**Compound K Rich Fractions Regulate NF-κB-dependent Inflammatory Responses and Protect Mice from Endotoxin-induced Lethal Shock**

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(Received September 18, 2008, Accepted December 11, 2008)

**Abstract:** In the previous studies, we isolated the compound K rich fractions (CKRF) and showed that CKRF inhibited Toll-like receptor (TLR) 4- or TLR9-induced inflammatory signaling. To extend our previous studies,1) we investigated the molecular mechanisms of CKRF in the TLR4-associated signaling via nuclear factor (NF)-κB, and in vivo role of CKRF for induction of tolerance in lipopolysaccharide (LPS)-induced septic shock. In murine bone marrow-derived macrophages, CKRF significantly inhibited the induction of mRNA expression of proinflammatory mediators such as tumor necrosis factor-α, interleukin-6, cyclooxygenase-2, and inducible nitric oxide synthase. In addition, CKRF significantly attenuated the transcriptional activities of TLR4/LPS-induced NF-κB. Nuclear translocation of NF-κB in response to LPS stimulation was significantly abrogated by pre-treatment with CKRF. Furthermore, CKRF inhibited the recruitment of p65 to the interferon-sensitive response element flanking region in response to LPS. Finally, oral administration of CKRF significantly protected mice from Gram-negative bacterial LPS-induced lethal shock and inhibited systemic inflammatory cytokine levels. Together, these results demonstrate that CKRF modulates the TLR4-dependent NF-κB activation, and suggest a therapeutic role for Gram-negative septic shock.

**Key words:** Compound K rich fractions, Toll-like receptor 4, nuclear factor κB, endotoxemia

**INTRODUCTION**

The innate immune system is the first line of immune defence against invading pathogenic microbes.2) When controlled, the innate immune responses have the power to eliminate infections.3) Toll-like receptors (TLRs) have been widely studied and are able to recognize microbial components during the innate immune responses.4) Together with other pattern-recognition receptors, TLRs are innate receptors which play a pivotal role in sensing a variety of invading pathogens and microbes. Although TLRs mediate key immune activation upon the recognition of pathogens, hyper-activation or dysregulation of TLR signaling is linked with a number of disease conditions. Therefore, targeting TLR signaling cascades could lead to novel therapeutic strategies in the treatment of infectious and inflammatory diseases.2)

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and improve survival in septic hosts. Ginseng, the root of Panax ginseng C. A. Meyer (Araliaceae), is one of the most widely used Chinese herbs with various pharmacological actions for a long times, however, the underlying mechanisms have not been largely understood. Ginsenoside is the major active ingredient of ginseng, and is one of the best known natural products with anti-inflammatory and anti-oxidative effects. Ginsenoside compound K (CK; 20-O-β-D-glucopyranosyl-20(S)-protopanaxadiol) is speculated to be produced through fermentation of protopanaxadiol-type ginsenosides from the intestine. In addition, CK was reported to be produced via transformation of ginsenosides using commercial enzyme preparations.

Ginsenoside metabolite CK is a promising natural product that could be applied for various pharmaceutical purposes. Several reports have shown the effects of CK on the anti-metastatic and anti-allergic activities, apoptosis, regulation of plasma glucose levels, etc. Our recent studies have demonstrated a novel therapeutic role for CK in the treatment of lethal sepsis through the modulation of TLR4-associated signaling via glucocorticoid receptor (GR) binding. In another studies from our laboratories, we isolated the CK rich fractions (CKRF) and examined the effects of CKRF on the inflammatory signaling during TLR-induced cellular activation. In those studies, we found that CKRF specifically modulated TLR4- or TLR9-induced inflammatory signaling. These findings led us to hypothesize whether CKRF regulate the systemic inflammatory responses, for in vivo roles for potential therapeutic uses, as similar to those by CK demonstrated by our previous studies.

In this study, we describe a novel function for CKRF in the treatment of lethal sepsis through the modulation of TLR4-associated signaling via NF-κB. We demonstrate the functional significance of CKRF on the regulation of TLR4-dependent signaling pathways through NF-κB activation, and in vivo therapeutic effects against systemic inflammatory responses induced by endotoxin administration.

MATERIALS AND METHOD

1. Isolation of compound K rich fractions (CKRF) from red ginseng extracts

CKRF was made as described previously. Briefly, 100 g of alcohol extracts of red ginseng were incubated with 20 g of mixed enzymes Cellulase, Hemicellulase, Pectinase (Shin Nihon Chemical co., Japan) at 56°C in 10L reaction medium. After incubation for 48 hours, reaction was terminated without any termination treatment and CKRF was collected in No2 filter paper (Whatman, USA). The CKRF in filter paper was resuspended with ethyl alcohol and centrifuged at 3000 rpm in 10 min. CKRF was obtain from these supernatants with vacuum evaporating under 60°C. The yield of CKRF was 11.6 ± 0.1% (A dry weight basis) and the amount of CK in CKRF was 243.8 ± 16.1 mg/g. CKRF which was analyzed with HPLC. CKRF was dissolved in a mixture of dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO, USA): polyethylene glycol (PEG; Sigma) 400: DDW (1 : 4 : 5) and DMSO was added to the cultures at 0.1% (v/v) as a solvent control. The CK content was around 29% in CKRF.

2. LPS-induced endotoxin shock and histological analysis

All animal protocols and experiments were approved by the Institutional Animal Care and Use Committee, Chungnam National University College of Medicine (Daejeon, Korea), and complied with National Institutes of Health guidelines for the care and use of laboratory animals. All experiments described in this study were performed using C57BL/6 mice. The mice used for the lipopolysaccharide (LPS) challenge were age- and sex-matched 6-8 weeks old. Escherichia coli O26:B6 LPS (Sigma) was diluted in sterile phosphate-buffered saline (PBS) and injected into the animals intraperitoneally (i.p.).

For histopathological analysis, tissues were fixed with neutral-buffered formalin and sectioned for morphological evaluation using hematoxylin and eosin (H&E) staining. For immunohistochemistry, the spleens were fixed by inflating the tissues and then sectioned. The slides were assessed for COX-2 expression as previously described.

3. Cell isolation and culture

Bone marrow-derived macrophages (BMDMs) were obtained from 6-8 weeks old C57BL/6 female mice. Briefly, bone marrow cells from the femur and tibia were cultured for 4 days in macrophage colony-stimulating factor-containing media as described previously. 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% fetal bovine serum (FBS). Human embryonic kidney (HEK) 293 cells stably transfected with human TLR4, MD2, and CD14 (HEK/TLR4/MD2/CD14) was purchased from InvivoGen (San Diego, CA, USA) and grown in standard DMEM with 10% FBS supplemented with blasticidin (10 µg/ml).
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and Normocin (100 µg/ml).

4. Reagents, DNA, and antibodies
For in vitro experiments, ultrapure LPS (TLR4 agonist) and Dexamethasone (Dex) were obtained from InvivoGen and Sigma, respectively. DMSO was added to the cultures at 0.1% (v/v) as a solvent control. The NF-κB luciferase reporter plasmid was generous gifts of Dr. Gang Min Hur (Chungnam National University, Daejeon, Korea). Antibodies against IRF3 and NF-κB p65 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-COX-2 antibody was purchased from BD Transduction Laboratories (Franklin Lakes, NJ, USA).

5. Transfection and reporter assays
Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) as per the manufacturer’s instructions. The NF-κB luciferase activity was measured using the Luciferase Assay System (Promega, Madison, WI, USA) as described previously.

6. Reverse transcriptase-Polymerase chain reaction (RT-PCR) and chromatin immunoprecipitation (ChIP) assay
RNA was extracted from the cells using TRIzol (Invitrogen). Complementary DNA was reverse transcribed from 2 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen) and oligo-dT-3 primer in a total volume of 20 µl. Using PCR, 2 µl of cDNA was amplified. The primers and PCR conditions were as described previously. The PCR products were resolved on 1.5% agarose gels and were stained with ethidium bromide. ChIP assays were performed as described. The interferon-sensitive response element (ISRE) flanking region was amplified by PCR with the primers 5’-ATGGTCTGGAGACTTTCGAGGTT-3’ and 5’-TCAGGGCCCGAAAGCAAAACCA-3’.

7. Immunostaining
NF-κB p65 nuclear translocation in BMDMs was assessed by immunofluorescence microscopy. 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. NF-κB p65 was detected by incubation with a 1:100 dilution of the primary Ab for 1 h at 25°C, washing, and incubation with a 1:100 dilution of rabbit immunoglobulin G-Alexa Fluor 488 (Molecular Probes, Eugene, OR, USA) for 1 h. 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).

8. 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 nonparametric 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

1. CKRF modulate expression of proinflammatory cytokines, cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) by macrophages in response to LPS.
We recently reported that CKRF are regulated TLR4/LPS-induced inflammatory responses in macrophages. As shown in Fig. 1A, pre-treatment of BMDMs with CKRF resulted in specifically inhibited LPS-induced TNF-α and IL-6 mRNA expression in a concentration-dependent manner, in agreement with previous report. However, there was no significant changes in LPS-induced IL-10 mRNA expression by pre-treated with CKRF and those that were not (Fig. 1B). We further examined whether the effect of CKRF on LPS-induced proinflammatory mediators expression. As shown in Fig. 1B, pre-treatment with CKRF significantly attenuated LPS-induced COX-2 and iNOS mRNA expression in BMDMs, in a concentration-dependent manner. Cytokine mRNA expression peaked at 4 h post-LPS stimulation (data not shown). These results indicate that CKRF profoundly abrogated in LPS-induced pro-inflammatory signal activation in murine macrophages.

2. CKRF regulates NF-κB signaling by macrophages in response to LPS.
NF-κB transcription factors are rapidly activated in response to various stimuli, including cytokines and infectious agents, and regulate the expression of a variety of genes involved in immune responses and inflammation. TLR stimulation results in activation of different intracellular signaling cascades, generally resulting in the activation of NF-κB and activating protein (AP)-1 in MyD88-dependent pathways. Therefore, we next investigated the role of CKRF in the regulation of TLR4/LPS-induced NF-κB activation. We examined the effects of CKRF on
the modulation of NF-κB- and AP-1-luciferase gene activity in HEK/TLR4/MD2/CD14 cells after stimulation with LPS. Pre-treatment with CKRF significantly attenuated the transcriptional activities of NF-κB and the AP-1 in a concentration-dependent manner (Fig. 2A and data not shown). Furthermore, pre-treatment with CKRF significantly abolished the translocation of NF-κB p65 into the nucleus of BMDMs after LPS stimulation, thus confirming the role of CKRF in the modulation of LPS-mediated NF-κB activation (Fig. 2B). However, no inhibitory effects on TLR3/poly I:C-mediated translocation of IRF3 into the nucleus were observed upon pretreatment with CKRF (data not shown). Taken together, these data suggest that CKRF profoundly abrogated the activation of NF-κB signaling through MyD88-dependent pathways, but not TRIF-dependent pathways in response to LPS.

3. CKRF inhibits LPS-induced p65 recruitment to the interferon-sensitive response element (ISRE) flanking region

We next examined the molecular mechanism by which CKRF specifically modulates the response to LPS. Recent studies reported that GR specially inhibits the interaction of p65 with interferon regulatory factor (IRF) complexes to mediate TLR4/LPS-sepcific transcriptional responses, but not TRIF-dependent. Therefore, we asked whether p65/IRF3 complexes mediate TLR4/LPS signaling inhibited of transcriptional responses. To examine this, we performed chromatin immunoprecipitation assays (ChIP) analysis in RAW264.7 cells (Fig. 3).

As shown in Fig. 3, both IRF3 and p65 were interacted to the promoter regions of ISRE flanking region in response to LPS, confirming the previous findings of specific recruitment of p65 to the ISRE-containing promoter in response to LPS. Significantly, treatment with either CKRF or Dex attenuated the interaction of p65 to the ISRE in response to TLR4/LPS. However, in response to the TLR3 ligand, both p65 and IRF3 recruitment to the promoter regions of ISRE flanking region was not affected by treatment with either CKRF or Dex (data not shown). These data indicated that CKRF significantly inhibits the interaction of p65 with IRF3 in response to TLR4, but not TLR3, stimulation.

4. CKRF protected mice from LPS-induced lethal shock

We used murine sepsis model, as previously described. Oral administration of CKRF 24 h before endotoxemia and then injected (i.p.) with LPS (40 mg/kg body weight) in C57B/L6 mice, and survival was monitored for 5 days. Although 40 mg/kg of LPS killed 78% of the control mice within 5 days postinjection, oral administration of CKRF prevented the death of the LPS-injected mice in a concentration-dependent manner. At the highest dose (100 mg/kg) of pre-treated with CKRF, 90% of the mice were still alive, and almost 78% with 75 mg/kg of pre-treated with CKRF by day 5 post-injection of LPS (P < 0.001; Fig. 4).

We further examined histopathological evaluation of cellular infiltration and organ injury (Fig. 5). Paraffin-embedded sections were prepared from the liver and spleen of mice injected with vehicle or LPS for 24 h in the presence or absence of CKRF (75 mg/kg), and subjected to H&E staining. Our histopathological results show that bacterial lipopolysaccharide injection produced inflammatory changes (Fig. 5A), including acute inflammatory cell infiltration, congestion and marked germinal center reactions in the spleen. In the liver, acute inflammatory cell infiltrates were seen in the central vein surrounded by hepatocytes and sinusoids, and there was focal hepatocellular necrosis, Kupffer cell reactive hyperplasia and haemorrhage as well as a congested central vein. In the CKRF-

Fig. 1. Regulatory effect of CKRF on the TLR4/LPS-induced expression of proinflammatory mediators in murine macrophages. (A, B) Murine BMDMs were treated with increasing concentrations of CKRF or a solvent control for 45 min before LPS stimulation (1 μg/ml). The cells were harvested after 4 h, total RNA was purified and semiquantitative RT-PCR analysis of TNF-α, IL-6, IL-10, COX-2 and iNOS mRNA was performed. The data are representative of three separate experiments. D, solvent control (0.1% DMSO); M, media control.
Fig. 2. Regulatory effect of CKRF on the TLR4/LPS-induced NF-κB signaling in murine macrophages. (A) hTLR4-HEK293 cells were transiently transfected with pNF-κB-luc. At 24 h post-transfection, the cells were stimulated for 4 h with LPS (1 µg/ml) in the presence or absence of CKRF at the indicated concentrations. The cells were then harvested, and luciferase activity was measured and corrected for differences in transfection efficiency based on the β-galactosidase activity. The luciferase activities shown are the mean ± SD of three independent experiments. Significant differences from the control values (without CKRF pretreatment) are indicated as ** (P < 0.01) or *** (P < 0.001). (B) Murine BMDMs were stimulated with LPS (1 µg/ml) in the presence or absence of CKRF (10 µg/ml) and harvested 4 h later for immunofluorescence microscopy. The cells were fixed and stained with NF-κB Ab and PI, followed by examination under a confocal microscope (model LSM 510; Zeiss, Oberkochen, Germany). The data are representative of three separate experiments. Scale bar: 100 µm. D, solvent control (0.1% DMSO); M, media control.

Fig. 3. p65 recruitment to the proximal promoter region of ISRE is specifically induced by LPS and inhibited by CKRF. RAW264.7 cells were treated with LPS (1 µg/ml), Dex (10 µg/ml) or CKRF (10 µg/ml) for 1 h. ChIP assays were performed using antibodies against IRF3, p65, or IgG. Immunoprecipitated DNA was analyzed by PCR using primers specific for the promoter. A representative experiment of three independent replicates with similar results is shown.

treated mice, much less damage was seen than in the control mice after injection with LPS (Fig. 5A). When the COX-2 expression were compared in spleens from control and CKRF-treated mice with LPS injection, the COX-2
expression of CKRF-treated mice was found to be significantly lower than that of control mice (Fig. 5B). The pathological changes caused by LPS were significantly alleviated in CKRF-treated mice in all two organs. These data thus support a strong in vivo activity of CKRF for prevention of endotoxin-induced lethal shock.

**DISCUSSION**

Triggering of TLR signaling can lead to the production of inflammatory mediators, which promote the elimination of infectious agents. The ability to orchestrate biological activities of TLR agonists often results in diverse host responses to influence the outcome of the inflammatory
Ginsenoside metabolite CK is a promising natural product that could be applied for the pharmaceutical purpose or treatment of various pathologic conditions. Previous studies demonstrated that anti-metastatic and anti-allergic activities by the ginsenosides were mediated by CK. Our recent findings revealed that CK attenuated proinflammatory cytokine production by macrophages and significantly increased the survival rate of septic mice. However, in those studies, we found that CK can serve as an agonist ligand for GR and regulate the MyD88-dependent signaling via GR engagement. Currently available steroidal drugs generated enhanced binding to the GR could produce important and serious side effects. Therefore, new compounds with reduced activities through GR engagement, and increased action on the anti-inflammatory effects should be desirable for both clinical applications and basic research.

In the current study, CKRF was isolated from Korean red ginseng. Red ginseng was reported to contain higher amounts of protopanaxadiol-type ginsenosides (Rb1, Rb2, Rc, Rd, Rg3) than white ginseng. Recent studies have reported that the propanaxadiol-type ginsenosides are highly associated with anti-inflammatory responses in various types of cells. Therefore we were to investigate the effects of CKRF including a variety of protopanaxadiol-type ginsenosides, which could contribute to develop effective anti-inflammatory agents containing reduced amounts of purified CK. As CKRF contained around 29% of CK in their contents, CKRF significantly regulate inflammatory signaling during TLR4- or TLR9-induced cellular activation, whereas CK significantly inhibits inflammatory signalling during TLR2- or TLR4-mediated activation. Therefore it may be useful as a therapeutic tool for the potential treatment of sepsis. In this study, we focused on the efficacy of CKRF using in vivo model of sepsis. We found that CKRF treatment effectively reduced organ inflammation and damage following injection with LPS, and COX-2 expression in the spleens, compared with those that received LPS but not CKRF. These data correlated with our recent findings with CK, and further demonstrate that CKRF can be used as a therapeutic purpose for endotoxin-induced lethal shock in vivo.

Nevertheless, these studies demonstrate that negative regulation of inflammatory responses induced by CKRF is thought to result, at least in part, from the ability of GR to interfere with the activities of NF-κB via transrepression. Our data showed that CKRF suppresses TLR4/LPS-induced inflammatory gene expression by inhibiting the recruitment of p65 to the ISRE in response to LPS (see Fig. 3). Previously, it was demonstrated that GR effectively disrupted the formation of an IRF3/p65 activator/coactivator complex required for the activation of ISRE-containing promoters in TLR4/LPS signalling. These findings may indicate that the binding of CK or other ginsenosides of CKRF to GR disrupts an IRF3/p65 complex required for the activation of ISRE-containing promoters by TLR4/LPS-dependent signaling. Further studies should clarify which components of CKRF modulate the GR-mediated transrepression.

Pretreatment with CKRF significantly inhibited the mRNA expression of TNF-α, IL-6, COX-2, and iNOS by BMDMs in response to LPS. As CKRF did not modulate the expression of IL-10, the suppressive effects of CKRF on the inflammatory mediator gene expression might be not due to an increase of anti-inflammatory responses. These data partly agree with our previous studies that CK substantially inhibited the secretion of inflammatory cytokines and activation of MAPK and NF-κB in BMDMs in response to TLR2/BLP or TLR4/LPS, but not TLR3/poly I:C. We also showed that CKRF affected the NF-κB signaling pathways initiated by TLR4/LPS. Of note, CKRF inhibited the transcription of NF-κB-luciferase in HEK/TLR4/MD2/CD14, and translocation of NF-κB in BMDMs, when stimulated with LPS. In supporting the important role of NF-κB activity on systemic inflammation, Ye et al. have recently reported that endothelial NF-κB activity is critically required for the inflammatory and injurious responses leading to septic multiple organ inflammation and injury.

NF-κB activation is a key component of host immune response, but also drive to the progression of systemic inflammation and septic pathology. The pleiotropic transcription factor NF-κB is a central downstream element of TLR-dependent signalling and plays an important role in the regulation of multiple physiologic and pathologic phenomena, including apoptosis, cell growth, stress response, innate immunity and septic shock. In addition, patients...
with septic shock showed an increased NF-κB activity, which may help to predict the outcome of sepsis, as a potential early prognostic marker for severe sepsis. Moreover, specific blockade of NF-κB with peptide containing the NEMO-binding domain inhibited the LPS-delayed neutrophil apoptosis, which might affect the resolution of inflammation. A detailed understanding of the molecular basis of the regulation of NF-κB activation by CKRF is needed in order to specifically block inflammatory signaling during sepsis or severe inflammation, while avoiding adverse effects induced by GR stimulation. Future studies should reveal the precise components and molecular mechanisms by which CKRF regulates the individual TLR-induced inflammatory signaling pathways.

In summary, this study identified CKRF function as immunomodulatory factors with the capacity to inhibit or attenuate the TLR4/LPS-dependent inflammatory responses in vitro and in vivo. Our data strongly suggest a potential clinical utility of CKRF in the treatment of sepsis or severe inflammation through the modulation of excessive inflammatory responses.

ACKNOWLEDGMENTS

We thank Drs. Shengjin Li and Jin-Man Kim (Chungnam National University) for technical supports. This research was supported by the Korea Science & Engineering Foundation through the Infection Signaling Network Research Center (R13-2007-020-01000-0) at Chungnam National University, KT&G Central Research Institute (2006), and the Korean Society of Ginseng (2005).

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