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Original article

Comparative effects of schisandrin A, B, and C on *Propionibacterium acnes*-induced, NLRP3 inflammasome activation-mediated IL-1β secretion and pyroptosis

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ABSTRACT

Propionibacterium acnes, a common pathogen associated with acne, is also responsible for various surgical infections. Schisandrin A, schisandrin B and schisandrin C, the representative lignans of *Schisandra chinensis* (Turcz.) Baill. extract, inhibit *P. acnes*-induced inflammation. However, their effects on *P. acnes*-induced IL-1β secretion and pyroptosis mediated by NLRP3 inflammasome activation remain unknown. In this study, we compared the effects of schisandrin A, B, and C (Sch A, B, and C) on IL-1β secretion and pyroptosis in *P. acnes*-infected THP-1 cells. As NLRP3 plays important roles in *P. acnes*-mediated inflammation and pyroptosis, we also investigated the effects of Schs on *P. acnes*-induced NLRP3 inflammasome activation by measuring the levels of NLRP3, active caspase-1, and mature IL-1β, and activity of caspase-1. Our results showed that Sch A, B, and C suppressed *P. acnes*-induced pyroptosis. Further, the three lignans significantly suppressed NLRP3 inflammasome activation, with the following potency: Sch C > Sch B > Sch A. Three lignans also inhibited the production of mitochondrial ROS and ATP release. Additionally, Sch B and C almost completely prevented the efflux of K^+, whereas Sch A had a relatively weak effect. Collectively, our novel findings showed that Sch A, B, and C effectively suppressed IL-1β secretion and pyroptosis by inhibiting NLRP3 inflammasome activation in *P. acnes*-infected THP-1 cells. Thus, Schs may be promising agents for the treatment of *P. acnes*-related infections.

1. Introduction

Propionibacterium acnes (*P. acnes*), is a human skin-resident, gram-positive, anaerobic bacterium with low virulence and pathogenicity. Several lines of evidence have implicated that *P. acnes* plays a key role in eliciting host inflammatory responses, which are thought to be essential for the pathogenesis and responsible for the clinical manifestation of acne vulgaris. *P. acnes* contributes to the inflammatory nature of acne by inducing innate immune cells to secrete pro-inflammatory cytokines, such as IL-1β [1–3]. In addition, these low-virulence skin parasites are involved in various surgical infections in almost all clinical disciplines, including ophthalmology, orthopedics, neurosurgery, and blood transfusion, and the post-operative infection rates show an upward trend [4–8].

The innate immune system, the first line of defense against external microbes, utilizes pattern recognition receptors (PRRs), such as nucleotide oligomerization domain (NOD)-like receptors (NLRs) and Toll-like receptors (TLRs), to recognize conserved motifs called pathogen-associated molecular patterns [9,10]. PRRs are expressed by innate immune cells, such as monocytes, macrophages, neutrophils, natural killer cells, and dentritic cells [11]. TLR2 plays an important role in immune responses to *P. acnes*. Immune cells recognize *P. acnes* via TLR2 on the membrane and synthesize inflammatory factors by activating the downstream MAPK and NF-κB signaling pathways [12,13]. Unlike TLR2, NLR is a cytoplasmic recognition receptor, which upon recognizing *P. acnes* activates the inflammasome and caspase-1, thus cleaving the IL-1β precursor into its biologically active form and causing pyroptosis [14,15].

Pyroptosis, a type of programmed cell death mediated by caspase-1/11, is mainly documented in immune cells, such as monocytes, macrophages, neutrophils, natural killer cells, and dentritic cells. Pyroptosis is associated with the production of several inflammatory cytokines, such as IL-1β and IL-18. The proteolytic processing of IL-1β and IL-18 substrates and the secretion of their bioactive forms require the activation of caspase-1 [16,17]. Caspase-1 is activated by certain inflammasomes, such as the NLR family pyrin domain-containing 1 (NLRP1), NLRP3, NLR family CARD-containing 4 (NLRC4), and absent in melanoma 2 (AIM2) [18,19]. NLRP1 inflammasome is activated by

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muramyl dipeptide, *Bacillus anthracis* lethal toxin, and *toxoplasma gondii*. NLRC4 inflammasome is activated by the intermediary molecules NAIP1, NAIP2, and NAIP5/6. AIM2 inflammasome can be directly activated by doubl-stranded DNA. In contrast to the other known inflammasomes, NLRP3 inflammasome can detect and respond to a wide range of agonists, including pathogens, pore-forming toxins, environmental irritants, and endogenous damage-associated molecular patterns. These responses occur only in the context of pre-stimulation with TLRs or tumor necrosis factor prestimulus [20–23].

Schisandra chinensis (Turcz.) Baill. is a traditional herbal, distributed in East and Southeast Asia and southern North America. It possesses several pharmacological activities, including anti-inflammatory, hepatoprotective, anticancer, and immunostimulant activities, and effects the respiratory, cardiovascular, and central nervous systems [24,25]. Dibenzocyclooctene lignans are the main active constituents of *S.chinensis*, and Schisandrin A, B, and C (Sch A, B, and C), the representative dibenzocyclooctene lignans of this plant, have been shown to prevent lipopolysaccharide-induced inflammation [26–30].

In our previous study, we demonstrated the inhibitory effect of *S. chinensis* extract on *P. acnes*-induced IL-1β secretion [31]. However, the effects of its main active constituents namely Sch A, B, and C on *P. acnes*-induced NLRP3 inflammasome activation, IL-1β secretion, pyroptosis, and related mechanisms remain unknown. In this study, we compared the effects of Sch A, B, and C on NLRP3 inflammasome-activation mediated IL-1β secretion and pyroptosis in *P. acnes*-infected THP-1 cells.

2. Material and methods

2.1. Materials

Sch A, B and C were purchased from PureOne Biotechnology (Shanghai, China). Anti-caspase-1 antibody and anti-NLRP3 antibody were purchased from Cell Signaling (MA, USA) and Proteintech Group (IL, USA) respectively. The Bradford protein assay kit, BCA protein assay kit, FITC-conjugated secondary IgG, ECL luminescence reagent, IL-1β ELISA kit, and 4′, 6-diamidino-2-phenylindole (DAPI) were obtained from Weiao biotech (Shanghai, China). Animal DNA isolation kit, Caspase-1 activity assay kit, Cell mitochondria isolation kit, Annexin V-FITC/PI detection kit and ATP assay kit were purchased from Beyotime biotechnology (Jiangsu, China). Fluorometric mitochondrial ROS assay kit was purchased from Genmed Scientifics (MA, USA). DNA loading buffer were purchased from Yeasen biotech (Shanghai, China). Total RNA extraction kit was purchased from KeyGen biotechnology (Nanjing, China). Reverse-transcription kit was purchased form Takara (Shiga, Japan). Ac-YVAD-cmk and Mito TEMPO were purchased from Sigma-Aldrich (MO, USA), which were a selective irreversible inhibitor of caspase-1 and a specific scavenger of mitochondrial superoxide, respectively.

2.2. Cell culture maintenance and treatment

Human monocytic THP-1 cells (Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI 1640 medium (Gibco, NY, USA) supplemented with 10% heat-inactivated FBS (Hyclone, UT, USA) at 37 °C in a humidified incubator with 5% CO_2 and passaged every 3–4 days. *P. acnes* (ATCC 6919) was grown in a cooked meat medium (Rishui biotechnology, Qingdao, China) containing one-third volume of dried beef particles in an anaerobic environment. After anaerobic incubation for 72 h, the culture was harvested via centrifugation, washed with cold phosphate-buffered saline (PBS), and immediately used to stimulate THP-1 cells after resuspension in serum-free medium. The THP-1 cells were pretreated with Sch A, Sch B, or Sch C (10 μM), Mito-TEMPO (40 μM) or Ac-YVAD-cmk (25 μM) for 2 h and then infected with *P. acnes* at a cell-to-bacteria ratio of 1:100.

2.3. Cell viability assay

The toxicity of Schs, Ac-YVAD-cmk and Mito TEMPO at optional concentration to THP-1 cells was evaluated by MTT assay. Cells were adjusted to approximately 2×10^5 cells/well in 96-well plates, and treated them with Sch A, Sch B, or Sch C (10 μM), Mito-TEMPO (40 μM) or Ac-YVAD-cmk (25 μM) for 36 h at 37 °C. Then, 20 μL MTT (5 mg/mL) was added into the culture medium of each hole. 150 μL dimethyl sulfoxid was added to solubilize the formazan crystals after additional 3-h incubation. Absorbance was measured at 570 nm excitation and 630 nm emission by using a spectrophotometer.

2.4. Phagocytosis of P. acnes in THP-1 cells

The infected THP-1 cells were lysed in 1.5% Triton X-100 for 5 min, after washing 5× with RPMI 1640 medium containing antibiotics to remove *P. acnes* attached to the cell surface. The lysates were plated on cooked meat medium agar. The plates were incubated at 37 °C for 24 h in an anaerobic environment. We then determined the number of colony-forming units (CFUs).

2.5. Flow cytometry analysis

THP-1 cells in the exponential growth phase were inoculated into 6-well plates at a density of 5×10^5 cells/mL and incubated with 10 μM of Sch A, B, or C for 2 h. The cells were then infected with *P. acnes* for 24 h and subjected to flow cytometry after staining with annexin V-FITC/PI according to the kit manufacturer's instructions. Briefly, the cells were suspended in a solution containing annexin V-FITC and incubated in the dark at 25 °C for 15 min. The cells were then collected by centrifugation, resuspended in the binding solution containing PI, incubated in the dark on ice for 2 min, and analyzed by flow cytometry immediately.

2.6. DNA gel electrophoresis

The DNA was extracted from THP-1 cells that were pretreated with Schs and infected with *P. acnes* according to the DNA isolation kit manufacturer's instructions. The samples were mixed with loading buffer and resolved by electrophoresis on a 1.5% agarose gel at 100 V. The gel was then stained with ethidium bromide dye and observed under ultraviolet light.

2.7. Western blot analysis

After the cells were pretreated with Schs and stimulated with *P. acnes*, the cytoplasmic proteins were extracted on ice and quantified using a BCA protein assay kit. The samples were then denatured by boiling with loading buffer, separated on a 10% SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 3% skim milk for 2 h and reacted with rabbit anti-caspase-1 or goat anti-tubulin antibodies at 4 °C overnight. Finally, the membranes were incubated with a horseradish peroxidase-conjugated secondary antibody for 1 h at 25 °C, and visualized using an enhanced chemiluminescence kit after washing.

2.8. Immunofluorescence analysis

The Sch-pretreated, *P. acnes*-infected THP-1 cells were harvested, fixed in 4% paraformaldehyde for 30 min, treated with 0.3% Triton X-100 for 10 min, blocked with 5% BSA for 1 h and incubated with primary anti-NLRP3 antibody overnight at 4 °C. After rinsing with cold PBS, the cells were incubated with the corresponding secondary antibody labeled with FITC for 1 h in the dark, followed by staining with DAPI for 2 min. The slides were then sealed with antifade mounting medium and observed under a fluorescence microscope.