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Research Article

Compound K, a ginsenoside metabolite, plays an antiinflammatory role in macrophages by targeting the AKT1-mediated signaling pathway

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ABSTRACT

Background: Compound K (CK) is an active metabolite of ginseng saponin, ginsenoside Rb1, that has been shown to have ameliorative properties in various diseases. However, its role in inflammation and the underlying mechanisms are poorly understood. In this report, the antiinflammatory role of CK was investigated in macrophage-like cells.

Methods: The CK-mediated antiinflammatory mechanism was explored in RAW264.7 and HEK293 cells that were activated by lipopolysaccharide (LPS) or exhibited overexpression of known activation proteins. The mRNA levels of inflammatory genes and the activation levels of target proteins were identified by quantitative and semiquantitative reverse transcription polymerase chain reaction and Western blot analysis.

Results: CK significantly inhibited the mRNA expression of inducible nitric oxide synthase and tumor necrosis factor-α and morphological changes in LPS-activated RAW264.7 cells under noncytotoxic concentrations. CK downregulated the phosphorylation of AKT1, but not AKT2, in LPS-activated RAW264.7 cells. Similarly, CK reduced the AKT1 overexpression-induced expression of aldehyde oxidase 1, interleukin-1β, interferon-β, and tumor necrosis factor-α in a dose-dependent manner.

Conclusion: Our results suggest that CK plays an antiinflammatory role during macrophage-mediated inflammatory actions by specifically targeting the AKT1-mediated signaling pathway.

1. Introduction

Inflammation is one of representative host defense responses. Inflammatory biological processes mediated by myeloid lineage immune cells such as macrophages protect the body from infection with pathogens to increase danger signals. These processes are characterized by five hallmarks: redness, heat, pain, swelling, and loss of function [1–3]. Inflammatory responses are triggered by recognition of pathogen-associated molecular patterns of the invading pathogens by the corresponding receptors and pattern recognition receptors equipped in macrophages [2]. Inflammation-inducing signaling reactions are subsequently continued to

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stimulate the activation of nuclear factor-kappa B (NF-κB), activator protein-1, and interferon-regulatory factors [4–7]. Toll-like receptors (TLRs) are a family of pattern recognition receptors expressed on the macrophage surface. TLR4 is a receptor for lipopolysaccharide (LPS), a strong agonist of gram-negative bacteria. Binding of LPS to TLR4 generates inflammatory signaling cascades through increasing the activation of a variety of intracellular signaling molecules in inflammatory cells including macrophages. This is resulted in mRNA expression of inflammatory genes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2, and the production of inflammation-regulatory molecules, such as nitric oxide (NO), prostaglandin E_2, tumor necrosis factor-α (TNF-α), interferon-β, interleukin (IL)-1β, and IL-6 [4–8].

Ginseng (*Panax ginseng*) is a perennial plant that belongs to the family Araliaceae and genus *Panax*. Ginseng is being popularly used in various countries in Eastern Asia, North America, and Europe, including Korea, China, Japan, America, United Kingdom, France, Germany, and Austria [9]. Multiple ginseng species in the *Panax* genus have been used as pharmacological and phytochemical remedies to treat various human diseases [10–14]. Although ginseng has not been approved as a drug, it is the most frequently consumed herbal supplement worldwide [15]. Ginseng contains various bioactive constituents, including ginsenosides, polysaccharides, and flavonoids. The major pharmacological activities of ginseng are attributed to the secondary derivative ginsenosides and steroidal glycosides. Recent studies have successfully characterized various types of ginsenosides.

Compound K (CK), a protopanaxadiol-type minor ginsenoside, is a primary metabolite of ginsenoside Rb1. CK has demonstrated antidisease roles, including antitumor, antidiabetes, and hepatoprotective activities [10,16–20]. CK has also demonstrated an antiinflammatory role toward macrophages in inflammatory diseases [21–25]; however, the molecular and cellular mechanisms of CK-mediated antiinflammatory activity are poorly understood. In present report, we looked over the antiinflammatory action of CK with mechanistic understanding in macrophage-mediated inflammatory responses.

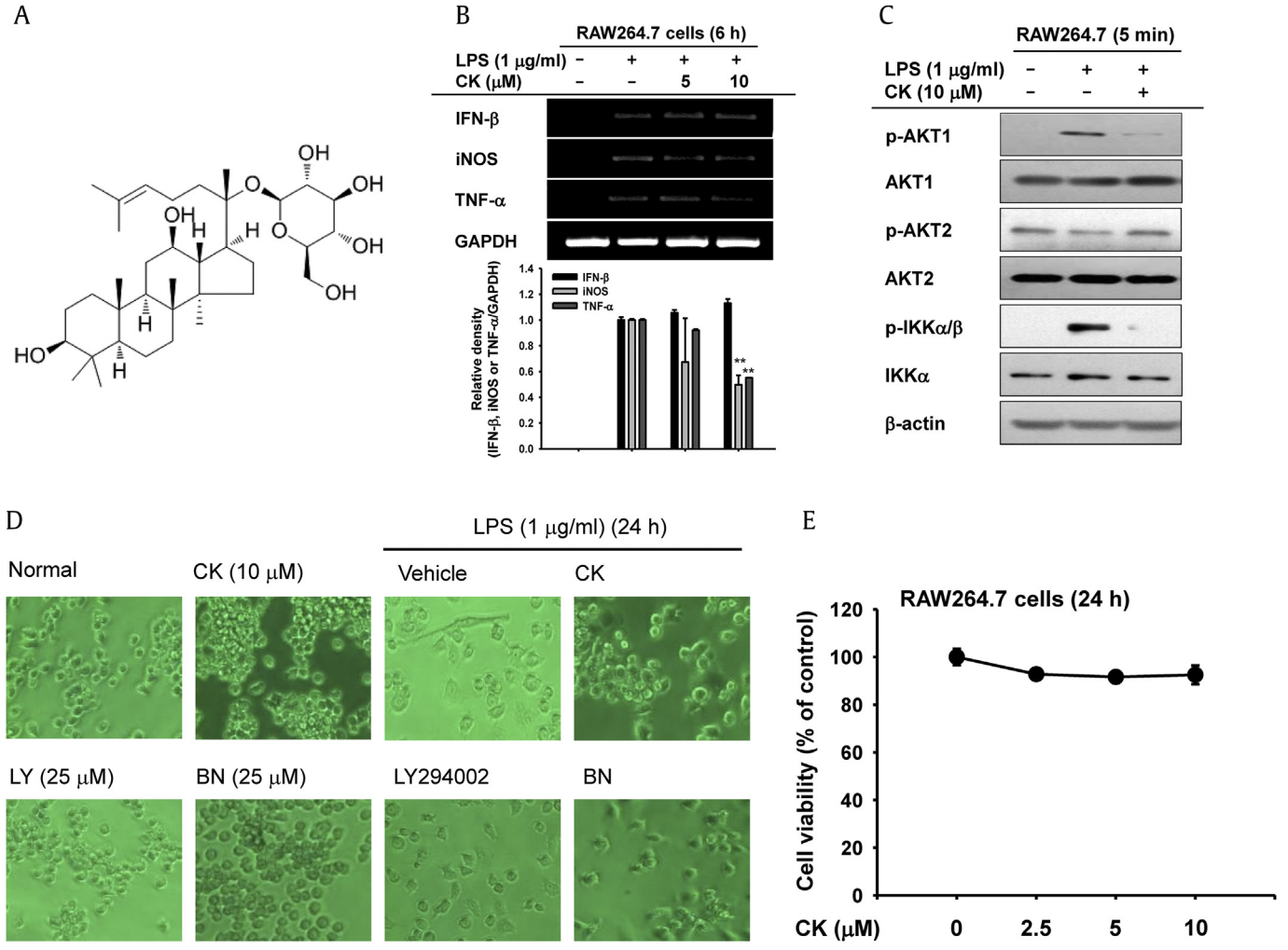

Fig. 1. Cytotoxic effect of CK on RAW264.7 and HEK293 cells. (A) Chemical structure of CK. (B) mRNA expression of iNOS, IFN-β, and TNF-α was determined by semiquantitative RT-PCR in RAW264.7 cells stimulated with LPS (1 μg/ml) in the presence or absence of CK. Relative intensity was calculated by densitometric scanning values of inflammatory genes relative to using the DNR Bio-imaging system. (C) Phospho-forms or total proteins of AKT1, AKT2, IKKα/β, IKKα, and β-actin were identified by Western blotting analysis from total cell lysates of RAW264.7 cells stimulated with LPS (1 μg/ml) in the presence or absence of CK (10 μM). β-actin was used as an internal control. (D) RAW264.7 cells were stimulated with LPS (1 μg/ml) for 24 h in the presence or absence of CK (10 μM), LY (25 μM), or BN (25 μM). Cells were incubated for a further 24 h, and morphological changes were observed under an optical microscope by taking photos with a digital camera. (E) RAW264.7 cells were treated with the indicated doses of CK (0–10 μM) for 24 h. Cell viability was determined by an MTT assay. $^{**}p < 0.01$ compared to control.
BN, BN82002; CK, compound K; iNOS, inducible nitric oxide synthase; IFN-β, interferon-β; IL-1β, interleukin-1β; LPS, lipopolysaccharide; LY, LY294002; MTT, (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor-α; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

2. Materials and methods

2.1. Materials

CK (Fig. 1A, purity: 97%) was obtained from the Ambo Institute (Daejeon, Korea). RAW264.7 cells and HEK293 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). LY294002 (LY), BN82002, (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS, polyethylene imidazole, and MuLV reverse transcriptase were from Sigma-Aldrich Co. (St. Louis, MO, USA). Dulbecco's Modified Eagle's medium, Roswell Park Memorial Institute 1640, and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). Plasmid expressing NF-κB-luciferase was used according to a previous report [26]. Antibodies against Src, AKT1, AKT2, IKKα/β, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). qPCRBIO SyGreen Mix Lo-ROX for quantitative real-time polymerase chain reaction (PCR) was from PCR Biosystems (London, United Kingdom).

2.2. Cell culture

RAW264.7 and HEK293 cells were maintained as reported previously [27].

2.3. Semiquantitative RT-PCR and quantitative real-time PCR

Total RNA was prepared from LPS-treated RAW264.7 cells or HEK293 cells transfected with HA-Src, HA-AKT1, or HA-AKT2 for 48 h in the presence or absence of CK (0–10 μM) for 24 h. cDNA synthesis and PCR reaction (semiquantitative RT-PCR and quantitative real-time PCR) were carried out according to previous report [28,29]. The primer (from Bioneer, Daejeon, Korea) sequences are listed in Table 1.

Table 1
Primer sequences used for PCR in this study.

Name	Direction	Sequence (5′ to 3′)
RT-PCR		
iNOS	F	CCCTTCCGAAGTTTCTGGCAGCAG
	R	GGCTGTCAGAGCCTCGTGGCTTTGG
IFN-β	F	TCCAAGAAAGGACGAACATT
	R	TGAGGACATCTCCCACGTCA
TNF-α	F	TTGACCTCAGCGCTGAGTTG
	R	CCTGTAGCCCACGTCGTAGC
GAPDH	F	CACTCACGGCAAATTCAACGGCA
	R	GACTCCACGACATACTCAGCAC
Real-time PCR		
AOX1	F	CAA CCT TCC ATC CAA CAC TG
	R	CCA CAT TTG ATT GCC ACT TC
iNOS	F	GGA GCC TTT AGA CCT CAA CAG A
	R	TGA ACG AGG AGG GTG GTG
IL-1β	F	ATT TGA ATT CCC TGG GTG AG
	R	CCT CAT CCT GGA AGG TCC AC
IFN-β	F	AAGAGTTACACTGCCTTTGCTATC
	R	CACTGTCTGCTGGTGGAGTTCATC
TNF-α	F	TGC CTA TGT CTC AGC CTC TT
	R	GAG GCC ATT TGG GAA CTT CT
GAPDH	F	CAA TGA ATA CGG CTA CAG CAA C
	R	AGG GAG ATG CTC AGT GTT GG

AOX1, aldehyde oxidase 1; iNOS, inducible nitric oxide synthase; IFN-β, interferon-β; IL-1β, interleukin-1β; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor-α

2.4. Western blot analysis

For Western blot analysis, total cell lysates prepared from RAW264.7 cells activated by treatment of LPS (1 μg/mL) in the presence or absence of CK (10 μM) or HEK293 cells transfected with HA-Src, HA-AKT1, or HA-AKT2 in the presence or absence of CK (10 μM) were subjected to sodium dodecyl sulfate gel electrophoresis and immunoblotting with antibodies to detect total and phosphorylated proteins as reported previously [30].

2.5. Morphological changes

Macrophage-like RAW264.7 cells were exposed with LPS (1 μM) in the presence or absence of CK (10 μM), LY (25 μM), or BN82002 (25 μM) for 24 h. Morphological changes were confirmed by taking photos with a digital camera as reported previously [31].

2.6. Cell viability assay

Effect of CK (0–10 μM) on the viability of RAW264.7 and HEK293 cells for 24 h was evaluated by an MTT assay as previously reported [32].

2.7. NF-κB-luciferase reporter gene assay

HEK293 cells were transfected with plasmids expressing NF-κB-luciferase (0.5 μg/mL), β-galactosidase (0.1 μg/mL), and either Flag-MyD88 (0.5 μg/mL) or HA-TAK1 (0.5 μg/mL) for 24 h using polyethylene imidazole and subsequently treated with CK (0–10 μM) for 24 h. Cells underwent three rounds of freezing and thawing. Cell lysates were used to measure NF-κB–mediated luciferase activities with a luciferase assay system as reported previously [33].

2.8. Statistical analysis

Statistical significance of all data (mean ± standard deviation) done by at least three independent experiments was evaluated by analysis of variance/Scheffe's post hoc test and Kruskal-Wallis/Mann-Whitney test using the SPSS program (SPSS Inc., Chicago, IL, USA).

3. Results and discussion

We explored the antiinflammatory role (Fig. 1B) and underlying mechanism (Figs. 1 and 2) of CK in macrophages during inflammatory responses using *in vitro* cellular models. Cytotoxicity is a critical hurdle for drug development, regardless of the therapeutic efficacy of the drug. The cytotoxicity of CK was examined before investigating its antiinflammatory activity. RAW264.7 cells were treated with increasing doses of CK (0–10 μM) for 24 h, and the cell viability was determined by an MTT assay. CK did not significantly reduce the viability of RAW264.7 cells from a low dose (2.5 μM) to the highest dose (10 μM) (Fig. 1E). Similarly, CK did not reduce the viability of HEK293 cells at concentrations of 2.5–10 μM (Fig. 2C). Previous studies demonstrated that CK exhibited immunomodulatory activities at doses ranging from 5 to 10 μM [21,29], which indicates that the minimal CK cytotoxicity was not due to inactive low doses. Our results suggest that CK had minimal cytotoxicity at pharmacologically and physiologically functional doses.

We first investigated the antiinflammatory action of CK using LPS-exposed RAW264.7 cells. As shown in Fig. 1B, CK blocked the expression of iNOS and TNF-α at a concentration of 10 μM (Fig. 1B). The effect of CK on NF-κB pathway activation was confirmed in previous reports [34]. HEK293 cells were transfected with MyD88, an intracellular adaptor molecule that activates NF-κB [35]. Cells were treated with increasing doses of CK (0–10 μM), and NF-κB–mediated luciferase reporter gene activity was determined. CK inhibited the NF-κB–mediated luciferase activity induced by

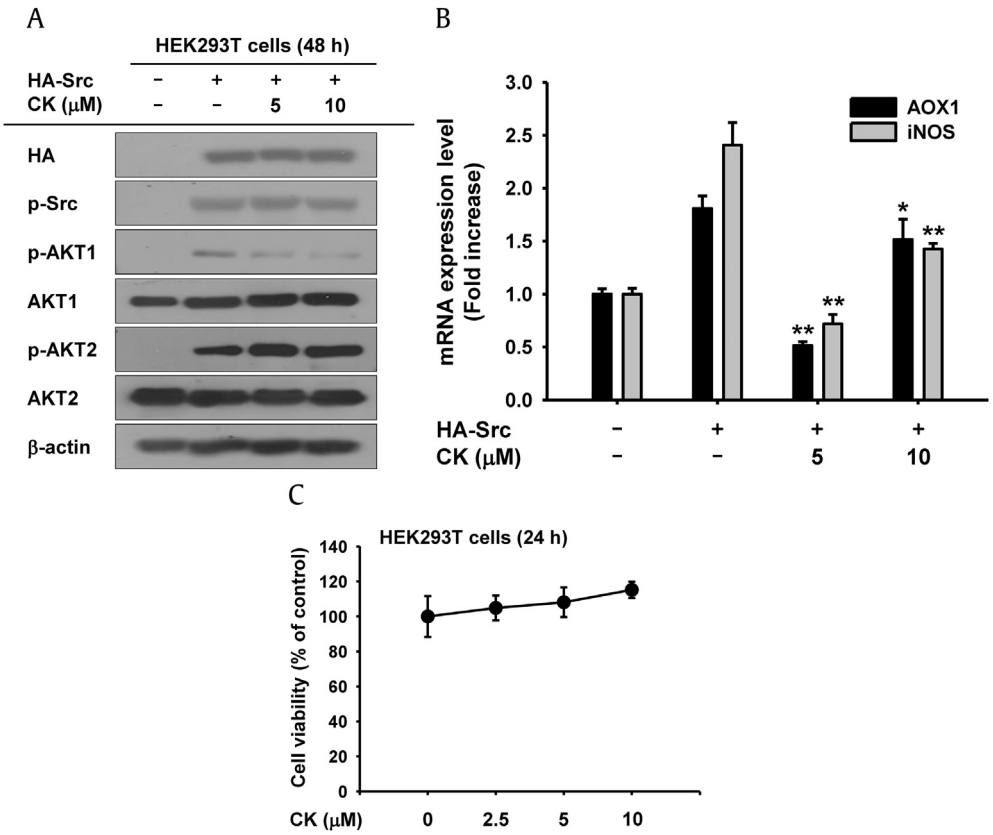

Fig. 2. Effect of CK on the activation of AKTs under Src overexpression conditions. (A and B) HEK293 cells were transfected with HA-Src for 48 h and treated with the indicated doses of CK (0–10 μM) for 24 h. (A) The levels of phosphorylated and total HA, Src, AKT1, and AKT2 proteins in the total cell lysate were determined by Western blotting analysis. (B) mRNA expression levels of AOX1 and iNOS were determined by quantitative real-time PCR. (C) HEK293 cells were treated with the indicated doses of CK (0–10 μM) for 24 h. Cell viability was determined by an MTT assay. β-actin was used as an internal control. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control.
AOX1, aldehyde oxidase 1; CK, compound K; iNOS, inducible nitric oxide synthase; MTT, (3-4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; HA, influenza hemagglutinin.

MyD88 at the 10 μM dose (data not shown). This result was consistent with our previous observation that NF-κB–mediated luciferase activity was significantly inhibited by BIOGF1K, a fraction of Korean ginseng rich in CK [36]. The effect of CK on AKT was examined because AKT activation is a hallmark of NF-κB signaling pathway activation [7]. Activation of AKT1 and AKT2 was clearly observed after LPS stimulation in RAW264.7 cells, and, as expected, phosphorylation of AKT1, but not AKT2, and phosphorylation of IKKα/β were inhibited by CK (10 μM) (Fig. 1C). A similar pattern was observed in an experiment confirming morphological change. LPS induced morphological changes of RAW264.7 cells after 24-h stimulation while CK and the specific AKT inhibitor LY clearly blocked this change (Fig. 1D), implying that CK ameliorates LPS-induced morphological changes through suppression of AKT activity. To confirm this finding, we employed an overexpression strategy using constructs with genes encoding known AKT activation upstream proteins [37] and constructs with the *AKT1* or *AKT2* gene. For this experiment, we transfected HEK293 cells with Src, an upstream intracellular kinase, to activate AKTs and the NF-κB signaling pathway [38,39] and further treated the cells with increasing doses of CK (0–10 μM). AKT1 and AKT2 activities were determined by Western blot analysis. CK inhibited the

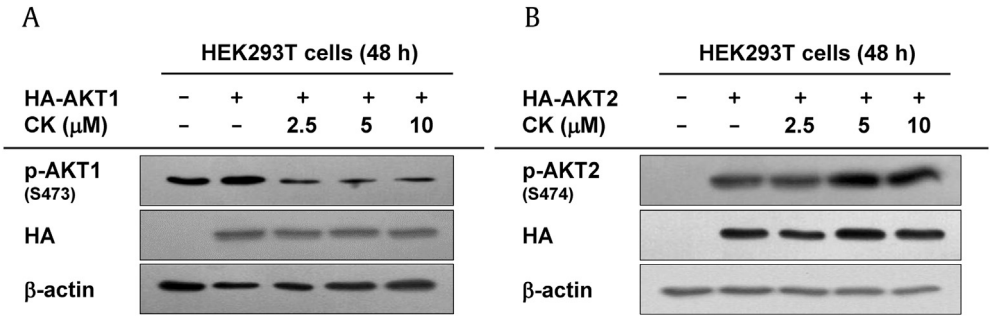

Fig. 3. Effect of CK on the activation of AKT1 under AKT overexpression conditions. (A and B) HEK293 cells were transfected with either HA-AKT1 or HA-AKT2 for 48 h and treated with the indicated doses of CK (0–10 μM) for 24 h. The level of phosphorylated and total HA, AKT1, and AKT2 proteins in the total cell lysates were determined by Western blotting analysis. β-actin was used as an internal control.
CK, compound K.

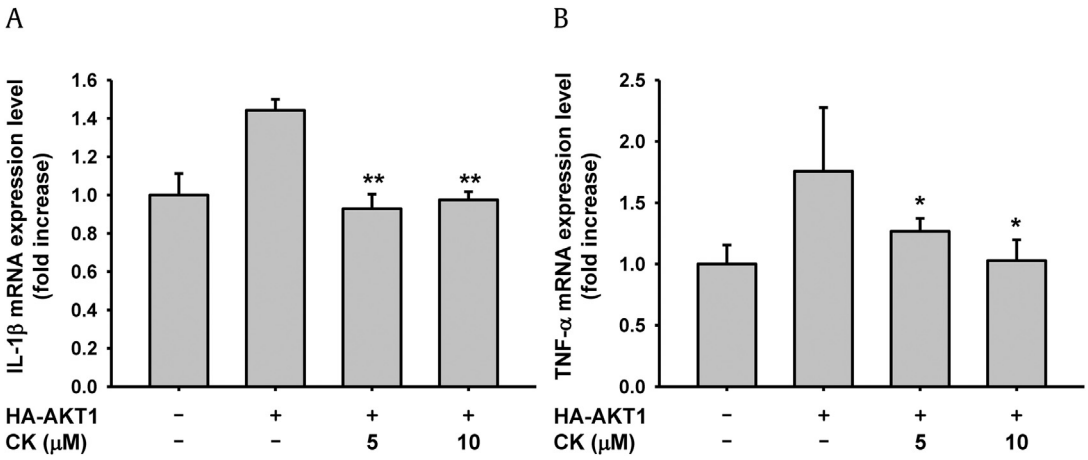

Fig. 4. Effect of CK on cytokine expression under AKT1 overexpression conditions. (A and B) HEK293 cells were transfected with HA-AKT1 for 48 h and treated with the indicated doses of CK (0–10 μM) for 24 h. (A) The mRNA expression of IL-1β was determined by quantitative real-time PCR. (B) The mRNA expression of TNF-α was determined by quantitative real-time PCR. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to control.
CK, compound K; IL-1β, interleukin-1β; PCR, polymerase chain reaction; TNF-α, tumor necrosis factor-α.

phosphorylation of AKT1 at 5 and 10 μM but did not affect phosphorylation of AKT2 in Src-transfected HEK293 cells (Fig. 2A). We also examined the effect of CK on mRNA expression of aldehyde oxidase 1 (AOX1) and iNOS. AOX1 is a molybdenum-containing hydroxylase in humans [40] that catalyzes NO production via the reduction of nitrite [41–43]. iNOS is an inflammatory enzyme that produces NO, and its expression is induced under inflammatory conditions [44]. HEK293 cells were transfected with the inflammatory kinase Src to activate the NF-κB signaling pathway [7] and treated with CK (0–10 μM). The mRNA expression of AOX1 and iNOS was determined by quantitative real-time PCR. CK significantly reduced Src-induced mRNA expression of both AOX1 and iNOS at 5 and 10 μM (Fig. 2B). These results were further confirmed in HEK293 cells that were transfected with either AKT1 or AKT2 and treated with increasing doses of CK (0–10 μM). CK markedly inhibited the phosphorylation of AKT1 in the AKT1-transfected HEK293 cells (Fig. 3A) but did not inhibit the phosphorylation of AKT2 in the AKT2-transfected HEK293 cells (Fig. 3B). These results strongly suggest that CK suppresses activation of the NF-κB signaling pathway by targeting AKT1 rather than AKT2 during inflammatory responses. We finally investigated the effect of CK on AKT1-induced inflammatory gene expression. IL-1β and

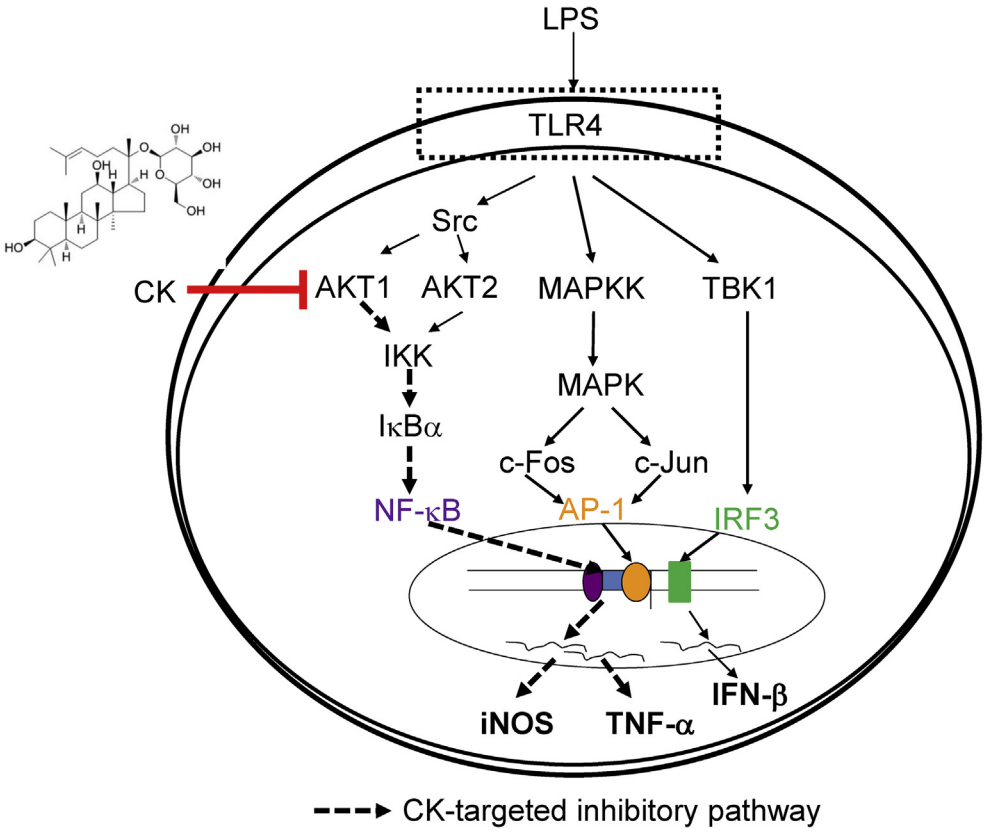

Fig. 5. Proposed model for CK-mediated anti-inflammatory activity by targeting AKT1 in NF-κB signaling pathway during macrophage-mediated inflammatory responses.
MAPKK, mitogen-activated protein kinase kinase; MAPK, mitogen-activated protein kinase; IKK, IkBa kinase; TBK1, TANK Binding Kinase 1; AKT, protein kiase B.

TNF-α are well-known proinflammatory cytokines [4,6,45]; therefore, we examined the effect of CK on their mRNA expression by quantitative real-time PCR. CK significantly suppressed the mRNA expression of IL-1β (Fig. 4A) and TNF-α (Fig. 4B) in the AKT1-transfected HEK293 cells, similar to the data in Fig. 1B. These results strongly indicate that AKT1 is a target of CK-mediated anti-inflammatory action. Since AKT1 is known to regulate various metabolic conditions and cell proliferation stages [46–48], the present data seem to indicate that the various activities of CK on diabetes, cancer, and vascular diseases might be mediated by reduction of AKT1. Further details in terms of the molecular mechanism of CK in cancer and diabetes will be further addressed in subsequent studies.

Conclusively, this report demonstrated the antiinflammatory activity of CK in macrophages. CK attenuated the inflammatory NF-κB signaling pathway by targeting AKT1 and IKKα/β with minimal cytotoxicity, as summarized in Fig. 5. CK suppressed the expression of inflammatory genes, including IL-1β, TNF-α, AOX1, and iNOS, under inflammatory conditions. Our results strongly suggest that CK, a ginseng constituent, is protective against macrophage-mediated inflammatory responses and provide insights for the development of potential drugs for the treatment and prevention of various inflammatory diseases.

Conflicts of interest

All authors declare no conflicts of interest.

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Special note: Jimmy Y. Cho is a 10th grade (second grade) student in Okemos High School. He was working in the Laboratory of Molecular Immunology, Department of Integrative Biotechnology, Sungkyunkwan University between June 25 and July 27, 2018. Fig. 1D in this study was the result of his project entitled “Actin cytoskeleton change in macrophages”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jgr.2018.10.003.

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