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Original Article

Comparison of Anti-Inflammation Effects of Specimens Before and After the Oil Extraction of *Raphanus sativus* L. Seed in RAW 264.7 Macrophage Activated by LPS

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ABSTRACT

Raphanus sativus L. has been reported to have anti-inflammatory and anti-tumor activity. However, the anti-inflammatory effect and mechanism of action of the *Raphanus sativus* L. seeds (RSS) with or without oil are still unknown. This study was undertaken to investigate the in-vitro anti-inflammatory effect with or without oil in the RSS on RAW 264.7 cells stimulated by lipopolysaccharide (LPS). Results showed the suppressed LPS-induced secretion of pro-inflammatory mediators such as nitric oxide (NO), inflammatory cytokine (IL-6, TNF-α). Additionally, a decrease in protein expression of iNOS was observed, but nuclear translocation of NF-κB p65 was not inhibited. To elucidate the underlying mechanism of the anti-inflammatory effect of RSS, the involvement of mitogen-activated protein kinase (MAPK) signaling pathways was examined. We also found that RSS blocked LPS-induced phosphorylation of c-Jun N-terminal kinase/stress-activated protein kinase (JNK) signaling but did not affect the phosphorylation of p38 MAPK and extracellular signal-regulated kinase (ERK) 1/2. These results suggest that RSS may have potential as an anti-inflammatory agent through the inhibition of LPS-induced inflammatory cytokine production via regulation of the JNK pathway.

Keywords *Raphanus sativus* L. seed, anti-inflammation, macrophage, NO

INTRODUCTION

Inflammation on is one of the defense mechanism of a host against cell damages incurred by pathogens, bacteria, and biological, chemical, and physically noxious stimulation, and is controlled through the activation of various immunocytes.[1] Macrophage are well-known as immunocyte involved with inflammation and are reported to induce inflammation by being activated by exposure to lipopolysaccharide (LPS).[2] Activated macrophages produce proinflammatory cytokines such as interleukin (IL)-1β, IL-6, and TNF-α, and generate various inflammation-mediated molecules such as nitric oxide (NO) and prostaglandin E2 (PGE2) through the expression of enzymes, including inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).[3,4] It is known that excessive generation of these molecule and continued inflammation contribute to the development of a wide range of chronic diseases such as autoimmune disorders, cardiovascular disorders, and cancer.[5]

Three independent pathways, namely, extracellular signal-regulated kinase1/2 (ERK1/2), p38 kinases (p38), and c-Jun NH2-terminal kinase (JNK) are known as mitogen-activated protein kinases (MARKs).[6] While inflammation is in progress, the expression of inflammatory cytokines is controlled by MARKs and nuclear factor kappa B (NF-κB) pathway.[7] NF-κB exists in inactivated form along with p50, p65, and inhibitory kappa B α (Iκ-Bα) in the cytoplasm. When stimulated by molecules such as LPS or cytokine, NF-κB dimer (p65/p50 subunits) translocate to the nucleus to accelerate gene expression related to immunity and inflammatory responses.[8]

Raphanus sativus L. (RS), a plant belonging to the Cruciferae family, is a vegetable cultivated and consumed widely throughout the world and has been used as a

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conventional herbal medicine for the treatment of digestion-related disorders such as indigestion, digestive inflammation, and abdominal disorders in Korea and China.[9] RS is equipped with various pharmacological activations including improvement of alcoholic fatty liver disorder and anti-tumor and anti-inflammation effects.[10] In addition, although there have been reports that the seeds of RSL have the effect of protecting mice from bacterial toxin-induced septicemic death by inhibiting the production of proinflammatory cytokines such as TNF-α, IL-6 and IFN-γ[11], there are insufficient reports on anti-inflammation in related to the presence and absence of the seed oil.

Although non-steroidal anti-inflammatory drugs (NSAIDs) are currently used widely to alleviate the pain and inflammation in a wide range of diseases related to acute and chronic inflammation conditions, side effects including indigestion, high blood pressure, and renal and cardiovascular abnormalities, etc. have also been reported.[12] Therefore, the discovery of natural substances that display anti-inflammation efficacy and can be substituted for the improvement of the efficacy of NSAIDs could lead to the development of better treatment drugs. Therefore, this study aims at the measurement of the generation of NO and proinflammatory cytokines (TNF-α, IL-6) and the disclosure of their mechanism of suppressing inflammation in RAW 264.7 cells stimulated by LPS to investigate anti-inflammatory efficacy in relation to the presence and absence of oil of RSS.

MATERIALS AND METHODS

Preparation of *Raphanus sativus* L seed

The *Raphanus sativus* L seed used in this study was purchased from CK pharm Co., Ltd. and was made into specimens A & B by simple pulverization and pulverization after oil extraction, respectively, at JBK Lab, Inc., for use in the experiments.

Cell culture

RAW 264.7 macrophage cell line was obtained from the Korea Cell Line Bank (Seoul, Korea) and cultured in the Dulbecco's modified Eagle's medium (DMEM, Welgene, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS, Welgene, Seoul, Korea) and 100 μg/mL penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a humidified atmosphere containing 5% CO2.

Cell viability assay

RAW 264.7 cells were seeded into 96-well plates at a density of $2x10^4$ cells/well and incubated at 37°C for 24 hr. The cultured cells were treated with *Raphanus sativus* L seed A or B at the indicated concentrations for 24 hr. After adding of WST-8 solution (Biomax Inc., Seoul, Korea) to each well, cells were incubated for 2 hr at 37°C. The absorbance at a wavelength of 450 nm was measured using a microplate reader (Thermo Scientific, Waltham, MA, USA).

Nitric oxide (NO) production assay

RAW 264.7 cells were seeded into 48-well plates at a density of $2x10^5$ cells/well and incubated at 37°C for 24 hr. Cells were stimulated with Lipopolysaccharide (LPS, Sigma-Aldrich, USA) for 24 hr in the absence or presence of *Raphanus sativus* L seed A or B. The levels of nitric oxide in the culture supernatant were determined using a nitric oxide (NO) detection kit (iNtRON BioTechnology, Korea) according to manufacturer's instruction.

Cytokine assays

RAW 264.7 cells were seeded into 48-well plates at a density of $5x10^4$ cells/well and incubated at 37°C for 24 hr. Cells were stimulated with LPS (1 μg/mL) for 24 hr in the absence or presence of *Raphanus sativus* L seed A or B. The levels of IL-6 and TNF-α in the culture supernatant were determined using a commercially available ELISA kit (Invitrogen, Carlsbad, CA, USA).

Western blot analysis

RAW 264.7 cells were seeded into 60mm dish at a density of 8x105 cells/dish and incubated at 37°C for 24 hr. Cells were stimulated with LPS (1 μg/mL) for 15 min in the absence or presence of *Raphanus sativus* L seed A or B. Total cell lysate were harvested using RIPA buffer containing protease inhibitor cocktail (Sigma-Aldrich), 1 mM phenylmethylsulfonyl fluoride, protease inhibitor, and phosphatase inhibitor cocktail set III (Sigma-Aldrich).

Cells were stimulated with LPS (1 μg/mL) for 1 hr in the absence or presence of *Raphanus sativus* L seed A or B. Nuclear extracts were obtained using a Nuclear Extract kit (Active Motif, Carlsbad, CA, USA), according to the manufacturer's recommendations. Proteins were electrophoresed using 10% SDS-PAGE and transferred to PVDF membranes (Amersham Biosciences, Piscataway, NJ, USA) and blocked with 5% skim milk in TBS/T buffer (Tris buffered saline in 0.1% Tween-20) for 1 hr. The membrane was incubated with specific antibodies against iNOS, COX-2, phosphate(p)- JNK, JNK, p-p38, p38, ERK, p-ERK, IκB, NF-κB (p65) (Cell Signaling Technology, Beverly, Ma, USA). Blots were incubated with HRP-conjugated secondary antibody. The blots were

developed using enhanced chemiluminescent (ECL) western blotting detection reagent (Amersham Biosciences).

Statistical analysis

All data were expressed as means ± SD. The data presented were one representative experiment of three independent experiments. One-way analysis of the variance (ANOVA) with Tukey's multiple comparison test was used to analyze the differences between the LPS treatment group and experimental groups. Differences with $p<0.05$ were considered statistically significant.

Result

Effect of RSS on RAW264.7 Cell Viability.

A RAW 264.7 cells were treated with various concentrations of RSS-A and RSS-B to examine the cytotoxicity, and cell viability was measured after 24 hours thereafter. As a result, there was no evidence of cytotoxicity in the concentration range of 50-500 μg/ml. Therefore, the maximum effective concentration was set at 500 μg/ml in this study (Table 1).

Table 1. Effect of RSS on RAW264.7 Cell Viability.

Dose (μg/ml)	RSS-A	RSS-B
0	100 ± 2.43	100 ± 2.43
50	136.05 ± 7.02***	125.56 ± 2.00***
100	156.77 ± 1.56***	131.62 ± 10.29***
200	175.39 ± 0.51***	161.24 ± 2.45***
400	165.45 ± 1.60***	151.50 ± 5.15***
500	148.09 ± 1.18***	129.37 ± 5.61***
800	54.31 ± 8.82***	88.23 ± 5.12
1000	24.39 ± 0.78***	47.03 ± 2.83***

Cells were treated with various concentrations of RSS-A or RSS-B for 24 hr, and then cell viability was measured by WST-8 assay. Values are expression as mean ± SD triplicate determinations. *** $p<0.001$ vs. vehicle-treated group

Effects of RSS on the NO production and iNOS and COX-2 expression in LPS-stimulated RAW 264.7 cells.

The production of NO was highly increased by LPS (1 μg/mL) up to 38-fold compared to that of the normalgroup; however, treatment with RSS-A and RSS-B significantly blocked the production of NO in a dose-dependent manner (Fig. 1). RSS-A and RSS-B inhibited NO production by 70% and 55%, respectively, at the highest concentrations of RSS-A and RSS-B. Next, we investigated the effects of RSS-A and RSS-B on the expression of iNOS, which synthesizes NO. As shown in Figure 2A, the expression of iNOS increased upon LPS stimulation. However, treatment with RSS-A and RSS-B greatly blocked the increase in a dose-dependent manner. The expression of COX-2 was slightly reduced treatment with RSS-A and RSS-B (Fig 2B). In particular, RSS-A decreased the expression of iNOS and COX-2 more than RSS-B at the same treatment concentration.

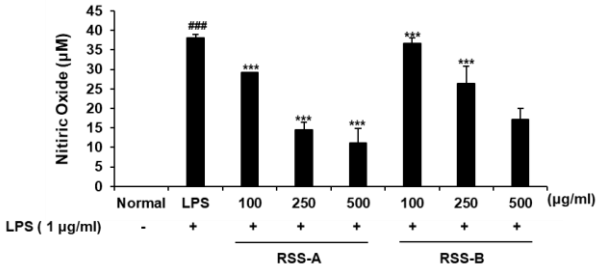

Fig 1. Effects of Raphanus sativus L seed on nitric oxide production in lipopolysaccharide (LPS)- stimulated RAW 264.7 cells. Cells were treated LPS (1μg/ml) in the absence or presence of Raphanus sativus L seed at different concentrations for 24 hr. The level of NO in the cell culture supernatant were measured by NO detection kit. Values shown in the graphs are mean ± SD of triplicate determinations. Statistical significance was analyzed by one-way ANOVA test. ###$p<0.001$ vs. vehicle-treated group. *** $p<0.001$ vs. LPS-treated group.

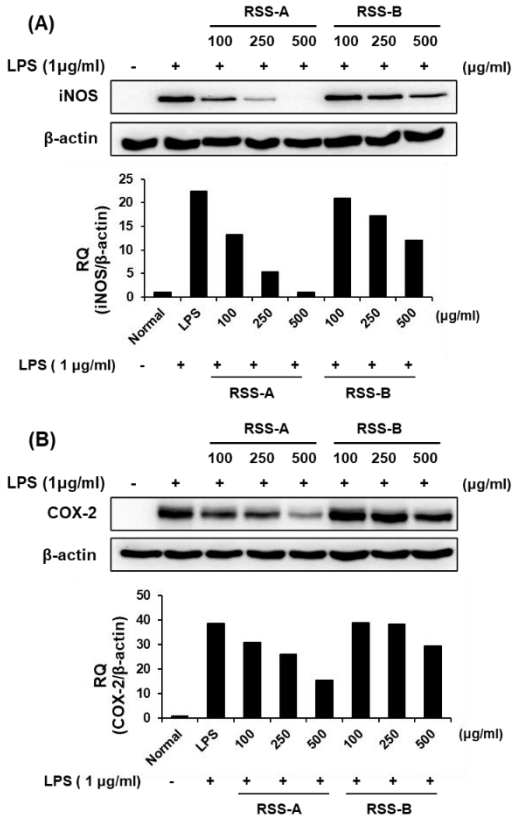

Fig 2. Effects of RSS on iNOS and COX-2 expression in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cells were treated with LPS (1 μg/ml) in the absence or presence of RSS-A or RSS-B at different concentrations for 24 hr. Expression of (A) iNOS and (B) COX-2 protein were determined by Western blot analysis. β-actin was used as loading control.

RSS inhibits TNF-α and IL-6 production in LPS-stimulated RAW 264.7 cells.

To investigate whether RSS-A and RSS-B suppress the production of proinflammatory cytokines, IL-6 and TNF-α were examined. LPS stimulation increased the level of IL-6 by approximately 8000 pg/ml, however treatment with RSS-A and RSS-B significantly inhibited the production of IL-6 in a dose-dependent manner. In particular, the production of IL-6 decreased by the low concentration treatment of RSS-A and RSS-B had a better effect with RSS-A (Fig. 3A). The level of TNF-α was greatly increased by the stimulants and suppressed by about 60% and 48%, respectively, at the highest concentrations of RSS-A and RSS-B (Fig. 3B).

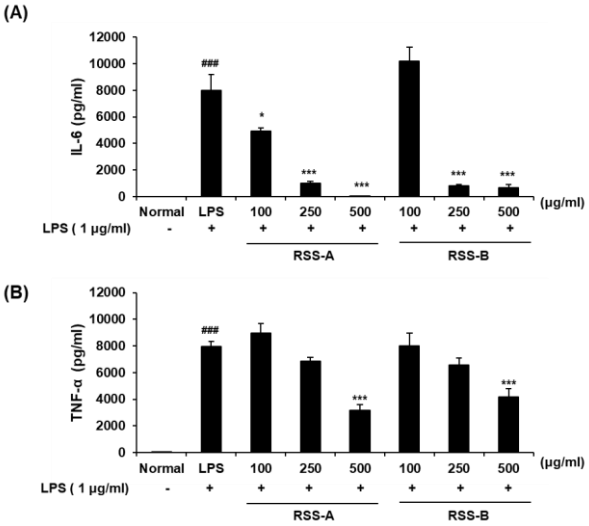

Fig 3. Effects of RSS on TNF-α and IL-6 production in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. (A-B) Cells were treated LPS (1μg/ml) in the absence or presence of Raphanus sativus L. (RS) seed at different concentrations for 24 hr. The level of TNF-α and IL-6 in the cell culture supernatant were measured by ELISA. Values shown in the graphs are mean ± SD of triplicate determinations. Statistical significance was analyzed by on-way ANOVA test. ###$p<0.001$ vs. vehicle-treated group. * $p<0.05$, *** $p<0.001$ vs. LPS-treated group.

Effect of RSS on the NF-κB and MAPK signaling pathway.

To examine the mechanism through which RSS-A and RSS-B inhibit the generation of proinflammatory cytokine, activation of ERK, p38, and JNK, which are NF-κB p65 and MAPKs, was checked. It is known that NF-κB is activated by LPS, and p65 accelerates gene expression related to inflammation by being translocated to the nucleus. As a result of western blot, although NF-κB p65 protein expression increased due to LPS treatment, there was no observation of change arising from treatment with RSS-A and RSS-B treatment (Fig 4). Phosphorylation of protein progresses when MAPKs are activated through LPS stimulation, and a western blot was carried out to confirm the effects of RSS-A and RSS-B treatment. As a result, when RAW 264.7 cells activated by LPS are treated with RSS, ERK and p38 was not inhibited, but phosphorylation of JNK was inhibited (Fig 5).

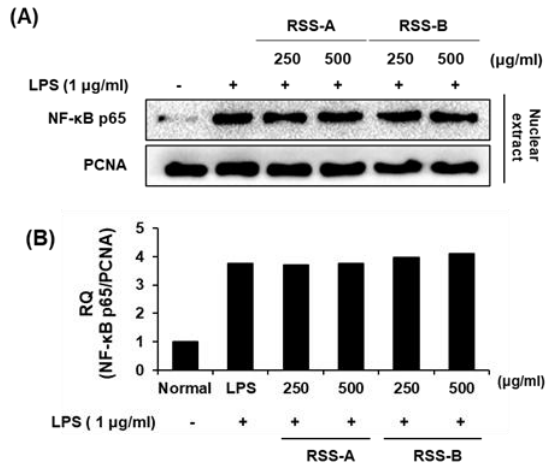

Fig 4. Effects of RSS on NF-κB p65 expression in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cells were treated with LPS (1 μg/ml) in the absence or presence of RSS-A or RSS-B at different concentrations for 1 hr. The expression of NF-κB p65 protein in the nuclear extract
was determine by western blot analysis. PCNA was used as loading control of nuclear extracts.

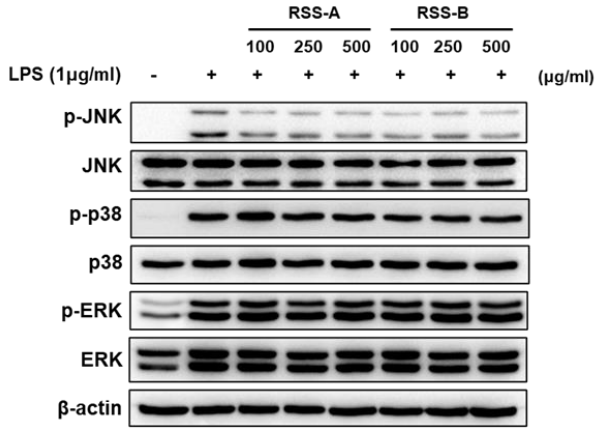

Fig 5. Effects of RSS on the phosphorylation of MAPKs in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Cells were treated with LPS (1 μg/ml) in the absence or presence of RSS-A or RSS-B at different concentrations for 0.5 hr. The MAPKs and phosphorylation of the MAPKs were detected by Western blot. β-actin was used as loading control.

Discussion

Although the leaves, seeds, and roots of RS are used extensively for the purposes of consumption and medical treatment, studies on RS were focused mainly on its roots[13]. Although anti-inflammation activation by vegetable oil obtained from rice bran, mustard, sesame seeds, sunflowers, and olives, among others, have been reported in numerous preceding studies[14], there are insufficient studies on the pharmacological anti-inflammation activation by oil extracted from RSS. Therefore, this study compared the activation of an anti-inflammation response in relation to the presence of oil from RSS. RAW 264.7 cell, which is a macrophage of the mouse, was treated with the specimen (RSS-A) and specimen (RSS-B), which are specimens prior to and after the extraction of oil from RSS, respectively, to select concentration that does not induce cytotoxicity by executing WST-8 (Table1). LPS promotes the generation of various inflammation factors in macrophages and it is deemed that substances that can inhibit such generation can be used beneficially in the treatment of various inflammation disorders associated with the activation of macrophages. Accordingly, an inflammation model using macrophages stimulated by LPS was also used in this study[15].

Treatment with RSS concentration-dependent manner inhibited the generation of NO, IL-6, and TNF-α by LPS (Fig 1-2). In addition, it was possible to confirm through western blot that treatment with RSS also decreases the protein level of iNOS (Fig. 3). In particular, it was confirmed that RSS-A prior to oil extraction show more dramatic effects in the reduction of generation of cytokine and NO, and the protein level of iNOS in comparison to that of RSS-B, the specimen following oil extraction. Then, the effects of RSS on the NF-κB pathway involved in inflammatory responses were examined. RSS did not impart any influence in nuclear translocation of NF-κB p65 by LPS (Fig. 4). MAPKs plays an important role in inflammatory responses such as cell proliferation, division and extinction, cytokine generation, along with the NF-κB pathway. Once MAPKs are activated due to LPS stimulation, the phosphorylation of protein proceeds, and the activated signaling pathway induces the various inflammation mediating factors such as proinflammatory cytokines and NO[16]. As a result of confirming the phosphorylation of MAPK with Western blot, the phosphorylation of JNK was reduced by RSS treatment. We suggest that RSS may have the potential to inhibit the production of various inflammatory mediators such as cytokines and NO through JNK regulation. In particular, *Raphanus sativus* L. (RS) seed extract prior to oil extraction had greater effects on the inhibition of NO or cytokine generation in comparison to that of the specimen

after oil extraction. It is deemed that these study results signify that oil from RSS is one of the key activation substances in inhibiting the inflammatory responses of macrophages stimulated by LPS and that RSS oil can be used as a functional substance in activating anti-inflammation response.

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CONFLICT OF INTEREST

The authors have no conflicting financial interests.

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