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Agrocybe chaxingu polysaccharide prevent inflammation through the inhibition of COX-2 and NO production

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The inhibition of nitric oxide (NO) and cyclooxygenase-2 (COX-2) production is considered to be a promising approach to the treatment of various diseases, including inflammation and cancer. In this study, we examined the effects of the *Agrocybe chaxingu* β-glucan (polysaccharide) on lipopolysaccaride (LPS)-induced nitric oxide (NO) and cyclooxygenase-2 (COX-2) expression in murine macrophage Raw 264.7 cells as well as 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced ear edema in mice. The polysaccharide significantly inhibited ($P < 0.01$) LPS-induced iNOS and COX-2 expression levels in the cells. Furthermore, topical application of polysaccharide resulted in markedly inhibited ($P < 0.01$) TPA-induced ear edema in mice. These results suggest that this polysaccharide may be used for NO- and COX-2-related disorders such as inflammation and cancer. [BMB reports 2009; 42(12): 794-799]

INTRODUCTION

The inflammatory process involves the activation of monocytes and macrophages, which secrete inflammatory mediators including nitric oxide (NO). Inducible nitric oxide synthase (iNOS) is one of three enzymes which generate NO, and mediate many physiological events. It is well known that the overproduction of iNOS is associated with various human diseases, including inflammatory and neuronal disorders (1-8).

Prostaglandins (PGs) are potent proinflammatory mediators derived from arachidonic acid metabolism by cyclooxygenases (COXs) which play important roles in modulating a number of pathophysiological conditions, including inflammatory and allergic immune response (9). The two isoforms of COX enzymes have been well studied. COX-1 is constitutively expressed and plays an important role in maintaining the normal physiological function of cells. COX-2 is markedly induced by a number of stimuli, including cytokines, during the inflammatory response (10-12).

Lipopolysaccharide (LPS) is the main component of endotoxin and is formed by a phosphoglycolipid that is covalently linked to a hydrophilic heteropolysaccharide (13). LPS arrests macrophage proliferation and activates them to produce pro-inflammatory factors, which play important roles in the immune response (14, 15).

Previous studies demonstrated that 12-O-tetradecanoylphorbol-13-acetate (TPA) promotes mouse skin carcinogenesis, which is closely linked to inflammatory responses. These responses include the development of edema and hyperplasis, as well as the induction of pro-inflammatory cytokines, reactive oxygen species (ROS), COX-2 and iNOS expression. Therefore, suppressing the induction of COX-2 and iNOS expression is a new paradigm in the prevention of skin diseases including inflammation and cancer (16-23).

Mushrooms are commonly known as a medically-potent natural product and have been widely used in Asian countries for hundreds of years in the treatments of various diseases including cancer. Different mushrooms are reported to have a number of biological activities including anti-tumor, anti-bacterial, and anti-viral activities (24-26). In addition, they have been reported to have anti-inflammatory and protective effects on the livers of rats (27, 28). It is well established from *in vitro* and *in vivo* studies that the polysaccharides fraction of mushrooms are largely responsible for their anti-tumor efficacy (25). Also, other studies have shown that the molecular mechanism underlying the anti-tumor and anti-inflammatoty effects of some compounds derived from edible or traditional medicinal plants is the inhibition of COX-2 and iNOS expression (23).

In the present study, we examined the inhibitory effects of β-glucan (polysaccharide) isolated from the edible mushroom *Agrocybe chaxingu* on NO production and COX-2 expression in LPS-induced murine macrophage Raw 264.7 cells and

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TPA-induced ear edema in mice. Our results showed that this β-glucan efficiently protect against LPS- and/or TPA-induced cell damage *in vitro* and *in vivo*. Therefore, we suggest that this polysaccharide may be useful as a potential therapeutic agent for inflammatory skin diseases including cancer.

RESULTS AND DISCUSSION

Effects of polysaccharide on cell viability and LPS-induced NO production

For hundreds of years, mushrooms have been widely used as a tonic to promote health and longevity in Asian countries. Also, the pharmaceutical effects of natural products are well known and there is a continuous demand for health-aids and natural drugs in the increasingly aged population. A variety of oligosaccharides, including the β-glucans found in mushrooms, have anti-inflammatory effects. However, the effect of the β-glucan found in edible mushroom *Agrocybe chaxingu* (polysaccharide) on inflammation is unknown (Fig. 1A). Therefore, we examined the protective effect of the polysaccharide against inflammation *in vitro* and *in vivo* in this study.

Since NO level is important in the assessment of the extent of inflammation and cell viability, the inhibitory effects of polysaccharides on NO production levels and cell viability was investigated. To determine the cytotoxic effect of polysaccharide on Raw 264.7 cells, the cells were treated with various concentrations (10-100 μg/ml) of polysaccharide and incubated for 12 h. As shown in Fig. 1B, the polysaccharide did not affect cell viability at the various concentrations (10-100 μg/ml).

It is well known that NO plays a key role in the pathophysiology of many diseases. To determine the effects of polysaccharide on NO production, Raw 264.7 cells were incubated for 12 h with LPS (100 ng/ml) in the absence or presence of various (10-100 μg/ml) concentrations of polysaccharide. Cell culture media were then collected and nitrite levels were determined. The polysaccharide reduced NO production in a dose-dependent manner (Fig. 1C).

Effects of polysaccharide on LPS-induced iNOS and COX-2 expression levels in Raw 264.7 cells

LPS is the main component of endotoxin, arrests macrophage proliferation and activates them to produce pro-inflammatory factors (14, 15). Therefore, we examined the effect of polysaccharide on COX-2 and iNOS expression levels with LPS exposure. Raw 264.7 cells were incubated for 12 h with LPS (100 ng/ml) in the absence or presence of polysaccharide (10-50 μg/ml). Our results showed that the polysaccharide suppresses LPS-induced COX-2 and iNOS protein expression levels in a dose-dependent manner (Fig. 2). When the effects of polysaccharide on COX-2 and iNOS mRNA expression levels were further examined in LPS-induced cells by RT-PCR, the polysaccharides inhibited LPS-induced mRNA expression levels of COX-2 and iNOS in a dose-dependent manner as shown in Fig. 3. These results suggests that the inhibition of COX-2 and iNOS mRNA expression by polysaccharide was responsible for the inhibition of COX-2 and NO production.

COX-2 and iNOS proteins have been reported to be closely associated with cutaneous inflammation, cell proliferation and skin tumor promotion and these can be rapidly induced by pro-inflammatory mediators (23, 29, 30). Also, reports suggest

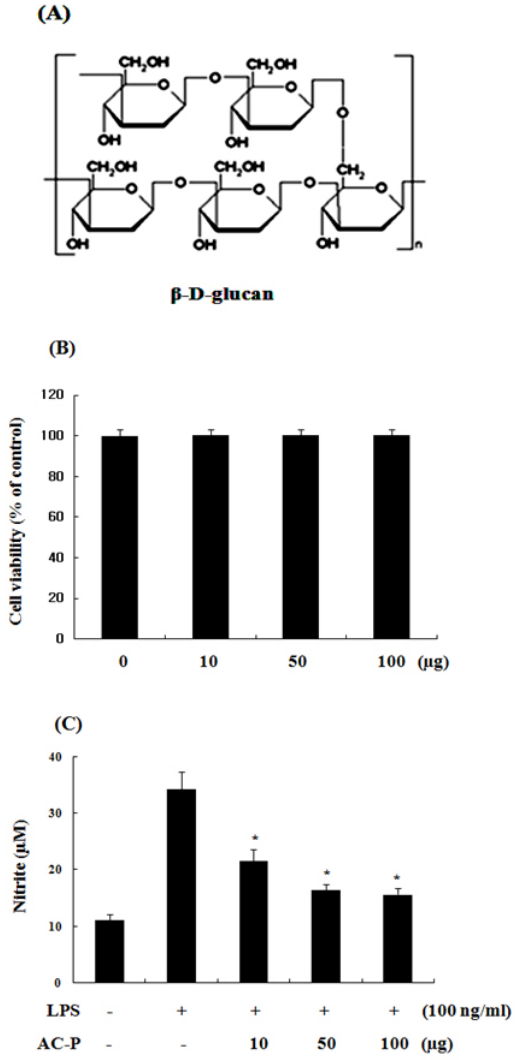

Fig. 1. Effects of polysaccharide on cell viability and LPS-induced NO production. The structure of the polysaccharide is depicted (A). Raw 264.7 cells were incubated with polysaccharide (10-100 μg/ml) for 12 h and cell viabilities were estimated by a colorimetric assay using MTT (B). Raw 264.7 cells were pretreated with polysaccharide (10-100 μg/ml) for 1 h before incubation with LPS (100 ng/ml) for 12 h. Nitrite levels were measured in the cultured media of LPS-stimulated cells by the Griess reaction (C). Each bar represents the mean ± SEM obtained from five experiments. *P < 0.01 compared with cells treated with LPS.

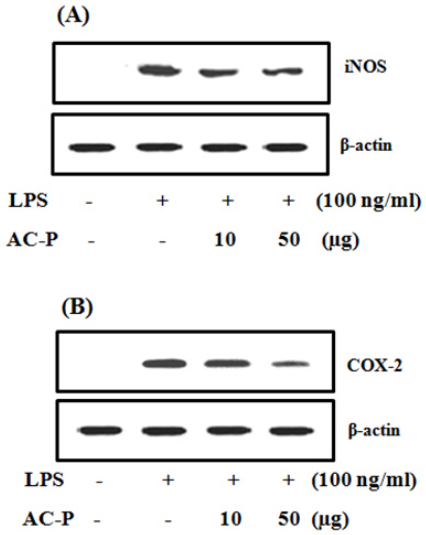

Fig. 2. Effect of polysaccharide on LPS-induced iNOS (A) and COX-2 (B) protein expression levels. Raw 264.7 cells were pretreated with polysaccharide for 1 h before incubation with LPS (100 ng/ml) for 12 h. Cells lysates were prepared and analyzed for iNOS and COX-2 protein expression levels by Western blotting.

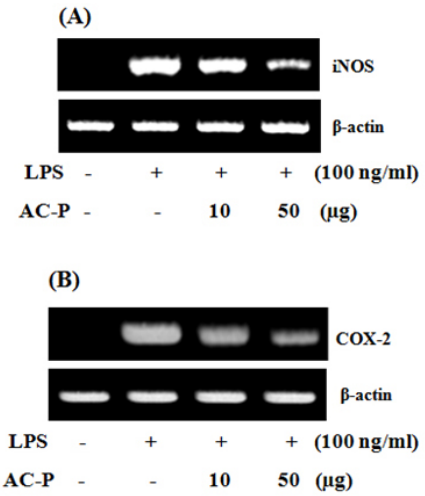

Fig. 3. Inhibitory effect of polysaccharide on LPS-induced iNOS (A) and COX-2 (B) mRNA levels in Raw 264.7 cells. The cells were pretreated with the polysaccharide for 1 h before incubation with LPS (100 ng/ml) for 12 h and total RNA was extracted. iNOS and COX-2 mRNA were analyzed by RT-PCR using specific primers.

that the inhibition of COX-2 and iNOS expression is important for alleviating inflammation as well as for the prevention of cancer (31, 32). Therefore, the inhibition of COX-2 and iNOS expressions may constitute an effective new therapeutic strategy for the treatment of inflammation and the prevention of inflammatory reactions and diseases.

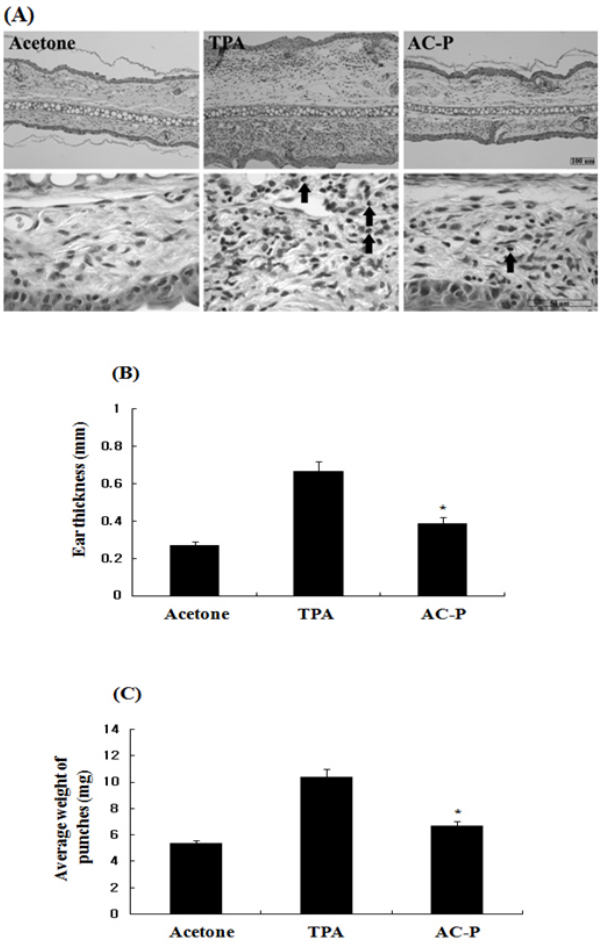

Fig. 4. Inhibitory effects of polysaccharide on TPA-induced ear edema. Mice ears were treated with TPA (1 μg) once a day for 2 days. The inhibition of TPA-induced ear edema by topical application of polysaccharide was analyzed by hematoxylin and eosin staining. Arrows indicate monocytes (A), measuring changes in ear thickness (B), and ear weight (C). *P < 0.01 compared with mice treated with TPA.

Effects of polysaccharide on TPA-induced ear edema in mice

Mouse ear edema induced by TPA has been commonly used as model for studies on inflammation. Multiple topical applications of TPA to mouse ears were shown to induce chronic types of skin inflammation consisting of edema, epidermal hyperplasia and infiltration of inflammatory cells (20, 33-35). Double application of TPA over 2 days to mouse ears induced inflammatory cell infiltration and increased ear thickness as well as ear weight. As shown in Fig. 4, topical application of polysaccharide to mice ears for 1 h after TPA application once a day for 2 days markedly inhibited TPA-induced increases in ear thickness and weight. In addition, polysaccharide inhibited

the infiltration of monocytes into the skin which represents one of the early steps in inflammation within the skin. In the TPA treated group, a number of monocytes were increased (7-fold) as compared with the control. However, application of the polysaccharide suppressed infiltration (2.3-fold). Although the exact mechanisms are still unclear and need to be further investigated, the polysaccharide efficiently suppressed TPA-induced ear edema.

In summary, we demonstrated for the first time that *Agrocybe chaxingu* polysaccharide can efficiently inhibit LPS- induced iNOS and COX-2 expression levels and cell death in Raw 264.7 cells. By assessing the protective effects of polysaccharide on TPA-induced skin inflammation, we have provided evidence of the topical anti-inflammatory effects of the polysaccharide. Although the detailed mechanism remains to be elucidated, the polysaccharide may be beneficial as a topical application against inflammatory skin disorders such as skin cancer.

MATERIALS AND METHODS

Materials

Fetal bovine serum (FBS), DMEM, and penicillin-streptomycin antibiotics were purchased from Gibco BRL (Grand Island, USA). Primary antibodies against COX-2, iNOS, and actin were purchased from the Santa Cruz Biotechnology company (Santa Cruz, CA, USA). *Agrocybe chaxingu* polysaccharide was kindly provided by Kangwon National University, Chunchon, Korea. All other chemicals and reagents were the highest analytical grade available.

Cell culture and MTT assay

The murine macrophage cells, Raw 264.7, were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES/NaOH (pH 7.4), 5 mM $NaHCO_3$, 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C under a humidified condition of 95% air and 5% CO_2.

The biological activity of the *Agrocybe chaxingu* polysaccharide was assessed by measuring the cell viability of Raw 264.7 cells treated with LPS (100 ng/ml) for 12 h. The cells were then seeded into a 6-well plate at 70% confluence. The cells were first pre-treated with polysaccharide (10-100 μg) for 1 h, followed by the LPS. Cell viability was estimated with a colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheyltetrazolium bromide (MTT) staining (36).

Determination of NO production

Raw 264.7 murine macrophage cells were incubated in 24-well plates at 70% confluence for 12 h. After incubation, the cells were pretreated with polysaccharide (10-100 μg) for 1 h before treatment with LPS (100 ng/ml) for 12 h, and the culture medium was harvested. NO production was determined by measuring nitrite, a metabolite of NO oxidation, as described previously (37). Briefly, 100 μl of cell culture medium was mixed with an equal volume of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) incubated at room temperature for 10 min, and then the absorbance at 540 nm was measured with a microplate reader. $NaNO_2$ was used as the standard.

Western blot analysis

Lysates from Raw 264.7 cells were prepared by incubating cells in a lysis buffer at 4°C for 30 min. The protein concentration was determined by Bio-Rad protein assay. Proteins (40 μg) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and then electrophoretically transferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat milk in Tris-buffered saline (TBS; 20 mM Tris, 0.2 M NaCl, pH 7.5) containing 0.05% Tween-20 (TBST) for 2 h and was then incubated for 1 h at room temperature with anti-COX-2, iNOS, and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1 : 500) in TBST. After washing, the membrane was incubated for 1 h with a proper secondary antibody conjugated to horseradish peroxidase diluted 1 : 10,000 in TBST. The membrane was incubated with a chemiluminescent substrate and exposed to Hyperfilm ECL (Amersham Biosciences, Piscataway, NJ, USA).

Determination of COX-2 and iNOS protein expression

Raw 264.7 murine macrophage cells were incubated in 24-well plates at 70% confluence for 12 h. After incubation, the cells were pretreated with polysaccharide (10-50 μg) for 1 h before treatment with LPS (100 ng/ml) for 12 h, and the culture medium was harvested. Then, the expression of COX-2 and iNOS protein levels were determined by Western blotting used anti-COX-2 and iNOS antibodies, respectively.

RT-PCR analysis

Total RNA was isolated from Raw 264.7 cells using a Trizol reagent kit (Invitrogen, Gaithersburg, MD, USA) according to the manufacturer's instructions (38). The RNA (2 μg) was reversibly transcribed with 10,000 U of reverse transcriptase and 0.5 μg/μl oligo-(dT) primer. PCR amplification of cDAN aliquots was performed with the following sense and antisense primers: COX-2 antisense, 5'-TGGACGAGGTTTTTCCACCAG-3'; sense, 5'-CAAAGGCCTCCATTGACCAGA-3'; beta-actin antisense, 5'-GGACAGTGAGGCCAGGATGG-3'; sense, 5'-AGTG TGACGTTGACATCCGTAAAGA-3'; iNOS antisense, 5'-CTGT CAGAGCCTCGTGGCTTT-3'; sense, 5'-ATGGCTCGGGATGT GGCTAC-3′. PCR was performed in 50 μl of 10 mM Tris-HCl (pH 8.3), 25 mM $MgCl_2$, 10 mM dNTP, 100 U of *Taq* DNA polymerase, and 0.1 μM of each primer and was terminated by heating at 70°C for 15 min. PCR products were resolved on a 1% agarose gel and visualized with UV light after ethidium bromide.

TPA-induced skin inflammation

Male 6-8 week old ICR mice were purchased from the Experi-

mental Animal Center, at Hallym University, Chunchon, Korea. The animals were housed at constant temperature (23°C) and relative humidity (60%) with a fixed 12-h light/12-h dark cycle and free access to food and water. The animals used in this experiment were treated according to the "Principles of Laboratory Animal Care" (NIH Publication No. 86-23).

Skin inflammation was induced in the right ear of each mouse by topical application of TPA. Five mice were in each group. TPA (1.0 μg) dissolved in 20 μl of aceton was applied to the inner and outer surfaces of the mice ears every 24 h for 2 days. Polysaccharide (300 μg) was topically applied to the ears of mice at 1 h after TPA treatment. 24 h after the last treatment with polysaccharide, the ear thickness was measured using a digital thickness gauge (Mitutoyo Corporation, Japan). Mice were sacrificed and ear biopsies were obtained with a punch (a diameter of 5 mm, Kai Industries Co. Ltd., Gifu, Japan) and weighed.

Histology

For histological analysis, ear biopsies were fixed in 10% (v/v) buffered formalin, embedded in paraffin, sectioned at 4 μm, and then stained with hematoxyline and eosin. Stained tissue sections were examined to analyze the infiltration of inflammatory cells using standard bright-field optics (Zeiss, AXIOIMAGER M1, Gottingen, Germany).

Statistical analysis

The results were expressed in mean ± S.E.M. from at least three independent experiments. The data were evaluated via one-way ANOVA, followed by Duncan's multiple range tests using GraphPad Prism 4.0 software (GraphPad Software Inc, San Diego, CA, USA). Differences were considered to be significant at $P < 0.05$.

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