

Spermidine mitigates lipopolysaccharide-induced acute lung injury by enhancing autophagic flux

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Abstract. The primary pathological characteristics of acute lung injury (ALI), comprise the dysfunction of the endothelial barrier, primarily due to the death of pulmonary microvascular endothelial cells (PMVECs), with pyroptosis identified as the principal mechanism underlying lipopolysaccharide (LPS)-induced endothelial cell death. Current research suggests that autophagy suppresses pyroptosis. Spermidine (SPD), an endogenous polyamine, is known for its potent ability to induce autophagy. Nevertheless, the impact of SPD on LPS-induced ALI and PMVEC pyroptosis remains unclear. In the present study, an ALI model was established through an intratracheal injection of LPS. SPD treatment significantly alleviated LPS-induced lung injury in mice and reduced caspase-1 activation and gasdermin D N-terminal expression in lung tissues. To further investigate the mechanism, PMVEC pyroptosis was modeled using LPS combined with nigericin. *In vitro* experiments revealed suppressed autophagic flux under pyroptotic conditions, while SPD restored autophagic flux and attenuated PMVEC pyroptosis. These findings indicated that SPD protects against endothelial cell damage, partially through autophagic flux restoration.

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are serious respiratory conditions characterized by hypoxemia, bilateral opacities on chest radiographs and reduced lung compliance (1,2). ALI/ARDS has a high annual mortality rate among critically ill patients. The underlying pathology of ALI/ARDS involves the disruption of the pulmonary vascular endothelial barrier function, due to endothelial cell death and

the loss of endothelial adhesion connections (3,4). The mechanism of cell death in pulmonary microvascular endothelial cells (PMVECs) is relatively complex, with pyroptosis being considered as the primary mode (5,6).

Pyroptosis is a form of programmed cell death primarily mediated by gasdermin D (GSDMD), resulting in the release of numerous pro-inflammatory cytokines (7). In addition, autophagy is a process essential for maintaining cellular homeostasis (8). Comprehensively, autophagy can negatively regulate pyroptosis by degrading, pathogen-associated molecular patterns and other cellular components involved in this process (9).

SPD, as an autophagic inducer, may exert protective effects against ALI. In the present study, the role and mechanism of SPD in LPS-induced ALI was investigated by establishing both an *in vivo* ALI mouse model and an *in vitro* pyroptosis model using PMVECs. The aim of the study was to demonstrate that spermidine (SPD) effectively suppresses PMVEC pyroptosis by promoting autophagic flux, thereby ameliorating LPS-induced ALI.

Materials and methods

Reagents and antibodies. LPS and SPD were obtained from Sigma-Aldrich (Merck KGaA). Nigericin (cat. no. HY-100381) and chloroquine (CQ) (cat. no. HY-17589A) were provided by MedChemExpress. Antibodies against caspase-1 (cat. no. ab207802), GSDMD (cat. no. ab219800), and sequestosome 1 (SQSTM1)/p62 (cat. no. ab109012) were supplied by Abcam. The antibody against microtubule-associated protein light chain 3 (LC3) I/II (cat. no. 12741) was supplied by Cell Signaling Technology, Inc. The HRP-conjugated β -actin antibody (cat. no. ET1702-67) and goat anti-rabbit IgG-HRP antibody (cat. no. HA1001) were supplied by HUABIO. Calcein/PI Cell Viability and Cytotoxicity Assay Kit (cat. no. C2015M), RIPA lysis buffer (cat. no. P0013B), Cell Counting Kit-8 (CCK-8) (cat. no. C0038), BCA Protein Concentration Assay Kit (cat. no. P0010S) were obtained from Beyotime Institute of Biotechnology. The ECL chemiluminescent substrate (cat. no. RM02867) was obtained from ABclonal. The CheKine™ Micro Lactate Dehydrogenase (LDH) Assay Kit (cat. no. KTB1110) was provided by Abbkine Scientific Co., Ltd.

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LPS-induced ALI model. Male C57BL/6 mice (n=30), weighing 20-25 g and aged 8-10 weeks, were obtained from Jinan Pengyue Experimental Animal Breeding Center (Jinan, China). The mice were housed in a controlled environment with regulated temperature (22-24°C) and humidity (50-60%), maintained on a 12-h light/dark cycle, and provided with a standard diet and tap water *ad libitum*. SPD was administered at doses of 10 mg/kg once daily, 10 mg/kg twice daily and 20 mg/kg once daily (10). Mice were pretreated with different concentrations of SPD for 3 days, followed by the induction of ALI via intratracheal instillation of LPS (5 mg/kg) for 24 h (11,12). Mice were anesthetized by an *i.p.* injection of 0.3% sodium pentobarbital (30 mg/kg), and then subjected to intratracheal instillation of LPS. After 24 h, mice were euthanized via an *i.p.* injection of an overdose of sodium pentobarbital (200 mg/kg). The present study was approved (approval no. KYDWLL-202212) by the Ethics Committee of Qilu Hospital of Shandong University (Qingdao, China). All animal experiments were conducted in accordance with the ARRIVE guidelines (13) and complied with the NIH Guide for the Care and Use of Laboratory Animals (14).

Protein concentration determination in bronchoalveolar lavage fluid (BALF). The collected BALF was centrifuged at 500 x g for 10 min at 4°C. The protein concentration of the supernatant was determined using the BCA Protein Concentration Assay Kit.

Lung tissue examination. The right lung lobe was fixed by soaking in 10% formalin buffer at room temperature for 48 h. It was then embedded in paraffin and sliced. The lung tissue sections (4 µm in thickness) were stained with hematoxylin for 2 min and eosin for 10 min at room temperature. Images were observed under a light microscope (Olympus Corporation).

Lung wet-to-dry ratio (W/D). Following the removal of the right lung, its wet weight was measured and then dried in an oven at 60°C for ~48 h. The W/D was calculated by dividing the wet weight by the dry weight.

Cell culture, cell cytotoxicity assay and LDH assay. The HULEC-5a (cat. no. C1402) immortalized pulmonary microvascular endothelial cell line (cat. no. CVCL_0A11) was purchased from Wuhan SUNNCELL Biotechnology Co., Ltd. To establish the pyroptosis model of PMVECs, the cells were stimulated with LPS (1 µg/ml) for 4 h, followed by treatment with nigericin (NIG) (20 µM) for 1 h. In the SPD group, the cells were pretreated with SPD for 12 h prior to stimulation with LPS and NIG. In the LPS + NIG + SPD + CQ group the cells were pretreated with CQ (20 µM) for 2 h prior to stimulation with SPD. Cells were incubated with CCK-8 working solution for 2 h, followed by cell viability detection. The necrotic cells were detected using the Calcein/PI Cell Viability and Cytotoxicity Assay Kit, according to the manufacturer's instructions. The cells were observed using a fluorescence microscope (Nikon, Ti-E Live Cell Imaging System; Nikon Corporation). LDH concentrations in cell homogenates were measured using the CheKine™ Micro Lactate Dehydrogenase (LDH) Assay Kit. After incubation, the cells were analyzed

using an automatic microplate reader (SpectraMax i3x; Molecular Devices, LLC).

Western blotting. Lung tissue homogenate and cell lysate were prepared in RIPA lysis buffer. The protein concentration of the extracted samples was determined using a BCA assay kit. Equal protein amounts (20 µg) were analyzed using 12% SDS-PAGE electrophoresis, followed by transfer to a PVDF membrane. The membrane was blocked with 5% non-fat milk at room temperature for 2 h and then incubated overnight at 4°C with primary antibodies against caspase-1 (1:1,000), GSDMD (1:1,000), SQSTM1/p62 (1:10,000), LC3 I/II (1:1,000) and β-actin (1:2,000). The membrane was then incubated with the secondary antibody (1:10,000) at room temperature for 60 min and then developed using ECL chemiluminescent substrate. Densitometric analysis was performed using ImageJ (version 1.54; National Institutes of Health).

Statistical analysis. All data are presented as the mean ± standard error of the mean. The experiments were repeated 3 times. Unpaired Student's t-tests were used for comparisons between two groups, and one-way ANOVA was used for comparisons among multiple groups. Post hoc Tukey's HSD test was applied following ANOVA to identify specific group differences. All statistical analyses were performed using GraphPad Prism (version 9.0.0, GraphPad Software; Dotmatics). P<0.05 was considered to indicate a statistically significant difference.

Results

SPD alleviates LPS-induced ALI. As shown in Fig. S1, SPD at 10 mg/kg twice daily significantly alleviated lung injury. Consequently, this dosing regimen (10 mg/kg twice daily) was selected for all subsequent experiments. Notably, mice treated with LPS exhibited significant lung damage, including diffuse alveolar and interstitial edema, when compared with the control group as revealed in Fig. 1A. SPD pretreatment significantly reduced alveolar and interstitial edema and inflammatory cell infiltration. Lung W/D and protein levels in BALF were also measured (Fig. 1B and C) to assess pulmonary vascular endothelial permeability. SPD pretreatment significantly reduced the lung W/D and protein in BALF. These findings indicated that SPD pretreatment effectively reduces LPS-induced lung damage.

SPD alleviates lung pyroptosis. To explore whether SPD alleviates lung damage through its effect on pyroptosis, the expression levels of pyroptosis-related proteins were detected in lung tissue. As shown in Fig. 2A and B, LPS administration significantly increased the levels of cleaved caspase-1 and the gasdermin D N-terminal (GSDMD^{NT}) in lung tissue, as compared with the control group. By contrast, SPD pretreatment decreased the levels of these proteins. This suggested that SPD reduces LPS-induced lung damage by inhibiting pyroptosis in lung tissue.

LPS- and NIG-induced pyroptosis in PMVECs. A concentration-dependent NIG screening (5, 10, 20, 30 and 40 µM) was performed on LPS-primed (1 µg/ml, 4 h)

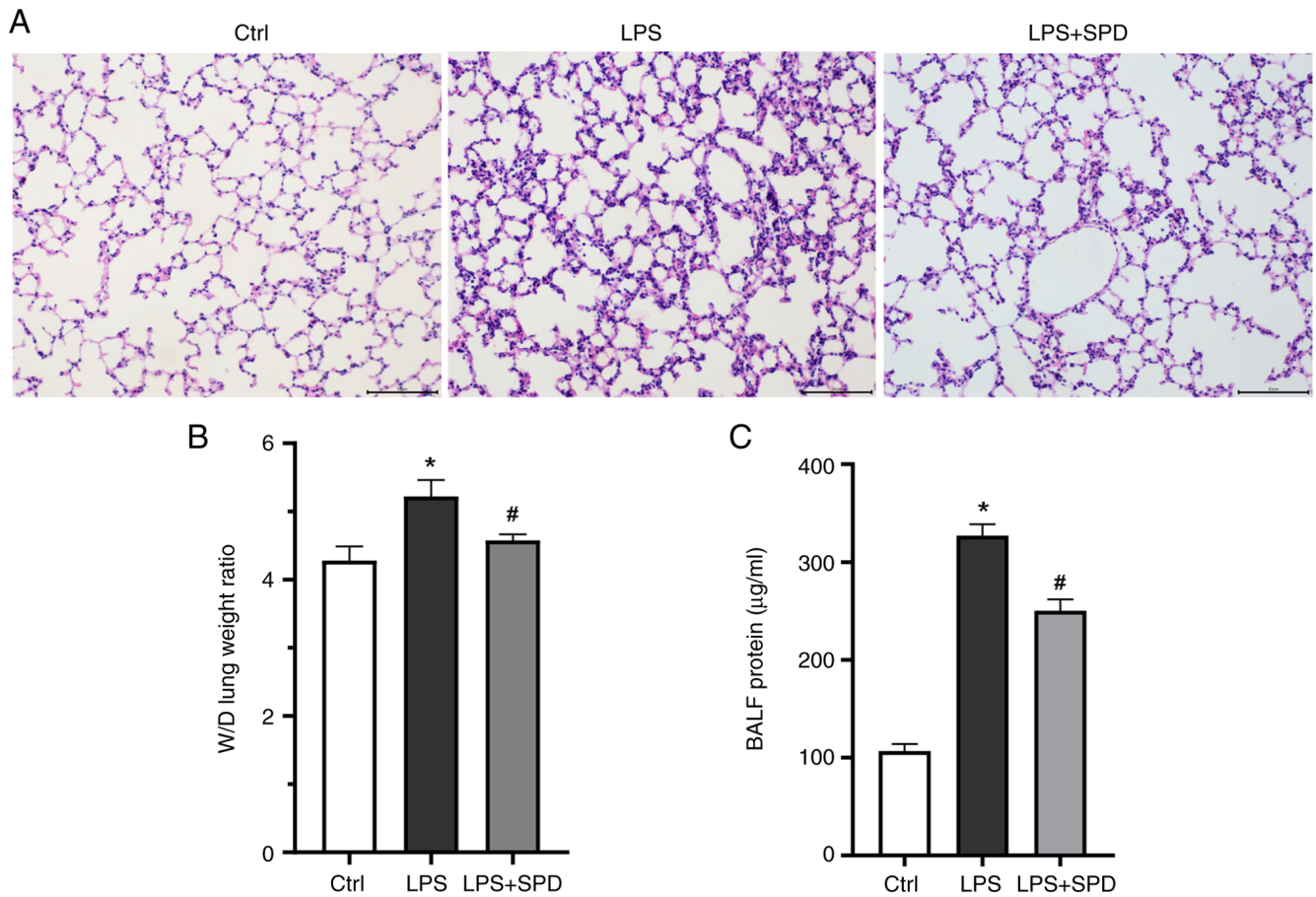


Figure 1. SPD alleviates LPS-induced acute lung injury. (A) H&E staining of lung tissue sections showing that SPD pretreatment alleviated LPS-induced lung injury. Scale bars, 50 μm. (B and C) W/D and BALF protein levels were measured to assess pulmonary vascular endothelium permeability. *P<0.05, the LPS group vs. the control group; #P<0.05, the LPS + SPD group vs. the LPS group. LPS, lipopolysaccharide; SPD, spermidine; H&E, hematoxylin & eosin; W/D, wet-to-dry ratio; BALF, bronchoalveolar lavage fluid; Ctrl, control.

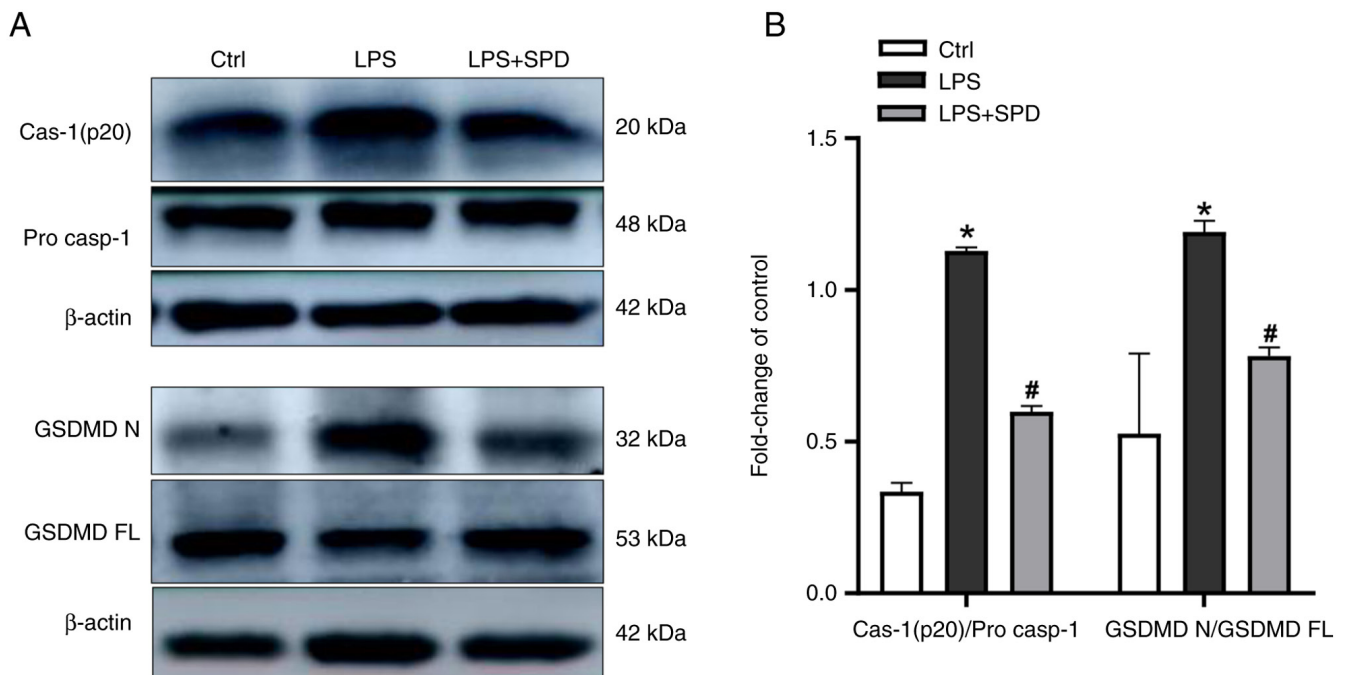


Figure 2. SPD alleviates lung pyroptosis. (A) Western blot analysis of caspase-1 and GSDMD^{NT} in mice that underwent various treatments. (B) Semi-quantitative analysis of (A). The results are presented as the mean ± SD. *P<0.05, the LPS group vs. the control group; #P<0.05, the LPS + SPD group vs. the LPS group. SPD, spermidine; GSDMD^{NT} or GSDMD N, gasdermin D N-terminal; LPS, lipopolysaccharide; Ctrl, control; Cas-1, caspase-1; pro-Casp-1, pro-caspase-1; GSDMD FL, gasdermin D full length.

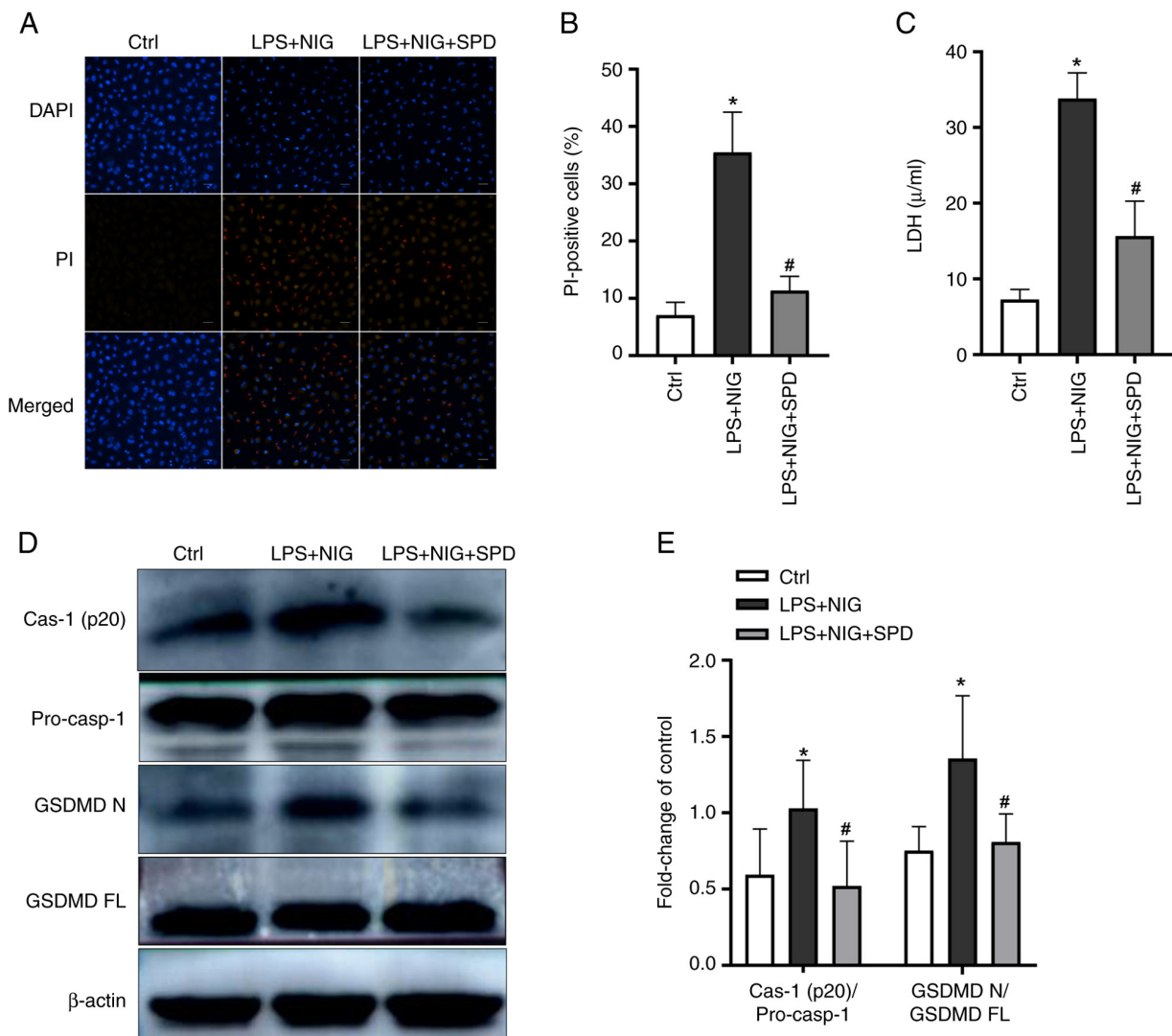


Figure 3. SPD reduces pyroptosis in PMVECs. PMVECs were first treated with 40 μ M SPD for 12 h, followed by exposure to LPS and NIG. (A) Pyroptotic cells were identified through PI staining, with nuclei stained blue using DAPI. Scale bars, 50 μ m. (B) Percentages of PI-positive cells. (C) LDH concentrations in cell homogenates were measured using the CheKine™ LDH Assay Kit. (D) Western blotting was performed to detect cleaved caspase-1 (p20) and GSDMD^{NT} in PMVECs. (E) Semi-quantification of (D). The results are presented as the mean $\pm$ SD. * $P < 0.05$, the LPS + NIG group vs. the control group; # $P < 0.05$, the LPS + NIG + SPD group vs. the LPS + NIG group. SPD, spermidine; PMVECs, pulmonary microvascular endothelial cells; LPS, lipopolysaccharide; NIG, nigericin; PI, propidium iodide; DAPI, 4',6-diamidino-2-phenylindole; LDH, lactate dehydrogenase; GSDMD^{NT} or GSDMD N, gasdermin D N-terminal; GSDMD FL, gasdermin D full length; Ctrl, control.

PMVECs (15-17). As shown in Fig. S2A, 20 μ M NIG was identified as the optimal concentration, balancing pyroptosis induction with preserved assay sensitivity, and was thus employed in further studies. Following SPD pretreatment (20, 40, 60, 80, 100 and 200 μ M) and sequential LPS/NIG challenge, CCK-8 analysis (Fig. S2B) revealed maximal viability enhancement at 40 μ M. Absence of additional benefit at higher concentrations (80-200 μ M) established 40 μ M as the optimal concentration for subsequent experimental applications.

SPD attenuates endothelial cell pyroptosis. To investigate the effect of SPD on endothelial cell pyroptosis, cells were treated with LPS and NIG. Pyroptotic cells form membrane pores that permit extracellular dyes, such as propidium iodide (PI), to enter and stain the nucleus (18). As revealed in Fig. 3A and B, a significant increase in the percentage of PI-positive cells was observed following stimulation with LPS and NIG. In

addition, the stimulation with LPS and NIG led to a significant increase in the release of LDH from endothelial cells (Fig. 3C). Following stimulation with LPS and NIG, the expression levels of cleaved caspase-1 and GSDMD^{NT} increased in endothelial cells (Fig. 3D and E). However, SPD reduced all LPS- and NIG-induced effects.

SPD enhances the disrupted autophagic process in endothelial cells undergoing LPS- and NIG-induced pyroptosis. SPD promotes autophagy, which can inhibit pyroptosis through different mechanisms. It was examined whether SPD restores autophagy in LPS- and NIG-treated endothelial cells. This was performed by measuring the levels of autophagic proteins LC3-II and p62. As shown in Fig. 4A and B, the levels of autophagy markers LC3-II and p62 increased in LPS- and NIG-treated endothelial cells. Following SPD treatment, the levels of LC3-II and p62 in endothelial cells

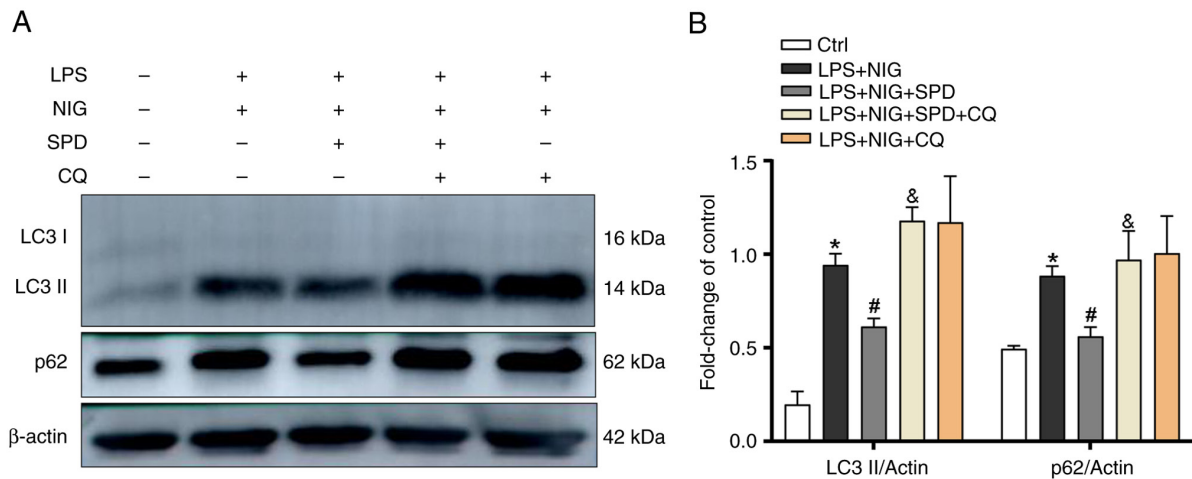


Figure 4. SPD improves the disrupted autophagic flow in PMVECs that are undergoing pyroptosis due to LPS and NIG. (A) Western blotting of LC3 II and p62 in the PMVECs. (B) Semi-quantification of LC3 II and p62. The results are presented as the mean $\pm$ SD. * $P < 0.05$, the LPS + NIG group vs. the control group; # $P < 0.05$, the LPS + NIG + SPD group vs. the LPS + NIG group; & $P < 0.05$, the LPS + NIG + SPD + CQ group vs. LPS + NIG + SPD group. SPD, spermidine; PMVECs, pulmonary microvascular endothelial cells; LPS, lipopolysaccharide; NIG, nigericin; LC3 I/II, microtubule-associated protein 1A/1B-light chain 3 I/II; CQ, chloroquine.

affected by LPS and NIG decreased. Notably, the addition of CQ to the LPS + NIG + SPD group further elevated LC3-II and p62 levels, suggesting that the protective effect of SPD was attenuated by CQ-mediated autophagy blockade. These results indicated that LPS and NIG inhibited autophagic flux in PMVECs, while SPD successfully restored this disrupted process.

SPD reduces pyroptosis in LPS- and NIG-treated endothelial cells by restoring the damaged autophagic flux. To better understand this process, the autophagy inhibitor CQ was used to block autophagic flux. As revealed in Fig. 5, the LPS + NIG + SPD + CQ group had more PI staining-positive cells and higher LDH release than the LPS + NIG + SPD group. In addition, this group exhibited increased levels of cleaved caspase-1 and GSDMD^{NT}. These findings indicated that by restoring autophagic flux, SPD mitigates pyroptosis in LPS- and NIG-treated PMVECs.

Discussion

SPD is a key metabolic regulator produced from the breakdown of ornithine in mammals, playing significant roles in various physiological and pathological processes (19). It has been shown to be a potent inducer of autophagy, contributing to anti-aging and antioxidant effects, inflammation reduction and apoptosis inhibition (20-22). However, the therapeutic potential of SPD for ameliorating ALI has not been previously reported. Hence, exploration of the therapeutic effects of SPD in ALI may provide novel insights for clinical applications.

Pyroptosis is recognized as the main mechanism through which LPS induces endothelial cell death. Early in the process of inflammation, endothelial cells undergo pyroptotic death to remove damaged cells. However, if this process is not controlled, it can damage the vascular endothelial barrier (23-25). Pyroptosis is a distinct form of programmed cell death that depends on caspases and gasdermins. The key effector, GSDMD, is cleaved by caspases into two fragments:

The N-terminal fragment (GSDMD^{NT}) and the C-terminal fragment (GSDMD^{CT}). The GSDMD^{NT} binds to cell membranes to create pores, resulting in cell destruction (26). The present findings were aligned with those of previous studies (23-26), showing an increase in GSDMD^{NT} and pro-caspase-1 expression in the lungs of mice with LPS-induced ALI and pulmonary vascular cells.

There are three main types of autophagy: Macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy is considered the primary form. During this process, the membrane of the autophagic vesicle encloses cellular components, leading to the formation of an autophagosome. The autophagosome then merges with a lysosome to create an autophagolysosome, where the cellular contents are broken down. This sequence of events is called autophagic flux (27). Studies have demonstrated that activating autophagy mitigates sepsis and attenuates organ dysfunction by suppressing pyroptosis (25,28,29). To date, therapies targeting both autophagy and pyroptosis for treating ARDS have not yet been reported in clinical practice.

p62, or SQSTM1, is a key target of autophagy; it interacts with LC3 on the autophagosome membrane, gets incorporated into the autophagosome, and is eventually degraded (8). The disruption of autophagy can cause the buildup of p62 protein. In the pyroptosis of LPS- and NIG-treated PMVECs, higher levels of p62 and LC3 II were observed, which suggested that autophagic flux was inhibited in PMVECs. Previous research has shown that autophagy negatively regulates cellular pyroptosis. Pu *et al* (30) found that autophagy inhibition increased macrophage pyroptosis in a *Pseudomonas aeruginosa*-induced sepsis model. In addition, a previous study showed that activating autophagy reduces neuronal cell pyroptosis in a mouse model of brain injury (31). To examine the effects of SPD, a potent autophagy inducer, on pyroptosis and autophagy in PMVECs, the cells were treated *in vitro* with SPD. It was observed that SPD attenuated LPS/nigericin-induced pyroptosis and rescued the suppressed autophagic flux in PMVECs. CQ has been revealed to block autophagic flux by inhibiting the fusion of

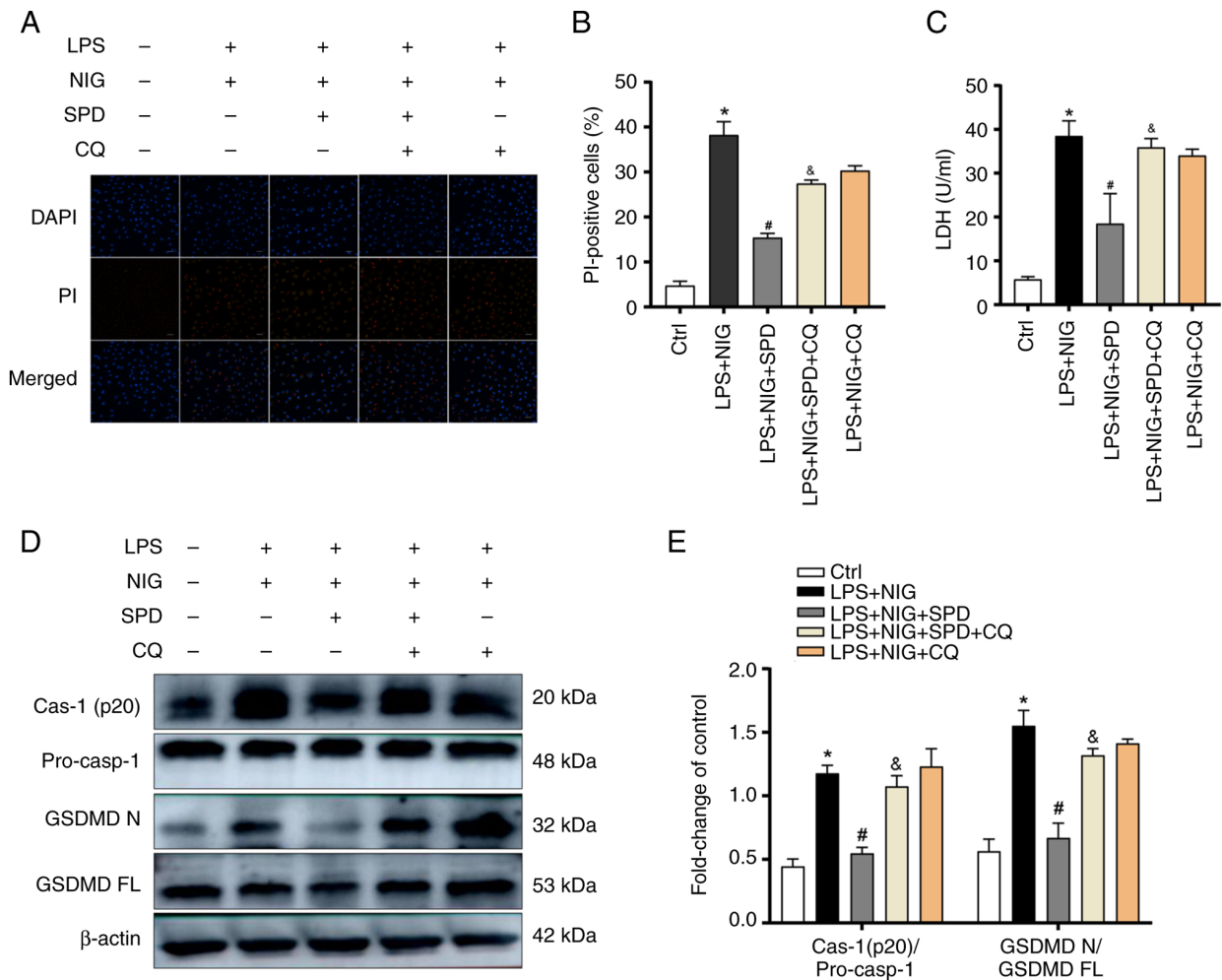


Figure 5. SPD reduces PMVEC pyroptosis caused by LPS and NIG by restoring the impaired autophagic flux. (A) Pyroptotic cells were labeled red using PI staining, while the cell nuclei were stained blue with DAPI. Scale bars, 50 μ m. (B) Percentages of PI-positive cells. (C) LDH concentrations in cell homogenates were measured using the CheKine™ LDH Assay Kit. (D) Western blotting was conducted to assess the levels of cleaved caspase-1 (p20) and GSDMD^{NT} in PMVECs. (E) Semi-quantification of (D). Results are presented as the mean $\pm$ SD. * P <0.05, the LPS + NIG group vs. the control group; # P <0.05, the LPS + NIG + SPD group vs. the LPS + NIG group; & P <0.05, the LPS + NIG + SPD + CQ group vs. LPS + NIG + SPD group. SPD, spermidine; PMVECs, pulmonary microvascular endothelial cells; LPS, lipopolysaccharide; NIG, nigericin; PI, propidium iodide; DAPI, 4',6-diamidino-2-phenylindole; LDH, lactate dehydrogenase; GSDMD^{NT} or GSDMD N, gasdermin D N-terminal; GSDMD FL, gasdermin D full length; CQ, chloroquine; Cas-1, caspase-1; pro-casp-1, pro-caspase-1; Ctrl, control.

autophagosomes with lysosomes (32). To investigate whether the SPD-mediated attenuation of PMVEC pyroptosis is dependent on the enhancement of autophagic flux, autophagic flux was inhibited using CQ in PMVECs. When autophagic flux was pharmacologically blocked by CQ, the protective effect of SPD against PMVEC pyroptosis was significantly abrogated. These findings collectively indicated that SPD mitigates PMVEC pyroptosis through the potentiation of autophagic flux.

In conclusion, in the present study it was demonstrated that SPD alleviates LPS-induced ALI by suppressing pyroptosis in PMVECs through an autophagy-dependent mechanism. These findings provide novel insights for the clinical management of ARDS. However, it should be noted that the present study has certain limitations. Interleukin-1 β (IL-1 β) is a well-established downstream effector of caspase-1 (33), and although it was demonstrated that SPD suppresses caspase-1 activation, additional validation for this specific cytokine within the scope of the present study was not performed. Evidence indicates that SPD can induce autophagy through the adenosine

monophosphate-activated protein kinase (AMPK)/mammalian target of rapamycin (mTOR) pathway to improve cardiac function and reverse B-cell senescence by controlling eIF5A hypusination (21,22). Future research should focus on elucidating whether SPD exerts its protective effects against ALI and PMVEC pyroptosis by inducing autophagy through the AMPK/mTOR signaling pathway, H5A phosphorylation or alternative mechanisms, along with its effects on IL-1 β maturation and secretion. Further clinical trials are warranted to validate the therapeutic efficacy of SPD in human patients.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

XX designed the study, critically reviewed and edited the draft, supervised the study, and acquired funding. XZ conducted the investigation, performed data collection and analysis and drafted the manuscript. HL worked on partial data collection and analysis, and participated in manuscript revision. XX and XZ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved (approval no. KYDWLL-202212) by the Ethics Committee of Qilu Hospital of Shandong University (Qingdao, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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