Isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate, A Novel Metabolite From *Salvia Miltiorrhiza*, Protects Against LPS-induced Acute Lung Injury in Mice by Attenuating the Canonical and Non-canonical Inflammatory Pathways of Pyroptosis

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Abstract

Background

The pathological characteristics of acute lung injury (ALI) or acute respiratory distress syndrome (ARDS) are pulmonary edema resulting from pulmonary permeability increasing. The main cause is uncontrolled inflammatory response leading to the damage of pulmonary vascular endothelial and alveolar epithelial barriers. However, there has not been effective drugs against ALI. In this study, we investigated the function of Isopropyl 3-(3, 4-dihydroxyphenyl)-2-hydroxypropanoate (IDHP), a novel metabolite of Danshen dripping pill having anti-inflammatory effect, in lipopolysaccharide (LPS) induced ALI in mice, and its underlying mechanisms.

Methods

Pretreatment of IDHP in LPS-induced acute lung injury in mice were observed on survival rate, pulmonary morphologic changes, total protein content in bronchoalveolar lavage fluid (BALF), and inflammatory cytokines in lung tissue and BALF. To further explore its mechanism on ALI, THP-1 macrophages was studied to analyse pyroptosis related proteins and co-culture with epithelial or endothelial cells to assess protection function of IDHP in vitro.

Results

As revealed by survival study, pretreatment with high dose of IDHP reduced the mortality of mice from ALI. IDHP pretreatment significantly improved LPS-induced lung pathological changes, reduced protein leakage and lung myeloperoxidase activity. IDHP also inhibited the release of inflammatory mediators TNF α , IL-1 β , IL-6 and IL-18 in BALF and lung tissue. Meanwhile, IDHP decreased the expression of active-caspase1 (in canonical pyroptosis pathway), caspase4/5 (non-canonical pyroptosis pathway), Nlrp3, mature IL-1 β , mature IL-18, Asc speck formation, and cleaved Gsdmd, all these are required for pyroptosis, in LPS stimulated THP-1 macrophages. Moreover, IDHP also decreased ROS production in LPS-stimulated THP-1 macrophages, inhibited the expression of tight junction proteins (Occludin, Zo-1) in endothelial cells, and decreased lactate dehydrogenase activity in supernatants of epithelial or endothelial cells, co-cultured with LPS-stimulated THP-1 macrophages.

Conclusions

Pretreatment of IDHP improves survival rate and ameliorates LPS-induced ALI in mice possibly via inhibiting canonical and non-canonical pyroptosis pathways.

Background

Acute lung injury (ALI) is a lung disease characterized by osmotic destruction of blood vessel walls leading to inflammatory cell infiltration and pulmonary edema in the alveoli[1]. Acute respiratory distress syndrome (ARDS) is a life-threatening acute lung injury, and the mortality rate of severe ARDS patients is

as high as 46%[2]. At present, there is no specific therapeutic drug for the treatment of ALI in the world, so exploring effective medicine and therapeutic targets for ALI is of great emergency. Danshen is the dried root of the plant *Salvia miltiorrhiza*, a traditional Chinese medicine, which is widely used in the treatment and research of cardiovascular diseases[3]. The biological and pharmacological effects of Danshen have been identified, including the improvement of microcirculation, anticoagulation, antioxidation, anti-myocardial ischemia, anti-inflammation, and anti-tumor[4-7]. IDHP was one of the main bioactive metabolites of Danshen after oral administration of compound Danshen dripping pills, which was developed by Northwest University's Chinese Herbal Medicine Modernization and Engineering Center. The structure of IDHP The IDHP structure is as follows. IDHP is the main metabolite of Danshensu, the water-soluble component of Danshen, with a purity of > 99%. Studies have found that IDHP exhibited anti-inflammatory effect on LPS-induced microglia cells by inhibiting nitric oxide (NO), tumor necrosis factor- α (TNF- α) and interleukin 1- β (IL -1 β) produces[8, 9]. However, whether IDHP can reduce ALI has not been studied.

Pyroptosis, manifested by cell swelling and lysis, is a novel mode of cell death which is characterized by both apoptosis and necrosis[10]. Including the Caspase1 mediated canonical pathway and the non-canonical pathway that directly act on caspase4/5/11, whereas caspases are the natural immune receptor of intracellular LPS [11]. Both pathways depend on the activation of Caspase1, which on the one hand cleaved IL-1 β and IL-18 maturation [12, 13], on the other hand, Gsdmd N-terminal oligomerization has perforating activity, which can be inserted into the cell membrane to form circular pores, destroy the integrity of the cell membrane, drive swelling and membrane rupture, release a large number of pro-inflammatory factors, thereby further inducing and amplifying the inflammatory response[14, 15]. Proteinases, cationic peptides and reactive oxygen species released by neutrophil aggregated during inflammation increase in permeability of the alveolo-capillary membrane[16]. The increased permeability of epithelial cells is due to the loss or redistribution of tight junction proteins[17]. Occludin and zonula occludens1 (ZO-1) are two important tight junction proteins, which play an important role in maintaining the integrity of epithelial and endothelial barrier functions[18]. Studies have shown that LPS stimulation significantly increases lung permeability and reduces the expression of connexin ZO-1 and Occludin. Targeting the caspase pathway can prevent the early disruption of tight junction proteins in ARDS and reduce the formation of lung oedema[19-21]. At the same time, up regulating of ZO-1 and Occludin protein can also improve the inflammatory response[22, 23]. Considering its anti-inflammatory effect, we designed LPS-induced acute lung injury model to evaluate the protective effect of IDHP and explored its potential targets to provide new insights in the treatment of acute lung injury.

Materials And Methods

Materials

IDHP is independently developed by the laboratory of Northwest University , LPS (*Escherichia coli* lipopolysaccharide, 055:B5), Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Company (St. Louis, MO, USA). Lactic dehydrogenase (LDH), Myeloperoxidase (MPO) were purchased

from the Jiancheng Bioengineering Institute (Nanjing, China). The primary Antibodies Nlrp3, Asc, Caspase1, Gsdmd, Hmgb1, Caspase4, Caspase5, IL-1 β , IL-18, IL-6, TNF α were purchased from Abcam (Cambridge, England), β -actin, Zo-1, Occludin were purchased from protentech (Chicago, USA). Enzymelinked immunosorbent assay (ELISA) kits of TNF α , IL-6 were purchased from R&D Corporation (R&D Systems Inc, MN, USA).

Animal grouping

C57 mice, 15-20 g, were obtained from the Animal Center of the Fourth Military Medical University (Xian, China). All studies were approved by the Institutional Animal Care and use committee of the Fourth Military Medical University. The mice were divided into control groups, lung injury model groups LPS (50 mg/kg), high dose of IDHP prevention groups IDHP (90 mg/kg)+LPS, medium-dose of IDHP prevention groups IDHP (60 mg/kg)+LPS and low-dose of IDHP prevention groups IDHP (30 mg/kg)+LPS (n=20 per groups). In the IDHP prevention groups, animals received IDHP by gavage, One hour later, the lung injury model groups (LPS) and the prevention groups (IDHP 90 mg/kg+LPS, IDHP 60 mg/kg+LPS, IDHP 30 mg/kg+LPS) were injected intraperitoneally with LPS 50 mg /kg, the control groups was intraperitoneally injected with physiological saline. Then mortality of mice was recorded every 6 h for 72 h after the LPS injection in each treated groups.

Bronchoalveolar lavage fluid (BALF)

The mice were anesthetized with intraperitoneal sodium pentobarbital. Each groups lungs (n=6) were lavaged twice with 1ml of PBS for five time, each time about 0.9 ml was recovered, and the recovery ratio of the fluid was approximately 90%, which is the collected whole lung alveolar lavage fluid. Centrifuge the BALF at 450 g for 10 minutes at 4°C, take the supernatant and use the BCA protein quantification method to determine the BALF protein content, and store the collected BALF at -80°C for subsequent Elisa detection.

Hematoxylin and eosin (H&E) staining and inflammation score

After the experiment, C57 mice were weighed (n=3). After anesthesia, the right lungs of each groups of mice were taken, fixed in 4% paraformaldehyde for 48 hours, than embedded in paraffin, cut into 4 μ m, hematoxylin and eosin (H&E) stains. Further through histopathological analysis, the degree of lung injury was evaluated from five aspects: pulmonary congestion, alveolar neutrophil infiltration, hyaline membrane formation, alveolar septum thickness and alveolar cavity fracture[24].

MPO activity

The mice were anesthetized with sodium pentobarbital (n=6), the whole lung tissue was taken to wash the blood stains with PBS, and the weight of the lung tissue was accurately weighed after the filter paper absorbed the water. According to the operation guide, the myeloperoxidase test was performed immediately.

Calculation formula: MPO activity (U/g) = (determined OD value-control OD value)/11.3 × sampling amount (g)

Elisa

Levels of TNF- α , IL-6, IL-1 β and IL-18 in the cells supernatant were determined by using commercially available Elisa kits according to the R&D manufacturer's instructions.

Western blotting

Weigh 20 mg of lung tissue and add 200 μ l lysate to homogenize, the supernatant was collected by centrifugation at 4°C, 12000 rpm 25 min, by pipetting on ice for 30 min, Protein concentrations were determined by BCA protein assay kit, and then added 1/4 SDS-PAGE sample loading buffer, 100°C, 5 min metal bath heating to denature the protein. Samples were separated on a denatured 8%SDS – polyacrylamide gel and transferred to polyvinylidene fluoride (PVDF) membrane, containing 5% non-fat dry milk at room temperature for 1 h, incubate the primary antibody overnight, and incubate the secondary antibody for 1 hour the next day. Use a chemiluminescence imaging system (Chemiscope 6100) for imaging, store at -20°C for subsequent Western blot experiments.

Cell culture

A human monocyte leukemia cell line (THP-1), adenocarcinoma human alveolar basal epithelial cells (A549) were presented by the Pathophysiology Laboratory of Fourth Military Medical University, and human umbilical vein endothelial cells (HUVEC) were purchased from Shanghai Zhongqiao Biological Co.Ltd. THP-1 cells are cultured in RPMI 1640 medium containing 10% fetal bovine serum and 1% double antibody at 37°C, 5% CO₂ in a cell incubator. When all cells are cultured with a density of 80%, pass them to a six-well plate containing 50 ng/ml PMA inducer. Grouped into control groups (Control), drug control groups (IDHP 90 mg/kg), model groups LPS (1 μ g/ml), drug prevention groups (IDHP+LPS), IDHP (90 mg/kg) was administered one hour in advance, then LPS (1 μ g/ml) was added, and various indicators were measured 12 hours later.

Apoptosis

Cell culture as described above, take each groups of cells, digest them with 2.5% EDTA trypsin for 3 minutes until the cells are separated from the six-well plate, stop the digestion and centrifuge at 1100 rpm for 5 min at 4°C, washed three times with PBS, save 50-100 μ l of cell suspension at the bottom, add FITC and PI staining solution, protect from light for 15 minutes, and immediately perform apoptosis detection on the flow cytometer.

Quantitative real-time PCR (qRT-PCR)

According to the instructions of TreliefTMRNAprep FastPure Tissue&Cell Kit, isolated the RNA from the six-well plate, then remove the genomic DNA according to the TAKARA kit and reverse transcription into

cDNA, and then apply the Thermal Cycler Dice™ Real Time System amplification instrument method for real-time fluorescence quantitative analysis. After the reaction, the amplification curve and melting curve are confirmed, and the PCR result is calculated using $2^{-\Delta\Delta CT}$.

Table 1 Primers used in this study for PCR

Gene	Forward	Reverse
IL-6	5'-CATCCTCGACGGCATCTCAG-3'	5'-TCACCAGGCAAGTCTCCTCA-3'
TNFα	5'-GCCCATGTTGTAGCAAACCT-3'	5'-GGACCTGGGAGTAGATGAGGT-3'
IL-18	5'-ATCGCTTCCTCTCGCAACAA-3'	5'-TCCAGGTTTTTCATCATCTTCAGC-3'
IL-1β	5'-AAATGATGGCTTATTACAGTGGCA-3'	5'-CCTTGCTGTAGTGGTGGTCG-3'
Asc	5'-GTCACAAACGTTGAGTGGCT-3'	5'-AAGTCCTTGCAGGTCCAGTT-3'
Caspase1	5'-ATGTCTGTGGGCAGGAAGTG-3'	5'-CCCTGTTTCTTCAGTGTGGGA-3'
Nlrp3	5'-CTGGCATCTGGGGAAACCT-3'	5'-AGTCCACATCCTCCAGGTCC-3'
Gsdmd	5'-CAGTTTCACTTTTAGCTCTGGGC-3'	5'-GACCCCATGCTCCGTGAC-3'
Caspase-4	5'-CAAGAGAAGCAACGTATGGCA-3'	5'-AGGCAGATGGTCAAACCTCTGTA-3'
Caspase-5	5'-TCACCTGCCTGCAAGGAATG-3'	5'-TCTTTTCGTCAACCACAGTGTAG-3'
Hmgb1	5'-CATCTCAGGGCCAAACCGAT-3'	5'-GCCCATGTTTAGTTATTTTTTCGTGC-3'
Pcaf	5'-GAATCGCCGTGAAGAAAGCG-3'	5'-TTACAAGACTCCTCGGCCTTG-3'

Western blot of inflammatory factors in the pyroptosis pathway

Plant the cells in a 25 cm plastic culture flask. After the THP-1 model is established, remove the cells from each groups, wash them with PBS three times, and add 200 ul of lysis solution. After lysis for 30 minutes, a centrifuge at 4°C, 12000 rpm, 20 min, supernatant for BCA protein quantification, boil at 100°C in metal bath for 5 min, samples are subjected to SDS-PAGE gel electrophoresis experiment.

Immunofluorescence

The induced THP-1 macrophages were inoculated into confocal dishes and divided into four groups. The drug control groups (IDHP) and drug prevention groups (IDHP+LPS) were given IDHP 90 mg / ml in advance, and the model groups (LPS) and prevention groups (IDHP+LPS) were given LPS 1 ug / ml one hour later. After 12 hours, PBS washed three times for 5 minutes, fix with 4% paraformaldehyde for 30 minutes, rupture the membrane with 0.2% TritonX-100 for 10 minutes, washed 5 minutes and add primary antibody at 4°C overnight. The next day, add the secondary fluorescent antibody to incubate at room

temperature for 1 hour, stain the nucleus with DAPI for 15 minutes, wash three times for 5 minutes, observed by confocal fluorescence microscope.

ROS detection

According to the instructions of the Biyuntian reactive oxygen detection kit, add the DCFH-DA probe, incubate at 37°C for 20 min, then wash three times with serum-free medium, try to clean the remaining probes, and immediately perform ROS on the flow cytometer detection.

Tight junction Western blotting

After the THP-1 cell model was established, the cell culture supernatant was taken to culture Human Umbilical Vein Endothelial Cells (HUVEC) and epithelial cells (A549). After 12 hours, cell proteins were extracted and Western blot determine the expression of connexin Zo-1 and Occludin.

LDH activity detection

After the THP-1 cell model was established, take the supernatant of the culture medium, and measure the absorbance with a 450-nanometer microplate reader according to the instructions of the Nanjing Jiancheng Lactic Dehydrogenase (LDH) Detection Kit.

Statistical analysis

Survival data was determined by Kaplan-Meier method, and log-rank test were used for comparison, used IBM SPSS Statistics 24 for statistical analysis. All data are Mean $\pm$ SEM. Differences between multiple groups are compared using one-way ANOVA. $P < 0.05$ is considered statistically significant. GraphPad Prism 6.02 is used as the drawing software.

Results

IDHP pretreatment improved the survival rate and ameliorated LPS-induced lung injury in mice.

Effect of IDHP on LPS-induced mortality and lung injury in mice.

To evaluate the protective effect of IDHP on mice with endotoxemia, IDHP (30, 60, or 90 mg/kg) was administrated orally prior to LPS injection. As shown in Fig.1. A, the accumulative mortalities during 72 hours were 15% in high dose (90 mg/kg), 40% in medium dose (60 mg/kg), 30% in low dose (30 mg/kg) IDHP pretreatment groups respectively. However, only mortalities in high dose (90 mg/kg) groups were significantly lower than that in LPS groups (65%, $P < 0.01$). The medium and high dose groups (30, 60 mg/kg) failed to protect significantly against death ($P > 0.05$). Total protein content in BALF reflects the severity of lung injury. LPS administration significantly increased the protein content in BALF (Fig.1.B), indicating that lung permeability increased. The high dose of 90 mg/kg IDHP efficiently decreased the total protein content in BALF of ALI in mice (Fig.1.B). To evaluate the neutrophil infiltration in lung, myeloperoxidase (MPO) activity in lung tissue were performed (Fig.1.C). LPS administration significantly

increased MPO activity in lung tissue, but IDHP pretreatment markedly reduced MPO activity compared with LPS groups. These data indicated that pretreatment with IDHP could significantly protect mice with endotoxemia from death. It was preliminaries proved that IDHP can protect the permeability of blood vessel walls , reduces the leakage of interstitial proteins in lung tissues and inhibit inflammatory. Consistent with the phenomenon of H&E staining section, the degree of injury in LPS groups was the most seriously , but the dose of 90 mg/kg in the drug groups was the lightest (Fig.1. D and E), which further proved that IDHP had a protective effect on ALI in mice. At the same time, we tested the inflammatory cytokines in BALF and lung tissue. It was found that the characteristic inflammatory cytokines exuded in BALF and lung tissue in LPS groups was significantly increased than the control groups, but IDHP+LPS 90 mg/kg groups was significantly decreased compared with LPS groups (Fig.1. F,G and H).

IDHP pretreatment inhibit the transcription and translation in the canonical and non-canonical pathway target proteins of pyroptosis of LPS-stimulated THP-1 macrophages

We established THP-1 cell model, and the cells induced by PMA are similar to macrophages derived from natural monocytes. With LPS stimulation for 12 hours after IDHP administration one hour, cell apoptosis was obvious in the LPS 1 ug/ml groups, and the maximum concentration of IDHP 90 mg/kg in animal experiments had nearly no damage to THP-1 macrophages, and it could reduce the apoptosis damage of macrophages (Fig.2. A and B). Therefore, LPS 1 ug/ml and IDHP 90 mg/kg can be used for the best effective concentration to explore its mechanism. Then we used qRT-PCR and Western blotting to detect the transcription and translation of the canonical pathway cell pyroptosis proteins, the mRNA of Asc did not significantly change among four groups. Nlrp3 mRNA increased significantly in LPS groups compared with control groups, but the IDHP+LPS groups had no difference between LPS groups. The Caspase1 and the Gsdmd gene transcription level were significantly inhibited in IDHP+LPS 90mg/kg groups compared with LPS (Fig.2. C). Protein expression analysis of the canonical pathway gene shows that compared with LPS groups, except for the little change in Asc, the other proteins in the IDHP+LPS groups had a significant decrease (Fig.2. D and E). Asc speck is the formation of dimer speck by Asc aggregates during the activation of inflammasomes, which are used to activate Nlrp3 and activate Caspase1. The red speck in the blank control groups and the drug control groups was weak and almost absent, the speck in the LPS groups was brighter and the area was obvious. The IDHP prevention groups was darker than LPS groups, but there were also red speck areas(Fig.2. F and G). Therefore, it can be judged that Asc assists the activation of Nlrp3 inflammasome to form red speck, which further proves that IDHP drugs have the effect of initially inhibiting the activation of Nlrp3. It affects that Caspase1 activity, at the same time, IDHP inhibits the transcription level and protein expression level of Caspase1, Gsdmd, and acts on the entire canonical signaling pathway.

The combination of high mobility groups box 1 (Hmgb1) and p300/CBP-associated factor (Pcaf) can mediate Hmgb1-LPS into the cytoplasmic lysosome, and LPS directly recognizes Caspase4/5/11 to activate the non-canonical pyroptosis pathway. Fluorescence images indicate that Hmgb1 (Green) groups are relatively weak, the LPS groups is relatively bright, and green fluorescence of the IDHP+LPS groups

does not change significantly with the LPS groups (Fig.3. D), there is no statistical difference(Fig.3. E). Therefore, it is still necessary to further explore the target of IDHP.

It can be seen from the figure that the transcription of Hmgb1, Pcaf, and Caspase4 proteins had little change, but the transcription level of Caspase5 increased significantly in the LPS groups, and decreased obviously in the IDHP (90 mg/kg)+LPS groups(Fig.3. A). It was speculated that IDHP may significantly inhibit the transcription level of Caspase5 in the non-canonical pathway. The non-canonical pathway Caspase4/5/11 in LPS groups expression increased successively, and significantly decreased in the drug groups(IDHP+LPS), but Hmgb1 PCR and Western blotting showed no obvious phenomenon (Fig.3. B and C). After Nlrp3 is specifically inhibited, IL-1 β and IL-18 released by the Caspase4/5/11 pathway are also reduce, and also Caspase1[25]. Therefore, our experimental results proved that IDHP may have an effect on both the canonical and non-canonical pathways. The final effect is to inhibit the activation of Caspase1, successively inhibit the activation of Gsdmd, and reduce the inflammatory factors IL-1 β and IL-18.

IDHP pretreatment reduce the release of IL-1 β and IL-18 in LPS- stimulated THP-1 macrophages

The expression of IL-1 β in THP-1 macrophages increased significantly in the LPS groups, and decreased significantly in the IDHP+LPS groups, IDHP inhibits transcription of IL-1 β , and that IL-18 was significantly increased in LPS groups, but not significantly decreased in IDHP+LPS groups (Fig.4. A and C). IDHP may not have an effect on the transcription of IL-18. However, when detecting the inflammatory factors in THP-1 macrophage culture supernatant by Elisa, we found that IL-1 β and IL-18 in LPS groups both increased significantly, and both decreased significantly in the IDHP+LPS drug groups (Fig.4. B and D). Consistent with the results of Elisa, IL-1 β in the LPS groups was significantly increased and was significantly decreased in the IDHP+LPS groups, IDHP inhibits LPS to activate IL-1 β transcription and translation (Fig.4. E and F).The IL-18 protein in the LPS groups also increased, and the release of IL-18 in the IDHP+LPS groups decreased. Therefore, we concluded that IDHP can inhibit the occurrence of pyroptosis and reduce the release of inflammatory factors IL-1 β and IL-18.

Reduction of inflammatory factors alleviate the damage of endothelial tight junction proteins

Excessive ROS can directly damage cellular macro-molecules, resulting in cell death. Our results showed that IDHP can significantly reduce the production of ROS by THP-1 macrophages (Fig.5. A and B). Tight junction down-regulates the expression of Zo-1 and Occludin in LPS- induced acute lung injury [22], and studies have shown that the up-regulation of Zo-1 and Occludin by IDHP contributes to the treatment of acute lung injury. THP-1 macrophages were co-cultured with endothelial cells, and western blotting as shown in Fig.5. C and D suggested that the expressions of Occludin and Zo-1 in LPS groups were reduced, and the expression of IDHP+LPS groups was higher than that of LPS groups. Therefore, it can be preliminarily judged that the reduction of the release of inflammatory factors reduces the damage of endothelial cell tight junction proteins Occludin and Zo-1. Take the supernatant of the co-culture medium to determine epithelial and endothelial the LDH activity. We found that LDH activity was the highest in the LPS groups, while the activity in the IDHP+LPS groups was decreased (Fig.5. E and F.). Therefore, it is

concluded that as the damage of the Occludin and Zo-1 is reduced, the permeability of the cell membrane is protected, the LDH release from the cell is reduced, and the cell damage is reduced accordingly.

Discussion

It is worth noting that IDHP has been confirmed to have anti-inflammatory effects on LPS-induced microglia, but the acute lung injury model and possible targets have not been explored in depth. In this study, IDHP pretreatment can reduce the pathological condition of the lung injury and improve the survival rate of mice. In addition, we also found that IDHP may reduce LPS induced THP-1 macrophage injury, reduce inflammatory factor release, ROS production and LDH activity, and protect cell injury, pulmonary microvascular permeability and pulmonary edema of preventing the occurrence of pyroptosis. LPS-induced acute lung injury is a model of replication-induced lung injury, which can destroy the vascular endothelial barrier and promote vascular permeability and pulmonary edema formation, was widely used by researchers[26, 27]. IDHP has been preliminarily verified to have anti-inflammatory properties. Our study shows that high-dose IDHP has a significant protective effect on the lethality of LPS mice, low-dose and medium-dose also have a certain protective effect, but the effect is not significant compared with high-dose groups. In addition, IDHP pretreatment also reduces LPS-induced BALF protein content, MPO activity, pathological levels of lung tissue sections, and the release of inflammatory factors including TNF α , IL-1 β , IL-18 and IL-6.

It has been speculated that IL-1 β and IL-18 may be released through pyroptosis. Pyroptosis includes two pathways, the canonical pathway and the non-canonical pathway. The canonical pathway cleaves Gsdmd by activating Caspase1, and the cleaved p30 amino terminal fragment Gsdmd is thought to be involved in the induction of pyroptotic cell death owing to its pore-forming capacity, is an event essential for induction of cell death [28]. The non-canonical pathway mediates LPS into the cytoplasm by Hmgb1 and directly activates Caspase11/4/5. Hmgb1 is a ubiquitous nuclear DNA binding protein, and the acetylase complex Pcaf mediates the translocation of Hmgb1[29]. Last, the pyroptosis executive protein Gsdmd is cleavage, Gsdmd-N-terminal oligomerization is activated, and it is inserted into the cell membrane to form ring-like structures lead to the membrane dissolved[30-33]. Our results indicate that LPS stimulates macrophages, and the cells necrosis through the pyroptosis pathway, IDHP may have a protective effect in the cells. Our preliminary analysis shows that IDHP pretreatment can inhibit the translation levels of Nlrp3, but has no effect on the transcription and translation of Hmgb1 and Pcaf. Therefore, we speculate that it is possible that IDHP did not inhibit the non-canonical pathway of pyroptosis through the Hmgb1 pathway.

Caspase family members have different regulatory mechanisms and functions[34]. Activation of Caspase1 and Gsdmd is essential in inducing cell pyroptosis[28], inhibition of Caspase1 activity, IL-1 β release and Gsdmd expression [35]. Caspase11 is a mouse gene, human caspase-4/5 has the highest homology with Caspase11. Caspase-4/5/11 is the receptor for cytosolic LPS, which is mediated by Hmgb1 and outer membrane vesicles (OMV) to conduct LPS into the cytoplasm[36, 37]. Studies have found that activation of Caspase4/5/11 induces pyroptosis, which results from maturation of IL-1 β

driven by the activation of Nlrp3 inflammasome. That is, the activation of the non-canonical pathway of pyroptosis eventually stimulates the activation of inflammasomes through a special pathway, and then activates the downstream Caspase1. The active Caspase1 cleavages the pre-IL-1 β and pre-IL-18 to mature, and through the protein Gsdmd is released from the cell[30, 31, 38-40]. Gsdmd is a substrate of Caspase1 and Caspase4/5/11[41]. Our results shows that in the drug prevention groups, IDHP can significantly inhibit the transcription and translation of Caspase1 and Caspase5, while the protein levels of Caspase4 and Gsdmd are significantly reduced. PCR results showed that IDHP significantly inhibited the transcription of IL-1 β , but not IL-18. It is suggested that the decrease of IL-18 in the supernatant may be due to that IDHP protects cell necrosis, inhibits the pyroptosis pathway, reduces the perforating activity of Gsdmd, and reduces its release.

Studies have found that in ALI patients, IL-18, a marker of Caspase1 activation, is elevated or associated with patient morbidity and mortality[42]. TNF α , ROS and IL-6 released by necrotic alveolar macrophages are the key initiators of neutrophil infiltration[43-46], and are considered to be the main triggers for the destruction of the alveolar capillary barrier function [47-49]. However, it is still uncertain which secondary mediators, such as TNF α , IL-1 β , IL-18 and other inflammatory factors released from macrophages through which receptors provide the necessary signals to destroy the vascular barrier function. But what we can be sure of is that both animal and cell experiments proved that the release of inflammatory factors including TNF α , IL-1 β , IL-18 and IL-6 in the IDHP pretreatment groups showed a decreasing trend. The main target of inflammatory factors and ROS released by pyroptosis is tight junction protein [50-55]. Endothelial cells are the key mediator of inflammatory response [56]. Destruction of endothelial tight junction protein and adhesion junction protein may change the alveolar capillary barrier function [57]. Studies have shown that it has a therapeutic effect on IL-1 β and TNF α induced acute lung injury in alveolar epithelial cells by reducing connexin and paracrine factors [58, 59]. Occludin and Zo-1 are two important tight junction proteins, which maintain the integrity of epithelium and endothelium through tight junctions. At present, a large amount of literatures have shown that protecting or up-regulating the expression of Occludin and Zo-1 can alleviate acute lung injury caused by LPS [18, 60, 61]. Our results proved that the expression of tight junction protein Occludin and Zo-1 in LPS groups was decreased by adding the culture supernatant of THP-1 cells to endothelial epithelial cells, and the expression of the drug prevention groups was increased. Compared with the LPS groups, the LDH activity in drug prevention groups was significantly lower. Although our present study indicated that the inhibition of pyroptosis pathway by IDHP contributed to LPS-induced inflammatory response, we should state that this effect of IDHP is not exclusively selective for the canonical and non-canonical pathway. NF- κ B, which is another crucial pathway in mediating LPS-induced inflammation, could also activate the pyroptosis pathway. In this study, we only observed that IDHP can alleviate LPS-induced acute lung injury, and then explored that IDHP's inhibition of pyroptosis may be one of its mechanisms to reduce inflammation. But the key target of IDHP inhibit pyroptosis pathway we need to address in future studies.

Conclusions

Collectively, we have demonstrated that pretreatment with IDHP significantly

attenuated the severity of LPS-induced lung injury in mice and greatly decreased the

production of IL-1 β and IL-18 both in vivo and in vitro, and inhibited the expression of tight junction proteins (Occludin, Zo-1) in endothelial cells. These observations further explained the anti-inflammatory effects of IDHP, which provides scientific basis for its application for the treatment of ALI/ARDS or sepsis.

Abbreviations

IDHP: Isopropyl 3-(3,4-dihydroxyphenyl)-2-hydroxypropanoate; LPS: Lipopolysaccharide; ALI: Acute Lung Injury ; ARDS: Acute respiratory distress syndrome; AM: Alveolar macrophages ; Zo-1: Zonula occludens1; PMA: Phorbol 12-myristate 13-acetate; LDH: Lactic dehydrogenase; MPO: Myeloperoxidase; ELISA: Enzyme-linked immunosorbent assay; BALF: Bronchoalveolar lavage fluid; PVDF: Polyvinylidene fluoride; qRT-PCR: Quantitative real-time PCR; OMV: Outer membrane vesicles.

Declarations

Acknowledgement

Not applicable.

Authors' contributions

ZCL, WN and MLZ have made significant contributions to the conception and design of the article. YL and XHZ provided IDHP. MLZ, MW, YJL, YCX and XYZ participated in the experiment process. MLZ, WN, ZCL and JC wrote the main manuscript text. All authors read and approved the final manuscript.

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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.

Ethics approval and consent to participate

All studies were approved by the Institutional Animal Care and use committee of the Fourth Military Medical University. At the same time, it is implemented in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Pretreatment of IDHP ameliorated LPS-induced lung injury in mice. (A) Mice were challenged by LPS (50 mg/kg) to induce acute lung injury with or without IDHP (30, 60, 90 mg/kg) pretreatment 1 h before LPS given. Survival was observed every 6 h during 72 h and the percent survival rate was expressed as Kaplan–Meier survival curves (n=20). (B) Total protein concentration in BALF were indicated the severity of lung injury (n=6). (C) MPO activity were reflected the neutrophil infiltration in the lung (n=6). Hematoxylin and eosin (H&E) staining were observed LPS-induced lung histopathologic changes (D) and performed Lung inflammation score (E): (a) Control; (b) LPS(50 mg/kg); (c) IDHP(30 mg/kg) + LPS; (d) IDHP(60 mg/kg) + LPS; (e) IDHP(90 mg/kg) + LPS. The scale bars are 200 μ m, n=10. (F) The content of inflammatory cytokines in BALF were detected by ELISA (n=6). (G) Western blotting analysis showed the protein expression of inflammatory cytokines in lung tissue (n=3). The dose of 90 mg/kg IDHP were used. (H) Densitometric analysis was used to quantify the expression of inflammatory cytokines (n=3). Results were presented as the Mean $\pm$ SEM, *p<0.05, **p<0.01, *** p<0.001 compared with the control groups; #p<0.05, ##p<0.01 compared with LPS groups.

Figure 2

IDHP inhibited the canonical pathway of pyroptosis activation in THP-1 macrophages stimulated by LPS.

(A) Cell survival were measured by flow cytometry. THP-1 macrophages were treated with 90 mg/kg IDHP for 12 hours before LPS administration. (B) Statistical analysis of the apoptotic rates (n=3). (C) Real-time PCR was used to analyze mRNA levels of pyroptosis canonical pathway related genes (Nlrp3, Asc, Caspase1, Gsdmd) (n=6). (D) The expression of pyroptosis canonical pathway related proteins were used

by western blotting (n=3). (E) Densitometric analysis was used to quantify the expression of pyroptosis canonical pathway related proteins (n=3). (F) Fluorescence staining of Asc speck (red). The nuclear (blue) were counter-stained by using the DAPI staining solution. (G) Quantitative analysis of Asc protein. Results were presented as the Mean±SEM. *p<0.05. ***p<0.001 compared with the control groups. #p<0.05, ##p<0.01 compared with LPS groups.

Figure 3

IDHP inhibited non- canonical pathway of pyroptosis activation in THP-1 macrophages stimulated by LPS. (A) Real-time PCR was used to analyze mRNA levels of pyroptosis non-canonical pathway related genes (Hmgb1, Pcaf, Caspase4, Caspase5) (n=6). (B) The expression of pyroptosis non-canonical pathway related proteins (Hmgb1, Caspase4, Caspase5) were analyzed by western blotting (n=3). (C) Densitometric analysis was used to quantify the expression of pyroptosis non-canonical pathway related proteins (n=3). (D) Fluorescence staining of Hmgb1 (green). The nuclear (blue) were counter-stained by using the DAPI staining solution. (E) Quantitative analysis of Hmgb1 protein (n=3). Results were

presented as the Mean±SEM. *p<0.05, ***p<0.001 compared with the control groups. #p<0.05, ##p<0.01, ###p<0.001 compared with LPS groups.

Figure 4

IDHP attenuated the production of the cytokines IL-1 β and IL-18 in THP-1 macrophages stimulated by LPS. (A) The mRNA levels of IL-1 β in THP-1 macrophages were analyzed by real-time PCR. THP-1

macrophages pretreated with IDHP for 1h and then incubated with 1 μ g/ml LPS for 12 h (n=6). (B) IL-1 β levels in supernatant of THP-1 macrophages were evaluated by ELISA assay (n=6). (C) The mRNA levels of IL-18 were analyzed by real-time PCR (n=6). (D) IL-18 levels in supernatant of THP-1 macrophages were evaluated by ELISA assay (n=6). (E) The protein expression of IL-1 β , IL-18 in THP-1 macrophages were analyzed by western blotting (n=3). (F) Densitometric analysis was used to quantify the relative expression of IL-1 β , IL-18 (n=3). Results were presented as the Mean $\pm$ SEM, *P<0.05, ***P<0.001 compared with the control groups, #P<0.05. ###P<0.001 compared with LPS groups.

Figure 5

IDHP alleviated injury in endothelial and epithelial cells co-culture with THP-1 macrophages stimulated by LPS. (A) Flow cytometry analyzed ROS production in THP-1 macrophages stimulated by LPS with or without IDHP pretreatment: (a) Control, (b) IDHP (90 mg/kg), (c) LPS (1 μ g/ml), (d) IDHP (90 mg/kg) + LPS (1 μ g/ml). (B) Statistical analysis of the ROS fluorescence intensity (n=3). (C) The expression of tight junction proteins, Occludin and Zo-1, were detected by western blotting in THP-1 macrophages pretreated with IDHP for 1 h and then incubated with LPS (1 μ g/ml) for 12 h. Tight junction proteins are the one of important components of the special structure of the capillary-alveolar barrier, they were reflected pulmonary permeability and severity in ALI (n=3). (D) Densitometric analysis was used to quantify the expression of Occludin and Zo-1 proteins (n=3). (E) LDH was measured in supernatants of endothelial cells (n=6). (F) LDH activity was measured in supernatants of epithelial cells (n=6). Results were presented as the Mean $\pm$ SEM, *p<0.05, **p<0.01, ***p<0.001 compared with the control. #p<0.05, ##p<0.01 compared with LPS.