Original Paper

Schizandrin A Alleviates LPS-Induced Injury in Human Keratinocyte Cell Hacat Through a MicroRNA-127-Dependent Regulation

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Key Words

Schizandrin A • Inflammation • miR-127 • P38MAPK/ERK pathway • JNK pathway

Abstract

Background/Aims: Inflammatory skin diseases are the most common problems in dermatology. Schizandrin A (SchA) has been reported to have anti-inflammatory properties. Herein, we aimed to investigate the protective effects of SchA on lipopolysaccharide (LPS)-induced injury in keratinocyte HaCaT cells. *Methods:* Inflammation injury in HaCaT cells was induced by LPS treatment. Cell viability, apoptotic cell rate, and apoptosis-related proteins were analyzed by cell counting kit-8 (CCK-8) assay, Annexin V-(fluorescein isothiocyanate (FITC)/ Propidium Iodide (PI) double staining method, and western blot, respectively. The pro-inflammatory factors were analyzed by western blot and quantified by enzyme linked immunosorbent assay (ELISA). Expression of miR-127 in SchA-treated cells was analyzed by qRT-PCR. The effects of SchA on activations of p38MAPK/ERK and JNK pathways were analyzed by western blot. *Results:* SchA protected HaCaT cells from LPS-induced inflammation damage via promoting cell viability, suppressing apoptosis. Meanwhile, SchA inhibited IL-1β, IL-6, and TNF-α expression. miR-127 expression was up-regulated in LPS-treated HaCaT cells but down-regulated after SchA treatment. Overexpression of miR-127 inhibited cell growth and induced expression of IL-1β, IL-6 and TNF-α. Additionally, miR-127 overexpression impaired the protective effects of SchA, implying miR-127 might be correlated to the anti-inflammation property of SchA and also involved in inactivation of p38MAPK/ERK and JNK pathways by SchA. *Conclusion:* miR-127 is involved in the protective functions of SchA on LPS-induced inflammation injury in human keratinocyte cell HaCaT, which might inactivates of p38MAPK/ERK and JNK signaling pathways in HaCaT cells.

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Introduction

The primary interface between the body and the environment is skin [1]. Inflammatory skin diseases are the most common problems in dermatology [2]. The mechanism of skin inflammation is complex and is still not completely understood [3]. For example, Lupus erythematosus (LE) is a kind of autoimmune disease and in this disease, immune system wrongly identifies self as non-self and executes a misdirected immune attack. Skin lesion occurs on most LE patients, and is often accompanied by skin inflammation [4] . Therefore, regulating the inflammatory response is important for the treatment of skin inflammation diseases.

Schizandrin (Sch) extracted from the fruit of *Schisandra chinensis (Turcz.) Baill* is a type of lignans, which are natural phenolic compounds with various biological activities. Schizandrin compounds have been widely reported to have antioxidant, anti-inflammatory, and anti-carcinogenic properties [5, 6]. For example, Schizandrin B (SchB) could protect skin cells from damage induced by ultraviolet radiation b (UVB)-irradiation [7]; Schizandrin C (SchC) was investigated to have the anti-neuro inflammatory property in microglia [8]. Schisantherin A (SchA) was also demonstrated to exhibit anti-inflammatory effects [9, 10]. Furthermore, SchA showed cytotoxicity in human cancer cells and enhanced the cytotoxic effect of the anticancer agent, and even reversed multidrug resistance [11]. SchA has diverse biological activities and thereby it was used as a treatment in skin inflammation model *in vitro* in this study and the possible underlying mechanism was also studied.

Nowadays, increasing evidence showed that some microRNAs (miRNAs) might be the potential therapeutic targets for treating inflammatory diseases and autoimmune diseases [12]. Meanwhile, miRNAs were also found to be novel regulators in skin inflammation [2]. Numerous reports illustrated the role of miRNAs in skin inflammation response and cytokine signaling pathways, such as miR-155 [13], miR-1246 [14], miR-145 and miR-224 [15]. Among all these identified miRNAs, abnormal expression of miR-127 was reported to be link to inflammatory disorders [16]. In addition, miR-127 was found to be involved in LPS-induced inflammation injury in ATDC cells [17] and in H9c2 cardiomyoblasts [18]. Of note, recent studies indicated that miR-127 had a potential role in autoimmune diseases, such as lupus, nephritis and oral pemphigus [19, 20]. Therefore, we intended to investigate whether it was also participated in skin inflammation under SchA treatment.

Human keratinocyte cell HaCaT was reported to be used for the study of LE *in vitro*. In addition, most of the inflammatory effects of infection due to bacteria was triggered by lipopolysaccharide (LPS), which is the bacterial cell wall of Gram-negative bacteria [21]. Importantly, study from Shiba *et al*. found that LPS was used for pro-skin inflammation agent [22]. Meanwhile, human adult keratinocytes were subjected to stimuli and were used in conditioned culture medium to mimic the natural micro-environment for skin study [23]. Therefore, we used LPS as the stimuli to induce human keratinocyte cell line HaCaT to construct *in vitro* cell skin inflammation model for our study.

In this study, the cell growth and pro-inflammatory cytokines levels were measured. Furthermore, we also explored the mechanism of SchA and then the role of miR-127 in regulation skin inflammation. We also determined the regulation of p38 mitogen-activated protein kinase (MAPK)/ extracellular signal‐regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) pathways.

Materials and Methods

Materials and Cell culture

Human keratinocyte cell line, HaCaT cells, purchased from the Chinese Academy of Sciences (Kunming, China), were maintained at 37°C in an incubator under a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. In addition, specific inhibitors of

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signaling protein (20 μM PD98059, ERK inhibitor, 20 μM SB203580, MAPK inhibitor, 20 μM SP600125; JNK inhibitor) was used for pretreated in HaCaT cells for 1 h [24, 25]. All these chemical substances were bought from Sigma-Aldrich (St. Louis, MO, USA).

Cell model construction

HaCaT cells were treated with LPS at different concentrations (2, 4, 8, or 10 μg/mL) for 12 h to induce inflammation injury. SchA solution was prepared by diluting SchA in methyl alcohol at the concentration of 10 mM. Cells were treated with SchA at 10, 20, 30, and 40 μM for 24 h. LPS and SchA (purity above 98%) were obtained from Sigma-Aldrich.

Cell toxicity test

The *in vitro* cytotoxicity analysis was carried out on the synthesized compounds to determine the maximum non-toxic dose (MNTD) to HaCaT cells. Since SchA was dissolved in methanol for testing, the cytotoxicity analysis is initiated with a control experiment using only methanol. Cell toxicity test method was according to Muhamad *et al.* [26].

Transfection

miR-127 mimic and the negative control (NC), synthesized by GenePharma Co. (Shanghai, China) were transfected into HaCaT cells using Lipofectamine 3000 reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

Cell Counting Kit-8 (CCK-8) assay

CCK-8 assay was performed to determine cell viability. HaCaT cells were seeded in 96-well plate, achieving the density of 5000 cells/well. After different treatment, cells were added with 10 μL CCK-8 solution (Beyotime, Shanghai, China) and further incubated at 37°C in a humidified incubator for 1 h. Finally, the absorbance was measured at 450 nm using a Microplate Reader (Bio-Rad, Hercules, CA, USA).

Flow cytometry analysis

Flow cytometry analysis was performed to determine apoptosis of HaCaT cells. Collected cells were washed with phosphate buffered saline (PBS) and then suspended in 195 μL binding buffer (Beyotime) and 5 μL Annexin V-fluorescein isothiocynate (FITC) solution (Beyotime). After incubation in the dark for 30 min, 10 μL propidium iodide (PI, Beyotime) was added in the cell suspension. After incubation in the dark for 30 min at room temperature, cells were analyzed by flow cytometry with FCM (FacsCalibur; Becton-Dickinson, Franklin Lakes, NJ, USA).

Quantitative real time polymerase chain reaction (qRT-PCR)

All RNAs was extracted from HaCaT cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA). To detect relative miR-127 expression, miRNA was isolated by using mirVana™ miRNA Isolation Kit. cDNA was synthesized using TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® MicroRNA Assays was used for qPCR reactions. All used kits were purchased from Thermo Fisher Scientific (Waltham, MA, USA). miR-127 expression was normalized to U6 and calculated by relative quantification $2^{-\Delta\Delta Ct}$ method [24].

Western blot

HaCaT cells were lysed in lysis buffer for 1 h on ice and the obtained lysates were clarified by centrifugation. The protein was quantitated by the Bradford assay (Life Science Co., CA, USA) using the reference standard of bovine serum albumin. The protein samples (35 μg/mL) were resolved by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore Co., MA, USA). After incubation with primary antibodies against Bcl-2 (ab32124), Bax (ab32503), pro caspase-3 (ab44976), cleaved caspase-3 (ab32042), pro caspase-9 (ab32539), cleaved caspase-9 (ab32539), p38 (ab170099), p-p38 (ab47363), ERK (ab196883), p-ERK (ab214362), JNK (ab179461), p-JNK (ab124956), c-Jun (ab32137), p-c-Jun (ab32385), interleukin-1β (IL-1β) (ab2105), interleukin-6 (IL-6) (ab6672), and Tumor necrosis factor (TNF)-α (ab9739), and β-actin (ab8227),

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(Abcam, Cambridge, UK), proteins were visualized by incubating with secondary antibodies marked by horseradish peroxidase, followed by ECL following the manufacturer's protocol (Amersham Life Science Co., Buckinghamshire, UK).

Enzyme-linked immuno sorbent assay (ELISA)

After HaCaT cells were treated with LPS or/and SchA, the supernatants after incubation were collected and IL-1β, IL-6, and TNF-α in the culture supernatant were quantified by ELISA kits (ab100562, ab46042, and ab181421, respectively; Abcam) according to the manufacturer's instructions.

Statistical analysis

All assays were repeated three times and obtained data are presented as the mean ± standard deviation (SD). Statistical analyses were performed by GraphPad Prism 6.0 software (GraphPad Software, San Diego, CA, USA) using A one-way analysis of variance or t test. Statistical significance was marked as * $P < 0.05$, ** $P < 0.01$, or *** $P < 0.001$.

Results

LPS induced pro-inflammatory response on HaCaT cells

Keratinocytes play a crucial role in the regulation of skin inflammation, responding to environmental and immune cells stimuli, such as LPS [27]. In addition, it has also been reported that LPS induces production of TNF-α, IL-6 and IL-1β [28]. Results revealed that the pro-inflammatory response on HaCaT cells was induced by LPS treatment. Cell viability was significantly inhibited after LPS was administrated at 4, 8, 10 μg/mL ($P < 0.05$, $P < 0.01$ or $P < 0.001$, Fig. 1A). The concentration of LPS 8 μg/mL was applied for subsequent assays. Apoptosis of HaCaT cells was significantly promoted by LPS ($P < 0.001$, Fig. 1B). In addition,
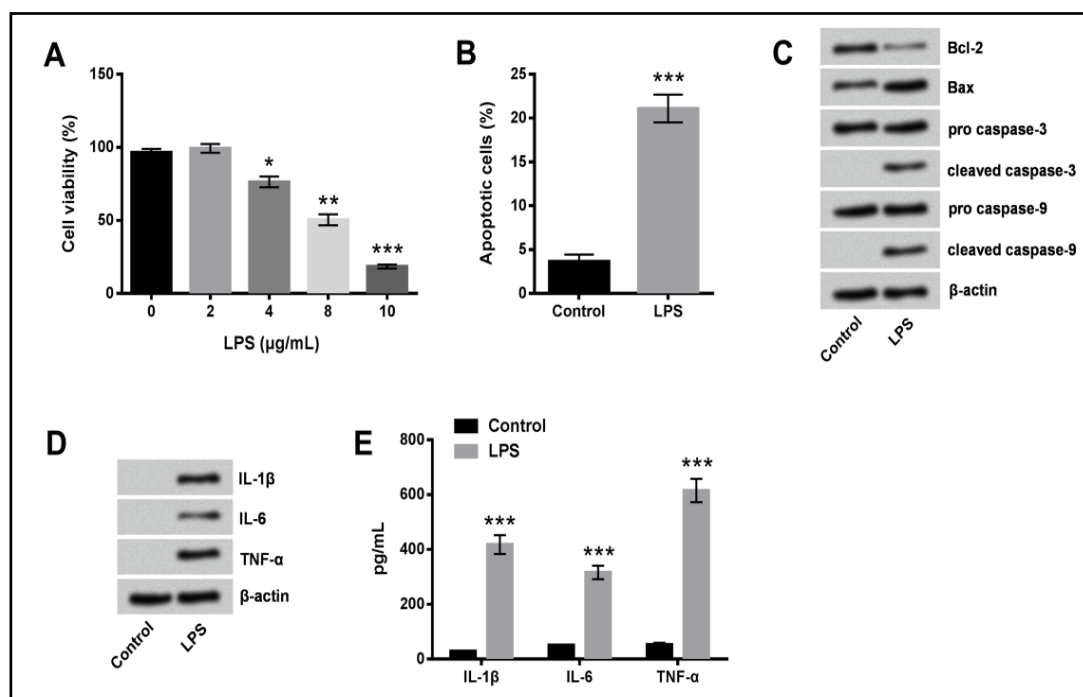

Fig. 1. Lipopolysaccharide (LPS) induced pro-inflammatory response on HaCaT cells. LPS treatment (A) inhibited viability, (B) accelerated apoptosis, (C) induced abnormal expressions of several apoptosis-related proteins, and (D and E) increased protein production of some pro-inflammatory factors. Each point represented the mean ± standard deviation (SD) of triplicates. Each experiment was performed in three times. * P<0.05, ** P<0.01, or *** P<0.001.

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up-regulation of the pro-apoptosis proteins, Bax, cleaved caspase-3, and cleaved caspase-9, as well as down-regulation of the anti-apoptosis protein, Bcl-2 was observed (Fig. 1C). According to analyses of Western blot and ELISA, the pro-inflammatory factors including IL-1β, IL-6, and TNF-α were up-regulated in LPS-treated HaCaT cells (all $P < 0.001$, Fig. 1D and E). These results demonstrated that LPS treatment induced cell inflammation injury in HaCaT cells.

SchA alleviated LPS-induced inflammation injury in HaCaT cells

Before this experiment, cell toxicity analysis was performed to choose concentration of methyl alcohol. As shown in Fig. 2A, concentration of 10 mM was no toxicity to cells and it is the maximum non-toxic dose (MNTD) of methanol. Then we used 10 mM methyl alcohol for diluting SchA and investigated the whether SchA had effects on LPS-induced inflammation injury in HaCaT cells. Results revealed that SchA had no significant effect on viability of HaCaT cells when the concentration was used ≤ 20 μM ($P > 0.05$, Fig. 2B). Thus, SchA concentration of 20 μM was used for the following experiments. The viability-inhibitory effect of LPS was significantly decreased by SchA ($P < 0.05$, Fig. 2C). Also, the apoptosis-promoting effect of LPS was significantly decreased by SchA ($P < 0.01$, Fig. 2D). Meanwhile, the synthesized apoptosis-related protein Bcl-2 was enhanced and Bax, cleaved caspase-3, and cleaved caspase-9 were suppressed after SchA treatment compared with only LPS administration group (Fig. 2E). Compared with LPS administration group, IL-1β, IL-6, and TNF-α expression were all down-regulated after SchA treatment based on Western blot results (Fig. 2F). Quantitative experiment of ELISA showed the significant decrease of IL-1β, IL-6, and TNF-α production (all $P < 0.001$, Fig. 2G). All data suggested that SchA prevented HaCaT cells from LPS-induced inflammation injury.

miR-127 was negatively regulated by SchA

As shown in Fig. 3A, miR-127 was significantly up-regulated by LPS treatment ($P < 0.05$). However, supplement of SchA significantly decreased the expression of miR-127 in LPS-treated HaCaT cells ($P < 0.05$, Fig. 3A). Then the subsequence experiment was carried out to investigate the effects of miR-127 in LPS-induced cell injury. Up-expression of miR-127 by transfection with miR-127 mimic implying the high transfection efficiency ($P < 0.01$,
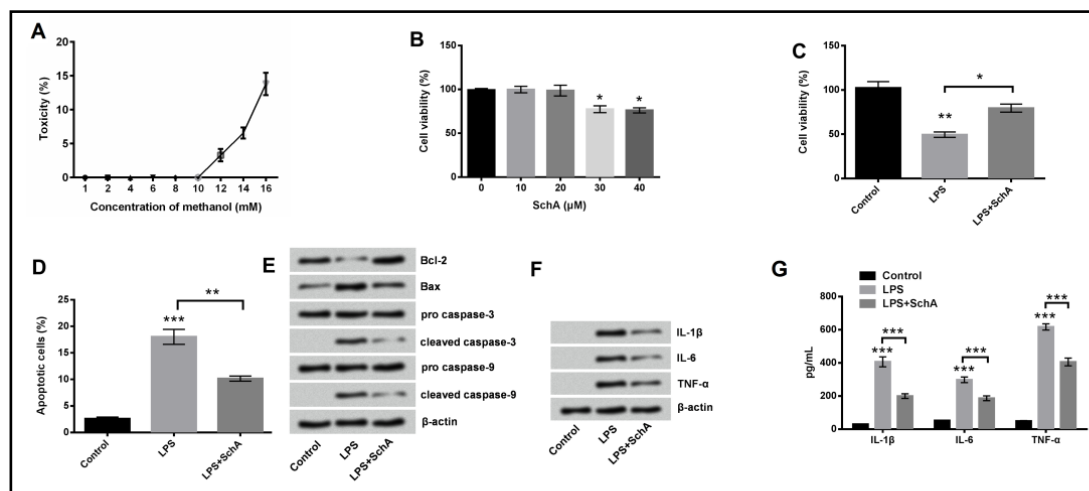

Fig. 2. Schizandrin A (SchA) decreased cell injury induced by lipopolysaccharide (LPS). (A) Cell toxicity test of methyl alcohol. (B) SchA had no cytotoxicity when applied at ≤ 20 μM. SchA inhibited inflammatory reaction in HaCaT cells by (C) enhancing cell viability, (D) suppressing apoptosis, (E) reversing the regulatory effect of LPS on apoptosis-related proteins, and (F and G) inhibiting expressions of the tested pro-inflammation factors. Each point represented the mean ± standard deviation (SD) of triplicates. Each experiment was performed in three times. * P<0.05, ** P<0.01, or *** P<0.001.

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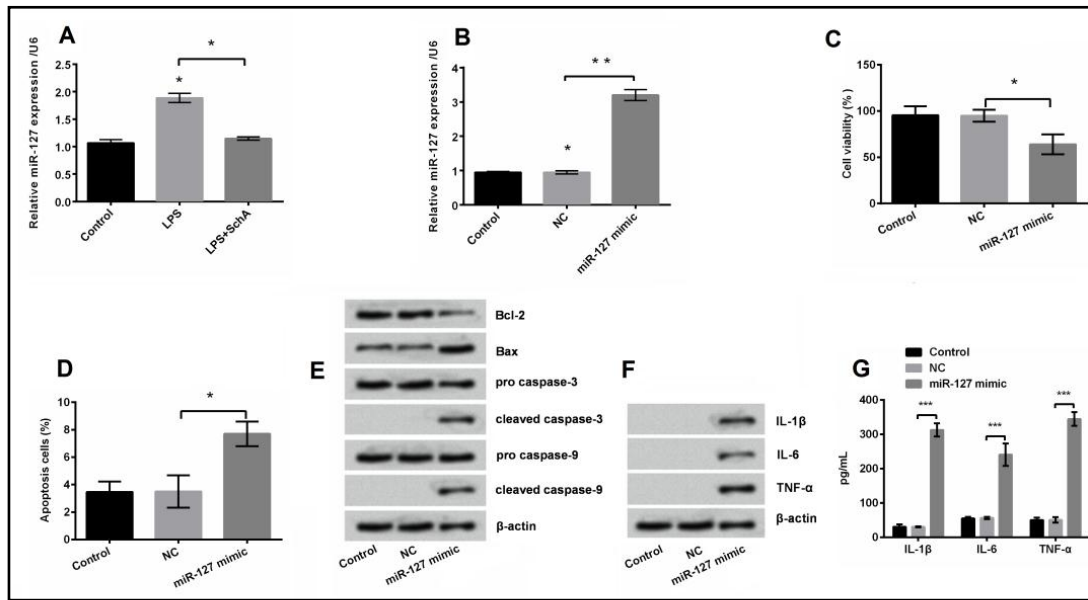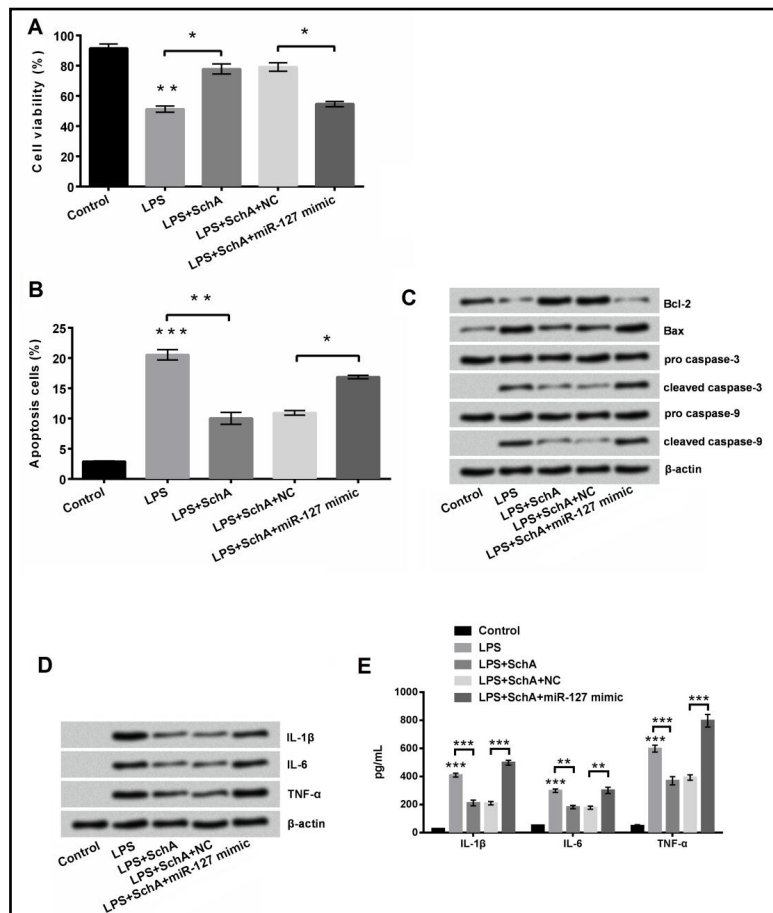Li et al.: The Effect of Schizandrin A on Hacat Cells

Fig. 3. SchA down-regulated miR-127 expression. (A) miR-127 expression by LPS and SchA. (B) transfection of miR-127 mimic. miR-127 overexpression increased cell injury through (C) decreased cell viability, (D) increased cell apoptosis. (E) expressions of apoptosis-related proteins. (F-G) synthesize of inflammation cytokines. Each point represented the mean ± standard deviation (SD) of triplicates. Each experiment was performed in three times. * P<0.05, ** P<0.01, or *** P<0.001.

Fig. 4. SchA decreased cell injury by down-regulation of miR-127. miR-127 mimic treatment (A) suppressed viability, (B) promoted apoptosis, (C) altered expressions of apoptosis-related proteins, (D and E) improved production of pro-inflammation factors. Each point represented the mean ± standard deviation (SD) of triplicates. Each experiment was performed in three times.* P<0.05, ** P<0.01, or *** P<0.001.

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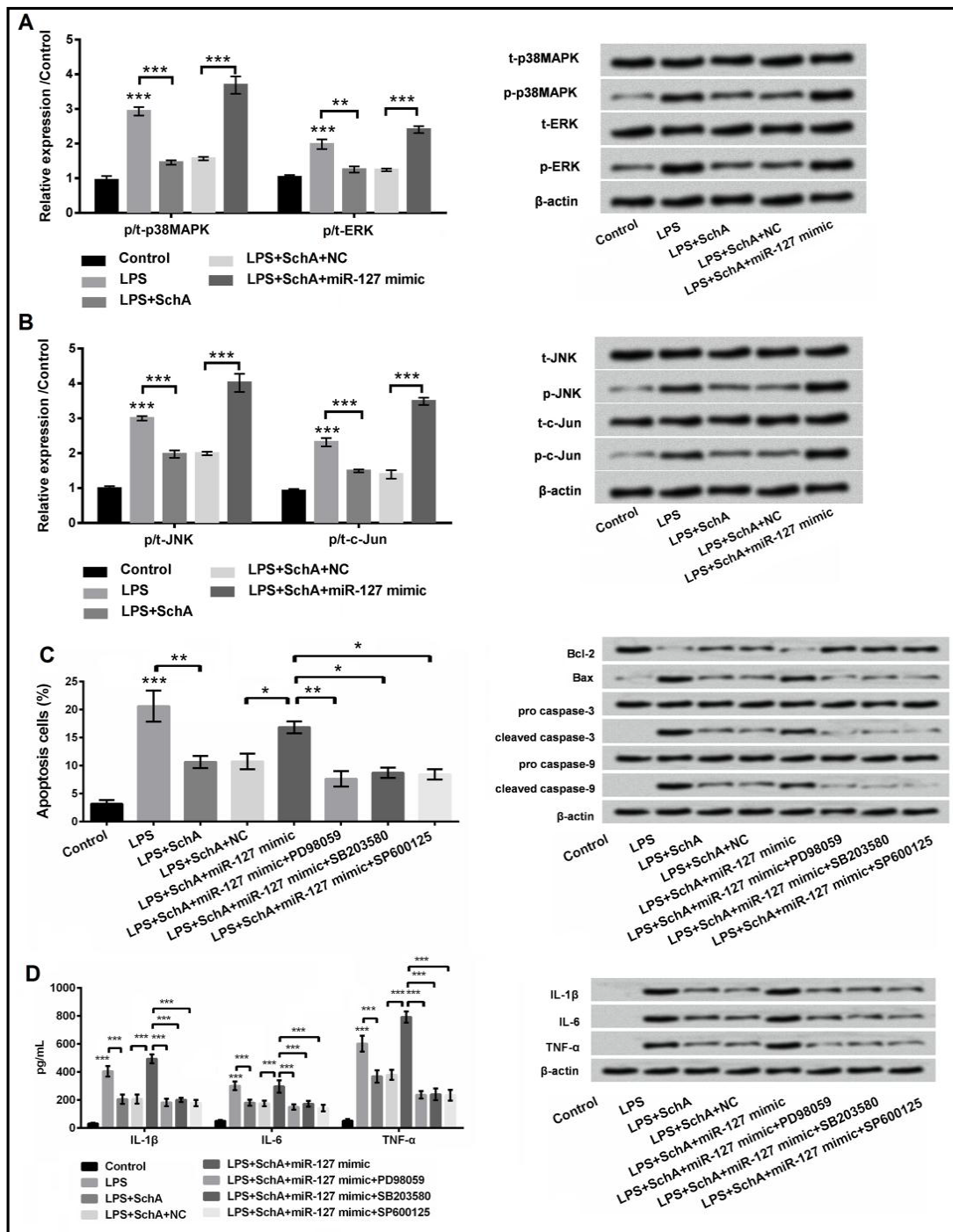Li et al.: The Effect of Schizandrin A on Hacat Cells

Fig. 5. SchA suppressed activations of (A) p38MAPK/ERK and (B) JNK signaling pathways possibly via inhibiting expression of miR-127. (C) Signal pathways related to cell apoptosis. (D) Signal pathways related to inflammation cytokines. Each point represented the mean ± standard deviation (SD) of triplicates. Each experiment was performed in three times. * P<0.05, ** P<0.01, or *** P<0.001.

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Fig. 3B). In addition, we found that transfection with miR-127 mimic decreased cell viability and increased cell apoptosis (both $P < 0.05$, Fig. 3C and 3D). Meanwhile, the apoptosis-related proteins in Fig. 3E confirmed the results in Fig. 3D. Furthermore, the synthesization of inflammatory cytokines IL-1β, IL-6, and TNF-α were up-regulated (all $P < 0.001$, Fig. 3F and 3G). This result demonstrated that SchA down regulated miR-127 expression and miR-127 promoted the LPS-induced inflammation in HaCaT cells.

miR-127 might be involved in protective effects of SchA for LPS-induced HaCaT cells

miR-127 overexpression impaired the cell-protective effects of SchA via inhibiting viability ($P < 0.05$, Fig. 4A), promoting apoptosis ($P < 0.05$, Fig. 4B). Furthermore, the synthesization of apoptosis-related proteins in Fig. 4C were confirmed the results in Fig. 4B. Additionally, according to Western blot data, under the treatment of SchA, synthesized IL-1β, IL-6, and TNF-α were advanced by transfection with miR-127 mimic in LPS-induced HaCaT cells compared with NC ($P < 0.01$ or $P < 0.001$, Fig. 4D and 4E). All data made us speculate that miR-127 plays an important role in protective functions of SchA in LPS-induced inflammation injury.

SchA inhibited activations of p38MAPK/ERK and JNK pathways in HaCaT cells

LPS treatment enhanced activations of p38MAPK/ERK and JNK signaling pathways by increasing expression levels of phosphorylated p38MAPK and ERK (both $P < 0.001$, Fig. 5A), as well as phosphorylated JNK and Jun (both $P < 0.001$, Fig. 5B). However, the expressions of phosphorylated p38MAPK, ERK, JNK and Jun were then suppressed by SchA ($P < 0.01$ or $P < 0.001$, Fig. 5A-5B). Nevertheless, under SchA treatment, miR-127 overexpression reversed the result compared with NC in LPS-induced cells. In addition, we explored the effects of the signal pathways on the cell apoptosis and inflammatory cytokines. Results showed that pretreatment with inhibitors of PD98059 (20 μM), SB203580 (20 μM), and SP600125 (20 μM) were all deceased the apoptosis-promoting effects and cytokines inducing effects caused by transfection of miR-127 mimic under SchA treatment in LPS-induced cells (Fig. 5C and 5D). All of these imply that SchA reduced inflammatory injury in HaCaT cells by inhibition of p38MAPK/ERK and JNK signaling pathways, which was possibly correlated with miR-127 expression.

Discussion

Inflammation is an innate immune response preventing tissue damage that generally leads to restoration of normal structure and function; conversely, uncontrolled and dysregulated inflammation leads to tissue damage, which is closely related with various disorders, including cancer and autoimmune diseases [9, 29]. Considering this, controlling inflammation might be an important method for the treatment of these diseases. In addition, chemical and physical stimuli trigger a cutaneous response by first inducing the main epidermal cells, keratinocytes, to produce specific mediators that are responsible for the initiation of skin inflammation [23]. However, relatively few studies have been assessed on new medicines for treatments of skin inflammation diseases. Meanwhile, the underlying mechanisms remain unclear.

In this study, potent inflammatory agent LPS was used to induce HaCaT cells. After we successfully established the *in vitro* model, studies were performed to investigate the effects of SchA on LPS-induced inflammation injury.

Previously, several studies have provided evidence that Sch compounds possess different physiological functions, such as antioxidant, antiviral, neuroprotective, and cancer chemopreventive activities [6, 30, 31]. The anti-inflammatory effect of SchA was also reported. SchA exerted neuroprotective effect through alleviating microglia-mediated neuroinflammation injury [32]. In another study, SchA significantly decreased the levels of TNF-α and IL-6 in bronchoalveolar lavage fluid [9].

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In our study, we found that SchA promoted cell growth in LPS-induced HaCaT cells: SchA improved cell viability and decreased apoptosis. In addition, previous studies revealed that pro-inflammatory cytokines play an important role in the mediation of inflammation and can cause death [33, 34]. For example, IL-6 protected pancreatic islets or β-cells from inflammatory cytokines-induced cell death and functional impairment both *in vitro* and *in vivo* [35]. In our study, SchA inhibited the accumulation of IL-1β, IL-6, and TNF-α, which might be correlative with the anti-inflammation functions of SchA.

Recent studies have indicated that miRNAs-mediated immune response regulation plays an crucial role in pathogenesis of LE [36, 37]. miR-127 down-regulation was found in LPS-treated cells, but up-regulation was confirmed after SchA administration, implying that miR-127 might be an important inflammation-regulatory factor in regulating LPS-induced HaCaT cell injury. miR-127 was reported to participate in regulation of important physiology functions, but one of its best described role is tumor suppressor [38]. So far, there is no study reporting the role of miR-127 in skin diseases. In our study, we found that miR-127 overexpression promoted LPS-induced cell inflammation injury and further studies revealed that miR-127 might be closely correlated with the protective effects of SchA in LPS-induced inflammation injury. Our study was consistent with the previous studies that miR-127 exaggerated pulmonary inflammation injury; and down-regulation of miR-127 restricted production of pro-inflammatory cytokines [16]

p38 MAPK was found to be involved in skin inflammation response [39], including in HaCaT cells [40]. In our study, SchA blocked p38MAPK/ERK and JNK signaling pathways and both the two signal pathways were related to apoptosis and inflammation cytokines. SchA showed apparent anti-inflammatory properties in an LPS-induced acute lung injury model by blocking the NF-κB and MAPK pathways [9]. The phosphorylation of NF-κB, p65, IκB-α, JNK, ERK and p38 in LPS-induced mouse acute respiratory distress syndrome were suppressed by SchA [10]. Taken together, we can infer that p38MAPK/ERK and JNK were important mediators in inflammation-regulatory effect of SchA.

Conclusion

We established cell injury model in keratinocyte cell HaCaT *in vitro* by LPS stimulation. SchA was found to effectively alleviate inflammation injury in HaCaT cells and miR-127 might be involved in this process; meanwhile, the p38MAPK/ERK and JNK signaling pathways were blocked by SchA. SchA may be a potential alternative in the treatment of skin inflammation diseases. However, further comprehensive studies are needed before it is used for clinical practice.

Disclosure Statement

The authors declare to have no competing interests.

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