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Chalcone Derivative L6H21 Reduces EtOH + LPS-Induced Liver Injury Through Inhibition of NLRP3 Inflammasome Activation

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Abstract

Background: Chronic alcohol intake increases circulating endotoxin levels causing excessive inflammation that aggravates the liver injury. (E)-2,3-dimethoxy-4'-methoxychalcone (L6H21), a derivative of chalcone, has been found to inhibit inflammation in cardiac diseases and nonalcoholic fatty liver disease. However, the use of L6H21 in alcoholic liver disease to inhibit exotoxin-associated inflammation has not been explored. In this study, we examined the effects of L6H21 on EtOH + LPS-induced hepatic inflammation, steatosis, and liver injury and investigated the underlying mechanisms.

Methods: C57BL6 mice were treated with 5% EtOH for 10 days, and LPS was given to the mice 6 hours before sacrificing. One group of mice was supplemented with L6H21 with EtOH and LPS. RAW264.7 cells were used to analyze the effects of L6H21 on macrophage activation.

Results: EtOH + LPS treatment significantly increased hepatic steatosis and serum levels of alanine transaminase (ALT) and aspartate transaminase (AST), which were reduced by L6H21 treatment. EtOH + LPS treatment increased hepatic inflammation, as shown by the increased hepatic protein levels of Toll-like receptor-4, p65, and p-I κ B, and increased oxidative stress, as

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AUTHOR CONTRIBUTIONS

W.F. and X.K. conceived the project and designed the experiments; X.K., G.W., and S.C. performed the majority of experiments and data analysis; L.Z., T.S., F.L., and L.R. participated in performing experiments and data analysis; H.Z., W.F., and S.C. drafted the manuscript; S.C. and C.M. provided critical discussion on the project and manuscript preparation.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

shown by protein carbonyl levels and reactive oxygen species formation, which were reduced by L6H21 treatment. In addition, L6H21 treatment markedly inhibited EtOH + LPS-elevated hepatic protein levels of NLRP3, cleaved caspase-1, cleaved IL-1 β , and caspase-1-associated apoptosis.

Conclusions: Our results demonstrate that L6H21 treatment inhibits EtOH + LPS-induced liver steatosis and injury through suppression of NLRP3 inflammasome activation. L6H21 may be used as an alternative strategy for ALD prevention/treatment.

Keywords

Alcoholic Liver Disease; Lipopolysaccharide; NLRP3 Inflammasome; L6H21

ALCOHOLIC LIVER DISEASE (ALD) is one of the major chronic liver diseases, mainly manifested in 3 forms: alcoholic fatty liver, alcoholic hepatitis, and alcoholic liver fibrosis (Lee et al., 2016; Li et al., 2013). Alcohol consumption-induced hepatic steatosis is initially benign and can be resolved by abstinence. However, it primes the liver to injury by a second hit or multiple hits. It has become clear that chronic alcohol consumption significantly changes gut microbiome homeostasis and increases Gram-negative bacteria-derived lipopolysaccharide (LPS) production in patients with ALD and in experimental ALD models (Kirpich et al., 2016). Previous studies demonstrate that chronic alcohol consumption increases gut permeability, leading to the elevation of portal LPS levels, which, in turn, serves as a “second hit” and causes liver inflammation, pathological changes, and liver injury.

Routine treatments for ALD patients, such as prednisolone and pentoxifylline, are utilized to suppress inflammation. However, their effectiveness remains controversial. It has been reported that pentoxifylline could suppress the regenerative effect of TNF- α and corticosteroids and may increase the risk of infection (Thursz et al., 2015). Therapies targeting gut microbiota regulation, such as probiotics and prebiotics, have shown promising results, but most of the studies are in animal models of ALD (Li et al., 2015). Nutritional supplementation to increase calorie intake, minerals such as zinc, and saturated fat have been suggested/attempted, but their efficacy remains to be evaluated. For severe ALD patients, liver transplantation is an ultimate choice, but it is realistically difficult due to the shortage of donors. Therefore, effective therapeutic strategies for ALD patients are urgently required.

The imbalance of the inflammatory response in the setting of alcohol exposure in the liver is one of the key mechanisms for the development and progression of ALD. Toll-like receptor (TLR) signaling represents a major innate immune regulation. LPS-induced activation of TLR4-mediated signaling plays a critical role in alcohol-induced liver injury (Kong et al., 2017; Petrasek et al., 2013; Szabo and Bala, 2010). Recent studies demonstrated that the NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome activation is one of the key events in ALD (Kanbe et al., 2017; Schroder and Tschopp, 2010; Wree et al., 2017). The NLRP3 inflammasome complex mediates the maturation of inactive pro-caspase-1 into active cleaved caspase-1 and promotes the maturation and activation of proinflammatory cytokine IL-1 β (De Nardo et al., 2014; Netea and Joosten, 2015; Yang et al., 2017). Obviously, targeting the inflammatory response is one of the major strategies for treating ALD.

Chalcone, a natural product, belongs to the flavonoid group and exists widely in many kinds of plants, such as fruits, vegetables, tea, and powdered soybean. A large number of studies reported that chalcone compounds and their derivatives have strong chemical and pharmacological anti-inflammatory activities (Kumar et al., 2011; Srinivasan et al., 2009; Yadav et al., 2011). It has been reported that chalcone reduced the phosphorylation and degradation of inhibitor of κ Ba ($\text{I}\kappa\text{B}\alpha$) and thus inhibited the inflammatory response induced by LPS (Kim et al., 2010). (E)-2,3-dimethoxy-4'-methoxychalcone (L6H21), a derivative of chalcone, is more potent than chalcone itself or several other synthetic chalcones for the inhibition of the inflammatory response (Wang et al., 2015; Wu et al., 2011). L6H21 could attenuate LPS-induced inflammatory response and sepsis in the lung (Wang et al., 2015) and inhibit LPS-induced cytokine release from macrophages (Fang et al., 2015). L6H21 inhibited AngII-induced formation of MD2/TLR4 and TLR4/MyD88 complex, which ameliorated cardiac remodeling and dysfunction (Fang et al., 2015). These studies led us to hypothesize that L6H21 might be used for the treatment of ALD.

In this study, we evaluated the protective effect and investigated the mechanisms of L6H21 function in mice exposed to 10-day alcohol and subsequent LPS challenge for 6 hours. We found that L6H21 protects the liver from alcohol and LPS-induced hepatic steatosis and inflammation mediated by the NLRP3 inflammasome pathway. This study provides a novel treatment strategy and mechanistic insights into protective effects of L6H21 for ALD.

MATERIALS AND METHODS

Animal Experiments

The 8- to 10-week-old male C57BL6 mice ($n = 36$, 8 mice in each group) weighing 25 to 30 g were obtained from Harlan (Indianapolis, IN). All mice were treated according to the protocols reviewed and approved by the Institutional Animal Care and Use Committee of the University of Louisville. The mice in the EtOH group and the EtOH + LPS group were treated as previously described (Kong et al., 2017). A group of mice was treated with L6H21 via oral gavage at a dose of 10 mg/kg/d on the same day as the start of alcohol feeding. The dose of L6H21 used in this study was based on previous studies (Fang et al., 2017b; Zhang et al., 2018). At the end of the experiment, plasma and tissue samples were collected for assays.

Liver Tissue Histology Analysis

For determination of lipid accumulation, hepatic tissues were stained with hematoxylin and eosin (HE) and frozen tissue sections were attained with Oil Red O. Liver tissue reactive oxygen species (ROS) accumulation was determined using dihydroethidium (DHE) staining. Hepatic tissue NF- κ B and F4/80 were evaluated by immunofluorescence analysis. We followed all those procedures described previously (Kong et al., 2017).

Biochemical Assays

Liver protein carbonyl contents were determined by a colorimetric assay following the manufacturer's instructions (Cayman Chemical, Ann Arbor, MI). The concentrations of TNF- α and IL-1 β in liver tissue homogenates were measured using commercial enzyme-

linked immunosorbent assay kits specific for mouse according to the manufacturer's instructions (Boster, Wuhan, China). Hepatic triglyceride (TG) concentrations and levels of serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined using assay kits from Thermo Scientific (Waltham, MA).

Cell Culture

RAW264.7 cells (mouse macrophage cell line) were obtained from American Type Culture Collection (ATCC). RAW264.7 cells were utilized for experimentation at 70 to 80% confluency. The cells were exposed to ethanol (EtOH; 100 mmol/l) for 48 hours prior to the treatment with LPS at 500 ng/ml for 6 hours. L6H21 was administered (10 or 20 μ mol/l) 2 hours before EtOH treatment. Cell viability was determined using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) following the manufacturer's instructions. Cellular apoptosis was evaluated by TUNEL assay as described previously (Liu et al., 2016). Intracellular ROS in culture cells was determined by using the cell-permeable fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA). Briefly, cells were treated with 10 μ M DCFH-DA for 30 minutes at 37°C in the dark, and then detached by gentle scraping with a cell scraper and analyzed by fluorescence microplate reader. The fluorescence of DCFH-DA was excited at 488 nm, and the emission was collected at 525 nm.

Western Blotting

Hepatic tissues or cultured cells were lysed in radioimmunoprecipitation assay buffer (Beyotime, P0013B) with PMSF lysis buffer. Cytoplasmic and nuclear protein extracts were prepared from liver tissue or RAW264.7 cells using nuclear and cytoplasmic protein extraction kits (Bio Basic, BSP002), according to the manufacturer's protocol. The quality of isolation of cytoplasmic and nuclear fractions was confirmed by Western blotting of β -actin and histone protein levels, respectively. The protein concentration was measured using a BCA assay kit (Beyotime, Shanghai, China). Equal amounts of protein (usually 90 μ g) were separated by 10 to 15% Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE). Proteins were then transferred to PVDF membranes (Immobilon, IPVH00010; Millipore, Billerica, MA). Blots were blocked and immunoblotted with NLRP3 (Cell Signaling, 15101; Danvers, MA), GAPDH (Bioworld, AP0063; Bioworld, Dublin, Ohio), Cleaved Caspase-3 (Cell Signaling, 9664), Caspase-1 (Cell Signaling, 2225), IL-1 β (Cell Signaling, 12426), NF- κ B p65 (Sigma, 510038; Sigma, St. Louis, MO), I κ B α (Abcam, ab32518; Cambridge, MA), p-I κ B α (Cell Signaling, 2859), TLR4 (Abcam, ab13556), Histone (Abcam, ab1791), and β -actin (Santa Cruz, sc-81178) at 4°C overnight, followed by HRP-conjugated secondary antibodies for 1 hour at room temperature. The results were analyzed with ImageJ analysis software (ImageJ 1.44p; Wayne Ras-band, National Institutes of Health, Bethesda, MD).

Statistics

All data are expressed as means $\pm$ SEM from at least 3 experiments. Student's *t*-test was used for statistical analysis for comparison of 2 groups. For more than 2 groups, statistical evaluation of the data was performed using a 1-way analysis-of-variance (ANOVA) test, followed by Tukey's post hoc test. *p*-value < 0.05 was considered to indicate a statistically significant difference.

RESULTS

L6H21 Ameliorates EtOH + LPS-Induced Liver Injury

Ten-day EtOH exposure alone produced an insignificant increase in hepatic lipid accumulation, which was markedly increased by LPS injection on the last day. The scores for steatosis were lower in the L6H21-EtOH + LPS group, compared with the EtOH + LPS group (Fig. 1A,B,G). Confirming the histology results, liver tissue TG concentration was significantly higher in EtOH + LPS-treated mice than in control mice ($p < 0.05$), and L6H21 treatment decreased hepatic TG concentration (Fig. 1C). Next, plasma ALT and AST activities were determined. EtOH + LPS treatment significantly increased ALT and AST levels compared to control mice. However, serum ALT and AST levels were markedly decreased in L6H21-treated mice (Fig. 1D,E). Furthermore, hepatic inflammation was analyzed by Chloroacetate Esterase (CAE) staining and scores for inflammation. EtOH alone produced slight infiltration of cytotoxic neutrophils and an insignificant increase in scores for inflammation, which was obviously increased by LPS injection. L6H21 treatment significantly decreased inflammation in liver tissue induced by EtOH + LPS (Fig. 1F,H). Taken together, our results suggested that LPS aggravated EtOH-induced hepatic steatosis and liver injury, which was improved with L6H21 pretreatment.

L6H21 Attenuates EtOH + LPS-Induced ROS Formation and TLR4–NF- κ B Activation

ROS production was evaluated by DHE staining of frozen sections of liver tissues. EtOH + LPS induced a marked elevation in ROS production, which was significantly decreased by L6H21 treatment (Fig. 2A,B). The contents of carbonylated protein, as an indicator of protein oxidation, were significantly increased in the EtOH group and further increased in the EtOH + LPS group, which was significantly reduced by L6H21 treatment (Fig. 2C).

Ten-day EtOH exposure did not change hepatic TLR4 protein concentration, but it was significantly increased in the EtOH + LPS group (Fig. 3A,B). EtOH exposure alone induced a slight increase in nuclear p65 and p-I κ B α protein levels in the liver, which was markedly increased by LPS treatment. EtOH + LPS treatment decreased I κ B α and increased p-I κ B α protein levels in the liver (Fig. 3A,C,D). F4/80 staining revealed a significant increase in macrophages in the liver (Fig. 3E). Importantly, all the inflammatory indices elevated by EtOH + LPS treatment were suppressed by L6H21 administration (Fig. 3), indicating an antiinflammatory effect of L6H21 in ALD. Since previous studies showed that L6H21 targeted MD2/TLR4 complex, we also measured MD2 protein levels. L6H21 administration did not change MD2 protein levels (data not shown).

L6H21 Decreases NLRP3 Inflammasome Activation

The NLRP3 inflammasome, expressed at high levels in macrophages, is one of the best understood members of the inflammasome family. NLRP3 inflammasome activates caspase-1, leading to maturation of IL-1 β and IL-18. We determined the expression of NLRP3 in liver tissues after EtOH and LPS treatment. As shown in Fig. 4A,B, EtOH-treated mice had higher level of NLRP3 protein in the liver tissues compared with the control mice. LPS injection further increased NLRP3 protein expression, which was significantly reduced by L6H21 treatment. Pro-caspase-1 was increased by EtOH alone, while Casp-1 p20 showed

no changes. However, Casp-1 p20 was markedly increased by LPS in the context of EtOH treatment, which was significantly attenuated by L6H21 (Fig. 4A,C). Liver tissue levels of TNF- α and IL-1 β were increased in EtOH + LPS-treated mice compared to the control group, which were decreased by L6H21 (Fig. 4D,E), suggesting that L6H21 treatment plays an important role in the inhibition of EtOH + LPS-induced inflammasome activation in the liver.

L6H21 Inhibits EtOH + LPS-Induced Apoptosis and Mitochondrial Damage in RAW264.7 Cells

To determine whether macrophage cells are responsible for the protective effects of L6H21 in EtOH + LPS-induced liver injury, we exposed RAW264.7 cells to EtOH (100 mmol/l) for 48 hours followed by 6 hours of incubation with LPS (500 ng/ml). L6H21 was added at doses of 10 and 20 μ Mol/l for 2 hours before EtOH treatment. Cell viability was detected by CCK-8. EtOH or LPS alone significantly decreased cell viability, which was exacerbated by the combined treatment (Fig. 5A). The loss of cell viability by EtOH + LPS was prevented by L6H21 pretreatment (Fig. 5A). Interestingly, a higher dose of L6H21 (20 μ M) treatment slightly decreased cell viability (Fig. 5A). Therefore, we used 10 μ Mol/l L6H21 in the following experiments. Cell apoptosis in RAW264.7 cells was detected by TUNEL staining (Fig. 5B). EtOH or LPS alone induced a moderate increase in the number of apoptotic cells, which was significantly increased in LPS-treated cells. L6H21 pretreatment markedly decreased apoptotic cell numbers (Fig. 5B). To determine oxidative stress involvement in the macrophage activation by EtOH + LPS exposure, we used the DCFH-DA kit to analyze ROS formation. EtOH and LPS individually increased DCF fluorescence, which was exacerbated by the combination of EtOH and LPS but was inhibited by L6H21 pretreatment (Fig. 5C). Bax/Bcl-2 ratio is one of the key indicators in mitochondria-dependent apoptotic signaling. EtOH or LPS alone significantly increased the Bax/Bcl-2 ratio, which was further increased by LPS treatment (Fig. 5D). L6H21 pretreatment completely inhibited EtOH + LPS-induced increase in Bax/Bcl-2 (Fig. 5D,E). Moreover, L6H21 pretreatment markedly decreased EtOH + LPS-induced elevation in cleaved caspase-3 protein (Fig. 5D,F), which was confirmed by immunofluorescence staining (Fig. 5G). These results suggested that L6H21 protects against oxidative stress and apoptosis induced by EtOH and/or LPS in RAW264.7 cells.

L6H21 Inhibits EtOH + LPS-Induced NF- κ B Activation in RAW264.7 Cells

We further examined the L6H21-mediated TLR4–NF- κ B pathway in RAW264.7 cells treated with EtOH and LPS. As shown in Fig. 6A, TLR4 protein level was not altered by EtOH but was increased by LPS treatment. The combination treatment with EtOH and LPS slightly increased TLR4 level (did not reach statistical significance). However, L6H21 treatment completely inhibited the increase in TLR4 induced by EtOH and LPS (Fig. 6A). Furthermore, NF- κ B p65 nuclear translocation was detected in RAW264.7 cells. As shown in Fig. 6B, NF- κ B p65 nuclear level was induced by EtOH or LPS alone and significantly increased by EtOH + LPS cotreatment, which was completely inhibited by L6H21 pretreatment. We did not observe alterations in MD2 protein expression in RAW264.7 cells by L6H21 treatment (data not shown). The results were confirmed by immunofluorescence

staining of NF- κ B (Fig. 6C). These results suggested that L6H21 protected against EtOH + LPS-induced TLR4–NF- κ B activation.

L6H21 Inhibits EtOH + LPS-Induced NLRP3 Inflammasome Activation in RAW264.7 Cells

The possible implications of EtOH and LPS in inflammasome activation in macrophages were examined in RAW264.7 cells. EtOH-treated cells showed slightly increased protein expression of NLRP3 and IL-1 β , which were significantly increased by EtOH + LPS cotreatment (Fig. 7A,C). Importantly, the elevated NLRP3 and IL-1 β protein expression was inhibited by L6H21 treatment (Fig. 7A,C). An active form of caspase-1, p20, was significantly increased by EtOH + LPS treatment and decreased by L6H21 pretreatment (Fig. 7B). These data suggested that L6H21 inhibited EtOH + LPS-induced activation of NLRP3 inflammasome in RAW264.7 cells.

DISCUSSION

In this study, we aimed to explore the effects and mechanisms of a chalcone derivative, L6H21, in EtOH + LPS-induced hepatic steatosis and liver injury. The results presented here demonstrated that L6H21 is effective in protecting against liver injury induced by LPS in the mice pretreated with alcohol. We showed that the protective effects of L6H21 are likely mediated by the inhibition of TLR4–NF- κ B- and NLRP3-mediated inflammasome inhibition.

Our previous studies have shown that EtOH feeding sensitizes the liver to LPS-induced lipid accumulation and liver injury through an autophagy-mediated signaling (Kong et al., 2017). LPS treatment damaged the autophagic response by EtOH and increased hepatic TG, ROS, NF- κ B-mediated inflammation, and liver injury, characterized by elevated ALT and AST. In this study, we further examined whether inflammasome activation is associated with EtOH + LPS-induced hepatic inflammation.

Inflammasomes sense danger signals from pathogens and damaged cells to mediate caspase-1 activation, which proteolytically activates IL-1 β and IL-18. Inflammasome-associated multiple-protein complex is involved in the formation of NLRP1, NLRP3, NLRC4, and NLRP6, which are stimulated by various proinflammatory factors. NLRP3 plays an important role in the immune response and disease process (Schroder and Tschopp, 2010; Zhang et al., 2016). Upon sensing danger signals such as LPS, NLRP3 proteins oligomerize and recruit caspase-1 through ASC (Yamamoto et al., 2004). Subsequently, pro-caspase-1 undergoes an auto-catalytic activation. Finally, mature caspase-1 cleaves pro-IL-1 β to produce cleaved IL-1 β . Full activation of the inflammasome needs 2 signals. LPS is the most characterized ligand in the TLR4-mediated signal 1, and signal 2 appears to be derived from injured cells to stimulate caspase-1 activation. Our study demonstrates that 10-day EtOH treatment activated neither signal 1 nor signal 2 in NLRP3 inflammasome in the liver. It was only activated when LPS was added to the EtOH treatment. This may be explained by the changes in microbiota homeostasis resulting from chronic alcohol exposure. Chronic Lieber–DeCarli alcohol diet feeding significantly changed microbiota only after 6 weeks of alcohol exposure in mice (Bullotterson et al., 2013). Prolonged alcohol exposure induces gut bacterial overgrowth leading to enhanced endotoxin production and

release through damaged gut barrier into the portal circulation (Ojeda et al., 2016), which, together with EtOH metabolism-induced ROS in liver, produces a variety of danger signals serving as signal 2 in inflammasome activation.

Our most important finding is that L6H21 protects the liver from EtOH + LPS-induced damage. L6H21 has been shown to elicit protective effects in various injury models (Fang et al., 2017a; Wang et al., 2015; Yu et al., 2017). Studies have shown that the cardioprotection provided by L6H21 reduced LPS-induced inflammation via targeting MD2 in the TLR4 signaling (Srinivasan et al., 2009; Wang et al., 2015). L6H21 effectively prevented high-fat diet (HFD)–induced hepatic lipid accumulation, profibrotic changes, and expression of proinflammatory molecules through the inhibition of MD2/TLR4 complex formation (Zhang et al., 2018). L6H21 also reduced HFD-induced fibrosis in the kidney and heart tissues by the inhibition of MAPK and NF- κ B signaling (Fang et al., 2017a, b). These findings suggest that L6H21 could be a potential therapeutic agent for the treatment of inflammation-related disease. This led us to test the effectiveness of L6H21 in the inflammatory response in the EtOH + LPS model. Our results clearly demonstrate that L6H21 protects the liver from EtOH + LPS-induced hepatic inflammation through the inhibition of inflammasome activation in macrophages.

The inhibitory effects of L6H21 on inflammasome activation are associated with decreased apoptosis. This caspase-1-associated apoptosis, pyroptosis, has been considered to be an important innate immune response to bacterial insults. Hepatocyte pyroptosis after alcohol consumption is known to participate in the pathogenesis of ALD. L6H21 pretreatment inhibited caspase-1-associated apoptosis in RAW264.7 cells, suggesting that the inhibition of pyroptotic cell death of macrophages may contribute to the protective effect of L6H21 in EtOH + LPS-treated mouse liver.

This study showed that L6H21, a chalcone derivative, effectively inhibited EtOH + LPS-induced hepatic fat accumulation and liver injury in vivo and in vitro. These protective effects are likely mediated by the inhibition of TLR4–NF- κ B signaling and NLRP3 inflammasome activation in macrophages. L6H21 would be a potential therapeutic strategy for the treatment of ALD and in other NLRP3 inflammasome-mediated inflammatory conditions.

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Fig. 1.

L6H21 inhibits EtOH + LPS-induced hepatic steatosis and injury. Male C57BL6 mice were fed a Lieber–DeCarli liquid diet containing 5% EtOH for 10 days followed by an LPS injection at a dose of 5 mg/kg i.p. on the last day. L6H21 was injected at a dose of 10 mg/kg/d via oral gavage before EtOH feeding. Six hours following LPS, the mice were sacrificed for analysis. (A) Liver sections were analyzed with H&E staining. (B) Hepatic steatosis was evaluated by Oil Red O staining of frozen liver tissue sections. (C) Hepatic tissue TG levels were determined using a TG kit. (D, E) Serum levels of ALT and AST were measured. (F) Neutrophil accumulation in the livers was assessed by chloroacetate esterase

staining. Total pathology score (**G, H**) of the liver was determined according to the scoring system as described in methods. Results are presented as means $\pm$ SEM (n = 6). * $p < 0.05$, ** $p < 0.01$.

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Fig. 2. L6H21 decreases EtOH + LPS-induced ROS formation. The superoxide anion-catalyzed ethidium red fluorescence was examined using confocal microscopy (400× magnification) (A) and quantified (B). (C) Protein carbonyl was detected by the spectrophotometric method. Data are expressed as mean $\pm$ SEM ($n = 6$). * $p < 0.05$.

Fig. 3.

L6H21 reduces EtOH + LPS-induced TLR4–NF-κB signaling. (A) Expression of protein TLR4 and nuclear NF-κB p65, p-IκB, and IκBα in the total hepatic fractions was detected by Western blotting. Quantification analysis of TLR4 levels (B) and p-IκB levels. Histone and actin were used as internal controls for nuclear and cytosolic fractions, respectively (C). (D) Quantification analysis of nuclear p65 and IκBα levels. (E) Macrophage infiltration was detected by F4/80 staining. Data are expressed as mean ± SEM ($n = 6$). * $p < 0.05$, ** $p < 0.01$.

Fig. 4.

L6H21 attenuates EtOH + LPS-induced expression of NLRP3 inflammasome. (A) Hepatic NLRP3 inflammasome protein levels were detected by Western blotting. Quantification analysis of protein NLRP3 (B) and Casp-1 p20 (C) levels. Levels of TNF-α (D) and IL-1β (E) in liver tissue were measured by ELISA. Data are expressed as mean ± SEM ($n = 6$). * $p < 0.05$, ** $p < 0.01$.

Fig. 5. L6H21 inhibits EtOH + LPS-induced apoptosis and mitochondrial damage in RAW264.7 cells. Cells were cultured in 96-well plates with or without L6H21 pretreatment. (A) Cell viability was detected by CCK-8 assay. (B) Apoptosis was detected by TUNEL staining. (C) ROS levels were analyzed by the DCFH-DA kit. Protein levels of Bax, Bcl-2, and cleaved caspase-3 were detected by Western blotting (D) and quantification analysis of Bax/Bcl-2 (E) and cleaved caspase-3 (F). (G) Fluorescence staining of cleaved caspase-3 (400× magnification). Data are expressed as mean $\pm$ SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$.

Fig. 6. L6H21 decreases EtOH + LPS-induced TLR4–NF-κB activation in RAW264.7 cells. **(A)** Protein level of TLR4 was detected by Western blotting and quantification analysis. **(B)** Nuclear protein p65 levels and quantification analysis. Histone was used as the internal control for nuclear fraction. **(C)** Immunofluorescence staining of p65 (**green**) using confocal microscopy (400× magnification). Data are expressed as mean ± SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$.

Fig. 7. L6H21 reduces EtOH + LPS-induced expression of NLRP3 inflammasome in RAW264.7 cells. Expression of NLRP3 (A), caspase-1 (B), and IL-1β (C) was detected by Western blotting. Data are expressed as mean ± SEM ($n = 5$). * $p < 0.05$, ** $p < 0.01$.