The ginsenoside metabolite compound K, a novel agonist of glucocorticoid receptor, induces tolerance to endotoxin-induced lethal shock

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Received: July 29, 2007; Accepted: November 12, 2007

Abstract

Compound K (C-K), a protopanaxadiol ginsenoside metabolite, was previously shown to have immunomodulatory effects. Here, we describe a novel therapeutic role for C-K in the treatment of lethal sepsis through the modulation of Toll-like receptor (TLR) 4-associated signalling via glucocorticoid receptor (GR) binding. In mononuclear phagocytes, C-K significantly repressed the activation of TLR4/lipopolysaccharide (LPS)-induced NF-κB and mitogen-activated protein kinases (MAPKs), as well as the secretion of pro-inflammatory cytokines. However, C-K did not affect the TLR3-mediated expression of interferon-β or the nuclear translocation of IRF-3. C-K competed with the synthetic glucocorticoid dexamethasone for binding to GR and activated glucocorticoid responsive element (GRE)-containing reporter plasmids in a dose-dependent manner. In addition, the blockade of GR with either the GR antagonist RU486 or a siRNA against GR substantially reversed the anti-inflammatory effects of C-K. Furthermore, TLR4-dependent repression of inflammatory response genes by C-K was mediated through the disruption of p65/interferon regulatory factor complexes. Importantly, pre- or post-treatment with C-K significantly rescued mice from Gram-negative bacterial LPS-induced lethal shock by lowering their systemic inflammatory cytokine levels and by reversing the lethal sequelae of sepsis. Collectively, these results demonstrate that C-K, as a functional ligand of GR, regulates distinct TLR4-mediated inflammatory responses, and suggest a novel therapy for Gram-negative septic shock.

Keywords: compound K • Toll-like receptor 4 • LPS • endotoxaemia • glucocorticoid receptor

Introduction

The incidence of Gram-negative sepsis, a major cause of death in hospital intensive care units, continues to rise worldwide due to the increased use of invasive procedures and therapies that result in immunosuppression. This syndrome, which is characterized by endothelial damage, coagulopathy, loss of vascular tone, tissue hypoperfusion and multiple organ failure, is caused by uncontrolled, overwhelming inflammatory responses that are triggered by microbial products [1–3]. Among these products, endotoxin, or lipopolysaccharide (LPS), a constituent of the outer membrane in Gram-negative bacteria, plays a central role by eliciting the production of pro-inflammatory cytokines [4, 5]. Apart from the use of antibiotics, the treatment of sepsis and septic shock is largely limited to supportive strategies.

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doi:10.1111/j.1582-4934.2007.00181.x
Interactions between pathogens and their multi-cellular hosts begin with the activation of pathogen recognition receptors, such as the members of the Toll-like receptor (TLR) family, which recognize specific pathogen-associated molecular patterns [6]. When stimulated, TLRs initiate signalling cascades that result in the production of a myriad of cytokines and effector molecules. As inducers of inflammation, TLRs are important triggers of sepsis and autoimmune disease exacerbation [7, 8]. TLR4 is involved in the activation of the immune system by LPS through the specific recognition of its endotoxic moiety (Lipid A). This is a critical event in the immune response to Gram-negative bacteria as well as in the aetiology of endotoxic shock [6]. Full activation of signal transduction via TLRs requires the co-operation of several signalling adaptors, including myeloid differentiation primary response protein 88 (MyD88) and the adaptors of the MyD88-independent pathway [9]. Whereas TLR2-dependent signals are entirely dependent upon MyD88, TLR4 can utilize either the MyD88/TIR domain-containing adapter protein (TIRAP) or Toll/interleukin-1 receptor (TIR) domain-containing adapter-inducing interferon-β (TRIF)/TRIF-related adapter molecule (TRAM) adapter pairs to generate distinct responses [10]. The latter pathway is critical to interferon (IFN)-β expression and the activation of IFN-regulated factor-3 (IRF-3), which also induces the late activation of nuclear factor-κB (NF-κB) [11].

There is great need for new therapies that can interrupt systemic inflammation and improve survival in septic hosts. Ginseng, the roots of Panax ginseng C. A. Meyer, has long been used as a health product and natural remedy in traditional medicine. Ginsenosides, the major components of ginseng, exhibit various biological activities, including anti-inflammatory and anti-tumour effects [12–14]. The protopanaxadiol ginsenosides Rb1, Rb2 and Rc are metabolized to compound K (C-K; Fig. 1A) by intestinal bacteria in humans and rats [15, 16]. C-K (20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol) shows various immunopharmacological activities in vitro and in vivo [17, 18], including an anti-inflammatory effect [19, 20]. Accumulating evidence suggests that some ginsenosides have glucocorticoid-like activity, raising the possibility that they activate glucocorticoid receptors (GRs) directly or indirectly [21, 22]. An understanding of the combinatorial control of homeostasis and immune responses by cross-talk between TLR4 and GR may lead to novel therapeutic strategies for the treatment of inflammatory diseases [23]. In this study, we describe a novel function for C-K in the treatment of lethal sepsis through the modulation of TLR4-associated signalling via GR. To our knowledge, this is the first report that demonstrates the functional significance of cross-talk between the TLR4- and GR-dependent signalling pathways in regulating excessive host inflammatory responses.

Materials and methods

Isolation of C-K

C-K (Fig. 1A), a ginsenoside metabolite, was isolated as follows: 1 g of protopanaxadiol-type saponins (ginsenosides Rb1, Rb2, Rc and Rd) was incubated with cellulase from Aspergillus niger at 37°C for 48 hr. The reaction was terminated by extraction with n-butanol. The butanol fraction was concentrated and loaded onto a silica gel column using chloroform–methanol (9:1) to isolate C-K. The C-K fraction was then separated by high-performance liquid chromatography (HPLC) system as follows: column, Discovery C18 (25 x 0.4 cm, 5 mm i.d., Supelco); elution solvent, gradient of solvent A (water) to solvent B (acetonitrile) from 80:20 to 10:90 over 0–90 min; detection, 203 nm. The purified C-K was identified by 13C nuclear magnetic resonance, 1H nuclear magnetic resonance, HPLC, and fast-atom-bombardment mass spectrometry. The purity of the sample exceeded 97%.

Mice, sepsis model and cells

All experiments described in this study were performed using C57BL/6 mice. All animal-related procedures and care were reviewed and approved by the Institutional Animal Care and Use Committee, Chungnam National University College of Medicine (Daejeon, Korea). The mice used for the LPS challenge were 8–10 weeks old. The experimental groups were age- and sex-matched. Escherichia coli O26:B6 LPS (Sigma, St. Louis, MO) was diluted in sterile phosphate-buffered saline (PBS) and injected into the animals intraperitoneally (i.p.). Cecal ligation and puncture (CLP) sepsis models were established as previously described [24]. Briefly, C57BL/6 mice were anaesthetized with pentobarbital (50 mg/kg, i.p.), a small abdominal midline incision was made, and the cecum was exposed. The cecum was mobilized and ligated below the ileocecal valve, punctured through both surfaces twice with a 21-gauge needle, and the abdomen was closed. Bone marrow-derived macrophages (BMDMs) were differentiated for 5–7 days in macrophage colony-stimulating factor-containing media as described previously [25]. The murine macrophage cell line RAW264.7 was purchased from the American Type Culture Collection (TIB-71) and grown in DMEM GlutaMAX supplemented with 10% foetal calf serum (FCS). Human embryonic kidney (HEK) 293 cells stably transfected with human TLR4, MD2 and CD14 (HEK/TLR4/MD2/CD14) or HEK 293 cells stably transfected with human TLR1/TLR2 (HEK/TLR1/TLR2) were purchased from InvivoGen (San Diego, CA). The 293/TLR clones were grown in standard Dulbecco's modified Eagle's medium (DMEM) with 10% FCS supplemented with blasticidin (10 µg/ml) and Normocin (100 µg/ml). In some experiments, adherent human monocytes were prepared as described [26] from peripheral blood mononuclear cells donated by healthy subjects. The study was reviewed and approved by the Institutional Research Board of Chungnam National University Hospital, and written informed consent was obtained from each participant.

Reagents, DNA, and antibodies

For in vitro experiments, ultrapure LPS (TLR4 agonist; InvivoGen) and poly I:C (InvivoGen) were used. The synthetic bacterial lipopeptide BLP (Pam3Cys-Ser-Lys4), derived from the immunologically active N terminus of bacterial lipoprotein, was purchased from InvivoGen. RNA-activated protein kinase (PKR) inhibitor and high-mobility group box 1 protein (HMGB1) were obtained from Calbiochem (San Diego, CA) and Sigma, respectively. Dimethyl sulfoxide (DMSO; Sigma) was added to the cultures at 0.1% (v/v) as a solvent control. The NF-κB luciferase reporter plasmid and the AP-1 luciferase reporter plasmid were generous gifts of Dr. Gang Min Hur (Chungnam National University, Daejeon, Korea). The pGL2-GRE
Fig. 1 Regulatory effect of C-K on the TLR4/LPS-induced secretion of proinflammatory cytokines in human monocytes. (A) Structure of the ginsenoside metabolite C-K. (B) Human monocytes were treated with increasing concentrations of C-K or a solvent control for 45 min before LPS stimulation (100 ng/ml). The supernatants were harvested after 18 hr and assessed for cytokine production by ELISA. The data shown are the mean ± SE of three experiments. Statistical differences (*, P<0.05; **, P<0.01; ***, P<0.001) compared to cell cultures without C-K pre-treatment are indicated. D, solvent control (0.1% Dimethyl sulfoxide [DMSO]); M, media control.
Transfection, reporter assays and siRNA studies

Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) as per the manufacturer's instructions. Luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI) as described previously [25]. RAW264.7 cells were transfected with four pooled small inhibitory (si)RNA duplexes (10 nM) directed against separate GR mRNA target sequences (SMARTpool; Dharmacon, Evry, France).

Enzyme-linked immunosorbent assays (ELISA), Western blotting, real-time polymerase chain reaction (PCR), chromatin immunoprecipitation (ChIP) assay and detection of nitrates

Cells were treated as indicated and processed for analysis by sandwich ELISA, Western blotting, and real-time PCR as described previously [22, 27, 28]. In the sandwich ELISA, serum and cell culture supernatants were analysed using DuoSet antibody pairs (Pharmingen, San Diego, CA) for the detection of interleukin (IL)-6 and tumour necrosis factor (TNF)-α. The IFN-β primers and PCR conditions used for quantitative real-time RT-PCR were as described previously [28]. Each PCR amplification was performed in triplicate, optical data were analysed using the default and variable parameters available in the iCycler iQ™ Optical System Software (ver. 3.0a, Bio-Rad, Hercules, CA), and samples were normalized to the reference reporter β-actin. ChIP assays were performed as described [23]. The interferon-stimulated response element (ISRE) flanking region was amplified by PCR with the primers 5’-ATG-STCTGGACTTTCGAGGTT-3’ and 5’-TCAGGGCCCGAAAGCAAAACA-3’. Nitric oxide production was also measured indirectly as nitrite accumulation in the cell culture medium using the Griess reaction [25].

Immunofluorescence microscopy for detecting IRF-3 nuclear translocation

Cells were fixed on coverslips in 4% (w/v) paraformaldehyde in PBS, followed by a 5-min permeabilization in 0.25% (v/v) Triton X-100 in PBS at 25°C. IRF-3 was detected by incubation with a 1:100 dilution of the primary Ab for 1 hr at 25°C, washing and incubation with a 1:100 dilution of rabbit immunoglobulin G-Alexa Fluor 488 (Molecular Probes, Eugene, OR) for 1 hr. Nuclei were visualized upon a 15-min incubation with 20 μg/ml propidium iodide (PI). Slides were examined with a laser-scanning confocal microscope (model LSM 510; Zeiss, Oberkochen, Germany).

Cellular fractionation

Nuclear and cytosolic protein extracts were prepared as described previously [29], with minor modifications. Briefly, cells were harvested by scraping in ice-cold PBS and lysed with Nonidet P-40 lysis buffer (50 mM Tris–HCl, 10 mM NaCl, 5 mM MgCl₂, 0.5% Nonidet P-40, pH 8.0) for 10 min on ice. Following centrifugation, the supernatant (or cytosolic extract) was collected and subjected to centrifugation at 15,000 x g for 30 min to remove cellular debris. The nuclear pellet was washed three times in the same lysis buffer and re-suspended by vortexing in high-salt buffer (20 mM HEPES, 0.5 M NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, pH 7.9). Fraction purity was tested by Western blotting using actin as a cytoplasmic marker and p84/N5 as a nuclear marker.

Histological analysis and cyclooxygenase (COX)-2 immunostaining

Immediately after the animals were sacrificed, their tissues were fixed with neutral-buffered formalin and sectioned for morphological evaluation using haematoxylin and eosin. For immunoassaying, the spleens were fixed by inflating the tissues and then sectioned. The slides were assessed for COX-2 expression as previously described [25].

GR competitor assay

Receptor-binding assays were carried out using RAW264.7 cells pre-incubated with C-K or dexamethasone (Dex) at various concentrations for 45 min before stimulation with Fluor-Dex (dexamethasone-fluorescein; Sigma). The cells were then harvested after 4 hrs and the OD485nm was recorded using a Fluoroscan Ascent (Thermo Electron Scientific, Waltham, MA).

GR competition assays were performed using a GR Competitor Assay Kit (Invitrogen) according to the manufacturer’s instructions. Briefly, GR was added to a fluorescent glucocorticoid ligand (Fluromone) in the presence of C-K in microwell plates for 4 hrs. C-K prevents the formation of a Fluromone/GR complex, resulting in a decreased polarization value. After 4 hrs, the shift in polarization value was assessed at an OD485nm using a Fluoroscan Ascent.

Statistical analysis

For parametric data, the results are expressed as the mean ± standard error of the mean (SE), and comparisons were made using the two-tailed Student’s t-test for paired samples. For non-parametric data, the results are expressed as the median ± quartiles, and comparisons were made using Wilcoxon’s signed-ranks test. Where indicated, an adjusted Bonferroni correction for multiple comparisons was used to reach an overall P value of < 0.05.

Results

C-K negatively regulates inflammatory cytokine secretion by LPS-stimulated macrophages

The hypothesis that C-K is the active metabolite responsible for the anti-inflammatory effect of ginseng saponins prompted us to investigate the effect of C-K on LPS-induced inflammation. As shown in Figure 1, pre-treatment of human monocytes (Fig. 1B) with C-K resulted in significantly reduced TNF-α, IL-6 and IL-8 production in a concentration-dependent manner, as determined by ELISA. As IL-10 negatively affects inflammatory cytokine
production by LPS-stimulated monocytes [30, 31], we investigated whether the decreased production of cytokines upon C-K treatment was the result of altered IL-10 expression. No significant differences in the levels of IL-10 were detected in cultures that were pre-treated with C-K and those that were not (Fig. 1B). These results imply that C-K affects the expression of several inflammatory mediators in LPS-stimulated BMDMs, except for IL-10.

C-K negatively regulates inflammatory signalling in LPS-stimulated macrophages

The activation of mitogen-activated protein kinase (MAPK) signalling is an essential part of the macrophage response to pro-inflammatory stimuli, such as LPS and cytokines [32]. Therefore, we investigated whether, in LPS-treated cells, C-K affects MAPK signalling cascades, including p38 MAPK (p38) and ERK1/2. LPS at 100 ng/ml induced peak activation of MAPKs (p38 and ERK1/2) within 30 min of stimulation (Fig. 2A). A second phase of p38 phosphorylation induction was observed after 6 hrs of LPS treatment, peaking at 9 hrs of stimulation (Fig. 2A), as recently described [33]. Notably, the phosphorylation of p38 and ERK1/2 was down-regulated by pre-treatment with C-K in LPS-stimulated cells in a dose-dependent manner (Fig. 2B). Treatment with C-K substantially inhibited the late phosphorylation of p38 at a 9 hrs of LPS stimulation, although the inhibition was not as potent as that induced by C-K in the early phase of p38 activation (Fig. 2C). In addition, pre-treatment of BMDMs with C-K significantly inhibited IκB attenuation and IκB kinase-α/β phosphorylation in a concentration-dependent manner, as shown by Western blotting (Fig. 2B). These results suggest that C-K preferentially mediates the negative regulation of inflammatory signalling in LPS-stimulated BMDMs.
C-K regulates TLR2- and TLR4-mediated but not TLR3-mediated macrophage inflammatory responses

We next investigated whether C-K affects the signalling pathways initiated by other TLR agonists that play key roles in innate immune signalling. Of note, pre-treatment with C-K significantly inhibited the secretion of TNF-α and IL-6 in human monocytes and the activation of MAPK and NF-κB in BMDMs in response to TLR2/BLP or TLR4/LPS, but not TLR3/poly I:C (Fig. 3A and B). Similarly, treatment with Dex (100 nM) significantly suppressed cytokine production and activation of MAPK and NF-κB in BMDMs in response to the agonists TLR2 or TLR4, but not in response to...
TLR3 (Fig. 3A and B). Of note, either C-K or Dex considerably inhibited the proinflammatory cytokine productions induced by HMGB1, a late cytokine mediator of lethal endotoxaemia and sepsis (Fig. 3A). In addition, we tested the effect of C-K on the transcription of NF-κB and AP-1-luciferase in HEK/TLR4/MD2/CD14 and HEK/TLR1/TLR2 cells stimulated with LPS and BLP, respectively. Pre-treatment with C-K inhibited the transcription of NF-κB and the AP-1 reporter in both HEK/TLR4/MD2/CD14 and HEK/TLR1/TLR2 cells, demonstrating that C-K acts on either TLR4 or TLR2 signalling (Fig. 3C and data not shown).

Furthermore, we examined the effects of C-K on the TRIF-dependent signalling pathway. As shown in Figure 4, C-K did not compromise the TLR3-mediated IFN-β mRNA expression or the translocation of IRF-3 into the nucleus. The poly I:C-induced IFN-β mRNA production and the translocation of IRF-3 into the nucleus were abolished upon pre-treatment with PKR inhibitor. However, no inhibitory effects on TLR3-mediated signalling were observed upon pre-treatment with Dex. Taken together, these data suggest that C-K plays a role in MyD88-dependent pathways but not TRIF-dependent pathways in TLR signalling.

C-K, a functional ligand of GR, promotes the LPS-induced pro-inflammatory response through GR

The molecular components of ginseng that are responsible for its activity are ginsenosides, triterpene saponins with a rigid steroidal skeleton and sugar moieties [31]. To test the effect of C-K on GR,
we examined the ability of C-K to compete with fluorescently labelled Dex (Fluo-Dex) for GR binding. RAW264.7 cells, which contain physiological levels of GR, were cultured for 2 hr in the presence of 500 μM Fluo-Dex and then with increasing concentrations of unlabelled Dex or C-K (Fig. 5A). Similar to Dex, C-K inhibited the binding of Fluo-Dex to GR in a dose-dependent manner (Fig. 5A). In addition, a competitive ligand-binding assay was performed to investigate the binding of C-K to GR using a Fluormone-recombinant human GR complex, as previously described [32]. Displacement of Fluormone from the GR-Fluormone complex by C-K resulted in decreased fluorescence polarization (Fig. 5B).

To determine the biological relevance of this result, we used a reporter plasmid to examine whether the binding of C-K to GR leads to the transcriptional activation of GR through GRE. RAW264.7 cells were transiently transfected with the reporter plasmid pGRE2-Luc. Similar to Dex, treatment with C-K also activated the transcription of the GRE reporter plasmid in a dose-dependent manner (Fig. 5C). Moreover, overexpression of GR caused by co-transfection of the GR expression vector and a GRE-luciferase vector into HEK 293 cells considerably enhanced the transactivation activity of the GRE promoter in the presence of C-K in a dose-dependent manner (Fig. 5D). These data indicate that C-K is a functional ligand of GR.

The above data prompted us to investigate whether GR has a role in the suppression of LPS-induced pro-inflammatory effects by C-K. We used either the specific GR antagonist RU486 (10 μM) or RAW264.7 cells transfected with siRNA against GR to block GR-related signalling. Both RU486 and the siRNA substantially reversed the C-K-induced suppression of LPS-stimulated TNF-α and NO production (Fig. 5E and F). These results suggest that the ability of C-K to modulate LPS-induced pro-inflammatory responses is mediated by GR.

**C-K mimics Dex by inhibiting the recruitment of p65 to the interferon-sensitive response element (ISRE) flanking region in response to LPS**

Having shown that C-K is an agonist ligand of GR, we next investigated the molecular mechanism by which C-K specifically modulates the response to the TLR4 agonist. Recent studies have demonstrated that GR represses inflammatory response genes by disrupting the p65/interferon regulatory factor (IRF) complexes required for TLR4-dependent, but not TLR3-dependent, transcriptional activation [23]. Therefore, we examined whether C-K specifically inhibits IRF3 target genes in response to TLR4/LPS signalling by targeting p65 using a ChIP assay with RAW264.7 cells (Fig. 6).

As shown in Fig. 6, both p65 and IRF3 were recruited to the promoter sequences of the ISRE flanking region in response to LPS, confirming the previous finding that p65 is recruited to ISRE-containing promoters in response to LPS [23]. Significantly, treatment with either C-K or Dex inhibited the recruitment of p65 to the ISRE in response to LPS, coincident with the ligand-dependent recruitment of GR to the promoters (Fig. 6A). However, in response to the TLR3 ligand, recruitment of p65 and IRF3 was unaffected by treatment with C-K or Dex (Fig. 6B). These findings suggest that C-K effectively diminishes the binding of IRF3 to p65 target promoters in response to stimulation by TLR4, but not TLR3.

**In vivo function of C-K during endotoxin-induced lethal shock**

To provide additional support for the idea that C-K can inhibit endotoxic lethal shock in vivo, we used a murine sepsis model [25]. C57/B6 mice were pre-treated with C-K orally 24 hr before endotoxaemia and then injected (i.p.) with LPS (40 mg/kg body weight), and survival was monitored for 5 days. Although 88% of the control mice died within 5 days post-injection, oral administration of C-K prevented the death of the LPS-injected mice in a dose-dependent manner. At 5 days post-injection, 78% of the mice that had received the highest dose of C-K (50 mg/kg) were still alive, as were nearly 55% of the mice that had received 30 mg/kg C-K (P<0.05; Fig. 7A). We next evaluated the effect of C-K on the production of inflammatory mediators that are mechanistically linked to endotoxaemia. C-K reduced the serum levels of endotoxin-induced inflammatory cytokines such as TNF-α and IL-6, as well as nitric oxide (systemic; Fig. 7B). Our histopathological results indicate that LPS injection produced a number of inflammatory changes (Fig. 7C), including acute inflammatory cell infiltration, congestion and marked germinal centre reactions in the spleen. In the liver, acute inflammatory cell infiltrates were seen in the hepatic lobules, and there was focal hepatocellular necrosis, Kupffer cell reactive hyperplasia and haemorrhage. In the C-K-treated mice, much less damage was observed following injection with LPS (Fig. 7C), and COX-2 expression was significantly lower in the spleens of the C-K-treated mice than in those that received LPS but not C-K (Fig. 7D). These data strongly suggest that C-K prevents endotoxin-induced lethal shock in vivo.

**C-K protects mice from endotoxin-induced lethal shock**

To further investigate the therapeutic effect of C-K during endotoxin-induced shock, several groups of mice were injected (i.p.) with 40 mg/kg LPS. Within 40 hrs, 100% of the control mice (n = 25) had died; however, when C-K was administered 6 hr after injection of 40 mg/kg LPS, 65% of the mice were still alive after 100 hr (Fig. 8A, left). In comparison, 70% of the mice that received 30 mg/kg LPS alone were dead within 100 hrs. C-K treatment at 6 hrs following the injection of 30 mg/kg LPS significantly increased the survival rate of the mice (P<0.01; Fig. 8A, right). In addition, CLP was performed on mice as described in the Materials and methods. C-K was administered 6 hrs after CLP. C-K provided significant protection against CLP-induced lethality at a dose of 30 mg/kg.
Regulation of the pro-inflammatory response by C-K requires the glucocorticoid receptor. (A) C-K or Dex was added to RAW264.7 cells at various concentrations 45 min before stimulation with Fluor-Dex (500 µM). The cells were then harvested after 4 hrs and the OD450nm was recorded using a spectrophotometer. (B) The affinity of C-K for human GR was assessed using a GR competitor assay kit. (C and D) RAW264.7 cells were transiently transfected with the GRE-luciferase plasmid pGL2-GRE-luc (C). HEK 293T cells were co-transfected with pGL2-GRE-luc and the GR expression plasmid (pRSH-GRµ). The transfected cells were stimulated for 4 hrs with LPS (1 µg/ml) in the presence or absence of C-K at the concentrations indicated. The cells were then harvested, and the level of luciferase activity was measured. The level of luciferase activity (mean ± SE) is presented as the fold activation relative to that in the untreated cells. The results are taken from at least three separate experiments. (E) RAW264.7 cells were pre-treated with either the GR antagonist RU486 (10 µM) or a solvent control (0.1% DMSO) for 30 min before stimulation with LPS (1 µg/ml) in the presence or absence of C-K (10 µg/ml). The supernatants were harvested after 18 hrs for cytokine assessment by ELISA (left) and for detection of nitric oxide production using nitrate and nitrite colorimetric assays (right). (F) RAW264.7 cells were transfected with GR-siRNA (10 nM) or non-specific siRNA (10 nM). After a 48-hrs incubation in normal culture medium, the transfected cells were stimulated with LPS (1 µg/ml) in the presence or absence of C-K (10 µg/ml) for 18 hrs. Following the harvest of the supernatants, the levels of TNF-α (ELISA) and nitric oxide were determined. In the upper panel, lysates of 5 × 10^5 cells from each transfectant (GR, GR-siRNA; NS, non-specific siRNA; at 10 and 20 nM) were immunoblotted with anti-GR Ab. The blots were stripped and re-probed with anti-actin mAb. The data shown are the means ± SE of three experiments. Statistical differences (*, P<0.05; ***, P<0.001) compared to cell cultures stimulated with LPS in the absence of C-K are indicated.
(P<0.001; Fig. 8B), with higher levels of protection afforded at 50 and 75 mg/kg (data not shown). Together, these data demonstrate an essential role for C-K in vivo and offer new possibilities for the treatment of Gram-negative septic shock.

Discussion

Significant efforts have been made to discover therapeutic or adjuvant therapeutic modalities that can interrupt systemic inflammation and improve survival in septic hosts. Alternative medicines, such as herbal products are increasingly being used for preventive and therapeutic purposes in inflammatory disorders. Ginseng is the best-known and most popular herbal medicine in the world, and it is currently under investigation for its ability to modulate inflammation and the immune response [34, 35]. The ginseng components glucan [36] and ginsan [37] reportedly decrease septic complications and enhance survival by modulating the innate immunity of the host [38–40]. Previous studies have demonstrated that the ginsenoside metabolite C-K may be biologically active. C-K, the major metabolite of ginsenosides, has antimitastatic and anti-carcinogenic effects due to its blocking tumour invasion and preventing chromosomal aberrations and tumourigenesis [17], but little is known about the role of C-K in regulating inflammation. We focused on the regulatory role of C-K in TLR4/LPS-induced inflammatory signalling. Our data demonstrate that C-K significantly inhibited the TLR4/LPS-induced secretion and mRNA expression of inflammatory mediators. In addition, C-K significantly repressed the TLR2-, but not TLR3-induced activation of inflammatory responses, in monocytes/macrophages.

Recent studies have revealed new layers of complexity and regulation in the pathways activated by TLRs. The signalling pathways that regulate inflammation are mediated by NF-κB and AP-1, which can both be modulated by glucocorticoids [41]. GR is prototypic of a subset of ligand-dependent nuclear receptors that integrate host immune responses with physiological circuits required to maintain necessary organ functions. The results of our fluorescence polarization assay and GRE-promoter assay revealed that C-K can indeed serve as an agonist ligand for GR. Treatment with the inhibitor RU486 or knockdown of GR using siRNA completely abolished the C-K-induced suppression of TNF-α and nitric oxide production in LPS-treated macrophages. These data have a partial correlation with previous findings regarding the ginsenosides Rg1 and Rb1 [4, 5, 21, 42]. Rg1 acts by binding to GR, and Rg1-induced eNOS phosphorylation and nitric oxide production are significantly reduced by RU486 and GR knockdown in human umbilical vein endothelial cells [42]. In our studies, both TLR2- and TLR4-mediated inflammatory responses through
Fig. 7 C-K treatment prevents lethal endotoxaemia. (A) Mice (n = 25 per group) received either C-K (10, 30 or 50 mg/kg; administered orally) or vehicle only at 24 hrs before a lethal dose of endotoxin (40 mg LPS/kg; intraperitoneal injection). Survival was recorded for the mice subjected to endotoxaemia. Viability was assessed every 5 hrs for the first 40 hrs and every 10 hrs thereafter. There was no further increase in death after 80 hrs. (B) Serum levels of TNF-α, IL-6 and nitric oxide were measured in mice that had received either C-K (30 mg/kg; administered orally) or vehicle only using ELISA (for TNF-α and IL-6) and a nitrate and nitrite colorimetric assay (for NO) at 18 hrs after LPS injection. The results are the mean ± SD of three experiments (n = 11). Statistical differences (**, P<0.01; ***, P<0.001) compared to the control mice are indicated. NS, non-specific. (C) Sections of liver (top) and spleen (bottom) from mice subjected to endotoxaemia 24 hrs after oral administration of either C-K (30 mg/kg) or vehicle. H&E staining was performed (scale bars: upper, 50 µm; lower, 200 µm). (D) COX-2 immuno-reactivity was compared in mice subjected to endotoxaemia 24 hrs after oral administration of either C-K (30 mg/kg) or vehicle. Spleen sections were stained with anti-COX-2 antibody (scale bar: 100 µm). The left figure is representative of the normal control mice. The middle and right figures show the liver and spleen injected with LPS. The images are representative of sections from five mice per group.
MyD88-dependent pathways are attenuated by C-K, which functions as an agonist ligand for GR. C-K failed to inhibit TLR3-dependent signalling, further supporting the TLR/GR cross-talk model in which GR ligands modulate the TLR signal through MyD88-dependent, but not TRIF-dependent, pathways [23]. Interestingly, C-K attenuated the production of proinflammatory cytokines induced by HMGB1, a therapeutic target for the treatment of lethal systemic inflammation. Recent studies reported that HMGB1 induced the inflammatory signals through TLR4 and TLR2 [43]. Together with these observations, our results indicate that

Fig. 8 Therapeutic effect of C-K on sepsis-related mortality. (A) Mice (n = 15 per group) received either C-K (30 mg/kg; administered orally) or vehicle 6 hrs after a lethal dose of endotoxin (left panel, 40 mg LPS/kg; right panel, 30 mg LPS/kg; intraperitoneal injection). Survival was determined in the mice subjected to endotoxaemia. (B) Delayed treatment with 30 mg/kg of C-K at 6 hr after Cecal ligation and puncture (CLP) still protected mice against CLP-induced lethality. Viability was assessed every 5 hrs for the first 40 hrs and every 10 hrs thereafter. There was no further increase in death after 80 hrs. Statistical differences compared to mice that received the vehicle only are indicated.
the ginsenoside metabolite C-K regulates MyD88-dependent signalling via GR engagement. Negative regulation of inflammatory responses is thought to result, at least in part, from the ability of GR to interfere with the activities of other signal-dependent transcription factors, including NF-kB and AP-1 family members, via transrepression [41]. Previous studies have suggested several models for GR-mediated transrepression, including direct interactions with NF-kB components [44, 45] or the regulation of the components of the pathways involved in NF-kB and AP-1 activation [46, 47]. In addition, certain genes that are sensitive to nuclear receptor-dependent repression when activated through TLR4 become resistant to repression when activated through TLR3 [23], indicating that the transrepression programs mediated by GR are regulated in a signal-specific manner. These studies also showed that GR effectively disrupted the formation of an IRF3/p65 activator/co-activator complex required for the activation of ISRE-containing promoters by TLR4-, but not TLR3-dependent, signalling [23]. Our data demonstrate that C-K suppresses TLR4/LPS-induced inflammatory gene expression by inhibiting the recruitment of p65 to the ISRE in response to LPS, coincident with the ligand-dependent recruitment of GR to these promoters (see Fig. 5). Thus, our data suggest that the binding of C-K to GR disrupts an IRF3/p65 complex required for the activation of ISRE-containing promoters by TLR4/LPS-dependent signalling.

Our data show that the activation of both p38 MAPK (early and late phase) and ERK1/2 in response to LPS is almost completely abrogated by pre-treatment with C-K, whereas the phosphorylation of p38, but not ERK1/2, is inhibited by Dex. These data partly correlate with recent studies showing that the early and late phases of LPS-induced p38 activation are attenuated by the stimulation of GR by Dex [33]. In addition, MAP kinase phosphatase-1 (MKP-1), a phosphatase induced by ligand-activated GR, suppresses p38 [48] and ERK1/2 [49] activation in HeLa cells and mast cells, respectively, and C-K significantly represses the phorbol 12-myristate 13-acetate (PMA)-mediated activation of p38 MAPK, ERK and c-Jun N-terminal kinase (JNK), which are upstream modulators of AP-1, in glioma cells [50]. However, the precise mechanisms by which C-K produces its anti-inflammatory effects via MAPK signalling remain to be determined. GR may interfere directly with Raf-1, which is downstream of Ras in the MAPK cascade, or via 14-3-3, an adapter protein that interacts with such proteins as protein kinase C and Raf-1 [51]. In addition, C-K suppresses the PMA-mediated activation of MAPKs, the expression of matrix metalloproteinase-9, and the in vitro invasiveness of glioma cells [50]. Future studies should reveal the precise molecular mechanisms by which C-K regulates the TLR4/LPS-induced MAPK signalling in the context of GR-dependent and -independent pathways.

The triggering of pattern recognition receptors and TLR signalling causes the secretion of pro-inflammatory mediators, which promote the elimination of infectious agents. Direct comparisons of the biological activities of TLR agonists have indicated a substantial divergence in their ability to influence the nature (pro- versus anti-inflammatory) and magnitude (absolute amounts) of the inflammatory response [52]. Therefore, excessive inflammation during bacterial infection can lead to marked tissue damage and lethal septic shock [1]. Our findings indicate that C-K attenuates pro-inflammatory cytokine production by macrophages and protects septic mice against hyperresponsiveness and death. Of note, post-injection of C-K significantly increased the survival rate of septic mice. Moreover, C-K was effective in our sepsis model that included CLP, which closely mimics human acute peritonitis and is regarded as the most clinically relevant animal model of sepsis. Taken together, these data suggest that C-K may be useful as a therapeutic tool for the treatment of sepsis by altering the inflammation induced by TLR4. However, the efficacy of C-K as compared to that of corticosteroids as a treatment for sepsis is unknown.

In summary, this work has identified C-K as a new immunomodulatory factor with the capacity to deactivate the inflammatory response. Our data strongly suggest the utility of C-K in the treatment of sepsis through the modulation of excessive inflammatory responses during endotoxin-induced lethal shock. Therefore, our findings provide new opportunities for the design and discovery of therapeutic drugs to treat infectious diseases and inflammation. In addition, these findings suggest that a clinical evaluation of the effects induced by C-K will be useful.

Acknowledgements

We thank Drs. H.-J. Sohn, Y.-S. Kim and J.-H. Do (KT&G Central Research Institute), Dr. G.-M. Hur (Chungnam National University), for kind provision of constructs and materials, and Dr. Y.-C. Cheng (Yale University) and Dr. K.-I. Kwon (Chungnam National University) for critical review of the paper. This research was supported by the Korea Science & Engineering Foundation through the Infection Signalling Network Research Center (R13-2007-020-01000-0) at Chungnam National University. The authors declare that they have no competing financial interests. Two of this paper’s authors (S.-R. Ko and B.-G. Cho) are listed as inventors of patents involving C-K.

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