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Activation of macrophages by polysaccharide isolated from *Paecilomyces cicadae* through toll-like receptor 4

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ABSTRACT

Paecilomyces cicadae have been reported to have immunomodulatory properties. In this study, we investigated the effect of polysaccharide (PCP) isolated from *P. cicadae* on the macrophages. PCP increased the production of nitric oxide (NO) and the gene expression of IL-1β, IL-6, and TNF-α in RAW 264.7 cells. To investigate the membrane receptor, we examined the effect of PCP on primary macrophages isolated from wild type C3H/HeN and C3H/HeJ mice having mutant-TLR4. PCP induced NO production and cytokine gene expression in macrophages from C3H/HeN, but not from tlr4-mutated C3H/HeJ mice, which suggests that TLR4 is the membrane receptor for PCP. PCP induced the phosphorylation of ERK, JNK, and p38, and the nuclear translocation of NF-κB p50/p65, which are the main signaling molecules downstream from TLR4. Among them, p38 and NF-κB signaling played a crucial role in PCP-induced NO production by macrophages. These results indicate that PCP activates macrophages through the TLR4 signaling pathway.

1. Introduction

Macrophages play important roles in the host defense system against microbial infections, and they produce various inflammatory mediators, cytokines, and phagocytic activities (Gopinath et al., 2006). Stimulation of macrophages by lipopolysaccharide (LPS) enhances the production of mediators, such as nitric oxide (NO), reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). NO mediates a number of the host-defense functions of activated macrophages. NO is a highly reactive signaling molecule and inflammatory mediator that acts as a cytotoxic agent and modulates immune responses (Korhonen et al., 2005). For these cellular events, various stimuli bind to pattern recognition receptors (PRRs) such as Toll-like receptor (TLRs), dectin-1, and complement receptor type 3 (CR3) on the surface of macrophages, and then trigger several signaling pathways including tyrosine kinase, phosphoinositide-3-kinase (PI3 K)/Akt, mitogen-activated protein kinase (MAPKs), and nuclear factor-κB (NF-κB) (Kim et al., 2011a; Lee and Hong, 2011).

Cordyceps species are fungal parasites of insects that often exhibit a high degree of host specificity. *Cordyceps* has been utilized in Chinese herbal medicinal prescriptions for thousands of years. One of the most important traditional medicines was *Cordyceps military*. It contains many kinds of active components, such as cordycepin, polysaccharides, ergosterol, and mannitol, and has various pharmacological activities (Ng and Wang, 2005). *Cordyceps cicadae* Shing (Ascomycetes) is a parasitic fungus on the larvae of *Cicada flammata* Dist. It has several putative active functions, including treatment of malaria, palpitation relief, enhancement of blood aggregation, antitumor activity, and general improvement of health (Kiho et al., 1989; Ukai et al., 1983; Weng et al., 2002). *Paecilomyces cicadae* Miquel Samson is the anamorph of *C. cicadae* Shing, which is within the Clavicipitaceae family (Fukatsu et al., 1997). *P. cicadae* has several biological activities, including increase of in interferon (IFN)-γ production by Peyer's patch cells, phagocytosis by macrophages, spleen cell proliferation, and dendritic cell maturation (Kim et al., 2011b; Takano et al., 2005; Yang et al., 2008). However, the effect of *P. cicadae* on macrophages is still unknown.

In our previous study, we demonstrated that a PCP isolated from *P. cicadae* induced dendritic cell maturation through TLR4/

Abbreviations: BM-Mac, Bone marrow-derived macrophage; CR3, complement receptor; ERK, extracellular signal-regulated kinase; IκB, inhibits NF-κB; JNK, C-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; NO, nitric oxide; PCP, *Paecilomyces cicadae* polysaccharide; PMB, polymyxin B; PRRs, pattern recognition receptors; SEAP, secetory alkaline phosphatase; siRNA, small interfering RNAs; TLR, toll-like receptor.

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NF-κB signaling pathways. Here, we demonstrated that immunostimulatory activity of PCP on macrophages.

2. Materials and methods

2.1. Materials

Female C3H/HeN and C3H/HeJ mice (6–8 weeks old) were obtained from the Korea Research Institute of Bioscience and Biotechnology (Chungbuk, Korea). Mice were housed in specific pathogen-free conditions at 21–24 °C and 40–60% relative humidity under a 12-h light/dark cycle. All animals were acclimatized for at least 1 week prior to the experiments. All experimental procedures were approved by the Animal Experimentation Ethics Committee of Chungbuk National University. Antibodies against extracellular signal-regulated kinase (ERK), p38, and C-Jun N-terminal kinase (JNK) MAPKs, IκBα/β, and NF-κB p65 were purchased from Cell Signaling Technology (Beverly, MA, USA). Lipopolysaccharide (LPS), polymyxin B (PMB), propidium iodide (PI), ERK inhibitor (PD98059), JNK inhibitor (SP600125), p38 inhibitor (SB203580) and NF-κB inhibitor (PDTC) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Isolation of PCP from P. cicadae

P. cicadae 3L was kindly offered by Dr. Aiying Liu from the Institute of Fungus Resource, Guizhou University (Gzuifr, China). All strains were maintained on YMA (Difco Laboratories, Detroit, MI, USA) slants and subcultured every month. After the slants were incubated at 27 °C for 7 days, and then stored at 4 °C for stock. YM broth was used as a seed and fermentation medium and contained, per liter, 10 g of glucose, 3 g of malt extract, 5 g of proteose peptone, and 3 g of yeast extract. For flask cultures, all strains were grown on YMA medium in a Petri dish for 10 days. Cells were then collected and homogenized at 8400 g for 1 min using a high-speed blender (SMT Co., Chiryu, Japan). Ten milliliters of the homogenized cell suspension was inoculated into 500-ml baffled flasks containing 200 ml of YM medium and cultivated for 11 days on a shaking incubator (Vision Biotech, Incheon, Korea) at 27 °C and 140 rpm. For 5-L jar cultures, 5% (vol/vol) of the homogenized culture solution was added to a 5-L fermenter (stirred type; Kobio Tech, Seoul, Korea) containing 4750 L of fresh YM medium. The fermenter was equipped with four equally spaced Rushton disk turbines with six flat blades. The pH was initially set at 6.5 with 1 N HCl or NH_4OH but was not controlled thereafter. The dissolved oxygen tension was initially set at 100% saturation with a constant air flow rate (1.5 vessel volumes per minute) and was maintained at 20% or higher during cultivation by controlling the agitation rate (200–800 rpm). Cultivations were then performed for 120 h. To obtain the heat-stable, nonpolymeric extract of cultured mycelia, mycelial masses were obtained by continuous centrifugation (Hanil Science Industrial, Incheon) at 10,447*g*. The mycelia were washed three times with distilled water, suspended in distilled water, heated for 6 h at 121 °C, filtered, and lyophilized. Water-soluble extracts mainly contained polysaccharide and were termed PCP. No endotoxin was detected at concentrations of up to 100 μg/ml PCP as determined by the Limulus amoebocyte lysate test (catalog number 291–53101, Wako Pure Chemicals, Osaka, Japan).

2.3. Cell culture

Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were plated and incubated in the presence of 5% CO_2 at 37 °C. Bone marrow-derived macrophages (BM-Mac) were prepared as previously described with some modifications (Kim et al., 2011b). In brief, BM cells were flushed from femurs and tibias. After the red blood cells were lysed, whole BM cells (4×10^5 cells/ml) were cultured in 100-mm^2 culture dishes in 10 ml per dish of complete medium containing 10 ng/ml macrophage colony-stimulating factor (M-CSF) (R&D Systems, Minneapolis, MN, USA). On day 3, another 10 ml of fresh complete medium containing 10 ng/ml M-CSF was added, and half of the medium was changed on day 6. On day 8, non-adherent and loosely adherent macrophages were harvested by vigorous pipetting.

2.4. Nitrite quantification

Nitrite accumulation was used as an indicator of NO production in the medium as previously described (Han et al., 2001). Cells were plated at 5×10^5 cells/ml in 96-well culture plates and stimulated with PCP or LPS for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethyl-enediamine dihydrochloride and 2% phosphoric acid) and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, the concentration of nitrite was measured by O.D. at 540 nm.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRI™ Reagent (Molecular Research Center, Cincinnati, OH, USA). For reverse transcription–polymerase chain reaction, single-strand cDNA was synthesized from 2 μg of total RNA (Kim et al., 2010c). The primer sequences used were as follows: inducible nitric oxide synthase (iNOS), sense, 5′-CCT TCC GAA GTT TCT GGC AGC AGC-3′, antisense, 5′-GGC TGT CAG AGC CTC GTG GCT TTG G-3′; interleukin-6 (IL-6), sense, 5′-TGC TGG TGA CAA CCA CGG CC-3′, antisense, 5′-GTA CTC CAG AAG ACC AGA GG-3′; interleukin-1β (IL-1β, sense, 5′-ATG GCA ATG TTC CTG AAC TCA ACT-3′, antisense, 5′-CAG GAC AGG TAT AGA TTC TTT CCT TT-3′; tumor necrosis factor-α (TNF-α), sense, 5′-AGG TTC TGT CCC TTT CAC TCA CTG-3′, antisense, 5′-AGA GAA CCT GGG AGT CAA GGT A-3′; and β-actin, sense, 5′- TGG AAT CCT GTG GCA TCC ATG AAA C-3′, antisense 5′-TAA AAC GCA GCT CAG TAACAG TCC G- 3′. Polymerase chain reaction products were fractionated on 1% agarose gels and stained with 5 μg/ml ethidium bromide.

2.6. Western blots

Lysates were prepared from total cells or nuclear as previously described (Kim et al., 2007). Detergent-insoluble materials were removed, and equal amounts of protein were fractionated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to pure nitrocellulose membranes. Membranes were blocked with 5% skim milk in Tween 20 plus Tris-buffered saline for 1 h and then incubated with an appropriate dilution of primary antibody in 5% bovine serum albumin (in Tris-buffered saline containing Tween 20) for 2 h. Blots were incubated with biotinylated antibody for 1 h and further incubated with horseradish peroxidase–conjugated streptavidin for 1 h. Signals were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.7. Analyses of NF-κB activation—SEAP reporter assay

RAW 264.7 cells containing pNF-κB-SEAP-neomycin phosphotransferase (NPT), an NF-κB-dependent (secetory alkaline phosphatase (SEAP) reporter construct (Moon et al., 2001), were stimulated with PCP or LPS for 20 h. Aliquots of culture media were heated to 65 °C for 5 min and then reacted with 4-methylumbelliferyl phosphate (500 μM) in the dark. SEAP activity was measured as RFUs with emission at 449 nm and excitation at 360 nm (Roh et al., 2011).

2.8. Small interfering RNA (siRNA) preparation and transfection

siRNAs to mouse TLR4 consisting of 21 nucleotides were synthesized from Bioneer Co., (Daejeon, Korea). The GeneBank accession numbers for mouse TLR4 were NM 021297.1. siRNA duplexes with the following gene-specific sense sequences were used; TLR4 sequence 1, 5′-GAA UUG UAU CGC CUU CUU AdTdT-3′; TLR4 sequence 2, 5′-CUG GAU UUU CAG CAC UCU AdTdT-3′; TLR4 sequence 3, 5′-GUG CAA CAC CUG UAG AGA UdTdT-3′. The following negative control siRNA (Bioneer Co, Korea) was used; 5′-CCU ACG CCA CCA AUU UCG UdTdT-3′. The 100 nM siRNA were transfected using lipofectamin 2000 (Invitrogen, Carlsbad, CA, USA) into RAW 264.7 cells following the manufacturer's protocol. Cell were incubated at 37 °C in a CO_2 incubator for 48 h for gene knock-down.

2.9. Statistical analysis

Data represent the mean ± STD of more than three samples and all experiments were performed more than three times. Standard deviations (STD) were calculated using the Student's t-test and p values were calculated using ANOVA software (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Activation of macrophages by PCP

We previously reported that PCP could activate dendritic cells though TLR4 signaling (Kim et al., 2011b). In the present study, we investigated the effect of PCP on macrophage activation. Upon exposure to PCP, NO production by macrophages increased in a dose-dependent manner (Fig. 1). To ensure that the effect of PCP was not due to endotoxin contamination, PCP was treated with polymyxin B (PMB), which inhibits the biological effects of LPS by binding to the lipid A moiety. As shown in Fig. 1, PMB abolished LPS-induced NO production in RAW 264.7 cells, whereas it did not inhibit PCP-induced NO production. These results demonstrated that the PCP used in this study was free of LPS contamination.

To investigate whether the increase of NO production by PCP was due to the induction of iNOS gene expression, we examined the effect of PCP on mRNA expression of iNOS using RT-PCR. As shown in Fig. 2, the cytokine gene expression of iNOS was dose-dependently increased by PCP. In addition, PCP increased the gene