Allergologia et immunopathologia
Sociedad Española de Inmunología Clínica, Alergología y Asma Pediátrica
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RESEARCH ARTICLE

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Coniferyl aldehyde alleviates LPS-induced WI-38 cell apoptosis and inflammation injury via JAK2-STAT1 pathway in acute pneumonia

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Received 4 June 2021; Accepted 21 June 2021
Available online 1 September 2021

KEYWORDS
coniferyl aldehyde (CA);
JAK2;
STAT1;
WI-38 cells

Abstract
Pneumonia is a kind of inflammatory disease characterized by pathogen infection of lower respiratory track. Lipopolysaccharide (LPS) is the main bioactive component of Gram-negative bacteria responsible for inflammatory response. Recently, coniferyl aldehyde (CA) has been reported to play a crucial role because of its anti-inflammatory activity. However, the effect and mechanisms of CA in ameliorating symptoms of acute pneumonia remain unknown. Evaluating and identifying the value and exploring the mechanisms of CA on LPS-mediated WI-38 apoptosis and inflammation were the aims of this study. Here, CCK-8 cell viability assay was applied on WI-38 after treatment with or without LPS at different doses of CA to verify that CA can increase LPS-induced cell viability. Then, quantitative polymerase chain reaction (qPCR) and enzyme-linked-immunosorbent serologic assays (ELISA) suggested that LPS treatment dramatically decreased the expression level of IL-10 (anti-inflammatory factor) while strikingly increasing the expression levels of IL-1β, IL-6, and TNF-α (tumor necrosis factor-α; proinflammatory factor) whereas CA treatment attenuates LPS-induced inflammation of WI-38. Further, flow cytometry and Western blot assay verified that LPS treatment dramatically promoted apoptosis of WI-38 cells, while administration of CA notably inhibited apoptosis of WI-38 cells. Moreover, the Western blot assay hinted that CA could inactivate LPS-induced JAK2-STAT1 signaling pathway. These findings indicated that CA could alleviate LPS-mediated WI-38 apoptosis and inflammation injury through JAK2-STAT1 pathway in acute pneumonia.

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https://doi.org/10.15586/aei.v49i5.464

Introduction

Pneumonia is a kind of inflammatory disease characterized by pathogen infection of lower respiratory track. It is the most common infection found in children and the elderly, accompanied by shortness of breath, cough, chest pain, fever, and even the typical clinical symptoms of heart failure and respiratory failure. Pneumonia is caused by the inflammatory stimulation of microbial pathogens (endotoxins). Lipopolysaccharide (LPS) is the main bioactive component of Gram-negative bacteria crucial for inflammatory stimulation. WI-38 cells are often used in the establishment of pneumonia models.[1] Janus kinase (JAK) family is expressed in almost all cells. JAK2 is the most crucial phenotype and the most conserved subtype of the JAK family, involved in various pathological processes. After cytokines or LPS induces cell surface receptor activation, the JAK2 protein is recruited and phosphorylated, resulting in the downstreaming of signal transducer and activator of transcription (STAT) phosphorylation. Phosphorylated STATs are dimerized and transported to the nucleus, where they bind to the promoters of target genes and activate their transcription, thereby participating in various pathological processes such as inflammation of various diseases.[2]

Coniferyl aldehyde (CA) is a phenolic compound extracted from plants such as cinnamon, vanilla, fig-tree leaf, or eucommia ulmoides. It is used to treat high blood pressure, strengthen muscles and bones, and restore damaged liver and kidney functions. CA has anti-inflammatory activity five times higher than aspirin, significantly alleviating radiation-induced acute intestinal injury and subacute enteritis.[3] In addition, CA has been reported to inhibit the inflammatory response of meningeal cells via inhibiting JAK2.[4] Moreover, CA alleviates ear edema induced by phorbol 12-myristate 13-acetate (TPA) in mice and inhibits excessive proliferation of epidermal and infiltration of white blood cells. Systemic application of CA significantly reduced carrageenan (CRG)-induced foot edema in rats, and CA had anti-inflammatory properties *in vitro* and *in vivo* by inhibiting JAK2–STAT1–iNOS signaling transduction.[5] In addition, CA also inhibited the LPS-induced apoptosis of macrophages.[6] However, the effect of CA on pneumonia remains unclear.

This study found that LPS-induced WI-38 cell apoptosis and inflammation were alleviated *in vitro* by CA by inhibiting extracellular signal-regulated kinase (ERK) and nuclear factor kappa light chain enhancer of activated B cell (NF-kB) pathways.

Materials and Methods

Cell culture

WI-38 cells were obtained from the Chinese Academy of Sciences. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS) (Gibco) and 1% penicillin–streptomycin (P-S; Gibco) in an incubator with 5% CO_2.

Lipopolysaccharide exposure

To study the effects of CA on WI-38, cells were separated into four different groups, including control, LPS, LPS+CA (10 μM), and LPS+CA (30 μM). WI-38 cells were pretreated with or without LPS (1 μg/mL; Sigma-Aldrich, St. Louis, MO, USA) for 24 h, and treated with or without various concentrations of CA (10 or 30 μM; Sigma-Aldrich) for 2 h.

CCK-8 cell viability assay

A total of 2.5×10^3 cells were seeded into plates and treated under different conditions. After treatment, optical density (OD) value of each sample was checked at 450 nm after incubation with CCK-8 solution (Beyotime) for 3 h.

Quantitative polymerase chain reaction (qPCR)

Trizol reagent (Invitrogen, Grand Island, NY, USA) was applied to collect total RNA from cells. NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Inc.) was applied to evaluate the quantity and integrity of RNA. SYBR Premix EX Taq (Takara, Japan) was used to detect expressions of IL-1β, IL-6, IL-10, and TNF-α (tumor necrosis factor-α; proinflammatory factor). Primer sequences are shown in Table 1.

Enzyme-linked-immunosorbent serologic assay (ELISA)

ELISA kits were used for detecting the IL-1β (ab100768; Abcam, Cambridge, MA, USA), IL-6 (ab100772; Abcam), IL-10 (ab100697; Abcam), Caspase 3 (KHO1091; Invitrogen), and Caspase 9 (BMS2025; Invitrogen) protein levels. Assays for the expressions of IL-1β, IL-6, IL-10, Caspase 3, and Caspase 9 in cells were performed with corresponding ELISA kits according to manufacturer's instructions.

Cell apoptosis

The cells were resuspended in Annexin V (Invitrogen) incubation solution and stained with Annexin V/FITC and PI

Table 1 Primers for IL-1ß, IL-6, IL-10, TNF-α, and reference genes.

Gene	Primer	Sequence (5′-3′)
IL-1β	Forward	ACCTTCCAGGATGAGGACATGA
	Reverse	CTAATGGGAACGTCACACACCA
IL-6	Forward	CACATGTTCTCTGGGAAATCG
	Reverse	TTGTATCTCTGGAAGTTTCAGATTGTT
IL-10	Forward	TTACCTGGTAGAAGTGATGCCC
	Reverse	GACACCTTGGTCTTGGAGCTTA
TNF-α	Forward	GCCACCACGCTCTTCTGTCTAC
	Reverse	GGGTCTGGGCCATAGAACTGAT
β-actin	Forward	GTGACGTTGACATCCGTAAAGA
	Reverse	GCCGGACTCATCGTACTCC

(Invitrogen). Then, cells were cultured in dark for 25 min and quantified by flow cytometry analysis (BD Accuri™ C6, USA).

Western blot assay

Cells were cleaned for three times in phosphate buffer solution (PBS), and the total protein was separated by radioimmunoprecipitation assay (RIPA) buffer (Beyotime). Bicinchoninic acid (BCA) protein assay kit (CoWin Biotechnology) was applied to detect protein concentration. Total proteins were electrophoresed to SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Boston, MA, USA) treated with 5% non-fat milk for 1 h. Protein were identified overnight at 4°C by specific primary antibodies: p-JAK2 (ab195055, 1:1000; Abcam), JAK2 (ab39636, 1:5000; Abcam), p-STAT1 (ab125685, 1:3000; Abcam), STAT1 (ab30645, 1:1200; Abcam), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab9485, 1:1,200; Abcam). Then the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody (ab205718, 1:2,000; Abcam), and the bands on membranes were visualized by the enhanced chemiluminescence (ECL) reagent (Beyotime). The analyzed samples were normalized by β-actin.

Statistical analysis

All data are expressed as mean ± standard error of the mean from three independent experiments. Differences between two groups were compared by Student's t-test, and differences in multiple groups were compared through one-way analysis of variance (ANOVA). $P < 0.01$ (two-tailed) was considered to indicate statistically significant difference.

Results

Coniferyl aldehyde increases LPS-induced cell viability

In order to evaluate the effects of CA on LPS-mediated WI-38 cell viability, the cell viability in WI-38 was evaluated after treatment with or without LPS at different CA doses through CCK8 assay. CCK8 assay indicated that LPS treatment inhibited the cell viability of WI-38 cell lines compared with the control group, while administration of CA significantly promoted the cell viability of WI-38 cell lines in a dose-dependent manner, with significant inhibitory effects at high doses (Figure 1). This result revealed that CA increased LPS-induced cell viability.

Coniferyl aldehyde alleviates LPS-induced cell inflammation

In order to determine the effects of CA on LPS-mediated WI-38 cell inflammation, the mRNA and protein expression level of inflammatory cytokines in WI-38 were evaluated after treatment with or without LPS at different CA doses through qPCR and ELISA. These results indicated that LPS treatment dramatically decreased the mRNA and protein expression level of IL-10 (anti-inflammatory factor) while

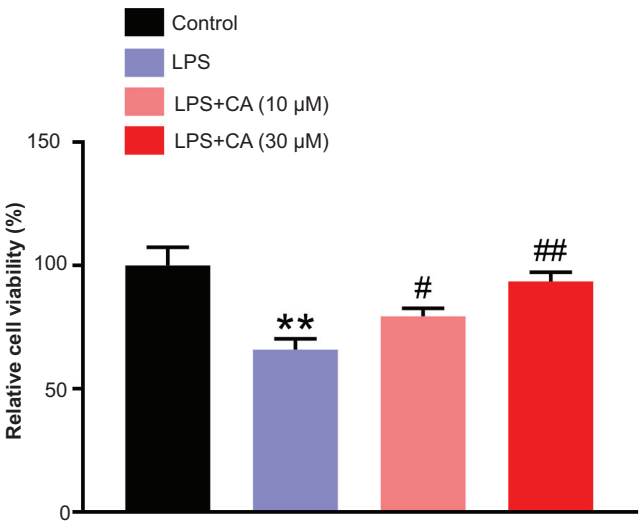

Figure 1 Coniferyl aldehyde increases LPS-induced cell viability. Serial CCK-8 assays detected the WI-38 cell viability after treatment with or without LPS at different CA doses. Data are presented as mean ± SD with three independent experiments. $^{**}P < 0.01$ versus control group, $^{\#}P < 0.05$, and $^{\#\#}P < 0.01$ versus the LPS-treated group.

strikingly increasing the expressions of IL-1β, IL-6, and TNF-α. However, administration of CA markedly reversed the expression of inflammatory cytokines, suggesting that CA treatment attenuates LPS-mediated WI-38 cell inflammation in a dose-dependent manner, with significant inhibitory effects at high doses (Figures 2A and B). These results demonstrated that CA alleviated LPS-induced cell inflammation.

Coniferyl aldehyde alleviates LPS-induced apoptosis

In order to explore the role of CA in the apoptosis of WI-38 cells, flow cytometry assays were applied in each group. The flow cytometry assay indicated that LPS treatment dramatically promotes apoptosis of WI-38 cells, as evidenced by increased apoptotic cells with LPS administration than the control group. Meanwhile, the flow cytometry assay also proved that administration of CA notably inhibits apoptosis of WI-38 cells, as evidenced by decreased apoptotic cells with CA treatment than the LPS group (Figure 3A). Further, the ELISA kit also detected the protein expression level of apoptosis-related proteins (Caspase 3 and Caspase 9). The results also suggested that LPS treatment dramatically promotes apoptosis while CA administration notably inhibits apoptosis of WI-38 cell lines in a dose-dependent manner, with significant inhibitory effects at high doses (Figure 3B). These results proved that CA alleviated LPS-induced apoptosis.

Coniferyl aldehyde regulates the JAK2-STAT1 signaling pathway

In order to estimatethe mechanism of CA on the alleviation of LPS-mediated WI-38 apoptosis and inflammation

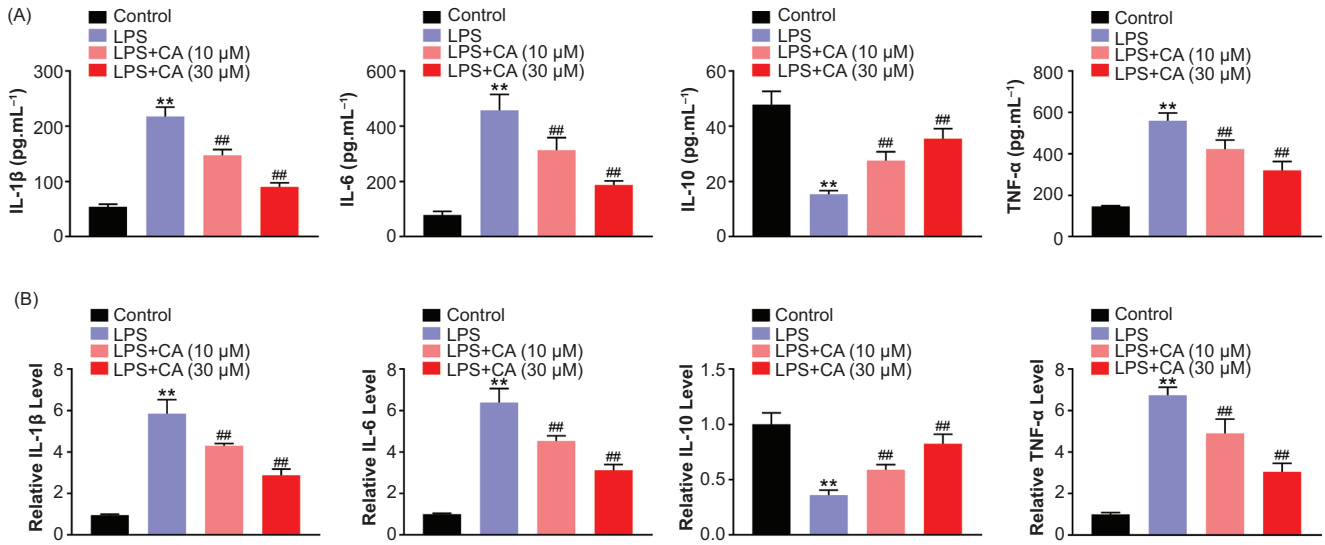

Figure 2 Coniferyl aldehyde alleviates LPS-induced cell inflammation. (A) The mRNA expression levels of IL-1β, IL-6, IL-10, and TNF-α in WI-38 cells in each group. (B) The protein expression levels of IL-1β, IL-6, IL-10, and TNF-α in WI-38 cells in each group. Data are presented as mean ± SD with three independent experiments. $^{**}P < 0.01$ versus the control group and $^{\#\#}P < 0.01$ versus the LPS-treated group.

injury by JAK2 and STAT1 pathway, Western blot analysis was applied to detect the expression of p-JAK2, JAK2, p-STAT1, and STAT1 in WI-38. Results of the Western blot analysis hinted that the phosphorylation levels of JAK2 and STAT1 demonstrated dramatic upregulation in WI-38 cells after LPS treatment. However, the phosphorylation levels of JAK2 and STAT1 were reversed in the LPS group administrated with CA in a dose-dependent manner, with significant inhibitory effects at high doses, which indicated that CA could be inactivated in JAK2-STAT1 signaling pathway (Figure 4). This result hinted that CA regulated the JAK2-STAT1 signaling pathway.

Discussion

In spite of advancements in medical science, the molecular mechanism of acute pneumonia is not clear. Recently, some studies have suggested that CA can play an essential anti-inflammatory role.[4] However, CA is rarely studied in acute pneumonia, and understanding the molecular mechanism of CA in acute pneumonia could provide novel therapeutic targets for acute pneumonia. Nevertheless, the molecular mechanism of CA in determining acute pneumonia remains poorly understood.

Coniferyl aldehyde is a phenolic compound extracted from plants such as cinnamon, vanilla, fig-tree leaf, or eucommia ulmoides, and is used to treat high blood pressure, strengthen muscles and bones, and restore damaged liver and kidney functions. Several studies have reported that CA markedly ameliorated symptoms of pulmonary as well as other diseases. Specifically, studies have also found that CA can attenuate Alzheimer's pathology by Nrf2 pathway.[7] The relationship between CA and neurite outgrowth has been recently confirmed to be mediated by ERK1/2 signaling pathway.[8] Moreover, CA was found to inhibit LPS-induced apoptosis through PKC α/ß II/Nrf-2/HO-1 pathway.[6] Owing to the multi-targeted actions of CA, it could have a broad prospect in the development of new and safe drugs as a promising natural product. However, a few research papers had concentrated on the pharmacological value of CA in acute pneumonia, but the potential mechanism has not been reported yet. This study disclosed that LPS treatment inhibited the viability of WI-38 cell lines, while administration of CA significantly promoted the viability of WI-38 cell lines in a dose-dependent manner, with significant inhibitory effects at high doses. In addition, the fact that CA treatment attenuates LPS-induced inflammation of WI-38 cells was also confirmed. Further research indicated that LPS treatment dramatically promotes apoptosis of WI-38 cells, while administration of CA notably inhibits apoptosis of WI-38 cells. These results reveal that CA may act as a promoting factor in ameliorating symptoms of acute pneumonia.

A growing number of research papers have hinted that CA carries out its functions by regulating the expression of target mRNAs. A previous study proved that CA analog induces distinctive apoptosis by downregulating MEK/ERK signaling pathway in hepatocellular carcinoma cells.[9] Moreover, CA was reported to ameliorate glucose and lipid metabolism in HepG2 by LKB1/AMPK.[10] In addition, CA was also reported to prevent destruction of articular cartilage in a murine model by Nrf2/HO-1.[11] Another novel discovery of this study was that JAK2 and STAT1 proteins were the targets of CA. JAK2 is a human tyrosine kinase protein that is essential for the signal transduction of specific cytokines. STAT1 is a member of the STAT protein family which plays a key role in multiple cellular processes, including cell growth, proliferation, and migration. Changes in the protein expression levels of JAK2 and STAT1 induced by LPS

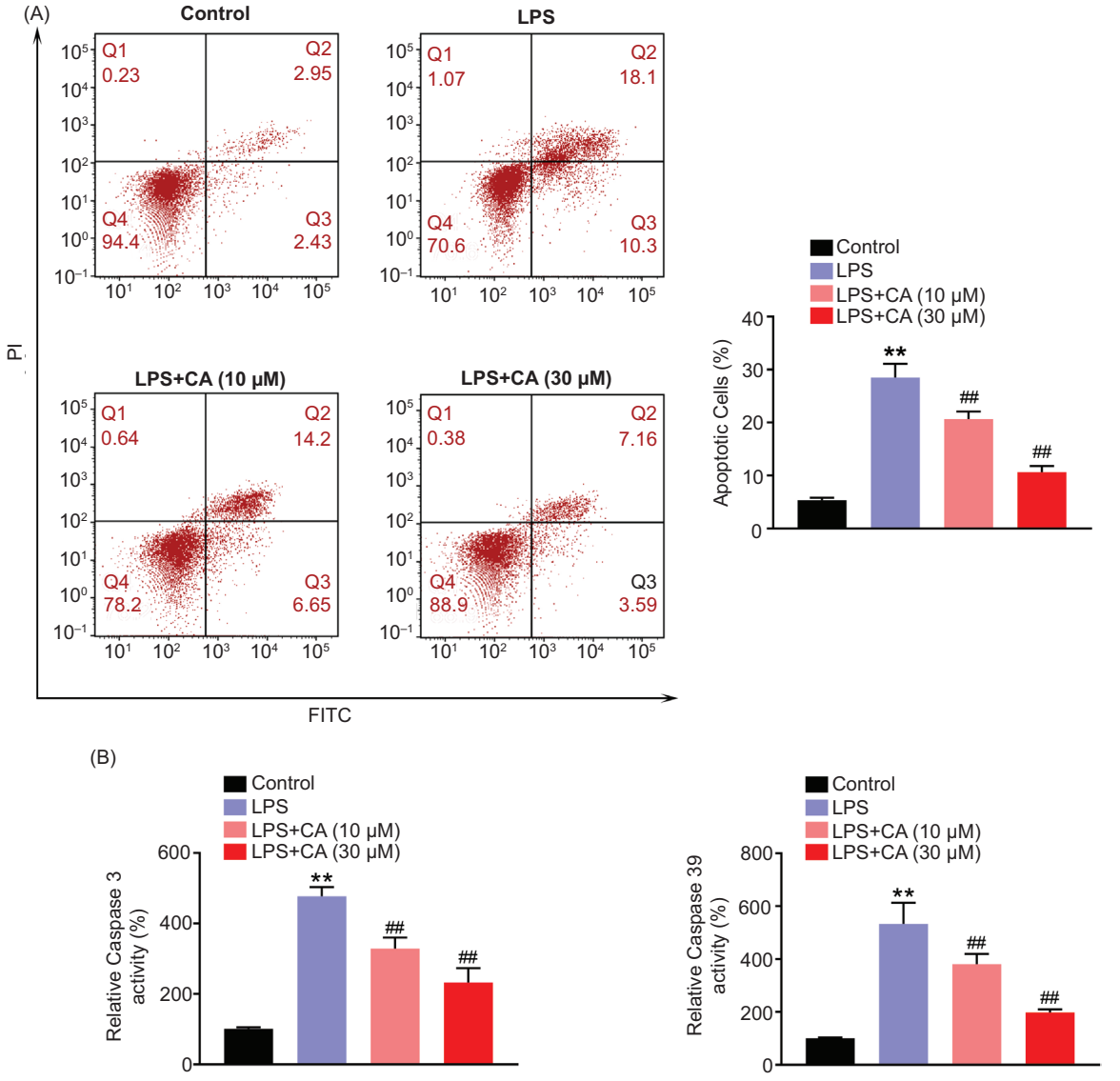

Figure 3 Coniferyl aldehyde alleviates LPS-induced apoptosis. (A) Serial flow cytometry assays detected apoptosis of WI-38 cell line after treatment with or without LPS at different CA doses. (B) Protein expression levels of Caspase 3 and Caspase 9 in WI-38 cells in each group. Data are presented as mean ± SD with three independent experiments. $^{**}P < 0.01$ versus control group and $^{\#\#}P < 0.01$ versus the LPS-treated group.

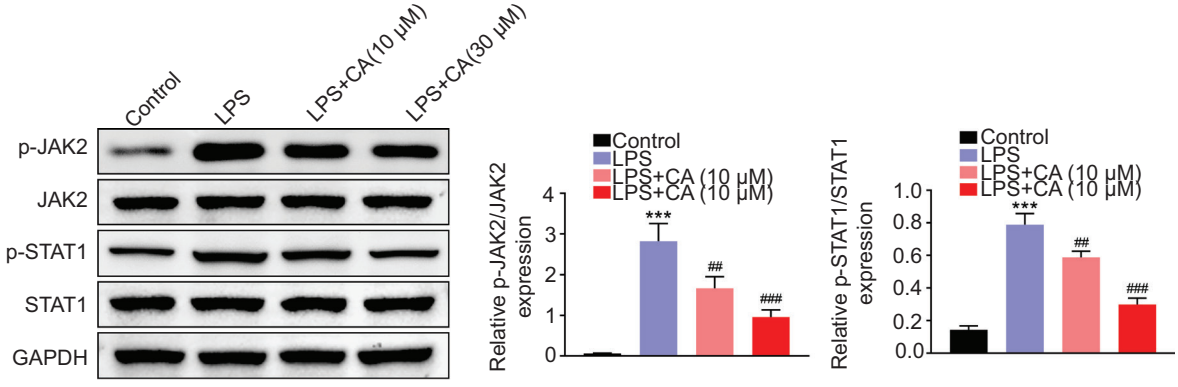

Figure 4 Coniferyl aldehyde regulates the JAK2-STAT1 signaling pathway. The protein expression levels of p-JAK2, JAK2, p-STAT1, and STAT1 in WI-38 cells in each group. Data are presented as mean ± SD with three independent experiments. $^{***}P < 0.001$ versus control group, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ versus the LPS-treated group.

gradually changed with increasing CA dose, indicating that CA ameliorates acute symptoms of pneumonia by activating JAK2-STAT1 pathway.

In conclusion, LPS treatment inhibited the viability of WI-38 cell lines, while administration of CA significantly promoted the viability of WI-38 cell lines in a dose-dependent manner, with significant inhibitory effects discovered at high doses. Moreover, treatment with CA attenuates LPS-induced inflammation of WI-38 cells. Meanwhile, LPS treatment dramatically promotes apoptosis of WI-38 cells, while administration of CA notably inhibits apoptosis of WI-38 cells. Finally, it is also confirmed that CA ameliorates symptoms of acute pneumonia by inactivating JAK2-STAT1 pathway. All these results figured out the role of CA-JAK2-STAT1 signaling pathway in ameliorating symptoms of acute pneumonia, which could conceivably pave a path for advanced therapeutic targets in acute pneumonia.

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