Research Article

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

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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...
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 E2, 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.
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), BNB2002, (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 MyD88 (0.5 μg/mL) were obtained from the American Type Culture Collection (Rockville, MD, USA). Antibodies against Src, AKT1, AKT2, IKKα/β, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). pCRBIO 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. The primer (from Bioneer, Daejeon, Korea) sequences are listed in Table 1.

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 μg/mL) in the presence or absence of CK (10 μM), LY (25 μM), or BNB2002 (25 μM) for 24 h. Morphological changes were confirmed by using 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).

### Table 1

| Name               | Direction | Sequence (5' to 3') |
|--------------------|-----------|---------------------|
| RT-PCR             |           |                     |
| iNOS               | F         | CTTCCGAAGTTTGCAGGCAGAG |
| IFN-β              | F         | GGCTGCAAGCCGCTGGCTGTTGG |
| TNF-α              | F         | TTCGCACTCTCAGCACGTCG |
| GAPDH              | R         | GGCTGTCAGAGCCTCGGTCTGG |
| AOX1               | F         | CAA CCA CCT TTC AAC CAC TG |
| IFOS               | R         | GCC CCTT TTG AGA CCT CAA CAG A |
| IL-1β              | F         | TGA ACC AGG AGG GTG GTG |
| IL-1β              | F         | CCT CAT CCT GGA ACC TCC AC |
| IL-1β              | F         | GAG GCT GTC TGC GAA CTT CT |
| TNF-α              | R         | CAA TGA ATG CCA TGA CAG C |
| GAPDH              | R         | AGG GAG ATG CTG ACT 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.
MyD88 at the 10 \( \mu M \) dose (data not shown). This result was consistent with our previous observation that NF-\( \kappa \)B-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-\( \kappa \)B 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 IKK\( \alpha/\beta \) were inhibited by CK (10 \( \mu M \)) (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-\( \kappa \)B signaling pathway [38,39] and further treated the cells with increasing doses of CK (0–10 \( \mu M \)). AKT1 and AKT2 activities were determined by Western blot analysis. CK inhibited the

![Fig. 2](image_url)

**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 \( \mu M \)) 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 \( \mu M \)) for 24 h. Cell viability was determined by an MTT assay. \( \beta \)-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.

![Fig. 3](image_url)

**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 \( \mu M \)) 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. \( \beta \)-actin was used as an internal control.

CK, compound K.
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 iNOS gene expression were measured in HEK293 cells transfected with either AKT1 or AKT2 and treated with increasing doses of CK (0-10 μM) (Fig. 4A and 4B). 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
TNF-α are well-known proinflammatory cytokines [4,45]; 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 IκKζ/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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Appendix A. Supplementary data

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

References

[1] Janeway Jr CA, Medzhitov R. Innate immune recognition. Annu Rev Immunol 2002;20:197–216.
[2] Yi YS. Caspase-11 non-canonical inflammatory signaling. Cytoskeleton 2017;152:207–17.
[3] Yi YS. Folate receptor-targeted diagnostics and therapeutics for inflammation. Mol Neurobiol 2014;53:2529–40.
[4] Murohara H. Proof of the mysterious efficacy of ginseng: basic and clinical trials: metabolic activation of ginsenoside: deglycosylation by intestinal bacteria and esterification with fatty acid. J Pharmacol Sci 2004;95:153–7.
[5] Yayeh T, Jung KH, Jeong HY, Park JH, Song YB, Kwak YS, Kang HS, Cho JY. Hepatoprotective effect of ginseng: basic and clinical trials: metabolic activation of ginsenoside: deglycosylation by intestinal bacteria and esterification with fatty acid. J Pharmacol Sci 2004;95:153–7.
[6] Lee SH, Choi JH. Involvement of immune cell network in aortic valve stenosis: communication between valvular interstitial cells and immune cells. Immune Netw 2016;16:28–32.
[7] Yun TK. Panax ginseng—a non-organ-specific cancer preventive? Lancet Oncol 2002;1:49–55.
[8] Wakabayashi C, Murakami K, Hasegawa H, Murata J, SaiKI I. An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. Biochem Biophys Res Commun 1998;246:225–30.
[9] Cho JY, MaK, Sakurai H, Kim DH, Saiki I. A ginseng saponin metabolite suppresses tumor necrosis factor-alpha-promoted metastasis by suppressing nuclear factor-kappaB signaling in murine colon cancer cells. Oncol Rep 2008;19:395–600.
[10] Jia L, Zhao Y. Current evaluation of the millennium phytomedicine—ginseng (I): etymology, pharmacognosy, phytochemistry, market and regulations. Curr Med Chem 2009;16:2475–84.
[11] Park JS, Shin JA, Jung JS, Hyun JW, Van Le TK, Kim DH, Kim HS. Anti-inflammatory mechanism of compound K in activated macrophage and its effect on liver inflammation. Biomed Pharmacother 2017;91:1739–53.
[12] Hong YH, Kim D, Nam G, Yoo S, Han SY, Jeong SG, Kim E, Jeong D, Yoon K, Kim JH, Kim SH, Kim S, et al. Photographic protective effects of BIOFIFG, a compound-K-rich fraction prepared from Panax ginseng. J Ginseng Res 2018;42:81–9.
[13] Yu T, Yang Y, Kwak YS, Song GC, Kim MY, Rhee MH, Cho JY. Ginsenoside Rc from Panax ginseng exerts anti-inflammatory activity by targeting TANK-binding kinase 1/interferon regulatory factor-3 and p38/ATF-2. J Ginseng Res 2017;41:127–33.
[14] Lee JE, Kim E, Kim JH, Hong YH, Kim HG, Jeong D, Kim J, Kim SH, Park C, et al. Antimelanogenesis and skin-protective activities of Panax ginseng calyx ethanol extract. J Ginseng Res 2017;41:298–306.
[15] Kim E, Kim D, Yoo S, Hong YH, Han SY, Jeong SG, Kim E, Jeong D, Yoon K, Kim JH, Cho JY. Ginsenoside Rc from Panax ginseng calyx ethanol extract suppresses UV-induced mitogen-activated protein kinase (MAPK) activation. J Ginseng Res 2017;41:298–306.
[16] Kim E, Cho J, Choi B, Bae EA, Han MJ, Kim NJ, Kim DH. Hepatoprotective effect of ginsenoside Rb1 and compound K on tert-butyl hydroperoxide-induced liver injury. Liver Int 2005;25:1069–73.
[17] Joh EH, Lee IA, Jung IH, Kim DH. Ginsenoside Rb1 and its metabolite compound K inhibit IκB kinase activation—the first step of inflammation. Biochem Pharmacol 2011;82:278–83.
[18] Shin DJ, Kim JE, Lim TG, Jeong EH, Park G, Kang NJ, Park JS, Yeom MH, Oh DK, Bode AM, et al. 20-O-beta-D-glucopyranosyl-20(S)-protopanaxadiol suppresses UV-Induced MMP-1 expression through AMPK-mediated mTOR inhibition as a downstream of the PKA-LKB1 pathway. J Cell Biochem 2014;115:1702–11.
[19] Lim TG, Jeon AJ, Yoon JH, Song D, Kim JE, Kwon JY, Kim JR, Kang NJ, Park JS, Yeom MH, et al. 20-O-beta-D-glucopyranosyl-20(S)-protopanaxadiol, a metabolite of ginsenoside Rb1, enhances the production of hyaluronic acid through the activation of ERK and Akt mediated by Src tyrosine kinase in human keratinocytes. Int J Mol Med 2015;35:1388–94.
[20] Lee HU, Bae EA, Han MJ, Kim NJ, Kim DH. Hepatoprotective effect of ginsenoside Rb1 and compound K on tert-butyl hydroperoxide-induced liver injury. Liver Int 2005;25:1069–73.
[21] Ahn SH, Park JS, Shin JA, Jung JS, Hyun JW, Van Le TK, Kim DH, Park EM, Kim HS. Anti-inflammatory mechanism of compound K in activated macrophage and its neuroprotective effect on experimental stroke in mice. J Pharmacol Exp Ther 2012;341:59–67.
[22] Park EK, Shih YW, Lee HU, Kim SS, Lee YC, Lee BY, Kim DH. Inhibitory effect of ginsenoside Rb1 and compound K on NO and prostaglandin E2 biosyntheses of LPS-induced RAW264.7 cells. J Ginseng Res 2002;20:187–96.
[35] Baek KS, Yi YS, Son Yj, Yoo S, Sung NY, Kim Y, Hong S, Aravintan A, Kim JH, Cho JY. *In vitro* and *in vivo* anti-inflammatory activities of Korean Red Ginseng-derived components. J Ginseng Res 2016;40:437–44.

[36] Hossen MJ, Hong YD, Baek KS, Yoo S, Hong YH, Kim JH, Lee JO, Kim D, Park J, Cho JY. *In vitro* antioxidative and anti-inflammatory effects of the compound K-rich fraction BIOGF1K, prepared from Panax ginseng. J Ginseng Res 2017;41:43–51.

[37] Yoo S, Kim MY, Cho JY. Syk and Src-targeted anti-inflammatory activity of aripiprazole, an atypical antipsychotic. Biochem Pharmacol 2018;148:1–12.

[38] Yang WS, Ratan ZA, Kim G, Lee Y, Kim MY, Kim JH, Cho JY. 4-isopropyl-2,6-bis(1-phenylethyl)aniline 1, an analogue of KTH-13 isolated from Cordyceps bassiana, inhibits the NF-kappaB-mediated inflammatory response. Mediators Inflamm 2015;2015:143025.

[39] Hossen MJ, Jeon SH, Kim SC, Kim JH, Jeong D, Sung NY, Yang S, Baek KS, Yoon DH, Song WO, et al. *In vitro* and *in vivo* anti-inflammatory activity of Phyllanthus acidus methanolic extract. J Ethnopharmacol 2015;168:217–28.

[40] Garattini E, Mendel R, Romao MJ, Wright R, Terao M. Mammalian molybdoflavoenzymes, an expanding family of proteins: structure, genetics, regulation, function and pathophysiology. Biochem J 2003;372:15–32.

[41] Li H, Kundu TK, Zweier JL. Characterization of the magnitude and mechanism of aldehyde oxidase-mediated nitric oxide production from nitrite. J Biol Chem 2009;284:33850–8.