

Original Research Article

Jaceosidin inhibits NLRP3-mediated pyroptosis by activating SIRT1/NRF2 and ameliorating intestinal epithelial cell injury

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Abstract

Purpose: To investigate the effect of Jaceosidin in ulcerative colitis (UC).

Methods: An ulcerative colitis cell model was established by stimulating normal human colon mucosal epithelial cell lines (NCM460 cells) with lipopolysaccharide (LPS). The cells were treated with 5, 10, 20 or 40 μ M Jaceosidin. Cell viability was performed using cell counting kit 8 (CCK8) assay. Oxidative stress was measured with superoxide dismutase (SOD), lipid peroxidation MDA, reduced glutathione (GSH), oxidized glutathione (GSSG), and human myeloperoxidase enzyme-linked immunoassay (ELISA) kits. The mRNA levels were determined by quantitative polymerase chain reaction (qPCR) assay, while protein levels of SIRT1, NRF2, NLRP3, caspase-1, TNF- α , IL-1 β , and IL-6 were determined by western blotting.

Results: Jaceosidin significantly inhibited oxidative stress and accumulation of inflammatory cytokines in LPS-induced NCM460 cells, as well as NLRP3-mediated cell pyroptosis ($p < 0.05$). Jaceosidin also inhibited activation of NLRP3 inflammasome by activating SIRT1/NRF2 pathway, thereby preventing NCM460 cell pyroptosis.

Conclusion: Jaceosidin inhibits NLRP3-mediated pyroptosis, thus suggesting that jaceosidin is a potential lead for UC secondary to NLRP3 inflammasome.

Keywords: Jaceosidin, Pyroptosis, SIRT1/NRF2, Intestinal epithelial cell injury

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INTRODUCTION

Epidemiological statistics from different regions suggest that global incidence of ulcerative colitis (UC) is approximately 20 - 250 per 100,000 people [1]. Oxidative stress and inflammation are key players in UC [2]. Diffuse infiltration of inflammatory cells in colitis stimulates excessive

production of ROS, leading to colon cell damage [3].

Inflammasomes are protein complexes that play important roles in inflammatory responses. Activation of NLRP3 inflammasome further activates Caspase-1 and promotes secretion of pro-inflammatory cytokines, thereby inducing inflammatory reactions and subsequent tissue damage [4,5]. The NLRP3 inflammasome plays

an important role in UC. The NLRP3 levels are elevated in UC patients and are associated with severity and progression of the disease [5-8]. In addition to promoting pro-inflammatory cytokines release, the NLRP3 inflammasome also participates in cell death through pyroptosis in various cell types in UC, including immune and epithelial cells [9,10]. Studies have also indicated that NLRP3 inflammasome exacerbates intestinal inflammation by affecting survival and death of various cell types in UC [9,10]. Jaceosidin is a flavonoid compound extracted from *Artemisia princeps*, and it possesses antioxidant, anti-inflammatory, and anticancer activity [11]. Some studies have shown that Jaceosidin inhibits acute myocardial cell oxidative stress and inflammation induced by DOX, via the activation of the SIRT1 signaling pathway [12].

Jaceosidin was investigated in LPS-induced intestinal epithelial cell damage using intestinal epithelial cell line NCM460 as an *in vitro* model. The effects of Jaceosidin on cell viability, oxidative stress, and accumulation of inflammatory factors induced by LPS were studied, in addition to its regulation of the SIRT1/NRF2 pathway and its inhibitory effect on NLRP3-mediated cell pyroptosis.

EXPERIMENTAL

Cell culture and treatment

The NCM460 was frozen in the laboratory, grown in high glucose Dulbecco's Modified Eagle medium (DMEM, Code no. 11965092, Gibco) and induced with 5 µg/mL LPS (Code no. 00-4976-93, Invitrogen) for 24 h. The NCM460 cells were further treated with 5, 10, 20 or 40 µM Jaceosidin for 24 h.

Cell viability and proliferation assays

The NCM460 cells were seeded in 96-well plates, and viability was measured by Cell Counting Kit-8 (Code no. C0038, Beyotime).

Western-blotting assay

Total protein was extracted from NCM460 cell pellets with radioimmunoprecipitation assay (RIPA) buffer (Code No. P0013B, Beyotime). Proteins were separated by electrophoresis using 8 % sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) electro-transferred onto polyvinylidene difluoride (PVDF) membranes and then blocked with 5 % skim milk. Membranes were probed with primary antibodies: β-actin (Code no. ab8226, 1:5000,

Abcam), SIRT1 (Code no. ab110304, 1:5000, Abcam), NRF2 (Code no. ab62352, 1:2000, Abcam), NLRP3 (Code no. ab263899, 1:1000, Abcam), Caspase-1 (Code no. ab207802, 1:1000, Abcam). Goat anti-rabbit IgG H&L (HRP) (Code no. ab6721, 1:5000, Abcam) and goat anti-mouse IgG H&L (HRP) (Code no. ab205719, 1:5000, Abcam) were used as the secondary antibodies. Semi-quantitative analysis of western blotting results was performed by ImageJ software.

Quantitative polymerase chain reaction (qPCR) assays

Total RNA was extracted from NCM460 cells using TRIzol® RNA isolation reagents (Code no. 15596026, Invitrogen) according to manufacturer's protocol and reversely transcribed into complementary deoxyribose nucleic acid (cDNA) under the following conditions: reaction at 55 °C for 5 min, denaturation at 95 °C for 10 min, maintenance for 10 s, and annealing at 60 °C for 40 s. The primer sequences are shown in Table 1.

Table 1: Primer sequences used

Name	Primer sequence
GAPDH forward	5'-GTCTCCTCTGACTTCAACAGCG-3'
GAPDH reverse	5'-ACCACCCTGTTGCTGTAGCCAA-3'
TNF-α forward	5'-CTCTTCTGCCTGCTGCACTTTG-3'
TNF-α reverse	5'-ATGGGCTACAGGCTTGCTCACTC-3'
IL-6 forward	5'-AGACAGCCACTCACCTCTTCAG-3'
IL-6 reverse	5'-TTCTGCCAGTGCCTCTTTGCTG-3'
IL-10 forward	5'-TCTCCGAGATGCCTTCAGCAGA-3'
IL-10 reverse	5'-TCAGACAAGGCTTGCCAACCCA -3'

Oxidative stress determination

Oxidative stress for NCM460 treated cells was measured with superoxide dismutase (SOD), lipid peroxidation MDA, reduced glutathione (GSH), oxidized glutathione (GSSG), and human myeloperoxidase enzyme-linked immunoassay (ELISA) kits purchased from Beyotime.

Determination of cytokines

Supernatant cell culture of NCM460 treated cells were collected for cytokine measurement using human TNF-α, IL-6 and IL-10 ELISA kits purchased from Absin.

Statistical analysis

All data were presented as mean ± SD. Statistical significance was determined with t-test between the two groups. Statistical analysis was performed using GraphPad version 9.4.1. $P < 0.05$ was considered statistically significant.

RESULTS

Jaceosidin increased NCM460 cell viability stimulated by LPS

Jaceosidin (Figure 1 B) has no effect on viability of NCM460 cells without LPS stimulation (Figure 1 A). However, when NCM460 cells were stimulated with 5 µg/mL of LPS, cell viability was reduced by 30 %, and with the increased concentration of Jaceosidin, cell viability significantly recovered (Figure 1 C). These results demonstrated that viability of LPS-treated NCM460 cells increased with Jaceosidin treatment. Thus, Jaceosidin increases cell viability of intestinal epithelial cells in inflammatory state.

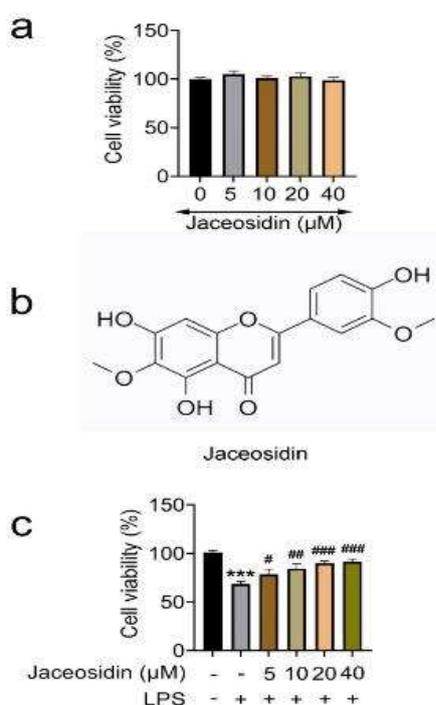


Figure 1: Jaceosidin increased cell viability induced by LPS. (a) Viability of NCM460 cells treated without or with 5, 10, 20, 40 µM Jaceosidin, (b) The chemical structure of Jaceosidin, (c) Jaceosidin attenuates cell damage induced by LPS (5 µg/mL)

Jaceosidin reduced LPS-induced oxidative stress

Treatment of NCM460 cells with 5 µg/mL LPS resulted in decreased SOD, and GSH levels and increased MDA as well as MPO levels indicating that oxidative stress was successfully induced. However, treatment with Jaceosidin exerted a dose-dependent increase in SOD and GSH levels as well as reduction in MDA and MPO levels. These results indicated that Jaceosidin reduces LPS-induced oxidative stress.

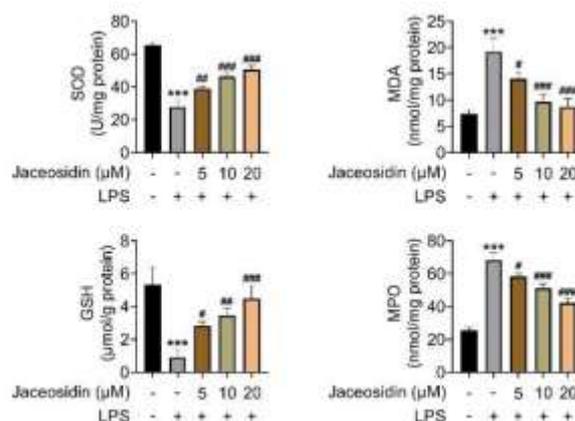


Figure 2: Jaceosidin reduced LPS-induced oxidative stress. NCM460 cells were stimulated without or with LPS, together with different concentrations of Jaceosidin

Jaceosidin reduced inflammatory factors stimulated by LPS

The mRNA levels of TNF-α and IL-6 increased, and IL-10 decreased after LPS induction (Figure 3 A). After treatment with Jaceosidin, mRNA levels of TNF-α and IL-6 decreased, and the mRNA level of IL-10 increased, indicating that Jaceosidin reversed expression of mRNA levels of inflammatory factors. This reversal effect was more obvious with increasing concentration of Jaceosidin. Similar results were replicated for cytokines. Jaceosidin decreased the secretion of pro-inflammatory cytokines and promoted the secretion of anti-inflammatory cytokines. These results demonstrated that Jaceosidin reduced levels of inflammatory factors stimulated by LPS.

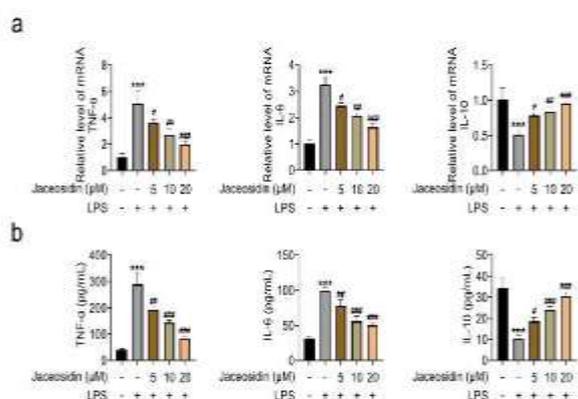


Figure 3: Jaceosidin decreased accumulation of inflammatory factors stimulated by LPS. NCM460 LPS-treated cells were subjected to different concentrations of Jaceosidin. (a) Levels of TNF-α, IL-6 and IL-10 mRNA measured with RT-PCR, (b) Secretion of TNF-α, IL-6 and IL-10 were measured with ELISA Kit

Jaceosidin activated SIRT1/NRF2 pathway and inhibited NLRP3-mediated NCM460 cell pyroptosis

Induction with LPS inhibited SIRT1 when compared to control group (LPS-/Jaceosidin-). However, Jaceosidin partially rescued protein expression of SIRT1 in a dose-dependent manner. After treatment with 20 μ M Jaceosidin, expression level of SIRT1 protein reduced to 75 % of the control group. Induction with LPS inhibited expression of NRF2, while Jaceosidin treatment partially restored its expression. Furthermore, LPS induction promoted NLRP3 and caspase-1 proteins, whereas Jaceosidin treatment inhibited NLRP3 and caspase-1 proteins. Jaceosidin activated the SIRT1/NRF2 pathway and inhibited NLRP3-mediated NCM460 cell pyroptosis in the LPS-induced NCM460 cell pyroptosis model.

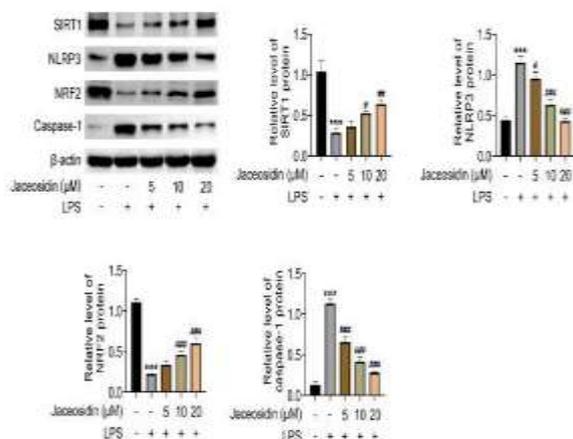


Figure 4: Jaceosidin activated SIRT1/NRF2 pathway and inhibited NLRP3-mediated NCM460 cell pyroptosis. The expression of SIRT1, NRF2, NLRP3 and caspase-1 was determined by western blot. β -actin was selected as reference protein. Semi-quantitative analysis was performed through image analysis software to evaluate the relative levels of SIRT1, NRF2, NLRP3 and caspase-1

Jaceosidin inhibited NLRP3-mediated cell pyroptosis through SIRT1

In order to directly investigate whether SIRT1 is key to the inhibition of Jaceosidin in LPS-induced cell pyroptosis, NCM460 cells were treated with Jaceosidin, SIRT1 inhibitor Sirtinol, or both after LPS induction. Changes in cell viability and levels of SIRT1, NRF2, NLRP3, and caspase-1 in different treatment groups were evaluated. Results showed that Jaceosidin treatment increased expression of SIRT1 and NRF2, and reduced expression of NLRP3 and caspase-1. When Jaceosidin and Sirtinol were simultaneously added, expression of SIRT1 and

NRF2 was further reduced, and NLRP3 as well as caspase-1 increased (Figure 5 A). Jaceosidin treatment attenuated LPS-induced cell pyroptosis (cell viability 88 vs. 69 %) (Figure 5 B).

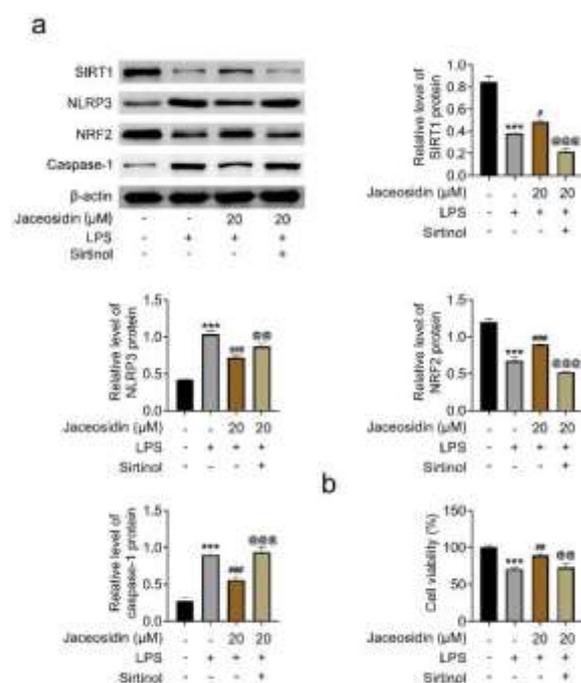


Figure 5: Jaceosidin inhibited NLRP3-mediated cell pyroptosis through SIRT1. (a) Expression of SIRT1, NRF2, NLRP3 and caspase-1 under different treatments, (b) Cell viability of NCM460 cells under different treatments

DISCUSSION

The NLRP3 inflammasome is considered a pathogenic mechanism of ulcerative colitis (UC) [5]. The inflammasome regulates host innate immunity and inflammatory response by activating pro-inflammatory cytokines, leading to inflammatory death of intestinal epithelial cells, as well as mucosal injury [8]. The SIRT1/NRF2 pathway is considered to play a key role in pathological processes such as inflammation. The SIRT1/NRF2 pathway regulates NLRP3-mediated cell pyroptosis through various mechanisms [13-15].

Jaceosidin possesses excellent anti-inflammatory effects including the ability to reduce levels of inflammatory cytokines [11]. However, the mechanism through which jaceosidin regulates NLRP3 inflammasome to exert its anti-ulcerative colitis effects is not clear. In this study, jaceosidin was demonstrated to be an inhibitor of NLRP3 inflammasome through *in vitro* cell experiments. Jaceosidin prevents NLRP3 inflammasome activation and inhibits intestinal epithelial cell pyroptosis by activating

the SIRT1/NRF2 pathway, so as to reduce cellular oxidative stress and inflammation [12]. Consistent with the mechanism identified in this study, Bian *et al* demonstrated that NRF2 activation inhibited oxidative stress and NLRP3/NF-Kb activation in colitis [16].

In this study, Jaceosidin inhibited activation of NLRP3 inflammasome, secretion of inflammatory cytokines, and cell pyroptosis. However, high concentrations of Jaceosidin still did not restore the NLRP3 or inflammatory cytokines to normal levels compared with control cells. This is because NLRP3 was already highly expressed and activated in LPS-treated NCM460 cells. Primary intestinal epithelial cells from postoperative colon tissues may be isolated for *in vitro* cell experiments in future studies. This provides a better model for investigating effect of jaceosidin in UC.

CONCLUSION

Jaceosidin prevents NLRP3 inflammasome activation and intestinal epithelial cell pyroptosis through activating SIRT1/NRF2 pathway. Thus, jaceosidin alleviates colitis by inhibiting expression of inflammatory cytokines. The inhibitory effect of Jaceosidin on NLRP3 inflammasome activation makes it a promising new candidate for NLRP3-associated ulcerative colitis. The modulating effect and safety of Jaceosidin on SIRT1/NRF2 pathway in regulation of NLRP3 inflammasome activation and pyroptosis needs further investigation by employing excision-based models.

DECLARATIONS

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None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Yifei Lv, Ting Qiu, Lu Niu designed the study and carried them out, supervised the data collection, analyzed and interpreted the data, prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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