Geniposide protects pulmonary arterial smooth muscle cells from lipopolysaccharide-induced injury via \( \alpha 7n\text{AChR-mediated TLR-4/MyD88 signaling} \)

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Abstract. Geniposide is a bioactive iridoid glucoside derived from \textit{Gardenia jasminoides} that has proven anti-inflammatory effects against acute lung injury. The aim of this study was to determine whether geniposide could protect pulmonary arterial smooth muscle cells (PASMCs) from lipopolysaccharide (LPS)-induced injury and to explore the participation of \( \alpha 7 \) nicotinic acetylcholine receptor (\( \alpha 7n\text{AChR} \)), which was previously reported to suppress pro-inflammatory cytokine production in LPS-stimulated macrophages. In the present study, rat PASMCs were isolated and stimulated using LPS. The effect of geniposide on LPS-induced PASMC injury was then explored. Geniposide exerted anti-apoptotic and anti-inflammatory effects on LPS-treated PASMCs, as demonstrated by the downregulation of pro-apoptotic proteins and pro-inflammatory cytokines, respectively. Furthermore, the \( \alpha 7n\text{AChR} \) agonist PNU282987 accentuated the protective effect of geniposide against LPS-induced injury in PASMCs by inhibiting toll-like receptor-4/myeloid differentiation primary response 8 (TLR-4/MyD88) signaling and downregulating nuclear factor (NF)-\( \kappa \)B expression. Conversely, methyllycaconitine, an inhibitor of \( \alpha 7n\text{AChR} \), attenuated the effects of geniposide. These findings collectively suggested that in conjunction with geniposide, the activation of \( \alpha 7n\text{AChR} \) may contribute to further mitigating LPS-induced PASMC apoptosis and inflammation. In addition, the underlying mechanisms critically involve the NF-\( \kappa \)B/MyD88 signaling axis. These results may provide novel insights into the treatment and management of lung diseases via geniposide administration.

Introduction

Pulmonary arterial hypertension is characterized by persistent airflow limitation and is severely harmful to human health. The pathophysiology of pulmonary arterial hypertension is characterized by hypoxic pulmonary vasoconstriction and vascular remodeling (1,2). Pulmonary artery smooth muscle cells (PASMCs) are not only involved in pulmonary vascular remodeling, but also act as immune cells that synthesize and secrete inflammatory factors to promote the development of pulmonary vascular inflammation. Among these factors, toll-like receptors (TLRs) are highly expressed in pulmonary macrophages, smooth muscle cells, epithelial cells and vascular endothelial cells (3). During airway remodeling in asthma, TLRs mediate nuclear factor (NF)-\( \kappa \)B signaling, which is an important pathway that regulates the synthesis and secretion of inflammatory factors in bronchial smooth muscle cells (4,5). In addition, specific mammalian TLRs, such as TLR-2/4, can be activated by lipopolysaccharides (LPS) to promote the production of cytokines, chemokines, adhesion molecules and acute-phase proteins to regulate inflammatory response (6).

Geniposide, an iridoid glycoside extracted from the fruit of \textit{Gardenia jasminoides} Ellis, is a popular medicine for the treatment of acute conjunctivitis, hepatic disorders, inflammatory diseases and hematuria (7-9). Evidence to date has identified the anti-tumor, anti-inflammation, and anti-oxidant properties of geniposide (10,11). Several reports have demonstrated that geniposide can protect rat hepatocytes and...
hippocampus against oxidative injury (12,13). Furthermore, geniposide attenuates LPS-induced over-release of pro-inflammatory cytokines in a mouse model of sepsis (14). However, whether geniposide could have and anti-inflammatory effect in pulmonary arterial hypertension remains unclear.

In the present study, the anti-inflammatory role of geniposide was investigated in relation to α7 nicotinic acetylcholine receptor (α7nACHR), which is a subtype of nAChRs with critical functions in the cholinergic system (15). α7nACHR is widely distributed in various neuronal and non-neuronal tissues (16), such as vascular smooth muscle cells (17), endothelial cells (18), and lung cells (19), and participates in various physiological and pathological processes, including inflammation and neurotransmitter release (16,20-22). α7nACHR was demonstrated to suppress LPS-induced placental inflammation in rats by inhibiting cytokine release and leukocyte infiltration (23). The expression of α7nACHR in normal lung cells and in a series of human lung cancer cells is ubiquitous and can be promoted by nicotine-derived nitrosamine ketone (24). Furthermore, α7nACHR exhibits anti-inflammatory effects in LPS-induced human airway epithelial cells (25).

The present study explored the effect of geniposide on LPS-induced injury in PASMCs. While LPS is usually used to stimulate inflammatory response in certain conditions, such as sepsis, ARDS and ALI, it has also been used to establish models of pulmonary hypertension (26,27). The present study aimed to investigate the involvement of TLR signaling in vascular remodeling through the study of LPS-induced inflammatory injury, which is representative of the pulmonary arterial hypertension phenomenon. It was previously revealed that geniposide can exert protective effects by regulating the expression of α7nACHR through the TLR/myeloid differentiation primary response 88 (MyD88) signaling pathway. The findings collectively revealed the role of geniposide and α7nACHR activation on LPS-induced PASMC apoptosis and inflammation, and provided novel insights into the treatment and management of lung diseases through treatment using geniposide.

Materials and methods

Materials. LPS, pentobarbital sodium, penicillin and streptomycin were purchased from Sigma-Aldrich; Merck KGAa. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific, Inc. Geniposide was purchased from MedChem Express. PNU282987 (PNU; α7nACHR agonist) and methyllycaconitine (MLA; α7nACHR inhibitor) were purchased from Bio-Techne. Primary antibodies against α7nACHR (cat. no. ab216485; 1:800), Bax (cat. no. ab32503; 1:2,000), Bcl-xL (cat. no. ab32370; 1:1,000), Bcl-2 (cat. no. ab59348; 1:500), cytochrome-c (Cyt-c; cat. no. ab76237; 1:200), tumor necrosis factor-α (TNF-α; cat. no. ab6671; 1:1,200), NF-kB (cat. no. ab16502; 1:2,000), TLR-2 (cat. no. ab108998; 1:5,000), TLR-4 (cat. no. ab13556; 1:500), MyD88 (cat. no. ab2068; 1:1,000), and GAPDH (cat. no. ab181602; 1:10,000) were purchased from Abcam. Secondary IgG antibody (cat. no. E030130; 1:10,000) was purchased from EarthOx Life Sciences. ELISA kits for interleukin (IL)-18 (cat. no. RA20058), TNF-α (cat. no. RA20035), and IL-1β (cat. no. RA20020) were purchased from Bioswamp Life Science Lab.

Cell treatment. To investigate the effect of geniposide on PASMCs, cells were pretreated for 2 h with geniposide at various concentrations (0-20 µM), followed by exposure to LPS (10 mg/l) for 2 h. Both geniposide and LPS were dissolved in ultrapure water. Then, the PASMCs were randomly divided into the following experimental groups: i) control; ii) LPS (10 mg/l); iii) LPS (10 mg/l) + geniposide (10 µM); iv) LPS (10 mg/l) + geniposide (10 µM) + MLA (10 nM); and v) LPS (10 mg/l) + geniposide (10 µM) + PNU (5 µM). Both MLA and PNU were dissolved in 100 mM DMSO and treatment time was 48 h at 37°C.

Identification of PASMCs. The morphology of PASMCs was observed by optical microscopy. In addition, isolated cells were identified by immunofluorescence staining of alpha-smooth muscle actin (α-SMA; cat. no. ab5694; Abcam). For immunofluorescence, cells were seeded at 1x10⁵ cells/ml onto 1.5-mm glass coverslips coated with 0.2% gelatin and washed with PBS. The cells were fixed with 4% paraformaldehyde for 15 min at room temperature and permeabilized in 0.2% Triton X-100 for 10 min at room temperature. After 1 h of blocking in 5% bovine serum albumin (cat. no. 10270-106; Gibco; Thermo Fisher Scientific, Inc.) at room temperature, the cells were incubated with the primary antibody...
against α-SMA (1:300 dilution) for 1 h at room temperature. The cells were then washed and incubated for 1 h at 37˚C with FITC goat anti-rabbit IgG (H+L) (1:200; cat. no. SAB43712; Bioswamp Life Science Lab) and 4'-6-diamidino-2-phenylindole as a nuclear counterstain. The coverslips were washed and imaged using a Zeiss Axioplan II microscope (Zeiss AG) at x100 magnification.

**PASMC viability.** After the PASMCs were subjected to various treatments, they were seeded in a 96-well plate at the density of 1x10^4 cells per well. Cell viability was determined using MTT assay (cat. no. PAB180013; Bioswamp Life Science Lab) as previously reported (29). MTT reagent (20 µl) was added into each well and incubated for 2-4 h at 37˚C. When the purple precipitate became visible, the medium was removed and 150 µl DMSO was added to each well. The microplate was shaken at a low speed for 10 min and the absorbance read at 570 nm on a microplate reader.

**PASMC apoptosis.** The apoptosis of PASMCs was measured using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) flow cytometry kit (BD Biosciences) according to the manufacturers’ instructions. PASMCs were washed twice with ice-cold PBS and resuspended in 200 µl of binding buffer at a concentration of 1x10^6 cells/ml. Annexin V-FITC and PI (10 µl of each) were added, and the cells were incubated for 30 min at 4˚C in the dark. Finally, 300 µl of binding buffer was added and the cells were analyzed by flow cytometry (Cytomics FC 500; Beckman Coulter, Inc.) within 1 h using CXP Analysis 2.0 software (Beckman-Coulter).

**ELISA.** The levels of IL-18, TNF-α and IL-1β secreted in PASMC culture medium were evaluated by ELISA according to the manufacturers’ instructions.

**Western blot.** Western blot was performed to determine the expression of α7nACHR, Bax, Bcl-xL, Bcl-2, Cyt-c, TNF-α, NF-xB, TLR-2, TLR-4 and MyD88. For total protein extraction, the cells were washed twice with PBS and lysed with radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology) containing protease inhibitors at 4˚C. Cell lysate was centrifuged at 12,000 x g for 15 min at 4˚C and the supernatant was collected. The protein concentration was determined by a bicinchoninic acid assay kit (cat. no. PAB180007; Bioswamp Life Science Lab). Proteins (30 µg) were separated by 10% SDS-PAGE and transferred onto PVDF membranes (EMD Millipore). The membranes were blocked for 2 h at room temperature with 5% skimmed milk in Tris-buffered saline (TBS; 20 mmol/l Tris, 500 mmol/l NaCl and 0.05% Tween 20). Subsequently, the membranes were incubated with primary antibodies against α7nACHR, Bax, Bcl-xL, Bcl-2, Cyt-c, TNF-α, NF-xB, TLR-2, TLR-4, MyD88, and GAPDH overnight at 4˚C. GAPDH was selected as an internal reference. The membranes were then washed with TBS and incubated with goat secondary antibody for 2 h at room temperature. Enhanced chemiluminescence reagent (EMD Millipore) was used to detect the signal on the membrane. Membranes were scanned with Gel Doc EZ imager (Bio-Rad Laboratories, Inc.). The gray values of the protein bands were quantified using ImageJ version 1.52a (National Institute of Health) and relative expression was calculated by dividing the gray value of each protein of interest with that of GAPDH.

**Statistical analysis.** Data were presented as the means ± standard deviation. Statistical analyses were performed with SPSS 19.0 software package (IBM Corp.) and data were compared using one-way analysis of variance followed by Tukey’s post-hoc test. P<0.01 was considered to indicate a statistically significant difference.

**Results**

**Observation and identification of rat PASMCs.** PASMCs were successfully isolated from rat pulmonary arteries. The cells
were elongated and spindle-shaped, exhibiting the typical hill and valley morphology (Fig. 1A). Positive immunofluorescence staining was observed for α-SMA, which was homogeneously distributed into the cytoplasm, as indicated by the green fluorescence (Fig. 1B-D). These observations confirmed that the cultured cells were smooth muscle cells.

Geniposide improves LPS-induced decrease in PASMC viability. Prior to experiments involving geniposide, the optimal duration of LPS treatment was determined in a preliminary experiment. PASMCs were treated with LPS (10 mg/ml) (17) for 0.5, 1, 2, 4 and 6 h and the cell viability was measured (results not shown). The results demonstrated that LPS inhibited cell viability in a time-dependent manner for up to 2 h; however, the cell viability did not further decrease thereafter. Subsequently, 2 h treatment was used in subsequent experiments with LPS stimulation. To determine the effect of geniposide on LPS-induced cell damage, PASMCs were pretreated with geniposide at various concentrations for different durations to determine the best treatment concentration and time for subsequent experiments. Then, cells were exposed to 10 mg/l LPS in the presence or absence of geniposide. The results from MTT assay demonstrated that geniposide increased the viability of LPS-treated PASMCs. Because there was no significant difference between 0.1, 1, 10, 20 and 50 µM (Fig. 2A), the middle concentration, 10 µM, was selected as the treatment dose. Subsequently, pretreatment with 10 µM geniposide for 2, 4, 12 or 24 h significantly ameliorated the viability of LPS-treated PASMCs. Because there was no significant difference between these time points (Fig. 2B), the shortest duration (2 h) was selected as the treatment time.

Geniposide inhibits LPS-induced apoptosis and inflammatory factor release in PASMCs. To verify the effect of geniposide on LPS-induced cell apoptosis, PASMCs were pretreated with 10 µM geniposide for 2 h and exposed to 10 mg/l LPS for 2 h. The results from flow cytometry demonstrated that PASMC apoptosis was increased after LPS treatment; however, preincubation with geniposide significantly decreased the percentage of apoptotic cells due to LPS (Fig. 3A). To further examine PASMC apoptosis, the apoptosis-related proteins Bax, Bcl-xL, Bcl-2, Cyt-c and TNF-α were detected by western blotting (Fig. 3B). The protein expression of the pro-apoptotic factors Bax, Cyt-c and TNF-α in LPS-treated PASMCs was higher than those in the control group, whereas the protein expression of the anti-apoptotic protein Bcl-2 and Bcl-xL was decreased. However, geniposide administration alleviated the stimulating effect of LPS on apoptosis by downregulating Bax, Cyt-c and TNF-α expression and upregulating Bcl-2 and Bcl-xL expression.

Subsequently, the levels of IL-18, TNF-α and IL-1β secreted in the cell culture medium were detected by ELISA (Fig. 3C). Compared with the non-treated cells, cells treated with LPS presented significantly higher levels of cytokines; however, geniposide pretreatment significantly decreased the levels of IL-18, TNF-α and IL-1β released by LPS-treated PASMCs. In addition, the protein expression of α7nAChR was evaluated in PASMCs (Fig. 3D). The results demonstrated that LPS significantly downregulated the protein expression of α7nAChR in PASMCs compared with non-treated cells; however, this decrease in α7nAChR expression was reversed by geniposide treatment.

Activation of α7nAChR enhances the effect of geniposide on LPS-induced injury. To verify the role of α7nAChR in geniposide treatment against PASMC damage, LPS-treated PASMCs were cultured with the α7nAChR agonist PNU282987 or the α7nAChR antagonist MLA. The apoptotic rate of LPS-treated PASMCs pretreated with geniposide and PNU282987 was significantly decreased compared with that of LPS-treated cells treated with geniposide only. Furthermore, the decrease in PASMC apoptotic rate following geniposide treatment was eliminated by the α7nAChR antagonist MLA (Fig. 4A). The activation of α7nAChR also increased the viability of LPS-treated PASMCs that were pretreated with geniposide, whereas inhibition of α7nAChR had the opposite effect (Fig. 4B).

The involvement of α7nAChR in apoptosis and inflammation was subsequently examined. The activation of α7nAChR downregulated the pro-apoptotic proteins Bax, Cyt-c and TNFα and upregulated the anti-apoptotic proteins Bcl-xL and Bcl-2, whereas inhibition of α7nAChR had the opposite

Figure 2. Geniposide prevented injury in PASMCs treated with LPS. Cell viability was measured by MTT. (A) PASMCs were pretreated with geniposide at 0.1, 1, 10, 20 or 50 µM for 2 h and exposed to 10 mg/l LPS for 2 h. (B) PASMCs were pretreated with geniposide for 1, 2, 4, 12 or 24 h before addition of 10 mg/l LPS for 2 h. All values were expressed as the means ± standard deviation (n=3). *P<0.01 vs. CON. \*P<0.01 vs. LPS. CON, control; LPS, lipopolysaccharides; PASMCs, pulmonary arterial smooth muscle cells.
effect (Fig. 4C). To assess whether activation of α7nAChR could decrease inflammation via the MyD88/NF-κB signaling pathway, the protein levels of NF-κB, TLR-2, TLR-4 and MyD88 were detected in LPS-treated PASMCs (Fig. 4D). In LPS-treated cells, geniposide decreased the protein expression of NF-κB, TLR-2, TLR-4 and MyD88. The treatment with PNU further downregulated the expression of these proteins, whereas treatment with MLA exerted the opposite effect.

Discussion

Geniposide is the major iridoid glycoside constituent of gardenia herbs and has been used as a component of traditional medical formulations because of its anti-inflammatory and antioxidant properties (30,31). It has been reported that geniposide can alter the NF-κB signaling pathway by controlling the overproduction of pro-inflammatory mediators in asthmatic lung tissues (32); however, its effects on pulmonary artery endothelial inflammation remain unclear. The present study demonstrated that geniposide attenuated LPS-induced PASMC injury and that activation of α7nAChR could enhance the protective effect of geniposide by inhibiting the stimulation of the MyD88/NF-κB signaling pathway.

Vascular inflammation and remodeling are important pathological features of chronic obstructive pulmonary disease and PASMCs are the main effectors of pulmonary vascular remodeling (33,34). Under stimulation induced by LPS, inflammation and oxidative stresses are activated.
Figure 4. Involvement of α7nAChR in the protective effect of geniposide against LPS-induced PASMC injury. (A) Flow cytometric detection and quantification of PASMC apoptosis after geniposide pretreatment, LPS stimulation and/or PNU/MLA administration. (B) PASMC viability assessed by MTT after geniposide pretreatment, LPS stimulation and/or PNU/MLA administration. Western blotting and quantification of the expression of proteins associated with (C) apoptosis and (D) MyD88/NF-κB signaling pathway. All values were expressed as the means ± standard deviation (n=3). *P<0.01 vs. LPS. **P<0.01 vs. LPS+GE. LPS, lipopolysaccharides; GE, geniposide; PNU, PNU282987; MLA, methyllycaconitine; Cyt-c, cytochrome c; TNF-α, tumor necrosis factor-α; PI, propidium iodide; FITC, fluorescein isothiocyanate; TLR, toll-like-receptor; NF-κB, nuclear factor-κB; PASMCs, pulmonary arterial smooth muscle cells.
and a complex network of antioxidants, including antioxidant enzymes and stress-response proteins, such as heme oxygenase-1, is triggered (35). The overall effects of geniposide on inflammation are primarily exerted by regulating the synthesis of various cytokines. In particular, TNF-α and IL-1β play critical roles in alveolar macrophages to induce the production of a large number of secondary inflammatory cytokines, such as IL-6 and IL-8. This subsequently results in the participation of neutrophils and CD8+ T lymphocytes in the inflammatory response involved in chronic obstructive pulmonary disease (36). Regarding apoptosis, the pro-apoptotic protein Bax can initiate cell death pathways, whereas Cyt-c can lead to apoptotic cell dismantling by mediating the allosteric activation of apoptosis-protease activating factor 1 to trigger caspase pathways (37). In the present study, the concentrations of the pro-inflammatory factors IL-18, TNF-α and IL-1β and the expression of the pro-apoptosis proteins Bax, Cyt-c and TNF-α were significantly elevated following cell treatment with LPS; however, geniposide mitigated these effects and exerted protection against LPS-induced injury in PASMCs.

Nicotinic acetylcholine receptors are neurotransmitter-gated ion channels of pentameric structure composed of α and β subunits (38). The activation of α7nAChR, which is a nicotinic acetylcholine receptor, has been implicated in the treatment of various diseases, including sepsis, atherosclerosis, and oxazolone-induced colitis (39-41). Administration of α7nAChR agonists can also suppress cytokine release and attenuate tissue damage during inflammation (42). Deficiency or impairment of α7nAChR signaling or the cholinergic anti-inflammatory pathway results in the overproduction of cytokines and enhanced tissue damage (42). In the present study, activation of α7nAChR by PNU enhanced the protective effect of geniposide on LPS-treated PASMCs by regulating the TLR-4/NF-κB signal pathway. Passive smoking and intratracheal instillation of LPS may cause lung injury similar to chronic obstructive pulmonary disease via the TLR-4/NF-κB signaling pathway (36). In lung tissues, upregulation of TLR-4 may activate NF-κB and induce the expression of inflammatory mediators. Furthermore, in macrophages from patients with chronic obstructive pulmonary disease, the TLR-4/MyD88 pathway is activated, and downstream inflammatory cytokines, such as TNF-α and IL-6, are upregulated (43). MyD88 is an adaptor molecule that is engaged by all TLRs, except for TLR-3. It triggers signaling via the MyD88-dependent pathway, which activates NF-κB to release cytokines, such as TNF-α and IL-1 (44). In the present study, the α7nAChR agonist PNU significant inhibited TLR-4/MyD88 signaling in LPS-treated PASMCs pretreated with geniposide, indicating that inflammation was further alleviated upon activation of α7nAChR.

In summary, the findings from the present study demonstrated that geniposide may attenuate LPS-induced PASMC injury, and that activation of α7nAChR could further contribute to the protective effects of geniposide. The mechanism responsible for these effects may involve the inhibition of TLR-4/MyD88 signaling and downregulation of NF-κB expression. These results suggested that the curative potential of geniposide may be associated with the modulation of inflammatory response, offering novel insights into the management of lung diseases. However, further investigation should be carried out to determine the therapeutic effects of geniposide before using it in clinical practice.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

Conceptualization: SYS, XQT and YHX; Development and design of methodology, and creation of models: SYS, LQR, HFZ and YHX; Conducting research and the investigation process, specifically performing the experiments and data collection: SYS, LQR, HDC, HFZ, DFZ; Application of statistical, mathematical, computational or other formal techniques to analyze or synthesize study data: SYS, BZ and YHX; Provision of study materials, reagents, materials, patients, laboratory samples, animals, instrumentation, computing resources, or other analysis tools: XQT and YHX; Preparation, creation and/or presentation of the published work, specifically visualization/data presentation: SYS; Supervision: YHX; Project administration: XQT and YHX; Funding acquisition: XQT and YHX; Writing-original draft: SYS; Writing-review and editing: SYS, XQT and YHX. SYS and YHX confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

Permission was granted from Wuhan Fourth Hospital, Pui Hospital to perform the animal experiments at Wuhan Myhalic Biotechnology Co., Ltd. (http://www.hlkbio.cn) and the study was approved by the Animal Ethics Committee of the Model Animal Institute at Wuhan Myhalic Biotechnology Co., Ltd. (approval no. HLK-20190611-01). All animal experiments were performed in accordance with the ‘Guidelines for Experimental Animals’ from the Ministry of Science and Technology (Beijing, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.
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