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# Molecular mechanisms of the anti-inflammatory effect of sinomenine on atopic dermatitis

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Sinomenine (SIN) is the major bioactive component of the Chinese medicinal herb *Sinomenium acutum* and its anti-inflammatory effects are well established. The purpose of this study was to investigate the effect of SIN on the progression of atopic dermatitis (AD) *in vitro*, and explore the underlying molecular mechanisms. A cell model of AD was established by stimulating RAW264.7 cells with 1 μg/ml LPS. The cells were treated with different concentrations of SIN (0.25, 0.5 or 1 mM), and cell viability was analyzed by MTT assay. The levels of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) were measured by ELISA. Furthermore, NO production in RAW264.7 cells was detected using the nitrate/nitrite assay kit, and the related gene and protein expression levels were determined by qRT-PCR or/and western blot, respectively. SIN inhibited LPS induced increase in the level of proinflammatory cytokines (TNFα, IL-1β and IL-6) and NO production in a dose-dependent manner. LPS increased the expression levels of iNOS and COX2, while pretreatment with SIN markedly decreased iNOS and COX2 expression. Moreover, LPS-induced activation of p38MAPK-NF-κB pathways was suppressed by SIN. Our results demonstrated the anti-inflammatory effect of SIN on AD via regulating the p38MAPK-NF-κB pathway.

## 1. Introduction

Atopic dermatitis (AD) is a highly pruritic chronic inflammatory skin disorder affecting 10-20% of children worldwide (Nomura and Kabashima 2016; Barton and Sidbury 2015). Symptoms can persist or begin in adulthood. The characteristic feature of AD is the infiltration of inflammatory cells, such as mast cells, eosinophils and macrophages, into lesioned skin (Nomura and Kabashima 2016; Barton and Sidbury 2015; Bieber and Simon 2011). The pathogenesis of AD is not completely elucidated, but is thought to be due to the complex interactions of genetic, immunological, and environmental factors (Akdis et al. 2000). In industrialized countries, the prevalence of this disease has increased by two- to three-fold during the past three decades. Currently, AD is a major public-health problem worldwide, with a lifetime prevalence of 10-20% in children and 1-3% in adults (Bieber 2008; Leung and Bieber 2003). Therefore, it is critical to identify new treatment therapies for AD.

Sinomenine (SIN) (7,8-didehydro-4-hydroxy3,7-dimethoxy-17-methyl-9α,13α, 14α-morphinan-6-one) is the major bioactive component of the Chinese medicinal herb *Sinomenium acutum*. In China, SIN is widely used in the treatment of mesangial proliferative nephritis and rheumatoid arthritis (Cheng et al. 2013; Xu et al. 2008). SIN was shown to possess various pharmacological properties, such as cytoprotective, anti-cancer, anti-endotoxin, immunosuppressive and anti-inflammatory activities (Sakumoto et al. 2015; Lu et al. 2013; Li et al. 2017; Hu et al. 2017; Cheng et al. 2009; Wang and Li 2011). However, its effects on AD development and the potential underlying molecular mechanisms remain largely unclear.

Macrophages play key roles in initiating and expanding inflammatory responses (Kanno et al. 2006). Lipopolysaccharide (LPS), the main component of the outer membrane of Gram-negative bacteria, is one of the most sthenic initiators of inflammation. LPS can activate monocytes and macrophages to produce pro-inflammatory factors (Fujihara et al. 2003).

Therefore, the present study investigated the effects of SIN on AD progression using RAW264.7 macrophage cell line stimulated with 1 μg/ml LPS as an inflammatory process model.

## 2. Investigations and results

### 2.1. Effects of SIN on the viability of RAW264.7 macrophages

After treatment with various concentrations of SIN (0.25, 0.5 or 1 mM), the cytotoxic effects of SIN on RAW264.7 cells were assessed by MTT assay. As shown in Fig. 1, no significant changes in cell viability were observed in the different groups, indicating that SIN had no cytotoxic effect on RAW264.7 cells.

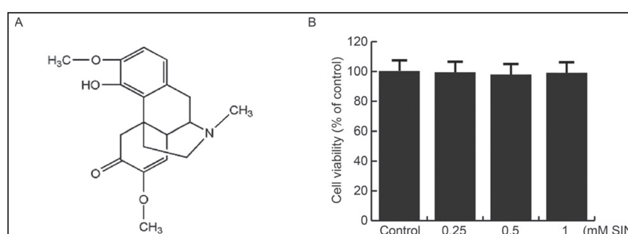


Fig. 1: Effects of SIN on cell viability in RAW264.7 cells. A: Chemical structures of SIN examined in the current study; B: RAW264.7 cells were treated with 0.25, 0.5 or 1 mM SIN for 24 h, and cell viability was determined by the MTT assay. Data are expressed as the mean±SD.

### 2.2. Effects of SIN on LPS-induced pro-inflammatory cytokine production

To investigate whether SIN has anti-inflammatory effects on AD, the production of TNF-α, IL-1β and IL-6 in the culture supernatants of RAW264.7 macrophages was measured. LPS-induced enhancement of TNF-α, IL-1β and IL-6 was inhibited by SIN in a dose-dependent manner (Fig. 2).

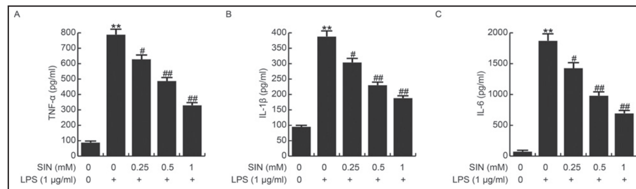


Fig. 2: Effects of SIN on LPS-induced pro-inflammatory cytokine production in RAW264.7 cells. After treatment, the levels of TNF-α, IL-1β and IL-6 in the culture supernatants of RAW264.7 macrophages were measured by ELISA. Data are expressed as the mean±SD. *p<0.05 vs. control group; #p<0.05 vs. LPS treatment alone group.

### 2.3. *Effects of quercetin and galangin on LPS-induced NO production*

After specific treatment, the effect of SIN on NO production in RAW264.7 macrophages was determined. As compared to the control group, NO production was significantly enhanced in the LPS-treated cells, and this effect was abrogated by SIN in a dose-dependent manner (Fig. 3A).

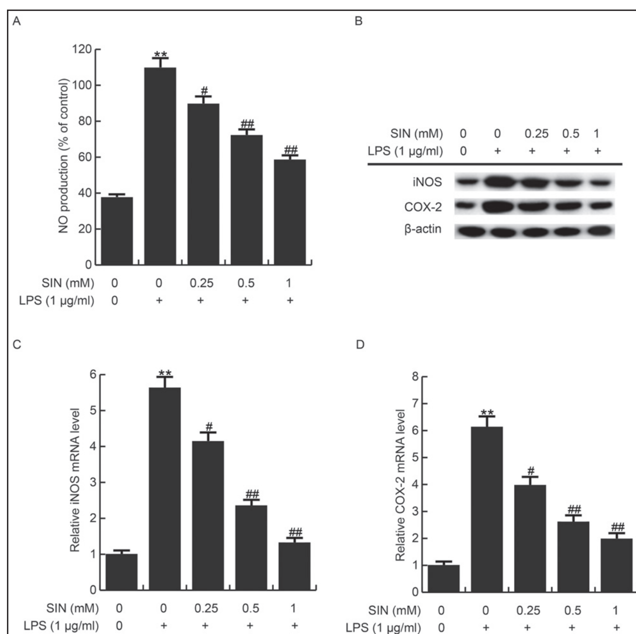


Fig. 3: Effects of SIN on LPS-induced NO production, and iNOS and COX2 expression in RAW264.7 cells. After treatment, the secretion of NO (A) was detected using the nitrate/nitrite assay kit and the protein (B) and mRNA (C, D) levels of iNOS and COX2 were measured by western blot and qRT-PCR, respectively. Data are expressed as the mean±SD. *p<0.05 vs. control group; #p<0.05 vs. LPS treatment alone group.

As iNOS and COX2 are associated with NO and PGE2 production, we examined the expression of iNOS and COX2. After treatment with LPS, the expression levels of iNOS and COX2 were significantly enhanced, while pretreatment with SIN markedly decreased iNOS and COX2 expression (Fig. 3B-D).

### 2.4. *Effects of SIN on the activation of p38MAPK-NF-κB in LPS-stimulated RAW264.7 macrophages*

Next, we investigated whether the p38MAPK-NF-κB pathway was involved in the anti-inflammatory effect of SIN. As shown in Fig. 4, LPS stimulation decreased IκB-α expression and increased p-p65 level in RAW264.7 cells. However, SIN pretreatment significantly attenuated the degradation of IκB-α and enhancement of p-p65. Moreover, the phosphorylation of p38 induced by LPS stimulation was markedly decreased by SIN in a dose-dependent manner.

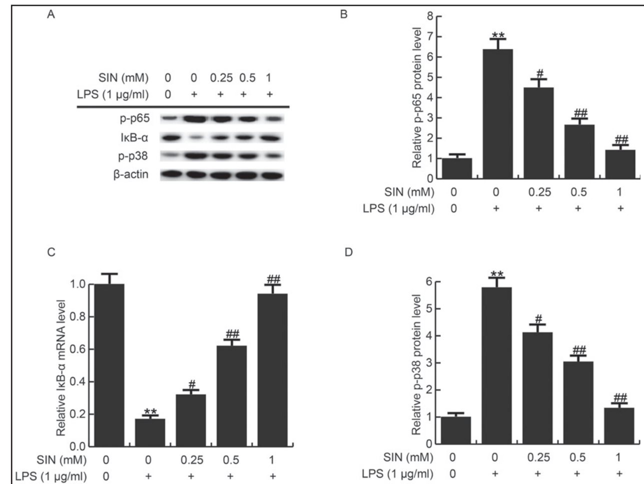


Fig. 4: Effects of SIN on degradation of IκB-α, phosphorylation of NFκB/p65 and p38MAPK protein in LPS-induced RAW264.7 cells. After treatment, the protein levels of IκB-α, p-p65 and p-p38 was detected using western blot analysis. Data are expressed as the mean±SD. *p<0.05 vs. control group; #p<0.05 vs. LPS treatment alone group.

## 3. Discussion

AD is an extremely pruritic and chronic inflammatory skin disease, and its incidence is increasing worldwide. Although some drugs, such as antihistamines, steroids, and immunosuppressants, are useful for the treatment of AD, their long-term efficacy is limited due to the side effects. Although significant advances have been recently made in the therapeutic strategy of AD, the efficacy remains unsatisfactory. Therefore, it is critical to find novel and effective drugs for AD treatment.

SIN is a common rattan drug, which has been used for the treatment of various arthritic diseases in traditional Chinese medicine clinics. It has many pharmacological effects including anti-inflammatory effect (Wang and Li 2011; Yang et al. 2017). However, whether SIN has an anti-inflammatory effect on AD and the underlying molecular mechanisms remain unclear, which was investigated in the present study.

Immune cells, especially macrophages, were activated during AD, and high levels of pro-inflammatory cytokines were released from macrophages (Kasraie and Werfel 2013). Macrophages are crucial immune cells that mediate allergic inflammatory responses, and LPS is the major activator of macrophages (Kanno et al. 2006; Fujihara et al. 2003). Thus, we used murine RAW264.7 macrophages stimulated with 1 μg/ml LPS to mimic AD inflammatory response *in vitro*.

To investigate the effect of SIN on AD, we first determined whether SIN (0.25, 0.5 or 1 mM) was cytotoxic to RAW264.7 macrophages and found that SIN exhibited no cytotoxicity towards RAW264.7 macrophages. Then, RAW264.7 cells were pretreated with or without various concentrations of SIN for 2 h, prior to stimulation with LPS (1 μg/ml). SIN inhibited LPS-induced increase in the levels of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and NO production in a dose-dependent manner. iNOS is a major producer of NO and plays important roles in the progression of inflammatory diseases, including AD. COX-2 expression is enhanced in inflammation-related cells after cytokine stimulation during the immune reaction, resulting in the secretion of PGD2 and PGE2, which are involved in inflammatory response (Orita et al. 2011; Hammad et al. 2003; Trebino et al. 2003). We then determined the expression of iNOS and COX2 in the present study, and found that LPS stimulation significantly enhanced the levels of iNOS and COX2, and these enhancements were inhibited by SIN. To further explore the underlying molecular mechanism of the anti-inflammatory effects of SIN on RAW264.7 macrophages, we investigated the p38MAPK-NF-κB pathway, which can regulate the expression of the inflammatory cytokines (Xing et al. 2017; Dai et al. 2017). LPS-induced

p-NF-κBp65 and p-p38 enhancement and IκB degradation was reversed by SIN treatment, which was consistent with a previous study (Wang et al. 2005).
This was the first study to prove that SIN ameliorated the LPS-induced inflammatory response in RAW264.7 macrophages by inhibiting the p38MAPK-NF-κB pathway.

## 4. Experimental

### *4.1. Cell culture and AD in vitro model conduction*

The RAW264.7 macrophage cell line was obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and cultured in DMEM containing 1% penicillin-streptomycin and 5% fetal bovine serum (FBS; HyClone, Utah, USA), and incubated at 37 °C in a 5% $CO_2$-humidified air environment. The cells were incubated for 24 h in DMEM medium supplemented with 10% FBS. Subsequently, the cells were treated with or without various concentrations of SIN (0.25, 0.5 or 1 mM) for 2 h in serum-free media, and then 1 μg/ml LPS was added to each well.
An AD cell model was established by stimulated RAW264.7 cells with 1 μg/ml LPS for 24 h.

### *4.2. Cell viability assay*

MTT assay was performed to measure the cell viability. Briefly, $3\times10^5$ RAW264.7 cells were seeded into the 96-well plates and treated with various concentrations of SIN (0.25, 0.5 or 1 mM) for 24 h. Then, 5 mg/ml MTT solution were added to each well and incubated at 37 °C for 4 h. After removing the supernatant, 200 μl DMSO was added to each well to solubilize the purple formazan crystals. Finally, the optical densities (OD) at 590 nm was measured using a microplate reader (BioRad Laboratories, Inc., Hercules, CA, USA).

### *4.3. Determination of IL-1β, IL-6, TNF-α production*

The levels of interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in the cell culture medium of different groups were measured by ELISA kits as per the manufacturer's instructions.

### *4.4. Determination of NO level*

After specific treatment, the NO production in the supernatants was measured using the nitrate/nitrite assay kit (Abnova, USA) according to the manufacturer's protocol.

### *4.5 Western blot analysis*

After specific treatment, total proteins from cells were isolated using the RIPA buffer (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China). The concentration of protein was detected by BCA assay (Thermo Fisher Scientific, Inc) per as the manufacturer's protocol. Equal amount of protein samples (30 μg) were separated on 10% SDS-PAGE and then transferred to a PVDF membrane. After blocking with 5% skimmed milk for 2 h at room temperature, the membranes were incubated with primary antibody (all Cell Signaling Technology Inc., Danvers, MA, USA) against p-p38 (dilution ratio: 1: 1000), p38 (dilution ratio: 1: 1000), p-p65 (dilution ratio: 1: 1000), IkB-a (dilution ratio: 1: 1000), iNOS (dilution ratio: 1: 1000), COX-2 (dilution ratio: 1: 1000) or β-actin (dilution ratio: 1: 2000) at 4 °C overnight. After washing thrice with TBST, the membranes were incubated with the horseradish peroxidase-conjugated secondary antibody, anti-rabbit IgG, HRP-linked antibody (1:5,000; Cell Signaling Technology Inc., Danvers, MA, USA), for 2 h at room temperature. Blots were visualized using a chemiluminescence detection kit (Cell Signaling Technology Inc.) as per the manufacturer's protocol.

### *4.6. QRT-PCR*

TRIzol (Invitrogen; Thermo Fisher Scientific Inc.) was used to extract the total RNA from cells according to the manufacturer's protocol. NanoDrop spectrophotometry (Thermo Scientific, Worcester, MA, USA) was used to determine the concentrations of total RNA. RNA was reverse-transcribed to cDNA using a cDNA synthesis kit (iNtRON Biotech, Sungnam, Korea) according to the manufacturer's protocol. Then, the TaqMan® Universal PCR Master Mix kit (Thermo Fisher Scientific Inc.) was used to analyze the synthesized cDNAs. Amplification conditions were as follows: 95 °C for 10 min, followed by 37 cycles of 95 °C for 10 s and 60 °C for 60 s. Expression levels of genes were normalized to GAPDH. All data were analyzed using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen 2001).

### *4.7. Statistical analysis*

The results are presented as the mean±standard deviation (SD). SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses. Relationship between two groups was analyzed by the Student's t-test. $P<0.05$ was considered to be statistically significant.

Conflicts of interest: None declared.

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