liver injury and inflammation.24,33,34 The major green tea polyphenol belongs to the family of catechins.35 Over the past few years, several authors have described that green tea polyphenols and isolated EGCG can ameliorate alcohol-induced liver damage.36,37 The most salient amino acid found in green tea is theanine. Research has demonstrated that theanine exerts hepatoprotective effects on alcohol-induced liver injury.38 Caffeine as the most important methylxanthine in tea can also alleviate alcohol-induced liver injury through the inhibition of oxidative stress and inflammation.39

NO is a crucial mediator of physiological and pathophysiological processes. As a prototypical endothelial relaxing factor, NO is a major determinant of blood vessel tone and hepatic blood supply.6,8 In our study, acute ethanol gavage caused a reduction in the plasma NO levels of the ethanol-treated group compared with the control group. Moreover, the plasma NO levels of the green tea-treated groups were significantly higher than those of the ethanol-treated group (Fig. 6A). The role of NO in liver injury is debatable. NO appears to play a paradoxical role or exhibit dual effects on liver injury due to various causes, depending on the experimental conditions, the amount of NO production, and NO synthase isoforms. The finding that two major isoforms of NOS are expressed in the liver has raised several questions regarding the role of NO in the liver. The overproduction of NO by the iNOS enzyme was implicated in liver injury.9 By contrast, eNOS deficiency resulted in reduced NO production and an increased severity of liver injury.18 Whether NO is beneficial or detrimental in alcoholic liver injury remains controversial. Accumulating evi-
evidence suggests that eNOS is a crucial regulator in various tissues, such as the liver, kidney, and heart, and that it is involved in oxidative stress, inflammatory processes, and cellular injury. Because of the key role of the liver in alcohol metabolism, several studies have demonstrated that hepatic eNOS plays a vital role in liver damage due to alcohol abuse. The levels of the eNOS protein and eNOS-derived NO of our ethanol-treated mouse model were lower than those of other groups (Fig. 6A and D). Therefore, the eNOS-controlled moderate upregulation of NO production may be involved in inhibiting liver injury and inflammation in ethanol-treated mice. Notably, we found that the ratio of p-eNOS to iNOS protein expression was highly correlated with the plasma NO level ($r = 0.9375$, $P < 0.01$). Based on these results, we speculate that the ramifications of this finding may involve protection against or contribution toward alcohol-induced liver injury. Although eNOS-derived NO is hepatoprotective in alcohol-induced liver injury, the role of iNOS-derived NO is much less clear and requires further research to shed light on the specific circumstances that determine the benefits or harms of NO generated from iNOS.

The PI3K/Akt pathway promotes eNOS activation; thus, we quantified the levels of phosphorylated PI3K and Akt. The results of western blot analysis indicate that green tea significantly amplified PI3K and Akt phosphorylation. Furthermore, the eNOS inhibitor significantly inhibited the increase in the levels of phosphorylated PI3K and Akt. These data suggest that green tea-induced PI3K/Akt activation promotes the activation of eNOS and consequently the induction of NO production. On the other hand, NO as a second messenger in cell signaling also involves the upstream regulation of the PI3K/Akt pathway. Whether NO synthesis interacts with the PI3K/Akt pathway in this manner warrants further investigation. In addition, the increase in cytoplasmic calcium levels activates CaM, which binds to the canonical CaM-binding domain of eNOS to promote the alignment of the oxygenase and reductase domains of eNOS, leading to efficient NO synthesis.

A recent study reported that CaM activates CaM-dependent protein kinase II (CaMK II), which also activates eNOS. Notably, recent studies have demonstrated that EGCG, a major constituent of green tea, can activate both PI3K/Akt and eNOS expression. Moreover, a recent study showed that the
cytoplasmic calcium levels and CaMK II were positively regulated by EGCG. This evidence suggests that in our study, EGCG was likely involved in the process of green tea-induced eNOS activation. Whether CaM and CaMK II are potential targets for the green tea-mediated activation of eNOS warrants further investigation.

Our data show that green tea significantly increased the plasma NO levels in ethanol-treated mice. Chemically, eNOS utilizes molecular oxygen and electrons from NADPH to oxidize the substrate L-arginine to form NO and L-citrulline. Notably, L-arginine can be biosynthesized from glutamic acid by endogenous enzymes. Furthermore, L-theanine (γ-glutamylethylamide), a non-protein amino acid, comprising 0.5%–2% of the dry weight of green tea, is rapidly absorbed and converted into ethylamine and glutamic acid in vivo. Based on these findings, we proposed one possibility that L-theanine may be a potential substrate of eNOS-derived NO synthesis. Two observations support this possibility: first, L-theanine promotes NO production in endothelial cells, and second, highly fermented black tea and non-fermented green tea had equal potency in stimulating NO production; however, chemical analysis revealed that black tea was almost devoid of catechins. In general, the theanine levels of tea leaves appear to be minimally affected by the fermentation process because both green tea and black tea contain similar theanine levels.

Although eNOS activation is regulated at multiple levels, an increasing body of evidence supports the view that post-transcriptional regulation plays a crucial role in the negative expression of eNOS in response to inflammation. NF-κB-activating stimuli, such as LPS, TNF-α, and IL-1β, reduce eNOS mRNA stability and protein levels; furthermore, NO production in human umbilical vein endothelial cells is effectively reversed by an inhibitor of NF-κB and specific knockdown of the NF-κB p65 subunit, indicating that the activity of the eNOS/NO axis is down-regulated by NF-κB activation. In fact,
NF-κB is significantly activated in hepatic inflammation and alcoholic liver injury, resulting in the increased levels of proinflammatory cytokines along with the increased production of reactive nitrogen and oxygen intermediates. These findings suggest that increased proinflammatory cytokine levels in plasma are associated with liver injury and inflammation by activating NF-κB and subsequently inhibiting the eNOS/NO axis activity. Notably, treatment with the eNOS inhibitor amplifies NF-κB expression, aggravates liver injury and inflammation, and increases proinflammatory cytokine production, which is a major risk factor for alcoholic liver diseases. These findings suggest the critical involvement of NF-κB in the

Fig. 5 The inhibitory action of green tea on ethanol-induced hepatic NF-κB activation was blocked by the eNOS inhibitor. The mice in each group were allowed free access to green tea infusion (1:50, w/v) or water for 14 consecutive days. In the three ethanol groups, ethanol (50% v/v, 6 g kg\(^{-1}\)) was administered intragastrically to the mice once a day over a 14-day period. The mice in the two eNOS inhibitor groups were injected intraperitoneally with L-NAME (10 mg kg\(^{-1}\)) once a day over a 14-day period. (A) Liver p-NF-κB/NF-κB protein. (B) Liver iNOS protein. (C) Liver COX-2 protein. (D) Liver iNOS mRNA levels. (E) Liver COX-2 mRNA levels. Data are presented as mean ± SEM (A–C, n = 4; D and E, n = 6).
Impairment of eNOS activation. Additional potential mechanisms linking green tea and reduction in alcoholic liver injury include modifications of lipid accumulation, oxidative stress, and mitochondrial dysfunction.\textsuperscript{1,2,3,24}

The present study has several limitations that need to be addressed in future studies. This study cannot fully explain the involvement of NO levels in the prevention of alcoholic hepatic injury, and the role of NO responsible for the beneficial effects of green tea needs to be further elucidated. We propose the possibility that L-theanine may be a potential substrate for NO synthesis, which requires further investigations. Finally, although the ethanol-treated mice model is a commonly used

![Graph showing plasma nitrite/nitrate level (μM) vs Alcohol, 2%GT, L-NAME, and their combinations.](Fig. 6)

Fig. 6  Influences of the eNOS inhibitor on the hepatic PI3K/Akt/eNOS pathway and NO level in the plasma of mice. The mice in each group were allowed free access to green tea infusion (1:50, w/v) or water for 14 consecutive days. In the three ethanol groups, ethanol (50% v/v, 6 g kg\textsuperscript{-1}) was administered intragastrically to the mice once a day over a 14-day period. The mice in the two eNOS inhibitor groups were injected intraperitoneally with L-NAME (10 mg kg\textsuperscript{-1}) once a day over a 14-day period. (A) Plasma NO levels. (B) Liver p-PI3K/PI3K protein. (C) Liver p-Akt/Akt protein. (D) Liver p-eNOS protein. (E) Liver eNOS protein. (F) Liver p-eNOS/eNOS protein. Data are presented as mean ± SEM (A, n = 6; B–F, n = 4).
laboratory practice, there remains a gap between the observations in this study and clinical applications to patients with alcoholic liver injury.

In summary, our data provide evidence that this beneficial effect of green tea is likely due to interference with NF-κB activation and down-regulation of eNOS and NO. The findings of the present study also offer a mechanistic explanation of the beneficial effect of green tea on alcoholic liver injury. Furthermore, green tea prevents alcohol-induced liver injury, oxidative stress and inflammation through the activation of the PI3K/Akt/eNOS pathway and inhibition of NF-κB expression.

Conflict of interest
The authors have no conflict of interest to declare.

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