Aloe-emodin inhibits Pam$_3$CSK$_4$-induced MAPK and NF-κB signaling through TLR2 in macrophages*

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ABSTRACT

Purpose: Aloe−emodin (AE), an ingredient of aloe, is known to exhibit anti−inflammatory activities. However, little is known about the underlying molecular mechanisms of its inflammatory modulatory activity *in vitro*. In the present study, we investigated the anti−inflammatory potential of AE using Pam$_3$CSK$_4$−stimulated macrophages. **Methods:** RAW 264.7 macrophages were treated with AE (0~20 mM) for 1 h, followed by treatment with Pam$_3$CSK$_4$ for 1 h. After incubation, mRNA expression levels of cytokines were measured. The effect of AE on TLR2−related molecules was also investigated in Pam$_3$CSK$_4$−stimulated RAW 264.7 macrophages. **Results:** AE attenuated Pam$_3$CSK$_4$−stimulated expression of proinflam− matory cytokines, including tumor necrosis factor−α (TNF−α), interleukin−6 (IL−6), and interleukin−1β (IL−1β) in RAW 264.7 macrophages. Two concentrations of AE (10 μM and 20 μM) effectively reduced mRNA expression of TLR2 by 41.18% and 54.43%, respectively, compared to that in control cells (p < 0.05). AE also decreased nuclear factor−kappa B (NF−κB) activation and mitogen−activated protein kinase (MAPK) phosphorylation. Phosphorylation levels of ERK1/2, p38, and JNK were markedly reduced by 20 μM AE. In particular, AE decreased phosphorylation of ERK in a dose−dependent manner in Pam$_3$CSK$_4$−stimulated RAW 264.7 macrophages. **Conclusion:** Our data indicate that AE exerts its anti−inflammatory effect by suppressing TLR2−mediated activation of NF−κB and MAPK signaling pathways in macrophages.

KEY WORDS: Aloe−emodin (AE), macrophage, mitogen−activated protein kinase (MAPK), nuclear factorkappa B (NF−κB), Toll−like receptor 2 (TLR2)

INTRODUCTION

Aloe leaves are used in the treatment of several medical conditions, including burns, cancer, and inflammatory bowel disease.[1-3] Aloe-emodin (AE), a major anthraquinone present in aloe, exhibits antibacterial, antiviral, anti-inflammatory, and anticancer effects.[4-6] The levels of AE and aloin, a C-glycoside derivative of AE, range from 0.1% to 21.5% of dry weight in leaf exudates of 68 Aloe species.[7,8] Previous studies have used *in vitro* and *in vivo* models to study the anti-inflammatory activity of emodin. Park et al. reported that AE dose-dependently inhibited the levels of nitric oxide (NO) and prostaglandin E2 (PGE2) by blocking the mRNA expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in LPS-stimulated macrophages.[9] Yin et al. reported that emodin

ameliorated lung injury via the inhibition of cytokine production and inhibition of the p38 mitogen-activated protein kinase (MAPK) pathway in an animal model.[10] Iwanowycz et al. have reported that emodin bidirectionally regulates macrophage phagocytosis and migration by blocking the nuclear factor kappa B (NF-κB)/interferon regulatory factor 5 (IRF5)/signal transducer and activator of transcription 1 (STAT1) and interferon regulator factor 4 (IRF4)/signal transducer and activator of transcription 6 (STAT6) signaling pathways.[11] However, few studies have investigated the biological activity of AE, and its exact mechanisms have not been fully elucidated.

Chronic inflammation is associated with several diseases. Since macrophages are implicated in the initiation of inflammatory responses, they play an important role in inflammatory diseases.[12,13] Therefore, the inhibition of

macrophage-mediated inflammatory responses is a useful therapeutic approach against several inflammatory diseases. Toll-like receptors (TLRs) are pattern recognition receptors that recognize several pathogen-associated molecular pattern (PAMP) molecules involved in pathogenic invasions.[14,15] PAMP-recognizing TLRs stimulate signaling pathways that involve NF-κB and MAPKs.

The aim of this study was to investigate the anti-inflammatory activity of AE against synthetic triacylated lipoprotein Pam_3CSK_4-stimulated RAW 264.7 macrophages.

MATERIALS AND METHODS

Reagents

AE and Pam_3CSK_4 were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Antibodies against extracellular signal-regulated kinase (ERK), p38, c-Jun N-terminal kinase (JNK), phospho (p)-ERK (Thr^{202}/Tyr^{204}), p-p38 (Thr^{180}/Tyr^{182}), and p-JNK (Thr^{183}/Tyr^{185}) were purchased from Cell Signaling Technology (Beverly, MA, USA). All other chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Cell culture

The mouse macrophage cell line (RAW 264.7) was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, and 1% penicillin/streptomycin. Cells were incubated at 37°C, 5% CO_2 for 5~7 days until monolayers of macrophages were observed.

Real-time reverse transcription-polymerase chain reaction analysis (RT-PCR)

Total RNA was extracted using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time quantitative polymerase chain reaction (PCR) was performed using a Quantitect[TM] SYBR Green PCR kit (Quantitect[TM] SYBR Green PCR, Qiagen, CA, USA). The specific primer sets were as follows: 5'-AAC-ATCCAACCTTCCCAAACG-3'/5'-CTCTTAACCCCC-GAATCCCAG-3′ for the tumor necrosis factor alpha (TNF-α) gene, 5'-TCACCTCTTCAGAACGAATTGACA-3'/5'-AGTGCCTCTTGCTGCTTTCAC-3′ for the interleukin 6 (IL-6) gene, 5'-ATTGGGATCATCTTGCTGGT-3'/5'-CCTGCTGTTCACAGTTGCC-3' for the interleukin 1β

(IL-1β) gene, and 5′-GAGCGCAAGTACTCTGTGTG-3'/5'-CGGACTCATCGTACTCCTG-3' for the β-actin gene used as an endogenous control. The relative mRNA expression levels of cytokines were normalized to that β-actin using the ΔΔCT method.[16]

NF-κB activity

To determine the NF-κB activity, the nuclears of macrophages were extracted using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA) and analyzed using a PathScan Total NF-κB p65 assay kit (Cell Signaling Technology, MA, USA) according to the manufacturer's instructions.

Western blotting

Whole cell extract was prepared by suspending in an extraction lysis buffer and the cellular proteins were extracted with Laemmli sample buffer. Proteins were separated on 10% sodium dodecyl sulfate (SDS) polyacrylamide gels electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membrane. The membranes were incubated with 5% nonfat dry milk, followed by incubation with appropriate primary antibodies in 5% nonfat dry milk in 0.05% Tris-buffered saline with Tween 20 (TBS-T) at 4°C overnight. The membranes were washed three times with TBS-T and then incubated for 1 h at room temperature with appropriate horseradish peroxidase-conjugated secondary antibodies. The membranes were visualized by chemiluminescence (ECL) and the density of the blots was quantified by ChemiDoc MP imaging system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All of the data are expressed as mean ± standard deviation (SD). We analyzed our data with the by one-way analysis of variance, followed by Duncan's multiple range tests. $P < 0.05$ was considered to be significant.

RESULTS

TNF-α mRNA expression

To evaluate whether AE can inhibit the gene expression of cytokines, we measured the TNF-α mRNA level in AE-pretreated RAW 264.7 macrophages after their stimulation with Pam_3CSK_4. We observed that Pam_3CSK_4 upregulated the TNF-α mRNA expression, but the overexpression was

inhibited by both 10 μM and 20 μM AE (Fig. 1).

TNF-α, IL-6 and IL-1β mRNA expression

Next, we measured the mRNA expression levels of pro-inflammatory cytokines in Pam_3CSK_4-stimulated RAW 264.7 macrophages by real-time PCR. Our results showed that AE at concentrations of 1-20 μM significantly inhibited the mRNA expression levels of all cytokines studied (Fig. 2; $p < 0.05$). AE significantly decreased the mRNA expression levels of IL-6 and IL-1β in a dose-dependent manner in this cell model (Fig. 2B, C; $p < 0.05$). Among the three proinflam- matory cytokines, TNF-α was most effectively blocked by AE treatment of macrophages (Fig. 2A; $p < 0.05$).

TLR2 mRNA expression

Next, we evaluated the inhibitory effect of AE on the Pam_3CSK_4-stimulated TLR2 mRNA expression in macro-phages. AE significantly inhibited the Pam_3CSK_4-stimulated TLR2 mRNA overexpression at both 10 μM and 20 μM
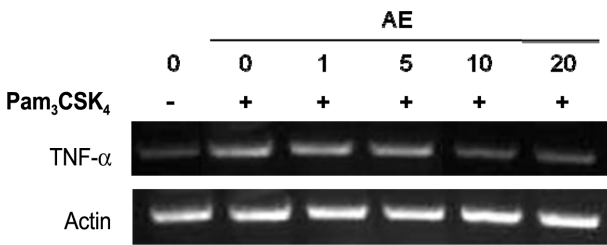
concentrations (Fig. 3; $p < 0.05$). Pretreatment with 10 μM and 20 μM AE decreased the mRNA expression of TLR2 by 41.18% and 54.43%, respectively, compared to that in the control cells (Fig. 3; $p < 0.05$).

NF-κB activation and MAPK phosphorylation

To understand the molecular mechanism(s) underlying the TLR2-blocking effect, NF-κB and MAPK activation were examined in RAW 264.7 macrophages. Our results showed that the NF-κB activity was dramatically upre-gulated by the Pam_3CSK_4 treatment compared with the activity in the Pam_3CSK_4-stimulated control group (Fig. 4A). AE at 10 μM and 20 μM reduced the NF-κB activity by 26.83% and 46.34%, respectively, compared with that in

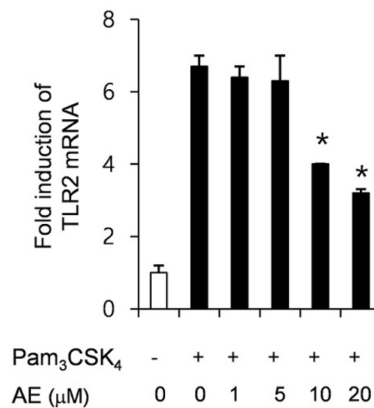**Fig. 1.** Effect of AE on Pam_3CSK_4-induced TNF-α mRNA expression in RAW 264.7 macrophages. RAW 264.7 macrophages were pre-treated with 0~20 μM AE for 1 h. The cells were further stimulated with Pam_3CSK_4 (1 μg/mL). After 1 h, the TNF-α mRNA expression was analyzed in an agarose gel.

Fig. 3. Effect of AE on the Pam_3CSK_4-induced TLR2 mRNA expression in RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with 0~20 μM AE for 1 h and further stimulated with Pam_3CSK_4 (1 μg/mL). After 1 h, TLR2 mRNA expression was determined. The data are expressed as a fold induction relative to the vehicle-treated cells. The values are the mean ± SD (n = 4). *Significantly different from the Pam_3CSK_4-stimulated control, $p < 0.05$.
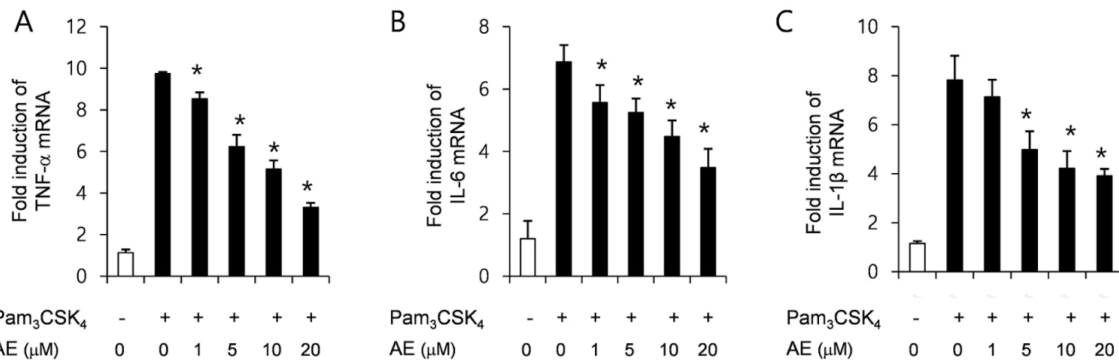

Fig. 2. Effect of AE on the Pam_3CSK_4-induced TNF-α (A), IL-6 (B), and IL-1β (C) mRNA expression in RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with 0~20 μM AE for 1 h and further stimulated with Pam_3CSK_4 (1 μg/mL). After 1 h, TNF-α, IL-6, and IL-1β mRNA expression was determined. The data are expressed as a fold induction compared with the vehicle-treated cells. The values are the mean ± SD (n = 3). *Significantly different from the Pam_3CSK_4-stimulated control ($p < 0.05$).
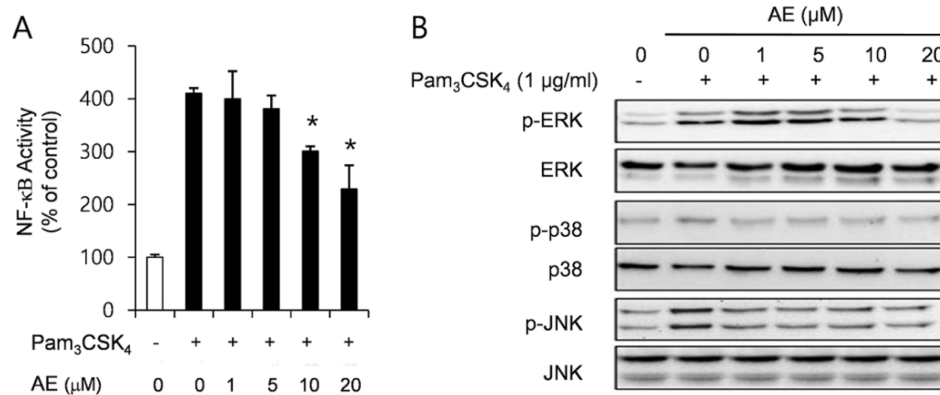
Fig. 4. Effect of AE on the NF-κB activation (A) and MAPK expression (B) in RAW 264.7 macrophages. RAW 264.7 macrophages were pretreated with 0~20 μM AE for 1 h and further stimulated with Pam₃CSK₄ (1 μg/mL). After 1 h, NF-κB activity was determined (A), and cell lysates were analyzed by MAPK immunoblotting (B). The NF-κB activity data are expressed as % activation compared with the vehicle-treated cells. The values are the mean ± SD (n = 4). *Significantly different from the Pam₃CSK₄-stimulatedcontrol, p < 0.05.

the Pam$_3$CSK$_4$-stimulated control cells (Fig. 4A; p < 0.05).

Next, we examined the MAPK phosphorylation in Pam$_3$CSK$_4$-stimulated RAW 264.7 macrophages. The phosphorylation of ERK1/2, p38, and JNK was reduced by 20 μM AE (Fig. 4B). In particular, AE decreased the ERK phosphorylation in a dose-dependent manner in the Pam$_3$CSK$_4$-stimulated RAW 264.7 macrophages. These results indicated that the anti-inflammatory efficacy of AE on RAW 264.7 is associated with the inactivation of NF-κB, as well as with blocking of MAPK phosphorylation.

DISCUSSION

TLRs are pattern recognition molecules, which represent major components of the innate immune response. Their specific signaling pathways are associated with several inflammatory diseases.[17] In particular, TLR2 is widely distributed on the surface of several types of immune cells, including macrophages, dendritic cells, and mast cells.[18-20] The receptor is the main sensor for PAMP recognition of gram-positive bacteria. Pam$_3$CSK$_4$ binds to the host TLR2 and leads to an inflammatory reaction.[21] In this study, we investigated whether AE can inhibit the Pam$_3$CSK$_4$-induced, TLR2-regulated signaling in RAW 264.7 macrophages.

To test the anti-inflammatory potential of AE, the mRNA expression level of the inflammatory marker TNF-α was measured in AE-pretreated RAW 264.7 macrophages after stimulation with Pam$_3$CSK$_4$. Our results showed that AE downregulated the TNF-α mRNA expression at concentrations of 10 μM and 20 μM (Fig. 1). Our results also showed that AE at concentrations of 5-20 μM effectively suppressed the mRNA levels for all the cytokines (TNF-α, IL-6, and IL-1β) in RAW 264.7 macrophages (Fig. 2). These data indicated that AE protects against the Pam$_3$CSK$_4$-induced inflammatory response in macrophages. Related *in vivo* studies have shown that emodin significantly ameliorated inflammatory responses through inhibition of cytokine overproduction. Nemmar et al. have suggested that emodin administration protects from diesel exhaust particle-induced lung inflammation via inhibition of TNF-α, IL-6, and IL-1β in mice.[22] According to Han et al., the treatment with emodin decreased the IL-1β secretion by blocking the activation of the NLRP3 inflammasome in alipopolysaccharide-induced endotoxin mouse model.[23]

To understand the mechanism of AE in regulating Pam$_3$CSK$_4$-induced cytokine gene expression, we examined the TLR2 mRNA expression in Pam$_3$CSK$_4$-stimulated macrophages. We found that AE effectively reduced upregulated expression of TLR2 (Fig. 3). These results indicate that AE down-regulates TLR2-mediated cytokine induction. There are a few studies evaluating the inhibitory effect of AE on TLR2-mediated signaling pathways. Li et al. reported that emodin from the Chinese herb Radix et Rhizoma Rhe significantly decreased the expression of cytokine genes by blocking TLR2 signaling pathways in rat kidney epithelial cells.[24]

The effect of AE in TLR-2 pathways may have associated with the inhibition of the NF-κB signaling pathway, which is the major transcription pathway for inflammatory responses.[25] The results showed that NF-κB activity was dramatically enhanced by Pam$_3$CSK$_4$ treatment compared

with the activity in the Pam$_3$CSK$_4$-negative control group; however, this enhancement of the activity was effectively alleviated in the 10 μM and 20 μM AE-treated groups (Fig. 4A). A previous study indicated that AE effectively decreased the activation of p38 and NF-κB in a concanavalin A-induced animal hepatitis model.[26] Although there are few data on AE, several *in vivo* studies have reported that emodin effectively ameliorates inflammatory diseases via inactivation of NF-κB.[27,28] This evidence indicates that AE is a potential anti-inflammatory agent that inhibits both NF-κB activation and gene expression of proinflammatory cytokines.

Because the activation of NF-κB as well as MAPK pathways are implicated in TLR2 signaling, we evaluated the protein levels of p-ERK1/2, p-p38, and p-JNK in Pam$_3$CSK$_4$-stimulated macrophages.

Pam$_3$CSK$_4$ stimulation upregulated the phosphorylations of the MAPKs in RAW 264.7 macrophages. We have also observed that 20 μM AE treatment prevented an increased expression of MAPKs (Fig. 4B), which is consistent with previous observations showing that emodin suppresses the activation of p38 and ERK1/2.[29,30] These results demonstrated that AE shows an anti-inflammatory action that is mediated by a TLR2-dependent MAPK signaling pathway in macrophages.

SUMMARY

Our data established that AE exhibited anti-inflammatory effects against Pam$_3$CSK$_4$-induced inflammation in macrophages. Its activity was partially due to the suppression of the NF-κB and MAPKs signaling pathways.

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