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Activation of macrophages by a laccase-polymerized polyphenol is dependent on phosphorylation of Rac1

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ABSTRACT

Various physiologically active effects of polymerized polyphenols have been reported. In this study, we synthesized a polymerized polyphenol (mL2a-pCA) by polymerizing caffeic acid using mutant *Agaricus brasiliensis* laccase and analyzed its physiological activity and mechanism of action. We found that mL2a-pCA induced morphological changes and the production of cytokines and chemokines in C3H/HeN mouse-derived resident peritoneal macrophages *in vitro*. The mechanisms of action of polymerized polyphenols on *in vitro* mouse resident peritoneal cells have not been characterized in detail previously. Herein, we report that the mL2a-pCA-induced production of interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1) in C3H/HeN mouse-derived resident peritoneal cells was inhibited by treatment with the Rac1 inhibitor NSC23766 trihydrochloride. In addition, we found that mL2a-pCA activated the phosphorylation Rac1. Taken together, the results show that mL2a-pCA induced macrophage activation via Rac1 phosphorylation-dependent pathways.

1. Introduction

Polyphenols and their derivatives reportedly have bioactive effects [1]: e.g. low molecular weight polyphenols show anti-inflammatory and antibacterial effects [2,3]. Many of these polyphenols are contained in natural plants and foods. Organisms such as plants have various polyphenol-oxidizing enzymes that act by polymerizing these polyphenols. These polymerized polyphenols, too, reportedly have bioactive effects, such as immunomodulatory effects on macrophages [4]. However, previous studies have never assessed the detailed molecular mechanisms of their activity in macrophages [5]. We revealed that the cold-water extract of *Agaricus brasiliensis* (AgCWE) exerts immunomodulatory effects and that polyphenol polymers are its major active component [6,7]. Therefore, in order to clarify the molecular mechanisms of the immunostimulatory effect of polymerized polyphenols on macrophages, we synthesized a high purity polymerized polyphenol, mL2a-pCA. We previously isolated laccase-encoding genes from *A. brasiliensis* cDNA [8]. Then, heat-stable mutant laccase (mutLac2a) was developed using a phylogeny-based design method. Recombinant mutLac2a was expressed in *Pichia pastoris* and was recovered from the supernatant [9].

Macrophages have varied functions and play an important role in both the induction and suppression of inflammation [10–13]. The activation of macrophages is of interest because of its role in the prevention of infections such as those by *Mycobacterium tuberculosis* [14]. RAS-related C3 botulinus toxin substrate 1 (Rac1), a Rho family GTPase, has been shown to regulate macrophage proliferation, cytokine production, migration, and morphological change [15–18].

Since macrophage-activating substances may be useful for preventing infections and developing vaccines, we aimed to clarify the effect of mL2a-pCA on macrophages. We investigated its activity by measuring cytokine and chemokine production in macrophages following mL2a-pCA treatment. The effect of mL2a-pCA on macrophage morphology was evaluated as well, and the molecular mechanism was analyzed using an inhibitor of Rac1 and the detection of the signal protein [19].

Abbreviations: CA, Caffeic acid; mL2a-pCA, mutLac2a-polymerized caffeic acid; p-Rac1, phospho-Rac1.

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2. Materials and methods

2.1. Animals

Male C3H/HeN mice were purchased from Japan SLC (Shizuoka, Japan). The mice were housed in a specific pathogen-free environment and were used at 6–10 weeks of age. All animal experiments were performed in accordance with the guidelines for laboratory animal experiments provided by the Tokyo University of Pharmacy and Life Sciences. Each experimental protocol was approved by the Committee for Laboratory Animal Experiments at Tokyo University of Pharmacy and Life Sciences (P17-46).

2.2. Synthesis of polymerized caffeic acid with mutLac2a

mL2a-pCA was synthesized by oxidative polymerization of caffeic acid (CA) catalyzed by mutLac2a. mutLac2a-catalyzed polymerization was achieved as follows: 45 mg of CA was dissolved in 50 mL of deionized water (DIW) and mixed with 1.2 mM $CuSO_4$ dissolved in DIW, 62.5 μg of mutLac2a dissolved in 12.5 mL of DIW, and 25 mL of 400 mM sodium phosphate buffer (pH 7.5). The mutLac2a solution was added to the CA solution and was incubated at room temperature for 24 h in the dark. After 24 h, the mixture was extensively dialyzed (MWCO: 50,000) against DIW for 2 days and then heated at 100 °C for 20 min to inactivate mutLac2a. Afterwards, the reaction solution was lyophilized. Samples were dissolved in dimethyl sulfoxide (20 mg/mL) and heated at 100 °C.

2.3. Cell preparation

Resident peritoneal macrophages were isolated from the peritoneal cavities by lavaging with 10 mL saline. After centrifugation, the cells were maintained in RPMI 1640 medium supplemented with 50 μg/mL gentamicin sulfate (Wako, Osaka, Japan) and 10% heat-inactivated fetal bovine serum (Biosera, Kansas City, MO, USA). The cells (225 μL) were then cultured in 96-well flat- (Sumitomo Bakelite Co., Ltd., Tokyo, Japan) or glass-bottomed plates (PerkinElmer, Inc., Waltham, MA, USA) at 37 °C for 24–48 h in a humidified atmosphere of 5% CO_2.

2.4. Macrophage morphological changes assay

To analyze morphological changes in macrophages, the cultured resident peritoneal macrophages (5×10^4 cells/well) were treated with a monomer polyphenol (CA), polymerized polyphenol (mL2a-pCA; 0–40 μg/mL), or lipopolysaccharides (LPS; 0–40 ng/mL; Sigma-Aldrich, St. Louis, MO, USA) in 96-well glass-bottomed plates for 24 h. The cells were stained as reported previously, with slight modifications [20]: After culture, the supernatant was removed, and the cells were fixed in 4% paraformaldehyde (Wako) for 15 min. After removal of paraformaldehyde, the cells were incubated with PBS containing 2 μg/mL DAPI (Santa Cruz Biotechnology, Dallas, TX, USA) and a 500-fold dilution of CellMask Deep Red plasma membrane stain (Thermo Fisher Scientific, Waltham, MA, USA) at room temperature for 1 h in the dark. The plate was washed twice and sealed with 200 μL of PBS. The plates were scanned, and images were collected with an Operetta all-in-one high contents imaging system (PerkinElmer) at 20× objective lens, with 5 fields of view/well. Images were then analyzed with Harmony Software (PerkinElmer). Morphological changes were determined by calculation of short length/long length or by cytoplasmic area (CellMask)/nucleus area (DAPI).

2.5. Cytokine and chemokine assay

For the cytokine and chemokine assay, culture supernatants were obtained from resident peritoneal macrophages stimulated with CA, mL2a-pCA, or LPS for 24 h and 48 h for MCP-1 and IL-6, respectively. The concentration of MCP-1 in the supernatants was determined using an OptEIA kit (BD Biosciences, Franklin Lakes, NJ, USA). IL-6 was determined using ELISA MAX Set (Biolegend, San Diego, CA, USA).

2.6. Rac1 inhibition assay

To analyze role of Rac1 in mL2a-pCA-stimulated macrophage activation, the Rac1 inhibitor NSC23766 trihydrochloride (300 μM; Wako) was added to the cell culture. After incubation for 24–48 h, the cells were harvested for further analyses.

2.7. Rac1 activation assay

Rac1 activation was assessed using western blotting, according to a previously reported method, but with slight modifications [21]. Briefly, resident peritoneal macrophages (5×10^4 cells) were incubated at 37 °C for 0, 5, 15, 30, or 60 min with mL2a-pCA (10 μg/mL) or LPS (10 ng/mL). After the incubation, the cells were lysed in SDS sample buffer (0.125 M tris-HCl (pH 6.8), 10% 2-mercaptethanol, 4% SDS, 10% sucrose, and 0.004% bromophenol blue) and boiled for 5 min. For SDS-PAGE, 10 μL of cell lysates were used. Cell lysates were separated by 12.5% SDS-PAGE in tris-glycine buffer (25 mM tris base, 0.192 M glycine, and 1% SDS). The proteins were transferred to nitrocellulose membranes at 100 V for 2 h in blotting buffer (25 mM tris-HCl (pH 8.3), 192 mM glycine, and 20% v/v methanol). The membranes were blocked with TBST (20 mM tris (pH 7.5), 150 mM NaCl, and 0.5% polyoxyethylene (20) sorbitan monolaurate) containing 1% bovine serum albumins (Sigma-Aldrich) at room temperature for 1 h and incubated with a 1:2500 dilution of phospho-Rac1 (p-Rac1; Cell Signaling Technology, Danvers, MA, USA) or Rac1 antibody (ThermoFisher Scientific) at room temperature for 1 h. After washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit IgG (Fc) for 1 h; the blot was developed using ImmunoStar Zeta (Wako). The signal intensity of the p-Rac1 band was analyzed by ImageJ software and quantified. The signal intensity at 0–60 min/0 min in each lane was calculated and evaluated as the phosphorylation activity of Rac1.

2.8. Statistical analysis

Data are expressed as means ± standard deviation ($n = 3$). At least 3 independent experiments were performed. The significance of the differences between the means was assessed using Student's t-test ($^{*}p < .05$; $^{**}p < .01$; $^{***}p < .001$).

3. Results

3.1. Activation of macrophages by mL2a-pCA

To confirm the immunomodulatory activity of mL2a-pCA, mouse resident peritoneal macrophages were stimulated with CA, mL2a-pCA, or LPS, and morphological changes were measured by the Operetta high-content analysis system. As shown in Fig. 1, mL2a-PCA induced morphological changes in macrophages. Next, we examined the effect of mL2a-PCA on the induction of cytokine and chemokine production by mouse resident peritoneal macrophages. As shown in Fig. 2, both IL-6 and MCP-1 production were significantly induced by mL2a-PCA. The optimal dose for MCP-1

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