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In Vitro Anti-Inflammatory Activity of *Russula virescens* in the Macrophage like Cell Line RAW 264.7 Activated by Lipopolysaccharide

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Abstract

Four different concentrations of *Russula virescens* (RVE) (0.5, 1, 2.5 and 5 mg/ml) were extracted by using ethanol. The results showed that the RVE concentrations of 5 mg/ml in the presence of lipopolysaccharide (LPS) (2 μg/ml) affected cell viability. The amount of nitrite, a stable metabolite of nitric oxide (NO) was used as the indicator of NO production in the medium. During the 24 h incubation, nitrite was 0.9 μM in control. When LPS (2 μg/ml) was added, NO production was 2.7 μM whereas, LPS-induced NO production was reduced in 1~5 mg/ml RVE treated groups. The tumor necrosis factor- α (TNF-α) mRNA expression in the RAW 264.7 cell decreased in the 5 mg RVE treated sample. Expressions of Signal transducers and activators of transcription (STAT) 1, pSTAT1, STAT6 and pSTAT6 were decreased by the RVE treatment in a dose-dependent manner in the RAW 264.7 cell. All these results suggest that the ethanol-extract of the RVE mushroom is a potent immune modulator in the RAW 264.7 cell.

Keywords: *Russula virescens*; RAW 264.7 cell; Anti-inflammatory; Nitric oxide; TNF-α; STAT

Introduction

In Asian countries, numerous mushrooms have been utilized as alternative medicine to remedy cancer, inflammation and the enhancement of immunity. Many constituents have been isolated from the mushroom, the major ones being polysaccharides [1]. Other constituents include a small amount of heterogalactan protein, the ganoderan A, B, C protein complex, volvatoxin, flamotoxin, ganoderol, ganoderic acid, eritadenin, and some nucleotides [1]. Yuminamochi et al. [2] reported that the immuno-modulatory or antitumor activity associated with mushroom intake has been suggested to be due to a number of isolated fractions from mushrooms including the β-D-glucans and other polysaccharides. Over the past decade, numerous studies have demonstrated that polysaccharide β-glucans extracted from mushrooms exhibited beneficial therapeutic properties, including immuno-stimulation, anti-infection, anti-tumor, wound-healing and other therapeutic aspects. The mushroom β-glucans have been shown to stimulate the mononuclear phagocyte system by binding to the complement receptor CR3 and some lymphocyte to produce cytokines such as interferons (IFNs) and interleukins (ILs) [3]. Wasser [4] reported that mushroom polysaccharides are known to stimulate natural killer cells, T-cells, B-cells, and macrophage-dependent immune system responses. Kim et al. [5] also showed immuno-stimulating activity of purified endo-polysaccharides extracted. *Russula virescens* (RVE) is also a basidiomycete mushroom of the genus *Russula* that can be found growing in deciduous forests. It has rarely been used for medicinal preparations although numerous mushrooms have been used as foods and medicinal preparations. Moreover, the mechanism of the anti-inflammatory effects of RVE has not been reported. Thus, we investigated the effects of RVE on the immune system, especially at the cellular level, and demonstrated that it selectively activated macrophages in this study.

Materials and Methods

Preparation of the plant extract

Dried *Russula virescens* was purchased from commercial grocery. The air dried *Russula virescens* (80 g) was extracted with 70% ethanol (800 ml) for 3 hr. The extract was dried by a rotary evaporator under vacuum at 40°C and stored at –20°C until use.

Reagents

LPS was purchased from Sigma Chemical Co. (St, Louis, MO, USA), Dulbecco's Modified Eagle's medium (DMEM) medium, and 3- (4, 5 dimethythiazol2-yl) -2, 5-diphenytetrazoleum (MTT) were purchased from Wako (Tokyo, Japan). Fetal Bovine Serum (FBS) was purchased from Gibco (Gaithersburg, MD, USA). The antibiotics were purchased from Gibco-BRL (Rockville, MD, USA).

RAW 264.7 cell line and sample treatment

The murine macrophage cell line (RAW 264.7) was obtained from the Korea Cell bank (Seoul, Korea). The cells were cultured and maintained DMEM containing 10% heat- inactivated FBS, penicillin (100 units/ml), and streptomycin sulfate (100 units/ml) in a humidified atmosphere of 5% CO_2 at 37°C. The extract was dissolved in PBS and applied to the cell cultures at final concentrations of 0.5, 1, 2.5 and 5mg/ml alone or with 1 μg/ml of LPS.

Assessment of cell viability

Cytotoxicity studies were performed in 96-well plates. RAW 264.7 cells were mechanically scraped, plated at $2x10^5$/well in 96-well plates containing 100 μl of DMEM with 10% heat-inactivated FBS and incubated overnight. After overnight incubation, the cells were treated with teat materials, incubated. Cells were washed once before adding 50 μl of FBS-free medium containing 5 mg/ml of MTT. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue

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that formed in the cells was dissolved in 100 µl of DMSO. The optical density was measured at 540 nm.

Nitrite oxide determination

The nitrite accumulated in culture medium was measured as an indicator of NO production based on the Griess reaction. Briefly, 100 µl of cell culture medium was mixed with 100 µl of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl], incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured in a microplate reader. Fresh culture medium was used as the blank in all experiments. The amount of nitrite in the samples was measured with the sodium nitrite serial dilution standard curve.

Western blot analysis

Cellular proteins were extracted from control and *Russula virescens*-treated RAW264.7 cells. Cells were collected by centrifugation and washed once with phosphate buffered saline. The washed cell pellets were resuspended in extraction lysis buffer (Thermo scientific) and incubated for 30 min at 4°C. Cell debris was removed by microcentrifugation, followed by quick freezing of the supernatants. The cellular protein from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation on 8-12% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution at 4°C, followed by incubation for dilution of polyclonal antibodies against IFN-γ and TNF-α (Santa Cruz Biotechnology Inc.). Blots were incubated with a dilution of horseradish peroxidase conjugated goat-anti rabbit IgG secondary antibody (Santa Cruz Biotechnology Inc.). Blots were developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, USA).

RNA isolation and RT-PCR

To determine the expressions of TNF-α mRNAs, RT-PCR was performed. Total RNA was isolated from DSS-induced mice cells using RNAzol™ B (TEL-TEST, Friendswood, TX). Two micrograms of RNA, Master mix (10 µl of 5x QIAGEN One-Step RT-PCR Buffer, 2 µl dNTP Mix (containing 10 mM of each dNTP), 10 µl of 5x Q-Solution, Primer A, Primer B, 2 µl QIAEN One-Step RT-PCR Enzyme Mix, RNase inhibitor) and Template RNA were added to the reaction mixture. And the final volume was brought up to RNase-free water. Mix the master mix thoroughly, and dispense appropriate volumes into PCR tubes. After initial denaturation for 2 min at 95°C, 30 amplification cycles were performed for TNF-α. PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea): TNF-α forward strand 5'-AANGTTCCCAANATGGCCTCCCTCTCATC-3', reverse strand 5'-GGAGGTTGACTTTCTCCTGGTATGAGA-3'; β-actin forward strand 5'–TACAGGCTTGTCACTCGAANTT-3', reverse strand 5'–CCTAGAANGCATTTGCGGTGCACGATG-3'. After amplification, portions of the PCR reactions were electrophoreses on 2% Agarose gel and visualized by ethidium bromide staining and UV irradiation.

Statistical analysis

Data are reported as mean ± S. D. values of three independent determinations. All experiments were performed at least three times, each time with three or more independent observations. Statistical analysis was performed using Student's t-test with one-way analysis of variance.

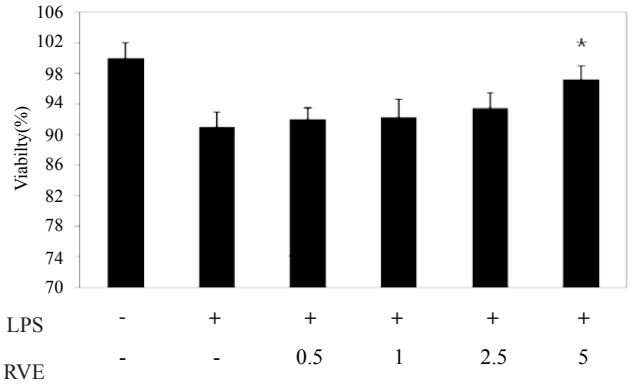

Figure 1: MTT assay of the ethanol-extract *Russula virescens* (RVE) with RAW 264.7 cell. Each cell was cultured in medium prepared with different concentrations (0.5-5.0 mg/ml) of the ethanol-extract for 24 hours.

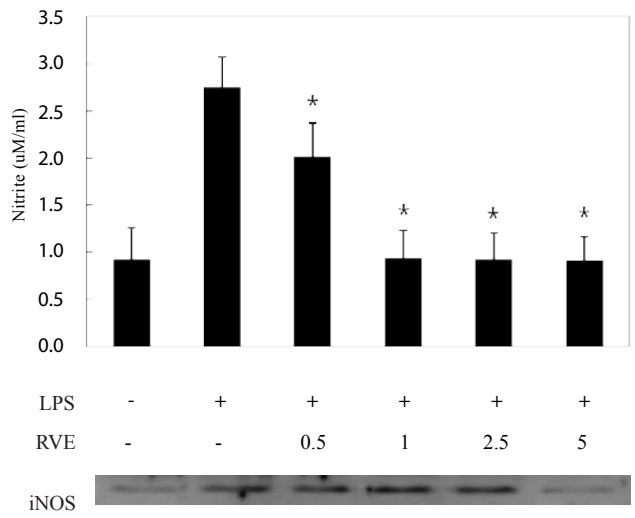

Figure 2: Concentration-dependent effects of *Russula virescens* (RVE) on NO production and iNOS protein in RAW 267.4 cells. The cells were left untreated or treated with LPS (1 g/ml) and RVE (0.5- 5.0 mg/ml) for 24 h, and the production of NO was evaluated by Griess reaction. Lysates were prepared from control or stimulated cells treated for 24 h with LPS (1 g/ml) alone or in combination with increasing concentrations (0.5- 5.0 mg/ml) of RVE. The experiment was repeated three times with similar results.

Results and Discussion

MTT assay of the ethanol-extract RVE with RAW 264.7 cell is shown in Figure 1. Four different concentrations of RVE were extracted by using ethanol. The results showed that the RVE concentrations of 5 mg/ml in the presence of LPS (2 µg/ml) affected cell viability. Inhibitory effects of *Russula virescens* extract on the nitrite in RAW 264.7 cell are shown in Figure 2. To assess the effects of *Russula virescens* on LPS-induced NO production in the RAW 264.7 cell, the cells were treated with LPS (2 µg/ml) and in the presence or absence of RVE (0.5, 1, 2.5, 5 mg/ml) for 24h. The amount of nitrite, a stable metabolite of NO, was used as the indicator of NO production in the medium.

During the 24 h incubation, nitrite was 0.9 µM in control. When LPS (2 µg/ml) was added, NO production was 2.7 µM whereas, LPS-induced NO production was reduced in 1~5 mg/ml RVE treated groups. Expressions of mRNA TNF-α and protein TNF-α in the RAW 264.7

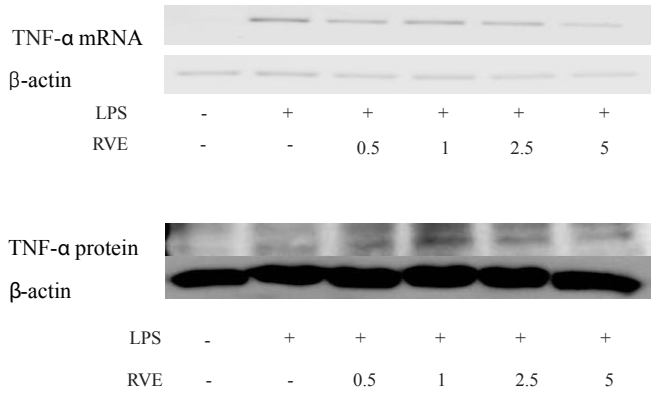

Figure 3: Concentration-dependent effects of *Russula virescens* (RVE) on LPS-induced TNF-α mRNA and protein expression in RAW 264.7 cells. Expression of mRNA level in macrophage treated for 0.5-5 mg/ml of RVE. RNA was extracted, and PCR was performed as described in the material and methods section.

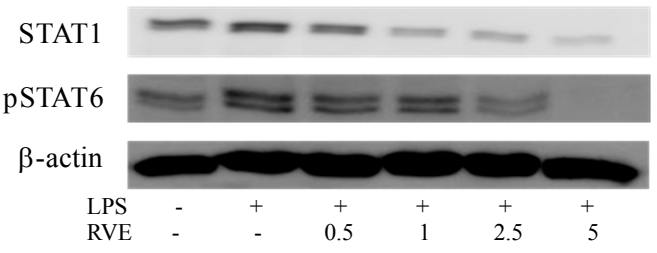

Figure 4: Concentration-dependent effects of RVE on LPS-induced STAT1 and Pstat1 proteins expression in RAW 264.7 cells. Lysates were prepared from control or stimulated cells treated for 24 h with LPS (1μg/ml) alone or in combination with increasing concentrations (0.5- 5.0 mg/ml) of RVE. The experiment was repeated three times with similar results.

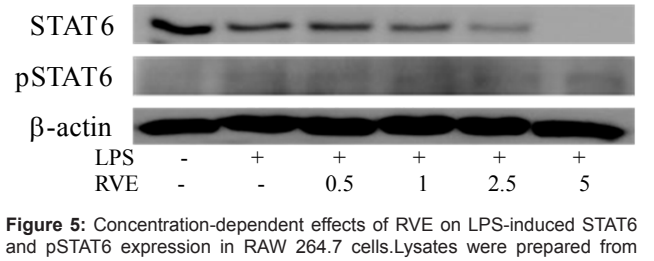

Figure 5: Concentration-dependent effects of RVE on LPS-induced STAT6 and pSTAT6 expression in RAW 264.7 cells.Lysates were prepared from control or stimulated cells treated for 24 h with LPS (1μg/ml) alone or in combination with increasing concentrations (0.5- 5.0 mg/ml) of RVE. The experiment was repeated three times with similar results.

cell are shown in Figure 3. The TNF-α mRNA expression in the RAW 264.7 cell decreased by RVE treatment in a dose-dependent manner. Expressions of STAT1, pSTAT1, STAT6 and pSTAT6 in the RAW 264.7 cells are shown in Figure 4 and 5. Expressions of STAT1, pSTAT1, STAT6 and pSTAT6 in the RAW 264.7 cell were also decreased by RVE treatment in a dose-dependent manner.

In the present study, we investigated the effects of RVE on the immune system, especially at the cellular level, and demonstrated that it selectively activated macrophages. NO play important roles in regulating inflammation and immune functions, and are formed by nitric oxide synthase; in inflammatory cells, nitric oxide is produced by an inducible isoform of nitric oxide synthase [6]. Excessive NO production has been closely associated with pathogenesis in several inflammatory diseases including septic shock, rheumatoid arthritis, and diabetes [7]. Because high concentrations of nitric oxide can be toxic, the inhibition of the overproduction of nitric oxide is an important goal [8]. In the present study, NO production was decreased in high RVE treatment samples (1, 2.5 and 5 mg/ml). This result indicates that RVE treatment can reduce NO production in the RAW 264.7 cell. Pacheco-sánchez et al. [7] also reported that polysaccharide extracted from *Collybia dryophila* treatment showed a down-regulation effect of NO production. Thus, we assume that RVE treatment could mitigate inflammation in the RAW 264.7 cell. TNF-α is not only a major inflammatory cytokine and a powerful anticancer cytokine but, also, TNF-α induces a pro-inflammatory response [9]. TNF-α has been the target of clinical investigations aimed at blocking its activity as a novel form of therapy [10]. In the present study, TNF-α was decreased by RVE treatment in a dose-dependent manner in the RAW 264.7 cell. This result indicates that RVE treatment can reduce inflammation in the RAW 264.7 cell. It is unclear by what mechanism the RVE treatment would reduced inflammation in the RAW 264.7 cell. However, we assume that RVE treatment can be a down-regulated TNF-α expression. This suppression effect may influence the inflammation. The STATs are a family of transcription factors that regulate a set of genes involved in the inflammatory response [11]. It has also been linked to susceptibility of several immune-mediated diseases including Graves' disease, systemic lupus erythematosus, and rheumatoid arthritis [12]. STAT6 plays a central role in exerting IL-4 mediated biological responses. IL-4 exerts the majority of its effect by signaling through STAT6 [13]. IL-4, through the STAT6 signaling pathway plays a crucial role in the down-regulation of proinflammatory cytokines such as TNF-α, IL-12 and possibly interferon gamma (IFN-γ), all of which have been implicated in intestinal mucosal tissue injury [14]. STAT1 was up-regulated in the peripheral blood of patients with sarcoidosis compared to healthy controls [12]. In the present study, RVE treatment down-regulated the STAT1, pSTAT1, STAT6 and pSTAT6 expressions. These results indicate that RVE suppresses the expression of STAT1, pSTAT1, STAT6 and pSTAT6, and consequently of STAT1, pSTAT1, STAT6 and pSTAT6-mediated gene expressions in the RAW 264.7 cell. The effect of down-regulation of these STAT proteins could influence anti-inflammation effects in RAW264.7 cells.

The best known of these is the immuno-modulatory effect of polysaccharides in mushroom belonging to the β-glucans [15]. Luhm et al. [16] reported that β (1→ 3) -D-Glucans from fungi were shown to be capable of having beneficial effects in pre-inflammatory responses, indicating that β-glucan can be a modulator of the anti-inflammatory response as interleukin mediators. In the present study, biological activities of RVE in the RAW 264.7 cell may be large influenced by β-glucan of RVE. Several studies suggested that the biological activities of polysaccharides would strongly depend on their structure, the degree of sulfation, the molecular weight, the sulfation pattern, and the glycosidic branches [17]. We do not exactly understand by what mechanism the RVE treatment would reduce inflammation in the RAW 264.7 cell. However, the possible explanation for these beneficial effects are that the mushrooms including RVE treatment may be associated with inflammatory cytokine such as TNF-α, and down-regulation of STATs or reduction of NO production. In conclusion, all these results suggest that the ethanol-extract of the RVE mushroom is a potent immune modulator in the RAW 264.7 cell. However, more research is needed to understand the effects of RVE treatment on anti-inflammation and how RVE relates to changes of cytokine, STAT and NO.

Acknowledgments

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