Short communication

Anti-inflammatory activity of caffeic acid phenethyl ester (CAPE) extracted from *Rhodiola sacra* against lipopolysaccharide-induced inflammatory responses in mice

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**A B S T R A C T**

A potent anti-inflammatory activity was detected from methanol extracts prepared from roots of *Rhodiola sacra*. In the preliminary test, methanol extracts from the roots of *R. sacra* could potently attenuate histological change in the lung tissue after lipopolysaccharide (LPS)-treated inflammatory stimulation. The active compound has been identified as 3-(3,4-dihydroxy-phenyl)-acrylic acid phenethyl ester (caffeic acid phenethyl ester, CAPE). *In vivo* study on protective effect of *R. sacra* CAPE against inflammatory response by endotoxin shock in the LPS-induced inflammatory mice showed significantly attenuated the intravenous LPS-induced increase in plasma TNF-\(\alpha\) and IL-1\(\beta\) concentration. *R. sacra* CAPE significantly inhibited LPS-induced nuclear transcription factor-\(\kappa\)B (NF-\(\kappa\)B) activation. It also down-regulates enhanced matrix metalloproteinase-9 (MMP-9) activity triggered by LPS in mouse lung. In the present study, the results indicate that the intake of *R. sacra* CAPE could provide health benefits, reducing the magnitude of the inflammatory process triggered by endotoxin shock and the production of inflammatory mediators.

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1. Introduction

Endotoxic shock, a severe inflammatory response triggered by systemic infection is characterized by hypoperfusion of major organ, leading to multiple organ failure, shock and death. The pathogenesis of sepsis involves a progressive and dynamic expansion of the systemic inflammatory response to bacterial infection [1]. Lipopolysaccharide (LPS), an integral part of the outer membrane of Gram-negative bacteria, is a major pathogenic factor in septic shock. The systemic effects of endotoxin occurred by a number of inflammatory mediators, the proinflammatory cytokines, TNF-\(\alpha\) and IL-1\(\beta\). In LPS-treated animals, synthesis of pro-inflammatory cytokines occurs through activation of nuclear transcription factor-\(\kappa\)B (NF-\(\kappa\)B). NF-\(\kappa\)B is normally present in the cytosol and exists as an active complex with a class of inhibitory proteins known as I\(\kappa\)B. Following an inflammatory stimulus, phosphorylation of I\(\kappa\)B triggers their degradation and NF-\(\kappa\)B translocation to the nucleus where it induces the expression of a wide variety of genes in inflammation that includes cytokines (e.g., IL-1, TNF-\(\alpha\)), enzymes (include nitric oxide synthase), adhesion molecules and acute phase proteins [2,3].

 Herbal medicines have been consumed over thousands of years in the Oriental nations such as China, India, Japan, and Korea as a health tonic or as anti-aging remedies. However, more scientific evidence is needed to verify a key role of bioactive components derived from the medicinal plants. Recently, much of the attention has been focused on the medicinal plants that have beneficial effects against various diseases. In view of these potential health benefits, reducing the magnitude of the inflammatory process triggered by endotoxin shock and the production of inflammatory mediators.
benefits, there has been intensive research on natural phenolic compounds (phenolic acids, flavonoids, and tannins) which are considered to be a major contributor to various biological activities, such as anti-oxidative, anti-inflammatory, anti-carcinogenic, anti-atherosclerotic activities, etc. [4,5]. *Rhodiola* plants, which are clinically used as a Chinese, Japanese, and Korean folk herbal medicine for treating diverse symptoms, including amnorrhea, climacteric syndrome and emotional imbalance, contain plenty of phenolic compounds such as caffeic acid, 4-hydroxychinnamic acid, 2-phenylethyl-β-d-glucopyranoside, etc., and demonstrate anti-allergy, anti-fatigue, anti-anoxia and beta-amylloid inhibitory activity related with the ability to improve memory ability [6,7]. Although the caffeic acids-structural amyloid inhibitory activity related with the ability to improve and demonstrate anti-inflammatory effects of bioorganic compounds derived from *Rhodiola* plants.

Our preliminary test for evaluation on pharmaceutical benefits of *Rhodiola sacra* showed that methanol extracts from the roots of *R. sacra* could potently attenuate histological change in the lung tissue after LPS-treated inflammatory stimulation. In the present study, we have confirmed chemical structure of a phenolic compound (3-(3,4-dihydroxy-phenyl)-acrylic acid phenethyl ester, caffeic acid phenethyl ester (CAPE)) isolated from *R. sacra* and investigated its anti-inflammatory effect against LPS-induced endotoxic shock using a murine model. Caffeic acid phenethyl ester, well known as the bioactive component of propolis, has strong antiviral, antimutural, anti-inflammatory, antioxidant, neuroprotective, antiatherosclerotic, and immunomodulatory properties in diverse systems. CAPE inhibits certain enzyme activities such as lipoxxygenase, cyclooxygenase, glutathione S-transferase, and xanthine oxidase, and potent and specific inhibitor of NF-κB activation [9]. For these reason, it is reasonable to expect that manipulations which diminish activation of NF-κB may decrease the intensity of NF-κB-mediated endotoxic or septic shock. While biomedical and pharmacological activities of CAPE have recently been elucidated, effects of CAPE on the endotoxic shock in mouse model have not been reported. Therefore, this study can identify our hypothesis that specific therapeutic targeting of NF-κB signaling could potentially contribute to the therapy of endotoxic shock without the toxic side effects that can be expected from general NF-κB inhibition with CAPE. In addition, it is possible to provide health benefits of *R. sacra* CAPE, reducing the magnitude of the inflammatory process triggered by endotoxin shock and of the production of inflammatory mediators.

2. Materials and methods

2.1. Preparation of *R. sacra* CAPE

The roots of *Rhodiola* plant (*R. sacra*) were obtained from herbal medicine market in Daegu, S. Korea, and a voucher specimen was deposited in co-author’s laboratory (W.-K. Choi). *R. sacra* CAPE was isolated according to Jin et al. [10] with slight modifications. Briefly, the plant sample (500 g) was extracted three times with methanol at 70°C for 5 h. Extracts were filtered through a 0.45 μm filter and lyophilized. The methanol extract (110 g) was suspended in water (500 ml) and successively re-extracted with 500 ml each (3 times) of hexane (yield: 7.9 g), chloroform (yield: 11.6 g), ethyl acetate (yield: 16.7 g), and n-butanol (yield: 36.8 g). All fractions, including the final remaining water fraction (yield: 39.7 g), were concentrated under reduced pressure using a rotary evaporator, then freeze-drying and lyophilization, the final yield (w/w) of CAPE was about 65 mg as off-white amorphous powder.

2.2. Animal treatments and histopathological examination

Specific pathogen-free female C57BL/6 mice were obtained from Charles River (Yokohama, Japan), and were kept in our animal facility for at least 2 weeks before use. All mice were used at 8–10 weeks of age. All experimental animals used in this study were under the protocol approved by the Institutional Animal Care and Use Committee of the Inje University College of Medicine. Endotoxic shock was induced in mice by i.v. injection of LPS at doses of 100 μg per mouse (Escherichia coli, serotype 055:B5; Sigma–Aldrich) as described by Han et al. [11]. *R. sacra* CAPE (1 mg kg⁻¹) and vehicle were administered intraperitoneally 16 h and 10 min prior to LPS injection. Ten minutes after LPS injection, the lungs and kidneys were removed and fixed in 10% neutral formalin, and embedded in paraffin. For histological examination, 4 μm sections of fixed and embedded tissues were cut on a Leica model 2165 rotary microtome (Leica, Nussloch, Germany), placed on glass slides, deparaffinized, and stained sequentially with hematoxylin 2 and eosin-Y (Richard-Allan Scientific, Kalamazoo, MI).

2.3. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce, Rockford, IL). As a probe for the gel retardation assay, an oligonucleotide containing the immunoglobulin κ-chain binding site (κB, 5′-CCGTTAAACA-GAGGGGCTTTCCGAG-3′) was synthesized. A non-radioactive method where the 3’ end of the probe was labeled with biotin was used for these experiments (Pierce). The binding reactions contained 10 μg of nuclear extract protein, buffer (10 mM Tris, pH 7.5, 50 mM KCl, 1 mM MgCl₂, 1 mM dithiothreitol, 0.05% Nonidet P-40, and 2.5% glycerol), 50 ng of poly (dl-dc), and 20 μM of biotin-labeled DNA. The reactions were incubated for 20 min at room temperature in a final volume of 20 μl. The competition reactions were performed by adding 200-fold excess of cold κB or irrelevant oligonucleotide (cAMP response element, CRE) to the reaction mixture. The reaction mixture was analyzed by electrophoresis on a 5% polyacrylamide gel in 0.5× Tris-borate buffer. The reactions were transferred to a nylon membrane. The biotin-labeled DNA was detected with LightShift chemiluminescent electrophoretic mobility shift assay kit (Pierce).

2.4. Determination of TNF-α and IL-1β in serum

Concentrations of TNF-α and IL-1β in sera were determined by sandwich ELISA, using capture and detection antibodies according to the manufacturer’s recommendations (R&D Systems, Inc., Minneapolis, MN). Sensitivity of the assay was 2.8 pg ml⁻¹ for TNF-α and 2 pg ml⁻¹ for IL-1β.

2.5. Gelatin zymography

Gelatinolytic activity of MMP-9 was measured by gelatin zymography, as described previously [12,13]. Briefly, a total of 10 μl of lung homogenate was subjected to electrophoresis in 10% polyacrylamide gel containing gelatin (1 mg ml⁻¹). The gel was washed in 2.5% Triton X-100 to permit renaturation of gelatinases, and stained with Coomassie blue after overnight incubation. Gelatinolytic activity was observed as clear bands against a blue background. Densitometry was performed using the Gel Logic 100 Imaging System and Kodak 1D software (Kodak Scientific Imaging Systems, Rochester, NY).

2.6. Western blot analysis

The lung tissues were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of lung proteins. Western blot analysis was performed as described previously [14]. The samples were loaded to 10% SDS-PAGE gels and were separated at 120 V for 90 min. The blots were incubated with an anti-MMP-9 antibody diluted at a ratio of 1:800 overnight at 4°C. The membranes were stripped and rebotted with anti-actin antibody (Sigma) to verify the equal loading of protein in each lane.

2.7. Statistical analysis

Data are expressed as mean ± S.D. Statistical comparison was performed using one-way ANOVA followed by the Fisher’s exact test. Significant differences between the groups were determined using the unpaired Student’s t-test. P < 0.05 was considered statistically significant.

3. Results and discussion

The molecular formula of phytochemical compound derived from *n*-BuOH fraction of the *R. sacra* methanolic extracts was deduced as C₁₇H₁₆O₃ based on the fragmentation [M+H]+ peak at m/z 284.1049 (C₁₇H₁₆O₃ m/z: 284.1049, Δ0.1 mmu) on FAB-MS.
The EI-MS spectrum (Shimadzu QP-5050A, Tokyo, Japan) yielded a molecular ion peak at m/z 284, and other prominent fragments at m/z 180, 163, 134, and 78. Infrared spectrum (Spectrum 2000 FT-IR, PerkinElmer, USA) of this compound exhibited the characteristic absorptions associated with hydroxyl (3289 cm\(^{-1}\)), ester (1719 cm\(^{-1}\)), and aromatic (1648, 1512 cm\(^{-1}\)) groups. Furthermore, it was confirmed by the following NMR spectra (1H NMR, 400 MHz, DMSO-\(d_6\)/13C NMR, 100 MHz, DMSO-\(d_6\); JEOL JNM ECP-400, Tokyo, Japan). As shown in Table 1, the phenolic compound was elucidated as 3-(3,4-dihydroxy-phenyl)-acrylic acid phenethyl ester (caffeic acid phenethyl ester), and protective effect of \(R.\ sacra\) CAPE against LPS-induced inflammatory responses was further studied in mice. Septic shock-induced morphological changes were examined in various organs. Massive congestion and cellular infiltration in pulmonary interstitium (Fig. 1) and considerable hemorrhage in renal medullae were observed. All these histological changes were prevented by pretreatment of CAPE before LPS injection (c and f). Lungs and kidneys were removed 10 min after LPS injection, fixed in 10% formalin, and hematoxylin and eosin (200×).

### Table 1

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Chemical shifts are referred to TMS. Multiplicities are indicated by usual symbols.

\(^a\) Coupling constants (Hz) are in parentheses.

![Diagram](image.png)
prior to LPS injection. The levels of TNF-α and IL-1β in serum at 1 h after injection were increased by LPS injection. In comparison to the saline-treated group, *R. sacra* CAPE (1 mg kg\(^{-1}\)) significantly decreased the concentration of TNF-α and IL-1β in mouse sera (Fig. 2).

We next examined the effects of CAPE on LPS-induced NF-κB activation. The induction of proinflammatory gene expression by bacterial LPS plays a crucial role in inducing septic shock with systemic Gram-negative bacterial infections, a major cause of morbidity and mortality. The transcription factor NF-κB is thought to be one of the central mediators of this process. The transcription factor NF-κB was observed to be ubiquitously expressed. Activation of NF-κB can occur through activation of the IkB kinase-2 (IKK), which promotes IkB degradation [15], and also through phosphoinositide-3 kinase-dependent phosphorylation of the transactivation domain of p65 [16]. NF-κB regulates the expression of proinflammatory cytokines, chemokines, enzymes that generate mediators of inflammation, immune receptors, and adhesion molecules, all of which play a key part in the initiation of inflammatory reactions. The inducers of NF-κB activation include proinflammatory cytokines, growth factors, microbial infections, endotoxin, and oxidant stress [17,18]. NF-κB plays a key role in the induction of these cytokines in vivo. The efficacy of CAPE in inhibiting NF-κB activation and proinflammatory production has been reported by Natarajan et al. [9] and Fitzpatrick et al. [19]. Injection of LPS resulted in NF-κB activation at 1 h after LPS injection.

The NF-κB binding activity levels detected in nuclear lung extract of LPS-induced mice were much higher than those found in nuclear extracts from saline control mice. Pretreatment of mice with CAPE significantly reduced the enhancement of LPS-induced NF-κB binding activity in lung (Fig. 3). The presence of NF-κB

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**Fig. 2.** Serum levels of TNF-a and IL-1β in LPS-induced mice. A pretreatment with *R. sacra* CAPE was performed as described in Fig. 1. Blood was collected and serum analyzed for TNF-α (A) and IL-1β (B) by ELISA. (A) Animals treated with LPS produced 910 ± 85 pg ml\(^{-1}\) of TNF-α. Pretreatment with CAPE caused a 54% reduction in LPS-induced TNF-α production (A). LPS-induced mice exhibited significantly increased IL-1β concentration (480 ± 35 pg ml\(^{-1}\)) in sera compared to saline control mice. CAPE (180 ± 12 pg ml\(^{-1}\)) administration significantly reduced the increase in IL-1β production in sera (B). Data represent means ± S.E.M. from 5 independent experiments. *P* < 0.05 vs. LPS.

**Fig. 3.** *R. sacra* CAPE inhibits LPS-induced NF-κB activation. Pretreatment with CAPE was performed as described in Fig. 1. Nuclear extracts from the lungs 20 min after LPS injection (100 mg/mouse) were incubated with a labeled κB oligonucleotide and electrophoresed on a 5% polyacrylamide gel. A 200-fold excess of κB or an irrelevant oligonucleotide (cyclic AMP response element, CRE) was added as competitor. Supershift assay was conducted with anti-p65 antibody. A representative of five independent experiments is shown.

**Fig. 4.** Effects of *R. sacra* CAPE administration on the expression of MMP-9 in the lungs of LPS-induced mice. LPS injection and pretreatment with CAPE were performed as described in Fig. 1. The cell extracts were prepared from the lungs of the experimental mice 6 h after challenge as well as from the lung tissue of a naive control mouse. The extracts were subjected to immunoblot analysis with antibodies to MMP-9; the blots were reprobed with antibodies to actin to confirm the consistent application of samples (A), densitometric analyses were also performed (B). Gelatin zymography of MMP-9 (C). Data represent means ± S.E.M. from 6 independent experiments. *P* < 0.05 vs. LPS.
components in the retarded complexes generated with the κB probe was evidenced by the supershift analysis using an antiserum against the p65 NF-κB subunit.

Significantly increased MMP activity has been observed in a variety of inflammatory disorders such as rheumatoid arthritis, multiple sclerosis [20] and in bacterial sepsis [21], implicating a role for the MMPs in the tissue injury that accompanies these diseases. Previous studies have demonstrated that MMP-9 deficiency protects against morbidity and mortality in endotoxic shock model in mice, and specific inhibition of MMP-9 was suggested as a possible new therapeutic approach for the sepsis and septic shock syndromes [22]. Therefore, we evaluated the activities of CAPE on LPS-induced MMP-9 expression in mice lung. The administration of CAPE significantly attenuated the increase in expression of MMP-9 protein in lung tissues (Fig. 4A and B). Also the administration of CAPE reduced the increased expression MMP-9 gelatinolytic activity (Fig. 4C).

These studies clearly suggest that CAPE derived from R. sacra treatment inhibits NF-κB activation and reduces the overexpression of cytokines involved in the acute inflammatory response. Our results provide further rationale for prospective clinical trials to determine whether R. sacra CAPE provides efficacy in a septic shock. Based on this observation, R. sacra CAPE represents a promising adjuvant agent for the treatment of septic shock and other inflammatory diseases. Furthermore, it is possible to provide a natural nutraceutical biomolecule with anti-inflammatory activity to protect immune disorder.

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References