ORIGINAL ARTICLE

Inhibition of Poly(I:C)-Induced Inflammation by Salvianolic Acid A in Skin Keratinocytes

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Background: Skin keratinocytes participate actively in inducing immune responses when external pathogens are introduced, thereby contributing to elimination of pathogens. However, in condition where the excessive inflammation is occurred, chronic skin disease such as psoriasis can be provoked. **Objective:** We tried to screen the putative therapeutics for inflammatory skin disease, and found that salvianolic acid A (SAA) has an inhibitory effect on keratinocyte inflammatory reaction. The aim of this study is to demonstrate the effects of SAA in poly(I:C)-induced inflammatory reaction in skin keratinocytes. **Methods:** We pre-treated keratinocytes with SAA then stimulated with poly(I:C). Inflammatory reaction of keratinocytes was verified using real-time polymerase chain reaction, enzyme-linked immunosorbent assay and Western blot. **Results:** When skin keratinocytes were pre-treated with SAA, it significantly inhibited poly(I:C)-induced expression of inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor-α, and CCL20. SAA inhibited poly(I:C)-induced activation of nuclear factor-κB signaling. And SAA also inhibited inflammasome activation, evidenced by decrease of IL-1β secretion. Finally, SAA markedly inhibited poly(I:C)-induced NLRP3 expression. **Conclusion:** These results demonstrate that SAA has an inhibitory effect on poly(I:C)-induced inflammatory reaction of keratinocytes, suggesting that SAA can be developed for the treatment of inflammatory skin diseases such as psoriasis. **(Ann Dermatol 31(3) 279~285, 2019)**

-Keywords-
Inflammasomes, Keratinocytes, Poly(I-C), Salvianolic acid A

INTRODUCTION

Skin is an organ that lies between organism and environment. It consists of three layers such as epidermis, dermis and subcutaneous fat. Skin protects organism from external stimuli such as microbes, harmful chemicals and ultraviolet light. Keratinocytes are the major cells in the epidermis that supply the fundamental protective role against harmful insults by making physical barrier. This protective barrier is produced by the sophisticated but well-ordered differentiation program of keratinocytes, in which many genes are spatiotemporally regulated to make solid water-impermeable structure called cornified cell envelope[1]. In recent years, another important defensive roles of keratinocytes have been recognized. That is, keratinocytes play a role as the immune surveillance cells in innate immunity. Keratinocytes sense the signals from environmental pathogen via the toll-like receptor (TLR) system and initiate the immune responses including the increase of gene expression for many cytokines[2,3]. In normal condition, the

activation of keratinocyte innate immune system helps to eliminate the external pathogens and to provoke adaptive immune response, thereby contributing to maintenance of homeostasis. However, in pathologic conditions such as psoriasis, keratinocyte activation results in abundant expression of cytokines including interleukin (IL)-1β, tumor necrosis factor (TNF)-α and IL-17, thereby exacerbating the inflammatory condition and then leading to onset of disease[4-6]. Considering the importance of these initial innate immune responses, the inhibition of excessive responses by keratinocytes can be a good target of drug development for inflammatory skin diseases such as psoriasis.

We attempted to screen the therapeutic materials for inflammatory skin diseases using an experimental model in which keratinocyte inflammatory reaction was induced using a double-stranded RNA (dsRNA) mimic polyinosinic: polycytidylic acid (poly(I:C))[7]. We found that salvianolic acid A (SAA) has an inhibitory potential on inflammatory reaction of keratinocytes. Previously, it has been reported that SAA protects against myocardial ischemia/reperfusion injury by reducing platelet activation and inflammation[8]. SAA also has been reported to alleviate the ischemic brain injury through the inhibition of inflammation and apoptosis in mice[9]. However, the effects of SAA on skin keratinocytes have never been addressed so far. In this study, we provide evidence that SAA has an inhibitory effects on poly(I:C)-induced inflammatory reaction of skin keratinocytes.

MATERIALS AND METHODS

Ethics statement

Skin tissues were obtained from volunteers under the written informed consent, and the present study was approved from the ethical committee of the Institutional Review Board of Chungnam National University Hospital (IRB no. 1011-135, 2016-07-009).

Cell culture and drug preparations

The immortalized SV40T-transformed human epidermal keratinocytes (SV-HEKs) were cultured using keratinocyte-serum free medium supplemented with bovine pituitary extract (BPE) and recombinant human epidermal growth factor (rhEGF) (Life Technologies Corporation, Grand Island, NY, USA)[10]. At about 70% confluency, culture medium was changed into MCDB153 (Welgene, Gyeongsan, Korea) supplemented with BPE and rhEGF. After overnight incubation, cells were treated with 1 μg/ml of poly(I:C) (InvivoGene, San Diego, CA, USA). SAA was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO).

Cytotoxicity test

For cytotoxicity test, well-established MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay was employed. After treatment with SAA for 24 hours, cells were incubated with MTT solution (0.5 mg/ml) and cell viability was validated by checking optical density at 570 nm.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was purified and reverse transcribed using moloney-murine leukaemia virus reverse transcriptase (MMLV-RTase; Elpis Biotech, Daejeon, Korea). The resulting cDNA was used in real-time PCR. The gene-specific primers were used as below: IL-1β, 5'-TTAAAGCCCGCCTGACAGA and 5'-GCGAATGACAGAGGGTTTCTTAG; IL-6, 5'-CTGC GCAGCTTTAAGGAGTTC and 5'-CCATGCTACATTTGCC GAAGA; IL-8, 5'-CCTTTCCACCCCAAATTTATCA and 5'-TT TCTGTGTTGGCGCAGTGT; CCL20, 5'-CCACCTCTGCGGC GAAT and 5'-TGTGTATCCAAGACAGCAGTCAAA; TNF-α, 5'-CTCCTTCAGACACCCTCAACCT and 5'-CGACCCTAA GCCCCCAATT; GAPDH, 5'-TGCACCACCAACTGCTTAGC and 5'-GGCATGGACTGTGGTCATGAG.

Enzyme-linked immunosorbent assay (ELISA)

The secreted cytokines were quantified by ELISA. The commercial kit for IL-8 was purchased from Life Technologies Corporation. The ELISA kits for IL-1β and TNF-α were obtained from R&D Systems (Minneapolis, MN, USA).

Western blotting

Cellular extracts were prepared using a lysis buffer (Intron, Daejeon, Korea). The cellular proteins were separated by gel electrophoresis and then transferred to nitrocellulose membranes. The membranes were incubated with primary antibody, and then sequentially incubated with peroxidase-conjugated secondary antibodies. The Western bands were obtained by enhanced chemiluminescence (Intron). The following primary antibodies were used: phospho-p65, phospho-IκBα, caspase-1 (Cell Signaling Technology, Beverly, MA, USA); IL-1β (Abcam, Cambridge, MA, USA); NLRP3 and ASC (AdipoGen, San Diego, CA, USA); actin (Sigma-Aldrich).
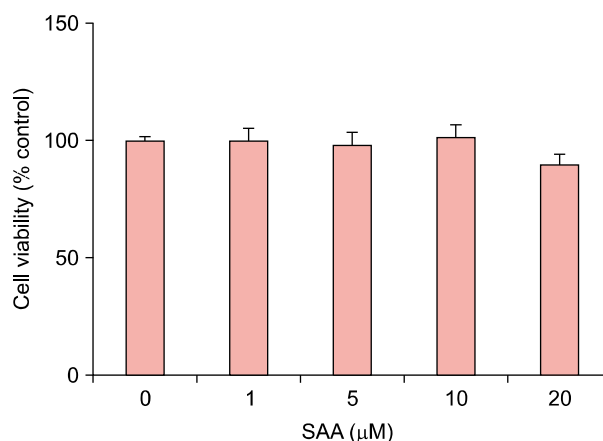
RESULTS

To screen the effective materials that can inhibit poly(I:C)-induced inflammation in skin keratinocytes, we initially used ELISA for IL-8 and single compounds commercially available. Among many compounds tested, SAA showed

A
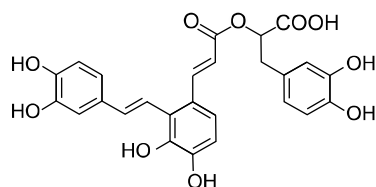
B

Fig. 1. (A) Structure of salvianolic acid A (SAA). (B) Cytotoxicity of SAA in keratinocytes. The immortalized SV40T-transformed human epidermal keratinocytes were treated with indicated concentrations of SAA for 24 hours. MTT assay was performed to measure cell viability. SAA did not show cytotoxicity up to 10 μM. The mean values$\pm$standard deviation are averages of triplicate measurements.

inhibitory potential on secretion of IL-8, thus we chose SAA for a further study. SAA is a polyphenolic compound (Fig. 1A), which has been originally extracted from Radix Salvia miltiorrhiza[11]. To determine the cytotoxicity, we treated SV-HEKs with different doses of SAA and performed MTT assay. As a result, SAA did not show the cytotoxic effect up to the dose of 10 μM, although insignificant slight decrease of cell viability was seen at the dose of 20 μM (Fig. 1B). Therefore, we treated keratinocytes with SAA up to 10 μM in the following experiments.

Poly(I:C) is a chemical mimicking the dsRNA of some viruses, which provokes innate immune reaction in a TLR3-dependent fashion in skin keratinocytes[12]. To examine the effect of SAA, we pre-treated SV-HEKs with SAA then stimulated with poly(I:C). Inflammatory reaction was first determined by qRT-PCR. In the absence of pre-treatment with SAA, poly(I:C) resulted in dramatic increase of mRNAs for inflammatory cytokines including IL-1β, IL-6, IL-8, TNF-α, and CCL20. By contrast, pre-treatment of SAA significantly inhibited poly(I:C)-induced expression of inflammatory cytokines (Fig. 2A). We next checked the secreted cytokines from keratinocytes using ELISA. Consistent with the data obtained from qRT-PCR, poly(I:C) treatment led to dramatic increase of secretion of inflammatory cytokines such as IL-1β, IL-8, and TNF-α from keratinocytes. Pre-treatment with SAA markedly inhibited poly(I:C)-induced secretion of inflammatory cytokines (Fig. 2B).

It has been well recognized that nuclear factor-κB (NF-κB) signaling is pivotal in innate immune responses of skin keratinocytes[13,14]. To delineate the potential mechanism, we investigated the effect of SAA on activation of NF-κB signaling. Consistent with previous data, poly(I:C) treat-

ment led to activation of NF-κB signaling that was evidenced by increase of phosphorylation of p65 subunit and IκBα. Pre-treatment of keratinocytes with SAA resulted in significant inhibition of poly(I:C)-induced phosphorylation of p65 and IκBα (Fig. 3A). In addition to NF-κB signaling, mitogen-activated protein kinases (MAPKs) are also implicated in poly(I:C)-induced inflammatory reaction in keratinocytes and other systems[15-17]. Thus, we examined whether SAA can also inhibit MAPK pathway using Western blot. As a result, SAA significantly inhibited poly(I:C)-induced phosphorylation of p38 MAPK, JNK, and ERK1/2 (Fig. 3B).

When skin keratinocytes were stimulated by poly(I;C), the large caspase-1-activating complex called inflammasome is assembled and then proteolytic activation of IL-1β is occurred. The active IL-1β is then released and participates in the induction of inflammation, contributing to establishment of inflammatory skin diseases such as psoriasis[7,18]. To examine whether SAA affects inflammasome activation, we collected cell culture medium and then checked the released IL-1β and caspase-1. As anticipated, treatment of keratinocytes with poly(I:C) resulted in remarkable increase of IL-1β secretion and inflammasome-activated caspase-1. Pre-treatment with SAA markedly inhibited the secretion of IL-1β and caspase-1 (Fig. 4A). As the inflammasome is a complex structure in which component proteins such as NLRP3 and ASC are involved[19], we next examined the protein levels of NLRP3 and ASC. Poly(I:C) increased the protein level of NLRP3, and this poly(I:C)-increased NLRP3 was obviously inhibited by SAA. However, there was no significant effect on ASC level by poly(I:C) treatment (Fig. 4B).
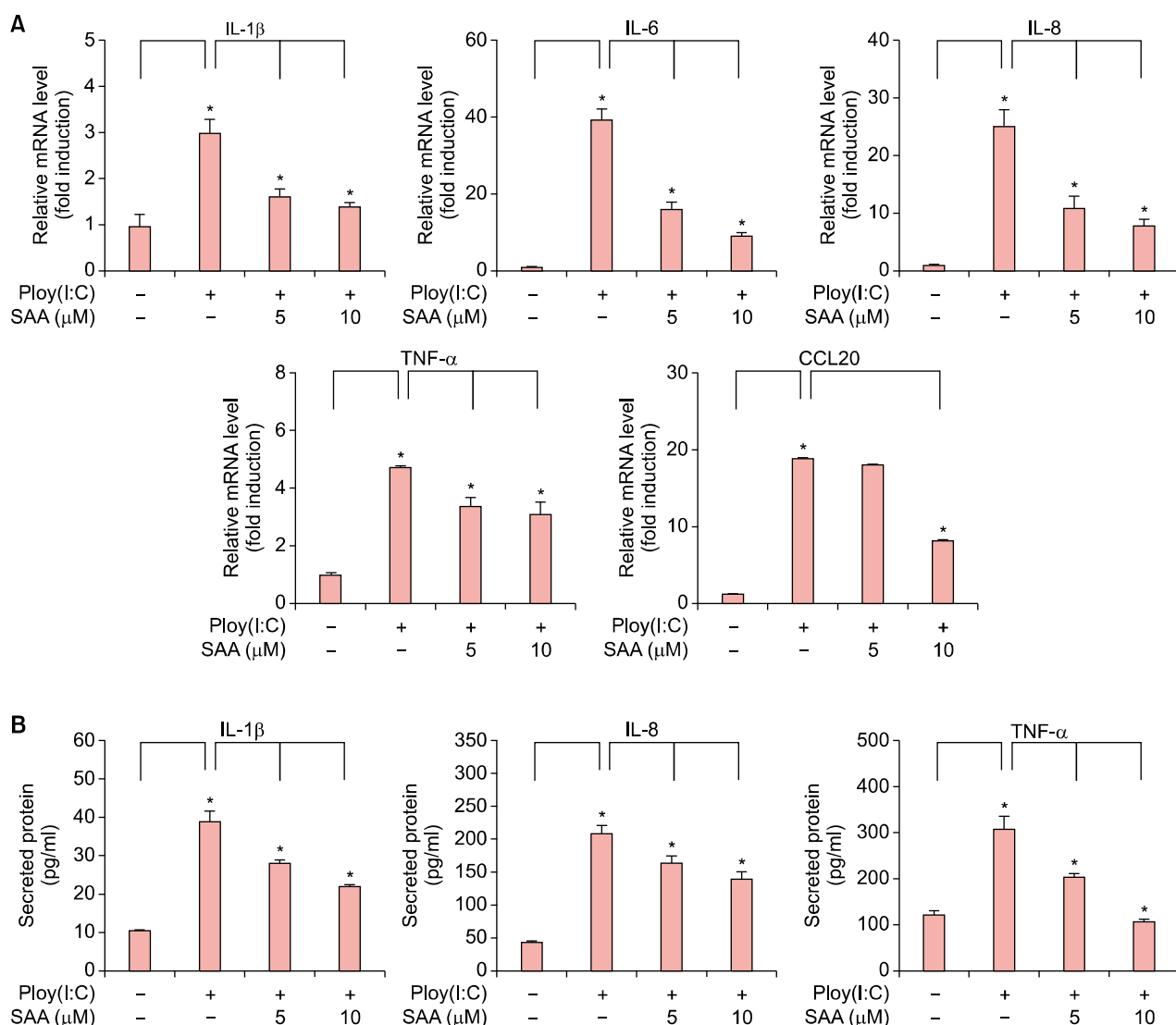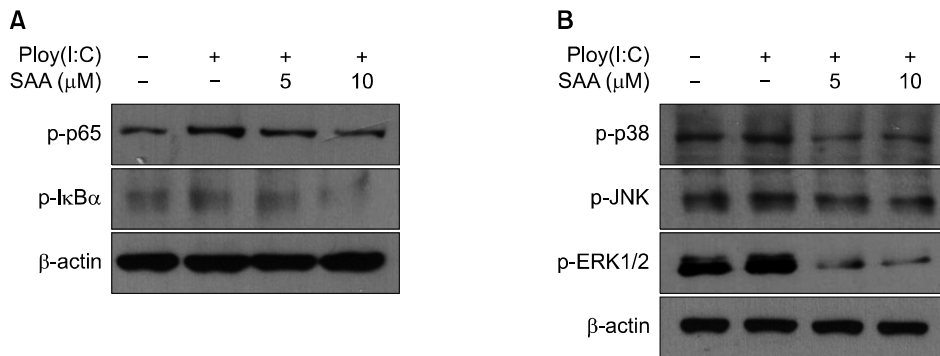
Fig. 2. Effect of salvianolic acid A (SAA) on poly(I:C)-induced inflammatory reaction in keratinocytes. (A) The immortalized SV40T-transformed human epidermal keratinocytes (SV-HEKs) were pre-treated with SAA at the indicated concentrations for 1 hour, and then stimulated with 1 μg/ml of poly(I:C) for 2 hours. The mRNA level was assessed by quantitative real-time polymerase chain reaction. SAA inhibited poly(I:C)-induced cytokine expressions. Data are expressed as fold induction. (B) SV-HEKs were pre-treated with SAA at the indicated concentrations for 1 hour, and then stimulated with 1 μg/ml of poly(I:C) for 24 hours. Secretion of cytokines was measured by enzyme-linked immunosorbent assay. SAA inhibited poly(I:C)-induced secretion of cytokines from SV-HEKs. The mean values±standard deviation are averages of triplicate measurements. IL: interleukin, TNF-α: tumor necrosis factor-α. *$p<0.01$.
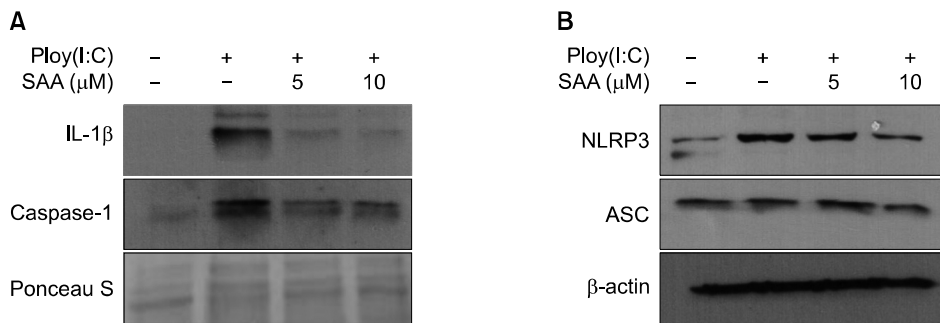
DISCUSSION

Skin keratinocytes play an important role in protection of organism by providing physical and functional defense systems. The one involves barrier structure that is composed of water-insoluble corneocytes and intercellular lipid molecules, the other includes immune modulatory potential in which many cytokines and chemokines are coordinately regulated by pathogen-sensing machinery. Especially for innate immunity, keratinocytes exert their important role as a primary guardian against non-self and/or self antigens through the activation of TLR systems. It has been demonstrated that keratinocytes express a line of functional TLRs including TLR3, 4, 5, 7 and 9[20,21]. Among them, TLR3 acts as a receptor for dsRNA that can be found in some viruses. In condition related to skin disease such as psoriasis, keratinocytes are susceptible to viral RNA intermediates, and activation of TLR3 leads to drastic increase of pro-inflammatory cytokines and chemokines including IL-1β, TNF-α, IL-6, IL-8 and CCL20[22]. The increased inflammatory cytokines in turn contributes to recruit and activate immune cells such as neutrophils and

A

| Ploy(I:C) | – | + | + | + |
| SAA (μM) | – | – | 5 | 10 |

p-p65

p-IκBα

β-actin

B

| Ploy(I:C) | – | + | + | + |
| SAA (μM) | – | – | 5 | 10 |

p-p38

p-JNK

p-ERK1/2

β-actin

Fig. 3. Effect of salvianolic acid A (SAA) on poly(I:C)-induced nuclear factor-κB (NF-κB) activation in keratinocytes. (A) The immortalized SV40T-transformed human epidermal keratinocytes were pre-treated with SAA at the indicated concentrations for 1 hour, and then stimulated with 1 μg/ml of poly(I:C) for 1 hour. The protein levels of phosphorylated-p65 (p-p65) and phosphorylated-IκBα (p-IκBα) were determined by Western blot. SAA inhibited poly(I:C)-induced activation of NF-κB. β-actin was used for internal control. (B) Effect of SAA on phosphorylation of mitogen-activated protein kinases (MAPKs). SAA inhibited poly(I:C)-induced phosphorylation of p38 MAPK, JNK, and ERK1/2.

A

| Ploy(I:C) | – | + | + | + |
| SAA (μM) | – | – | 5 | 10 |

IL-1β

Caspase-1

Ponceau S

B

| Ploy(I:C) | – | + | + | + |
| SAA (μM) | – | – | 5 | 10 |

NLRP3

ASC

β-actin

Fig. 4. Effect of salvianolic acid A (SAA) on poly(I:C)-induced inflammasome activation in keratinocytes. (A) The immortalized SV40T-transformed human epidermal keratinocytes were pre-treated with SAA at the indicated concentrations for 1 hour, and then stimulated with 1 μg/ml poly(I:C) for 24 hours. Culture medium was collected and concentrated, then subjected to Western blot to assess the secreted protein levels for interleukin (IL)-1β and caspase-1. SAA inhibited poly(I:C)-induced secretion of IL-1β and caspase-1. Ponceau S staining was used for loading control. (B) After treatment with SAA, cellular extracts were prepared. The protein levels of NLRP3 and ASC were determined by Western blot. SAA inhibited poly(I:C)-induced NLRP3 expression. β-actin was used for internal control.

activated T cells[23]. Thus, the drug screening against inflammatory reaction of keratinocytes is a good approach for the development of therapeutics for skin diseases such as psoriasis. In this study, we provide evidence that SAA inhibits poly(I:C)-induced inflammatory reaction of keratinocytes, suggesting that SAA can be applicable for the treatment of skin disease such as psoriasis.

SAA is a polyphenolic depside that is composed of three of monocyclic aromatic units linked by ester bond (Fig. 1A). It was originally isolated from the dried roots of Salvia miltiorrhiza[11]. SAA has a variety of biological and pharmacological activities, including anti-tumor, anti-metastatic, antioxidant, and anti-inflammatory effects. For example, SAA induces cell apoptosis and suppresses tumor growth in acute myeloid leukemia via the inhibition of PI3K/Akt sig-

naling[24]. Other evidence indicates that SAA inhibits the migration and invasion of human breast cancer cells by inactivating transgelin2[25]. In addition, SAA inhibits the high-fat diet-induced hepatic inflammation via a NLRP3 inflammasome-dependent way in rat liver[26].

In this study, we demonstrated that SAA has inhibitory potential on poly(I:C)-induced inflammatory reaction in skin keratinocytes. The effects of SAA were likely due to its inhibitory potential on NF-κB signaling, because that NF-κB is key player in the inflammatory reaction. As well recognized, NF-κB directly binds to promoter of many inflammatory cytokine genes, thereby provoking consequent relay of inflammatory process. It has been reported that SAA attenuates NF-κB activation in other systems such as mouse macrophages and rat renal tissues[27,28]. Thus, it is

plausible that the SAA inhibits the activation of NF-κB in skin keratinocytes thereby affecting downstream cellular events. In this regard, it is likely that the inhibition of inflammasome activation by SAA is also linked to NF-κB signaling. Inflammasome activation is an important process in innate immunity, and it has been also recognized that NF-κB activation plays a role in inflammasome activation. When pathogen-associated molecular patterns or damage-associated molecular patterns are recognized by defense cells, inflammasome component protein NLRP3 associates with the adaptor protein ASC. The assembly of NLRP3 and ASC results in the formation of protein complex that can activate the caspase-1 by proteolytic cleavage of pro-caspase-1[29]. The resultant active caspase-1 is then secreted from keratinocytes, thereby contributing to the pathogenesis of skin disease such as psoriasis[30]. In our study, SAA markedly inhibited poly(I:C)-induced activation of NF-κB signaling, and also inhibited inflammasome activation in terms of decreasing the NLRP3 level and the IL-1β secretion. Thus, our data provide evidence supporting that SAA can be used for treating innate immunity-related skin diseases.

In summary, we demonstrated that SAA inhibited poly(I:C)-induced inflammatory reaction of epidermal keratinocytes. Our data suggest that SAA would be a candidate for treatment of chronic inflammatory skin disease such as psoriasis.

ACKNOWLEDGMENT

CONFLICTS OF INTEREST

The authors have nothing to disclose.

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