Lycorine destabilizes lipid rafts to inhibit inflammation via LXRα signal in the lung of mice

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Abstract

Background and Purpose: Lycorine is an alkaloid that was in the bulb of the genus Lycoris. It has properties of anti-inflammatory. This study aimed to investigate the molecular mechanism by which lycorine can reduce acute lung injury (ALI). Experimental Approach: ALI model was established by intranasal injection of lipopolysaccharide (LPS). In vitro, A549 cells were treated with LPS and pretreated with lycorine for 1 hour. Key Results: The results showed that lycorine reduced histopathological changes in lung, myeloperoxidase (MPO) activity, and the production of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in mice. Lycorine dose-dependently inhibited the production of TNF-α, IL-1β, and IL-6. It also inhibited the transmission of TLR4/NF-ϰB passway in LPS-stimulated A549 cells. Lycorine increased cholesterol efflux through the activated LXRα-ABCA1/ABCG pathway. Lycorine has a good binding ability with LXRα. After adding the LXRα inhibitor, the anti-inflammatory effect of lycorine was eliminated. Conclusion and Implications: Lycorine can reduce ALI that was induced by lipopolysaccharide. The anti-inflammatory mechanism of lycorine is related to the up-regulation of the LXRα-ABCA1/ABCG pathway, which inhibits TLR4-mediated inflammation by increasing cholesterol efflux and reducing TLR4 transport to lipid rafts.

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Keywords: Lycorine; ALI; Lipid raft; TLR4; LXRα

Introduction

Acute lung injury (ALI) is a high morbidity and mortality disease. In recent years. Many people were died of ALI due to influenza and COVID-19(J. G. Zhang et al., 2021). ALI is the damage of alveolar epithelial cells and capillary endothelial cells caused by multiple direct or indirect injury factors. Alveolar epithelial cells are widely present in lung tissue. The damage of alveolar epithelial cells will cause diffuse pulmonary interstitial fibrosis and swelling(Shi et al., 2014). Lipopolysaccharide (LPS) is one of the important causes of ALI(Hu et al., 2021). LPS expressed its effects through the TLR family in cell membranes. The TLR family was related to inflammatory cytokines(Gross et al., 2020; Vitiello et al., 2021). TLR4 played an important role in natural immunity. LPS can promote the formation of lipid rafts. After LPS stimulation, TLR4 was recruited into the lipid raft(Kaelberer, Caceres, & Jordt, 2020). It interacted with some molecules on the lipid raft. It could activate the NF-ϰB signaling pathways and promoted the production of cytokines(Liao, Liu, & Zhang, 2021; S. Xu et al., 2023). Lots of cytokines caused the body's inflammatory response, leading to multiple organ failures in severe cases(H. C. Wang, Wu, & Kong, 2021).

The production of inflammatory factors required regulating signaling pathways(Alikiaii, Bagherniya, Askari, Johnston, & Sahebkar, 2021; Novoselova et al., 2015). Most signals were passed through something on the cell membrane, and lipid rafts were one of them(D'Aprile, Prioni, Mauri, Prinetti, & Grassi, 2021). Lipid rafts were the platform for protein docking, closely related to membrane signal transduction and protein sorting(Holani et al., 2020; Suzuki, 2012). The stability of lipid rafts played an important role in the TLR4/NF-ϰB pathway(Adebiyi, Soni, John, & Yang, 2014; B. Liu et al., 2018; Zhang et al., 2023). It was showed that the destruction of lipid rafts in lung epithelial cells could inhibit inflammatory response by LPS-induced(Colardo et al., 2021; Singh et al., 2021). The destruction of lipid rafts was associated with cholesterol metabolism on the cell membrane. Previous studies had found that cholesterol metabolism was related to nuclear receptors, liver X receptor (LXR), and had a regulatory effect on lipid metabolism. LXRs (Liver X Receptor α and β) are members of the nuclear hormone receptor superfamily of ligand-activated transcription factors (Hammer et al., 2021). Activation of LXRs could induce gene expression related to cholesterol excretion, such as ABCG(Song, Yan, Wang, & Lou, 2021; Wan et al., 2021). LXRs regulated the cholesterol metabolism of cells, affected the inflammatory response, and inhibited the expression of inflammatory genes(Kongkwamcharoen, Itharat, Pipatrattanaseree, & Ooraikul, 2021). It may be related to the pathogenesis of ALI. Currently, there is no very effective way to treat this disease. Some herbal medicines have been found to have anti-inflammatory effects and may be used to treat ALI.

Lycorine is an alkaloid in the bulb of Lycoris Radiata, a plant of the family Lycoris Radiata. It has effective anti-inflammatory, anti-viral and anti-tumor effects (H. Chen et al., 2020; Li et al., 2022; M.-H. Li et al., 2021). Does lycorine have a similar anti-inflammatory effect on ALI? Could it be used as a medicine in the clinical treatment of ALI? Previous studies had shown that lycorine can induce HSC-3 cell apoptosis and inhibit cell proliferation (W.-y. Liu et al., 2019). In addition, it has been found that lycorine can increase the production of reactive oxygen species (ROS) and trigger mitochondrial membrane potential (MMP) disorders(Jing, Zhang, Li, & Gao, 2020; Shang, Jang, Shi, & Ma, 2021). It was also found that lycorine

significantly inhibited the expression of CXCL1 and IL-1α in the senescence-associated secretion phenotype (SASP) of SIPS cells and slowed down senescence(Y. Xu, Li, Li, Deng, & Gao, 2023; W. N. Zhang et al., 2021). However, few reports show lycorine can reduce lung injuries caused by inflammation and oxidative stress. Whether lycorine has a protective effect on lung injury needs further study. Lycorine could reduce the inflammatory response of LPS-induced by destroying the lipid rafts was not yet clear. This mechanism remains to be explored.

Materials and methods

Reagents

Lycorine (L812279) was purchased from MACKLIN reagent, China. LPS (S11060) was purchased from Yuanye biotech, Shanghai. UItraRIPA kit for Lipid Raft (KA6023) was purchased from Bioleaf, Shanghai. The primers were purchased from Sangon Biotech, Shanghai. The antibodies were purchased from CST, USA. CCK-8 (BS350B) kit was purchased from Biosharp, China. MPO assay kit (EHJ-45871m) was purchased from HUIJIA BIOTECHNOLOGY, China. The ELISA kit of IL-1β, IL-6, and THF-α was purchased from LunChangShuo Biotech, China. LXR-Luc(11515ES) was purchased from Yeasen Bio, China. A549 cells were donated by Zhang Naisheng's team at Jilin University. GSK2033 (SML1617) was purchased from Sigma Aldrich, USA.

2.2. Animals and groups

Fifty C57 mice (6 weeks of age, 18-25 g) were divided equally between male and female. All mice were randomly divided into 5 groups: control group (CG), LPS group (LPS), and LPS+lycorine (20, 40, 80 mg/kg) group (20, 40, 80). LPS-induced acute lung injury and administration were as follows: In brief, ALI was induced by inhalation of 50 μl 2 mg/mL LPS through the nose in each mouse after injected lycorine (20, 40, 80 mg/kg) or saline intraperitoneally(F. Wang et al., 2009). The drug was injected twice in total intraperitoneally at 12-hour intervals. The control group was injected with saline. The lycorine group was injected with different doses of lycorine hydrochloride solution (20, 40, 80 mg/kg). All mice survived within 36 h after LPS intranasal infusion. All mice were raised at room temperature and anaesthetized with sodium pentobarbital 24 hours after the second intraperitoneal injection. They died of cervical dislocation and were quickly sampled and store in the -80 °C refrigerator. All the procedures in the present study were carried out following the Animal Care and Use Committee of Northeast Agricultural University (SRM-16).

2.3. Histology analysis

The fresh lung tissue of the mice was cut into 2mm-3mm tissue pieces, fixed with 10% neutral phosphate-buffered formalin, dehydrated and transparent, and then immersed in paraffin and cut into 3-5micron paraffin tissue sections. After staining with hematoxylin and eosin, the pathological changes in lung tissues were observed with a light microscope.

2.4. Detection of myeloperoxidase content

The double antibody sandwich method was used to determine the content of myeloperoxidase (MPO) in the sample. Coat the microtiter plate with purified mouse MPO antibody to make a solid phase antibody. Add MPO to the microporous of the coated monoclonal antibody, and then combine with the HRP-labeled MPO antibody to form an antibody-antigen-enzyme-labeled antibody complex. After thorough washing, add substrate TMB for color development. We calculated the content of MPO according to the OD value.

2.5. Immunofluorescence analysis of lipid rafts

Dewaxing paraffin sections to water. The tissue sections were placed in a repair box filled with EDTA antigen repair buffer (PH8.0) and repaired in a microwave oven. Excessive evaporation of buffer solution should be prevented during this process. BSA was dropped onto the tissue sections and incubated for 30min. The BSA was gently removed, and the prepared flotillin-1 antibody was dropped onto the sections. The sections were placed flat in a wet box at 4°C and incubated overnight. Add secondary antibody and incubate at room temperature for 50min. After the slices were shaken dry, DAPI dye was added to the ring and incubated

for 10min at room temperature, away from light. The self-quenching agent was added for 5min, and the water was rinsed for 10min. Tablets were sealed with anti-fluorescence quenching tablets. The sections were photographed under a fluorescence microscope.

2.6. Extraction of cell membrane protein

Add appropriate volume of ice-cold A-buffer to tissue samples (final concentration, 5 mg/ml total protein). Sonication is recommended to completely disrupt tissue debris and avoid contamination of the nucleus. Transfer tissue lysate to 1.5 mL tubes. Centrifuge samples at 10000 rpm for 5 min. Transfer supernatant to another tube. Add 0.5 mL of ice-cold A-buffer into the pellet (RIPA-insoluble fraction) and vigorously re-suspend the pellet with pipetting or voltas. Centrifuge samples at 10000 rpm for 5 min. Remove supernatant and add 100 μL of B-buffer into the pellet and vigorously re-suspended the pellet with pipetting or voltas at room temperature (option: sonication can be available on ice). Incubate for 5 min at room temperature. Centrifuge samples at 10000 rpm for 5 min. Collect the supernatant into a new tube.

2.7. Cholesterol levels assay in cell membrane

Measure the absorbance values of the calibration standard tube and the sample tube at 510nm respectively, and calculate the cholesterol content. Cholesterol esters are broken down into cholesterol fatty acids under the action of cholesterol esterase. Cholesterol and oxygen generate hydrogen peroxide under the action of cholesterol oxidase. Hydrogen peroxide, 4-AAP, and phenol will produce red quinone under the action of peroxidase, and its color is directly proportional to the content of cholesterol. Measure the absorbance values of the calibration standard tube and the sample tube at 510nm respectively, and calculate the cholesterol content.

2.8. cell culture and treatment

The A549 cells were cultured adherently in a DME/F-12 medium. This medium contains 10% heat-inactivated fetal bovine serum (FBS) or 1% 100 units of penicillin and 100ug/ml streptomycin. The cells were cultured in a sterile incubator containing 5% CO2 at 37 °C. When the cell coverage in the culture flask is 80%-90%, cell passaging is required. When passaging, discard the dead cells and the original medium. Added 2 ml trypsin for digestion, until the cells do not adhere to the wall. Then add the medium to terminate the digestion quickly, transfer the cells to a 14 ml centrifuge tube with a dropper, and centrifuge at 1000 rpm for 5 min. Discard the supernatant, add 2 ml of medium, and gently pipette to mix the cells. Divide into two new culture flasks to prepare the culture medium. After the passage to the third generation, when the cells had expanded to a sufficient number, they were cultured in 6-well plates and treated separately.

2.9. LXRα gene assay

A549 cells were cultured in a medium supplemented with LXR luciferase reporter plasmid (LXR-Luc) and β-galactosidase control vector for 24 h. Others were cultured in a medium supplemented with LXR inhibitor GSK2033. A549 cells were pretreated with lycorine (20,40,80 mg/L). After 1 hour, the culture medium was poured out and LPS (3mg/kg) was added for 24 hours. Luciferase activity was detected. The transcriptional activity of the LXR-Luc assay system was compared with that of the β-galactosidase normal group. Cell culture supernatants were collected and the levels of TNF-α, IL-1β, and IL-6 were determined by ELISA after transfection with A549.

2.10. Molecular docking analysis of LXRα and lycorine

ChemBioDraw Ultra 14.0 was used to draw small molecules, and the small molecules were imported into ChemBio3D Ultra 14.0 for energy minimization. The Minimum RMS Gradient was set to 0.001, and the small molecules were saved in MOL2 format. The optimized small molecules were imported into AutoDockTools-1.5.6 for hydrogenation, charge calculation, charge distribution, and rotatable bond setting and then kept in "PDBQT" format. Download the LXRα structure (PDB ID: 3FAL) from the PDB database. Pymol2.3.0 was used to remove the protein crystal water and original ligand. The protein structure was imported into AutoDocktools (V1.5.6) for hydrogenation, charge calculation, charge distribution, and atom type designa-

tion and saved in "PDBQT" format. AutoDock Vina1.1.2 was used for docking, and LXRα parameters were set as: center_x = 64.711, center_Y = 37.098, center_z = 23.658; The search space: size_x: 50, size_y: 50, size_z: 50 each lattice spacing is 0.375 (A) and exhaustiveness: 10, the rest of the parameters as the default Settings. PyMOL2.3.0 and LIGPLOT V 2.2.4 were used to analyze the interaction mode of the docking results.

2.11. Fluorescence quantitative PCR

Total RNA was extracted from mice lung tissue and A549 cells. The concentration and purity of the RNA solution were determined by ultraviolet spectrophotometry at 260 nm and 280 nm. A single cDNA template targeting TNF-α, IL-1β, IL-6, TLR4, NF-ϰB, LXRα, ABCA1, ABCG, specific primers were synthesized by reverse transcription design based on a known sequence of β-actin. Real-time quantitative PCR was performed using the ABI PRISM 7500 processing system. For each gene to be measured, a cDNA template and sample cDNA defining the expressed gene is selected for the PCR reaction. There are 40 cycles, such as 95 ℃ for 15 s, 60 for 60 s, and 72 degC for 20 s. Each experiment was repeated three times and each sample was repeated three times. The β-actin was used as an endogenous internal standard control.

2.12. ELISA assay

The double sandwich antibody method was used to detect the concentration of IL-1β, IL-6, IL-10, and TNF-α in mouse tissues and A549 cells. The tissues were weighed and rinsed with pre-chilled PBS. The tissue was ground with PBS (Simple: PBS=1:9) thoroughly with a glass homogenizer. The supernatant was taken after being centrifuged at 5000 rpm for 10 min. The cell culture supernatant was 2000rpm for 20 min to remove impurities and cell debris. The supernatant was tested. The anti-mouse antibody is coated on the ELISA plate. The cell factor in the sample was combined with the anti-mouse antibody. Then the horseradish peroxidase-labeled antibody is added, and the chromogenic substrate TMB is added. After the stop reaction solution was added. To measure the OD value with a microplate reader at 450nm wavelength. We can calculate the cytokine concentration in the sample by drawing a standard curve.

2.13. Western blot analysis

Total protein was extracted from mouse lung tissue and A549 cells. The protein concentration was determined by the BCA method. Separate the sample using an agarose SDS gel and transfer it to the NC membrane. The membrane was blocked with Tris-buffered saline (TBST) containing 5% skim milk at room temperature for 2 hours and then incubated with a specific primary antibody (1:1000) overnight. Subsequently, the membrane was washed with TBST and then incubated with a secondary antibody at room temperature for 1 hour. Wash the batch with TBST again, and then use BCL luminescent color developing solution to take pictures under the imaging system to analyze the brightness.

2.14. Statistical analysis

SPSS Statistical 19 was used for statistical analysis. The statistics are represented by an average of ± S.E.M. for three separate experiments. Differences between groups were analyzed by one-way ANOVA or Student t test. # $p < 0.01$ vs. the control group, * $p < 0.05$ vs. the LPS group, ** $p < 0.01$ vs. the LPS group.

3. Results

3.1. Lycorine attenuates LPS-induced ALI in mice

In this study, HE staining was used to evaluate pathological changes. ELISA was used to detect MPO activity and inflammatory factors. As shown in Fig. 1A, CG histopathological analysis showed standard lung structure. The lung slices of mice after LPS stimulation showed typical histological changes, including inflammatory cell infiltration, thickening of alveolar walls, interstitial edema, and pulmonary congestion (Fig. 1E). However, 20, 40, and 80 groups significantly alleviated the historical changes caused by LPS administration (Fig. 1B-D). And the higher the concentration of lycorine, the more significant the effect. It is shown in Fig. 1F. MPO activity was significantly increased in the LPS group compared with CG. The content of MPO in Groups 20, 40, and 80 was lower than the LPS group but still higher than CG. The

sandwich ELISA method measured the concentration of TNF-α, IL-1β, and IL-6 in lung tissue. Compared with CG, stimulating cells with LPS without lycorine treatment will significantly increase cytokine production (Fig. 1G-I). After the cells were treated with lycorine (20, 40, 80 mg/L), TNF-α, IL-1β, and IL-6 in the LPS group were significantly lower than in the LPS group. The binding capacity of lycorine to LXRα was calculated using the molecular docking technique.

3.2. Effect of lycorine on TLR4/NF-ϰB signaling pathway and lipid raft

We used the immunofluorescence method to analyze membrane lipid rafts and p65 protein. Western Blot and qPCR detected the level of proteins and mRNA related to the TLR4/NF-ϰB signaling pathway. The result is shown in Fig. 2. The level of lipid raft in lung tissue of mice in the LPS group was significantly higher than that in the CG. the degree of lipid raft in the group treated with lycorine was reduced considerably compared with the LPS group (Fig. 2A-F). As shown in Fig. 2G-I, mRNA levels of TLR4, IϰBα, and NF-ϰB p65 were not entirely different among all groups. Fig. 2J-Q shows the protein expression of each group. Compared with CG, the protein expression of TLR4 in the LPS group was increased, and the phosphorylation levels of IϰBα and NF-ϰB p65 were significantly increased. Compared with the LPS group, the phosphorylation level and TLR4 expression level of histones 20, 40, and 80 decreased successively. Translocation of p65 protein in lung tissue of mice in the LPS group was significantly higher than that in the CG (Fig. 2R-W). The translocation of p65 protein in the group treated with lycorine was considerably reduced compared with the LPS group. The results showed that lycorine could destabilize lipid rafts and reduce LPS-induced inflammatory response.

3.3. Effects of lycorine on membrane cholesterol and LXRα signal.

Lycorine interacts with LXRα mainly through hydrogen bond formation and hydrophobic force. It forms a hydrogen bond with His419 (B); the hydrogen bond length is 2.98 A. It has a hydrophobic effect with Met296 (B), Trp441 (B), Ile293 (B), Ala259 (B), Leu297 (B), Thr300 (B), Leu329 (B), Phe255 (B), Phe252 (B) and Val423 (B). The binding energy of lycorine to LXRα was -8.2kcal/mol, which proved that lycorine had a good binding effect (Fig. 3A). Cholesterol content was detected using the Nanjing Jiancheng cholesterol testing kit. Western Blot and qPCR were used to detect LXRα signal-related protein and mRNA levels. Cholesterol levels did not change significantly in the LPS group compared with CG but significantly decreased in the lycorine group (Fig. 3B). The protein expressions of LXRα, ABCA1 and ABCG in the lycorine group were significantly increased compared with CG and LPS groups (Fig. 3C). The higher the lycorine dose was, the more significant the effect was (Fig. 3D-F). As shown in Fig. 3G-I, the mRNA levels of LXRα increased in both LPS and lycorine groups (20, 40, 80) compared with CG. The mRNA of ABCA1 and ABCG were significantly increased in the lycorine group compared with CG and LPS. The results showed that lycorine up-regulated the expression of LXRα. And it activated ABCA1 and ABCG pathways and promoted the leakage of cholesterol.

3.4. Lycorine inhibits the secretion of inflammatory factors by regulating TLR4/NF-ϰB signaling pathway

The mRNA levels of TNF-α, IL-1β, and IL-6 in A549 cells and the concentrations of TNF-α, IL-1β, and IL-6 in cell culture supernatant were detected. LPS stimulation of cells without lycorine treatment resulted in a significant increase in mRNA levels and concentrations of cytokines compared to CCG. The mRNA levels and contents of TNF-α, IL-1β, and IL-6 in the lycorine group (C20, C40, C80) were significantly lower than those in the LPS group. The concentrations of TNF-α, IL-1β, and IL-6 in the supernatant of the A549 culture were determined by sandwich ELISA. LPS stimulation of cells without lycorine treatment resulted in a significant increase in cytokine production compared to CCG. The levels of inflammatory cytokines such as TNF-α, IL-1β, and IL-6 in the lycorine group (C20, C40, and C80) were significantly lower than those in the LPS group (Fig. 4A-B). As shown in Fig. 4C, there was no significant difference in mRNA expression of TLR4, IϰBα, and NF-ϰB p65 among all groups in cells. The protein expression levels of each group are shown in Fig. 4D-K. Compared with CG, the expression of TLR4 protein and the phosphorylation levels of IϰBα and NF-ϰB P65 were significantly increased in the LPS group. Compared with the LPS group, the phosphorylation level and TLR4 expression level of C20, C40 and C80 decreased successively. The results

showed that lycorine could reduce the inflammatory response induced by lipopolysaccharide by regulating TLR4/NF-κB signaling pathway.

3.5. Lycorine activates LXRα signal by increasing LXRα activity

In this study, qPCR and Western blot detected mRNA and protein levels of LXRα, ABCA1, and ABCG. Luciferase gene assay was used to determine whether lycorine could enhance LXRα activity. The result is shown in Fig. 5. LXRα mRNA and protein levels in lycorine group were not significantly increased compared with CCG (Fig.s 5A and 5E). The mRNA and protein expressions of ABCA1 and ABCG were increased dramatically in a dose-dependent manner compared with the control group (Fig. 5B-C, 5E). The activity of LXRα in the lycorine group was significantly increased compared with that of CCG, and the effect was more significant with increasing dose (Fig.s 5D). The protein ratio analysis of LXRα, ABCA1, and ABCG is shown in Fig. 5F-H. The results showed that lycorine activated the LXRα signal and promoted protein expression of ABCA1 and ABCG by enhancing LXRα transcriptional activity.

3.6. The anti-inflammatory effect of lycorine was weakened by inhibiting LXRα activity

In this study, we detected intracellular LXRα activity, protein levels of cholesterol, LXRα signaling pathway, TLR4/NF-κB signaling pathway, and inflammatory factors after the addition of GSK2033. The result is shown in Fig. 6. After the addition of GSK2033, the activity of LXRα in the lycorine group was significantly inhibited compared with CCG, but the cholesterol content was increased considerably(Fig. 6A-B). The protein level of LXRα in lycorine group was significantly increased compared with CCG (Fig. 6C-I). Protein expression of ABCA1 and ABCG was reduced considerably compared with CCG. The levels of IκBα and NF-κB p65 and phosphorylation were significantly increased. As shown in Fig. 7J, the contents of TNF-α and IL-1β increased considerably, and the range of IL-6 was significantly increased. The results showed that the anti-inflammatory effect of lycorine was weakened after the activity of LXRα was inhibited.

4. Discussion

Lycorine, an alkaloid found in the bulbs of Lycoris Radiata, has been proven to have anti-inflammatory effects(de Queiroz Souza et al., 2021; Ge et al., 2020). ALI is a respiratory disease with a high fatality rate that can be caused by a variety of reasons(L. Li et al., 2021; Zhu, Wang, Xing, & Xiong, 2021). In this study, we performed many tests to analyze whether lycorine has a protective effect on LPS-induced ALI in mice(Qiu, Luo, & Fang, 2021; Yao, Xu, Jia, Li, & Wei, 2021; S. M. Zhu et al., 2021). The results showed that lycorine significantly reduced the degree of LPS-induced ALI. The results of pathological analysis and molecular biological analysis proved that lycorine has a protective effect on LPS-induced acute lung injury(Cui, Xu, Lv, & Guo, 2023).

Lipid rafts are signal transduction platforms on cell membranes. The increase or decrease of lipid rafts will have a pronounced influence on TLR4 (Amine, Benomar, & Taouis, 2021; da Cruz et al., 2021; Park, Cha, Lee, Kim, & Choi, 2021)After LPS stimulation of lung tissues and cells, TLR4 is recruited into lipid rafts to induce downstream signal transduction(Y. Chen et al., 2020; Wei et al., 2016). It has shown that TLR4 recruitment into lipid rafts can be inhibited using drugs by interfering with lipid raft formation (cholesterol consumption)(Dolganiuc, Bakis, Kodys, Mandrekar, & Szabo, 2006). It reduces lipopolysaccharide-induced NF-κB activation and inflammatory cytokine production(Kang et al., 2020; R. Xu et al., 2023; T. Xu, Cui, Xu, Cao, & Guo, 2023). Our results show that lycorine can inhibit the transfer of TLR4 to lipid rafts by decreasing the formation of lipid rafts via consuming cholesterol. Next, we investigated the cause of lycorine lowering cholesterol levels in lipid rafts. We used AutoDock software to calculate the binding capacity of lycorine to LXRα. The results showed that lycorine had an excellent combining ability with LXRα. The results showed that LXRα plays a vital role in regulating cholesterol homeostasis. LXRα mediates the expression of ABCA1 and ABCG to regulate intracellular cholesterol levels.

We tested our hypothesis in cells that lycorine plays an anti-inflammatory role by inhibiting the activation of the TLR4/NF-κB signaling pathway through lipid rafts. The human non-small cell lung cancer cell line (A549) is the primary constituent cell of lung tissue. In this study, LPS stimulation of A549 induces

the activation of the TLR4/NF-ϰB signal pathway and promotes the secretion of inflammatory cytokines, which play an essential role in the process of ALI. This is similar to previous studies (Sivanantham et al., 2019). The results showed that TNF-α, IL-1β, and IL-6 were significantly increased in ALI of mice. We found that lycorine inhibits TNF-α, IL-1β, and IL-6 production in a dose-dependent manner. TLR4/NF-ϰB signal pathway plays a crucial role in inducing cytokine production. Our results show that lycorine inhibits LPS-induced cytokine production by inhibiting the activation of the TLR4/NF-ϰB signal pathway.

Therefore, we examined the effects of lycorine on the activation of LXRα, ABCA1, and ABCG. The results showed that lycorine induced the expression of ABCA1 and ABCG by activating LXRα. Is this pathway related to the anti-inflammatory effects of lycorine? We performed an LXRα activity inhibition assay. The results showed that lycorine altered the expression of ABCA1 and ABCG, membrane cholesterol levels, and LPS-induced inflammatory when LXRα activity was inhibited. It confirmed that inhibition of LXRα activity could reduce the anti-inflammatory effect of lycorine. These results indicate that lycorine binding to LXRα may promote the activation of LXRα. It confirmed that inhibition of LXRα activity could reduce the anti-inflammatory effect of lycorine. Lycorine depletes cholesterol by promoting the activation of LXRα. Cholesterol depletion causes lipid raft instability. It inhibits TLR4 transport to lipid rafts and LPS-induced inflammation.

In summary, our research shows that lycorine can inhibit the expression of TNF-α, IL-1β, and IL-6 in A549 cells stimulated by LPS. The anti-inflammatory mechanism of lycorine is related to the up-regulation of the LXRα-ABCA1/ABCG pathway, which leads to the consumption of cholesterol and reduces the transfer of TLR4 to lipid rafts thereby inhibiting the inflammatory response induced by LPS.

5.Conflict of interest

The authors declare that there are no conflicts of interest.

6. Acknowledgements

The authors gratefully acknowledge Professor Guo from Northeast Agricultural University for providing financial support and experimental technical analysis for this study.

7. Author contributions

Fuhan Wang: Conceptualization, Methodology, Software, Writing- Original draft preparation. Qirui Zhang: Data curation. Kan Li: Visualization, Investigation. Yao Xue: Supervision. Ziyi Li: Software, Validation. Xue-jiao Gao: Writing- Reviewing and Editing.

8. Data accessibility statement

All data, models, or code generated or used during the study are available from the corresponding author by request.

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Table 1

TLR4	Forward: 5'- GCCATCATTATGAGTGCCAATT -3'
	Reverse: 5'- AGGGATAAGAACGCTGAGAATT -3'
NF-ϰB p65	Forward:5'- CCATAGCCATAGTTGCGGTCCTTC -3'
	Reverse: 5'- CGTTCTTCCCTCCCTTTTCCTTTCC -3'
IϰB-α	Forward:5'- GAATCACCAGAACATCGTGAAG -3'
	Reverse: 5'- CAGTACTCCATGATTAGCACCT -3'
LXRα	Forward:5'- AGTTGTGGAAGACAGAACCTCAAGATG -3'
	Reverse: 5'- TGCTGACTCCAACCCTATCCCTAAAG -3'
ABCG	Forward:5'- CCTGACACATCTGCGAATCACCTC -3'
	Reverse: 5'- AACAGCATGGAGAAGAACAGGAAGC -3'
ABCA1	Forward:5'- TTGAATGACGAGGATGAGGATGT -3'
	Reverse: 5'- TTGTTGCCGCCACTGTAGTTA -3'
IL-1β	Forward: 5'- TTCCCA TTAGACAACTGC -3'
	Reverse: 5'- CTGTAGTGTTGTATGTGATC -3'
IL-6	Forward: 5'- CAGAACCGCAGTGAAGAG -3'
	Reverse: 5'- CAGAACCGCAGTGAAGAG -3'
TNF-α	Forward:5'- CTCA TTCCTGCTTGTGGC -3'
	Reverse: 5'- CACTTGGTGGTTTGCTACG -3'
β-actin	Forward:5'- CTACCTCATGAAGATCCTGACC -3'

TLR4	Forward: 5'- GCCATCATTATGAGTGCCAATT -3'
	Reverse: 5'- CACAGCTTCTCTTTGATGTCAC -3'

Fig. legends

Fig. 1: Histological analysis and inflammatory factor detection. (A-E) Histopathology of lung tissue. (A) Lung tissue control group (CG). (B-D) Lycorine administration group (80, 40, 20 mg/kg, respectively). (E) LPS treatment group (LPS). (F) Myeloperoxidase activity in lung tissue. (G) The TNF-α protein level in lung tissue. (H) The IL-1β protein level in lung tissue. (I) The IL-6 protein level in lung tissue. Data represent the contents of 1 mL of supernatant of lung tissue homogenate and are presented as mean ± SD (n = 10). *p < 0.05, significantly different from the CG; #p < 0.05, significantly different from the LPS group.

Fig. 2: Effects of allicin on TLR4/NF-ϰB signaling pathway and lipid raft in lung tissue. (A-E) Immunohistochemistry of flotillin-1 protein was performed on paraffin sections, enabling lipid rafts to be observed under electron microscopy. (A) Control group (CG); (B-D) lycorine administration groups (80, 40 and 20 mg/kg, respectively); (E) LPS treatment group (LPS). (F) Immunofluorescence relative intensity of Flotillin-1. The mRNA and protein levels of TLR4, IϰBα, and NF-ϰB p65 were detected by qPCR and Western blot. (G) The mRNA level of TLR4; (H) The mRNA level of IϰBα· (I) The mRNA level of NF-ϰB p65. (J) The protein levels of TLR4, IϰBα, phosphorylated IϰBα, p65, and phosphorylated p65 were detected. β-actin was used as a control; (K-Q) The relative intensities of TLR4 and IϰBα, p-IϰB α, p65, and p-p65. (R-V) Immunohistochemistry of NF-ϰB p65 protein was performed on paraffin sections so that the nuclear translocation of p65 protein could be observed by electron microscopy. (R) Control group (CG); (S-U) lycorine administration groups (80, 40 and 20 mg/kg, respectively); (V) LPS treatment group (LPS). (W) Immunofluorescence relative intensity of p65. *: p<0.05, **: p<0.01,***: p<0.001, ****: p<0.0001, significantly different from CG; #: p <0.05, ##: p<0.01,###: p<0.001, ####: p<0.0001. significantly different from LPS group.

Fig. 3: Effects of lycorine on membrane cholesterol and LXRα signal. (A) The binding sites of lycorine and LXRα. (B) Cholesterol levels of lipid raft in lung tissue. (C) The levels of LXRα, ABCA1, and ABCG proteins in lung tissues were detected by Western blotting. β-actin was used as a control. (D-F) The relative intensities of LXRα, ABCA1, and ABCG; (G)The mRNA level of LXRα· (H) The mRNA level of ABCA1; (I) The mRNA level of ABCG. CG, control group; lycorine administration groups (80, 40, and 20 mg/kg, respectively); LPS, LPS treatment group. *: p<0.05, **: p<0.01,***: p<0.001, ****: p<0.0001, significantly different from CG; #: p <0.05, ##: p<0.01,###: p<0.001, ####: p<0.0001. significantly different from LPS group.

Fig. 4: Lycorine inhibits the secretion of inflammatory factors by regulating TLR4/NF-ϰB signaling pathway. (A) The mRNA level of inflammatory factors; (B) The inflammatory factor levels; (C) mRNA level of TLR4/p65 pathway; (D) The levels of TLR4, IϰBα, phosphorylated IϰBα, p65, phosphorylated p65 were detected by Western blotting. β -actin was used as a control. (E-K) The relative intensities of TLR4 and IϰBα, p-IϰB α, p65, and p-p65 (n = 3). CCG, control group; lycorine administration groups (80, 40, and 20 mg/kg, respectively); LPS, LPS treatment group. *: p<0.05, **: p<0.01,***: p<0.001, ****: p<0.0001, significantly different from CCG; #: p <0.05, ##: p<0.01,###: p<0.001, ####: p<0.0001. significantly different from LPS group.

Fig. 5: Lycorine activates LXRα signal by increasing LXRα activity. (A) The mRNA level of LXRα. (B) The mRNA level of ABCA1. (C) The mRNA level of ABCG. (D) The activity of LXRα. (E) The levels of LXRα, ABCA1 and ABCG were detected by Western blotting. β -actin was used as a control. (F-H) The relative intensities of LXRα, ABCA1 and ABCG. CCG, control group; lycorine administration groups (80, 40, and 20 mg/kg, respectively). *: p<0.05, **: p<0.01,***: p<0.001, ****: p<0.0001, significantly different from CG; #: p <0.05, ##: p<0.01,###: p<0.001, ####: p<0.0001. significantly different from LPS group.

Fig. 6: The anti-inflammatory effect of lycorine was weakened by inhibiting LXRα activity. (A) Lipid raft

cholesterol level of A549 cells after the addition of inhibitors. (B) Effects of lycorine on LXRα activity after addition of inhibitor. (C) The protein levels of LXRα, ABCA1, ABCG, TLR4, phosphorylated IκBα, and phosphorylated p65 by western blot analysis in A549 cells after inhibitor addition. β-actin was used as the control group. (D-I) The relative intensities of LXRα, ABCA1, ABCG, TLR4,p-IκBα, and p-p65. (J) The inflammatory factors levels after the addition of inhibitors. CCG, control group; Lycorine administration group (80, 40, 20 mg/kg); LPS, LPS treatment group. *: $p<0.05$, **: $p<0.01$,***: $p<0.001$, ****: $p<0.0001$, significantly different from CCG.

Graphical Abstract:Schematic illustration of the mechanism by which Lycorine destabilizes lipid rafts to inhibit inflammation via LXRα signal in the lung. LPS induced acute lung injury in mice. Mechanism of lycorine's anti-inflammatory effect. Diagram of the mechanism of LPS-induced inflammation. GSK2033 inhibits the anti-inflammatory effects of lycorin by inhibiting LXRα activity.

Table 1 : Primer sequence table