Arch Pharm Res Vol 33, No 11, 1753-1760, 2010
DOI 10.1007/s12272-010-1107-3

Archives of Pharmacal Research
www.springer.com/12272

Mitogen Activated Protein Kinases are Prime Signalling Enzymes in Nitric Oxide Production Induced by Soluble β-Glucan from *Sparassis crispa*

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(Received June 20, 2010/Revised August 3, 2010/Accepted August 19, 2010)

Sparassis crispa (SC) is an edible mushroom that harbours β-glucans reported to possess immunostimulatory and anticancer properties. The role of SC in regulating the functional activation of macrophages is yet to be fully elucidated. The objective of this study was to investigate the molecular mechanism underlying the immune-stimulatory function of *Sparassis crispa* soluble β-glucan (Sc-SG) on macrophages. According to this study, Sc-SG was able to stimulate nitric oxide (NO) production as well as enhance the expression of inducible NO synthase (iNOS) from macrophage-like RAW264.7 cells. NO production was strongly suppressed by mitogen-activated protein kinase (MAPK) inhibitors such as U0126, extracellular signal-regulated kinase, SB203580, a p38 inhibitor, and SP600125, a c-Jun N-terminal kinase inhibitor. Thus, indicating that Sc-SG-induced NO release is possibly mediated by MAPK. Sc-SG induced phosphorylation of extracellular signal-regulated kinase, p38, and JNK in a time-dependent manner. Moreover, Sc-SG triggered the phosphorylation and translocation of c-Jun and c-Fos, components of the transcription factor AP-1, activated by MAPK. The results of this study suggest that MAPK may be a major signaling enzyme that regulates the Sc-SG-mediated NO production in macrophages.

Key words: *Sparassis crispa*, Immunomodulatory effects, Nitric oxide, Mitogen activated protein kinase, AP-1

INTRODUCTION

Macrophages constitute a major part in the fleet of immune cells providing anticancer, antibacterial and antiviral immune responses (Linton and Fazio, 2003). These cells are capable of directly or indirectly attacking the tumor cells, virus-infected cells and invaded bacteria by releasing cytotoxic molecules such as nitric oxide (NO) and reactive oxygen species (ROS), it also acts by engulfing target pathogens or infected cells. The activation of these cells therefore is considered as a potential means of enhancing body's defence mechanism. Considering that the thymus, an organ for T cell maturation and development, begins to atrophy with increasing age, the maintenance of immune defense system with the help of non-T cells such as macrophages should be compensated in this period. In view of this, macrophage-activating biomaterials are studied by researchers to develop immunostimulatory agents in the form of functional foods or food supplements.

Mushrooms are one among such food materials screened in many scientific studies for exploring its

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immunomodulatory potential. Some mushrooms such as *Lentinus edodes* (Shiitake) (Byeon et al., 2008) and *Inonotus obliquus* (Chaga) (Zjawiony, 2004) have also been studied for ethno-medicinal purposes to boost body's immune conditions. Traditionally, patients suffering from malignant diseases would maintain their anticancer defence response with the help of β-glucans (or polysaccharide fractions) obtained from medicinal mushrooms of Korea, China, and Japan. Various polysaccharide fractions such as PG101 (a water-soluble extract that consists of protein-bound polysaccharides), grifolan (an antitumor β-glucan), and PL (an acidic polysaccharide) have been identified as immunostimulating components (Choi et al., 2006) that function by activating the macrophages (Kodama et al., 2003; Jeong et al., 2008).

To our knowledge the exact mechanism behind β-glucan mediated activation of macrophages still remains elusive. Similar to other immunogens such as lipopolysaccharides, the activation of macrophages by these fraction is initiated following an interaction between surface receptors such as Toll-like receptors (TLR) and β-glucan, thus leading to subsequent upregulation of intracellular signaling of events mediated by enzymes such as Syk, phosphoinositide 3-kinases (PI3K) mitogen activated protein kinases (MAPKs) as well as transcription factors (e.g., nuclear factor [NF]-κB and activator protein [AP]-1). Consequently, the macrophages are compelled to express pro-inflammatory genes like inducible NO synthase (iNOS) and TNF-α (Burmester et al., 1997; Bresnihan, 1999; Gracie et al., 1999).

Although *Sparassis crispa* is an edible mushroom with medicinal properties in Korea and Japan, not many papers have reported the immunomodulatory roles of *Sparassis crispa* and its mechanism. Very few reports have suggested that this mushroom exhibits antitumor and antiallergic activities (Ohno et al., 2000; Yamamoto et al., 2009) and its major principles are identified as polysaccharide fractions composed of a backbone of β-(1→3)-linked D-glucopyranosyl residues with β-D-glucopyranosyl groups joined through O-6 and O-2 of D-glucose (Tada et al., 2007; Park et al., 2009). Interleukin (IL)-8, GM-CSF and interferon (IFN)-γ are reported to be produced by *Sparassis crispa* β-glucan (Harada et al., 2002; Nameda et al., 2003; Harada and Ohno, 2008). In addition, this mushroom has been also reported to contain some chemical constituents such as sequiterpenoids and benzoate derivatives that possess antifungal and antibacterial activities (Harada et al., 2005; Kodani et al., 2009). In this study, we explored the regulatory mechanism of *Sparassis crispa*-derived β-glucan in view of signaling cascade using macrophages to explain its immunostimulatory activity.

MATERIALS AND METHODS

Materials

Sc-SG was prepared by a method as reported previously (Park et al., 2009). Lipopolysaccharide (LPS, *E. coli* 0111:B4), hydroquinone (HQ), and (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) were purchased from Sigma Chemical Co. LY294002, SB203580 (SB), BAY11-7082 (Bay), and SP600125 (SP) were obtained from Calbiochem. Foetal bovine serum and RPMI1640 were obtained from GIBCO. RAW264.7 and U937 cells were purchased from ATCC. All the other chemicals were of Sigma grade. Phosphospecific or total antibodies to ATF-2, p65, c-Fos, c-Jun, CREB, extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), p38, Akt, and IκBα and antibodies to ERK, γ-tubulin, and β-actin were obtained from Cell Signaling. FITC-labeled dectin-1 antibody was purchased from Serotec.

Cell culture

RAW264.7, a murine macrophage cell line, was maintained in RPMI1640 supplemented with 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum. Cells were grown at 37°C and 5% CO_2 in humidified air.

The determination of NO production

After the preincubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, the cells were pretreated with Sc-SG (0 to 2500 μg/mL) for 30 min and were further incubated with LPS (1 μg/mL) for 24 h. The inhibitory effect of Sc-SG on NO production was determined by analyzing NO level using Griess reagents, as described previously (Cho et al., 2000).

Cell viability test

After the preincubation of RAW264.7 cells (1×10^6 cells/mL) for 18 h, Sc-SG (0 to 400 μg/mL) was added to the cells and incubated for 24 h. The effect of Sc-SG on the viability of RAW264.7 cells was then evaluated by a conventional MTT assay, as reported previously (Kim et al., 2010b).

Binding competition assay

Binding competition between dectin-1 antibody and Sc-SG was determined by flow cytometric analysis (Song et al., 2009; Lee et al., 2010). After incubation of RAW264.7 cells (1×10^6 cells/mL) with Sc-SG or curdlan, FITC-labeled dectin-1 antibody was treated to

the cells. The binding level of anti-dectin-1 antibody was determined with its fluorescence in cells on a FACScan (Becton Dickinson).

mRNA analysis by semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

To evaluate cytokine mRNA expression levels, total RNA was isolated from the LPS-treated RAW264.7 cells with TRIzol Reagent (Gibco BRL), as per the manufacturer's instructions. The total RNA was stored at −70°C until use. Semi-quantitative RT reactions were conducted as reported previously (Lee et al., 2009). The primers (Bioneer) used are indicated in Table I.

Western blotting (WB)

For total protein extraction: RAW264.7 cells were harvested, washed with cold PBS, and lysed as reported previously (Lee et al., 2008). Lysates were clarified by centrifugation at 16,000 × *g* for 10 min at 4°C. For nuclear protein extraction: Nuclear proteins were obtained through three steps. After Sc-SG treatment, cells were harvested and lysed in 500 μL of lysis buffer (50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, 20 μg/mL aprotinin, and 100 μM 1,4-dithiothreitol) on ice for 4 min. Cell lysates were centrifuged at 16,000 × *g* for 1 min at 4°C. In the second step, the pellet was washed with the washing buffer, which was the same as the lysis buffer but without Nonidet P-40. In the final step, the nuclei were incubated with extraction buffer (500 mM KCl, 10% glycerol, 10 mM HEPES, 300 mM NaCl, 0.1 mM 1,4-dithiothreitol, 0.1 mM PMSF, 2 μg/mL leupeptin, and 2 μg/mL aprotinin) and centrifuged at 16,000 × *g* for 5 min. Supernatant was collected as nuclear protein extract. Soluble cell lysates were immunoblotted and testing protein levels were visualized as previously reported (Kim et al., 2010a).

Table I. Sequences of primers used in RT-PCR analysis

Gene	Primer sequences
iNOS	F 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'
	R 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2	F 5'-CACTACATCCTGACCCACTT-3'
	R 5'-ATGCTCCTGCTTGAGTATGT-3'
GAPDH	F 5'-CACTCACGGCAAATTCAACGGCAC-3'
	R 5'-GACTCCACGACATACTCAGCAC-3'

ERK kinase assay

To determine the activation effects of Sc-SG or LPS on ERK activity, ERK prepared by immunoprecipitation (IP) with anti-ERK from RAW264.7 cells (5×10^6 cells/mL) was incubated with myelin basic protein (MBP), according to the manufacturer's instructions. The ERK kinase activity was determined with anti-phospho-MBP antibody after immunoblotting analysis.

Statistical analysis

The Student's *t*-test and one-way analysis of variance (ANOVA) were used to determine statistical significance. Data (Fig. 1 and 3) expressed as mean ± S.E.M. are calculated from at least three independent experiments performed in triplicate. The other data (Fig. 2 and 4) are repre sentative of three different experiments with similar results. $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The β-glucan obtained from *Sparassis crispa* exerts its immunostimulatory role by enhancing the production of IL-8, IFN-γ, and GM-CSF (Harada et al., 2002; Harada and Ohno, 2008). However, the exact mechanism of increase in cytokines and immune cell activation by the polysaccharides has not been fully understood till date. The results of this present study suggest that Sc-SG might be the major principle responsible for immunostimulatory property of *Sparassis crispa*. This study showed that Sc-SG significantly enhanced NO production in RAW264.7 cells up to 10 μM at 500 μg/mL, although the induction level of NO by Sc-SG was not higher than by LPS (Fig. 1A). Interestingly, higher concentrations (1 to 2 mg/mL) of Sc-SG suppressed NO release induced by LPS by 20%. Moreover, LPS-induced cell viability (35%) of RAW264.7 cells was abrogated by co-treatment of Sc-SG to an extent of 60 to 80% (Fig. 1B). It has also been observed that Sc-SG-mediated NO production was not due to endotoxin contamination. Thus, polymyxin B, a competitive binding inhibitor of LPS receptor molecule TLR4 (Espinoza et al., 1991), did not exhibit decrease in Sc-SG-mediated NO production (Fig. 1C). Similarly, the morphological change of RAW264.7 cells induced by LPS was not observed in Sc-SG treated group (Fig. 1D). Moreover, Sc-SG disrupted the binding of antibody of dectin-1, known as receptor of β-glucan (Fig. 1E) (Yadav and Schorey, 2006), indicating that dectin-1 acts as a receptor of Sc-SG, similar to other β-glucan (Cho et al., 2006; Kim et al., 2008).

To confirm whether NO production is linked to the induction of iNOS gene expression, mRNA level of the

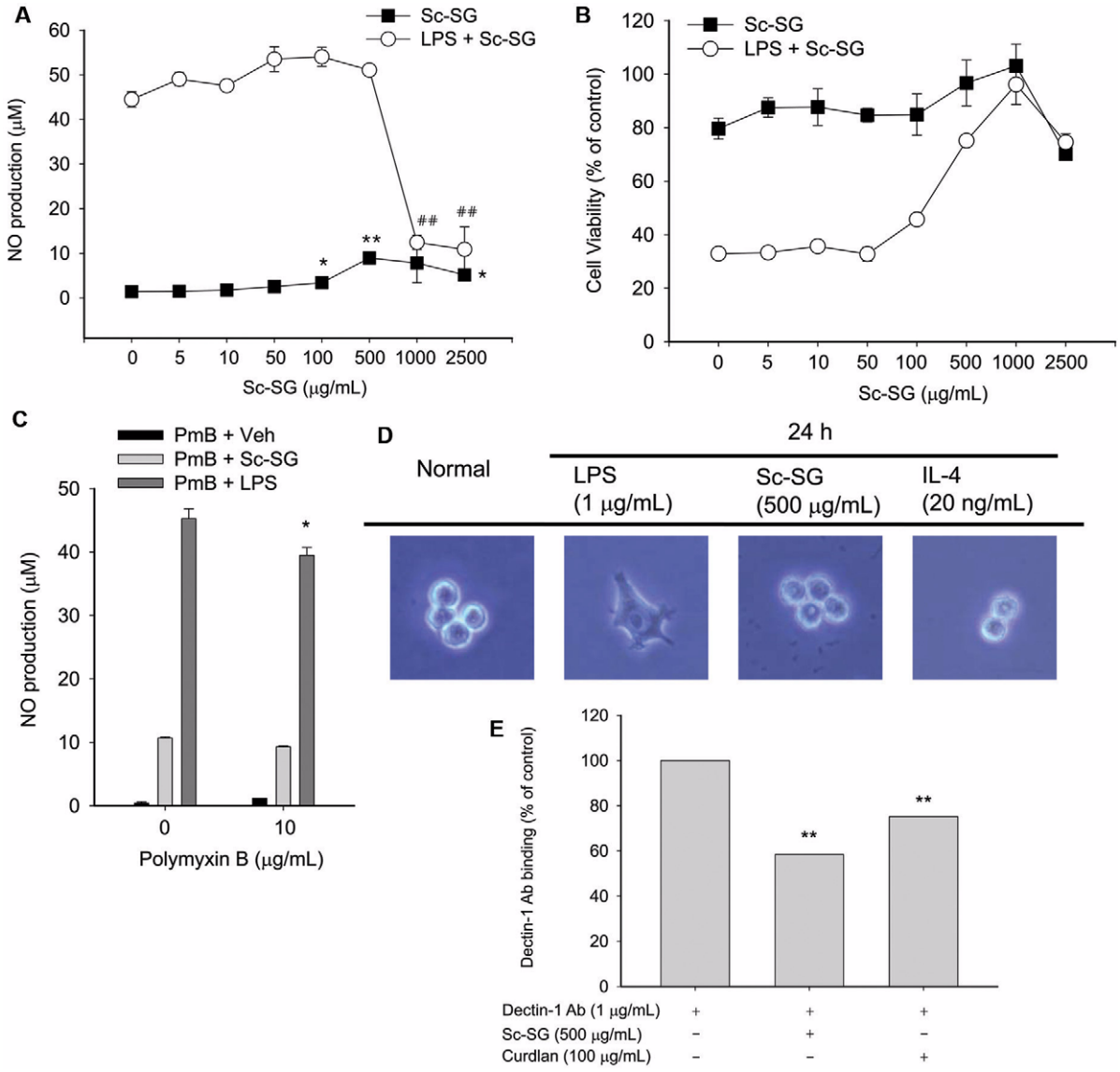

Fig. 1. Effect of Sc-SG on the activation of RAW264.7 cells. (**A**) The production level of NO was determined from culture supernatants of RAW264.7 cells treated with Sc-SG or LPS (1 μg/mL) for 24 h. (**B**) Cell viability was determined by MTT assay. (**C**) Endotoxin contamination was determined with polymixin B in the absence or presence of Sc-SG (500 μg/mL) or LPS. NO level was determined by Griess assay. (**D**) Morphological change induced by Sc-SG (500 μg/mL) or LPS (1 μg/mL) was checked by microscopic analysis. (**E**) Binding competition between Sc-SG to dectin-1 was determined by flow cytometric analysis with specific antibody to dectin-1. Data represent mean ± S.E.M. of three independent observations performed in triplicate. *$p < 0.05$ and **$p < 0.01$ compared to normal or dectin-1 Ab alone, and $^{\#\#}p < 0.01$ compared to LPS alone.

gene was determined by RT-PCR analysis. As in Fig. 2, Sc-SG induced the expression of iNOS but not COX-2, a PGE_2 producing enzyme, suggesting that Sc-SG can trigger transcriptional activation for NO production. Further to this, identification of the signalling enzymes that are responsible for the continuous iNOS gene expression and NO production by Sc-SG was elucidated in a step wise manner. First, two different types of enzyme inhibitors were employed, MAPK inhibitors (SB203580, SP600125, and U0126) for AP-1 activation and PI3K/Akt (LY294002, wortmannin) and IKK inhibitors (BAY 11-7082 and hydroquinone) (Kerzic et al., 2003) for NF-κB activation and their inhibitory activity was tested for Sc-SG-mediated NO production. Interestingly, ERK kinase inhibitor U0126 displayed the highest inhibitory effect and p38 inhibitor SB-203580 (SB), JNK inhibitor SP600125 (SP), and LY-294002 were also effective in significantly inhibiting the NO production without altering the viability of RAW264.7 cells (Fig. 3A and B). On the contrary,

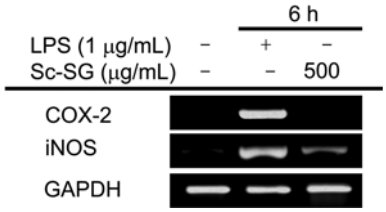

Fig. 2. Effect of Sc-SG on the mRNA expression of iNOS and COX-2. The mRNA levels of iNOS, COX-2, and GAPDH were determined by semi-quantitative RT-PCR. The results show one experiment out of three.

these MAPK inhibitors did not block LPS-induced NO production, indicating that the activation of MAPK could be distinct between dectin-1 and TLR4-mediated inflammatory signalling cascade.

Since NF-κB inhibitor hydroquinone and PI3K inhibitor wortmannin did not block NO production, the involvement of MAPK in Sc-SG was focused to be determined using Western blot analysis and ERK kinase assay. As Fig. 4A depicts, Sc-SG triggered the phosphorylation, indicative of activation, of ERK, JNK, and p38 from 5 min to 60 min. These phosphorylation patterns by Sc-SG was different from ones by LPS (Fig. 4A), indicating that the activation mechanisms are different. ERK kinase assay done with a commercially available kit also revealed its role during Sc-SG treatment. Thus, ERK kinase activity was enhanced by Sc-SG exposure and U0126 effectively blocked the kinase activity, as seen in the case of LPS (Fig. 4B).

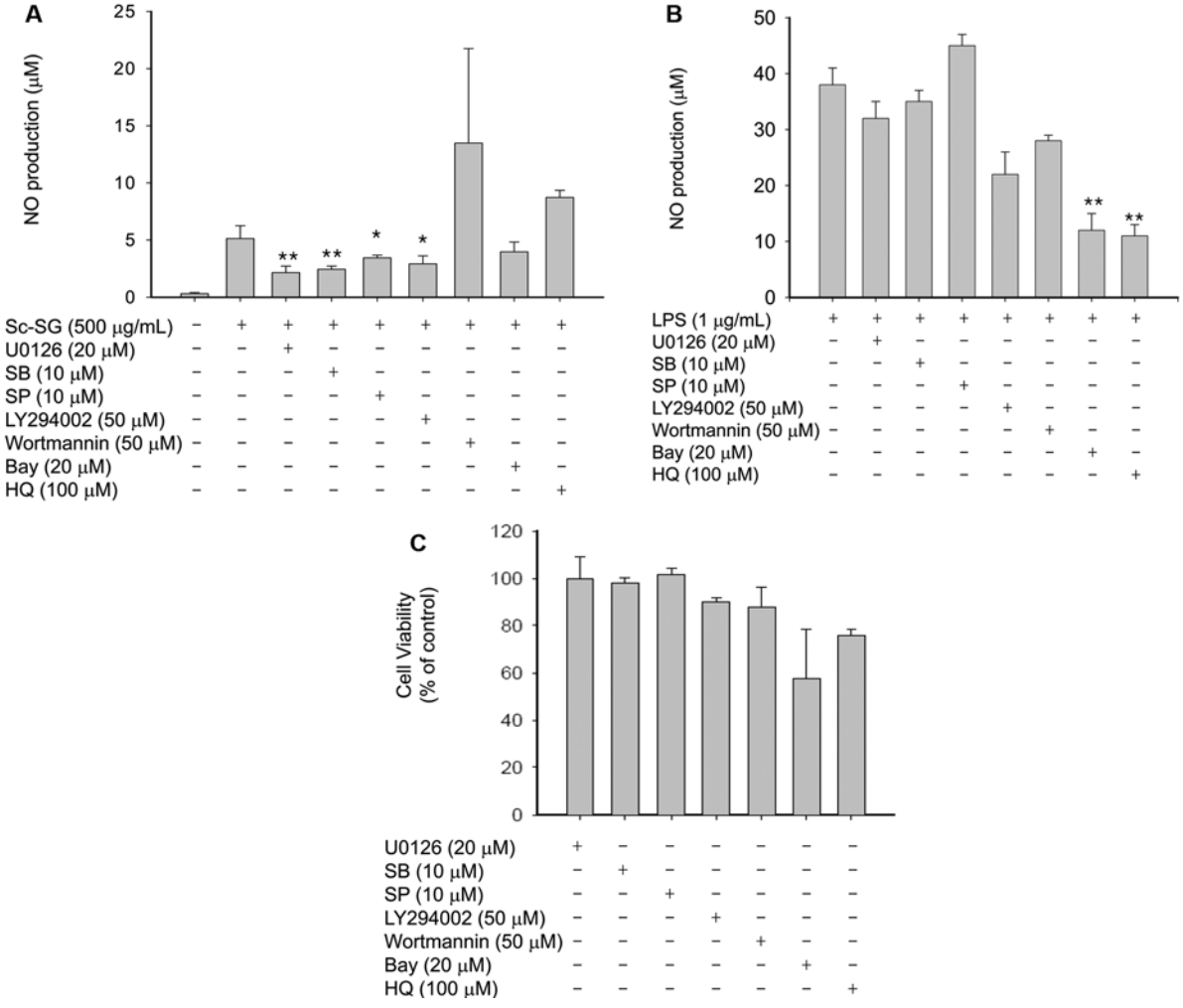

Fig. 3. The involvement of MAPK in Sc-SG-induced NO production in RAW264.7 cells. (**A**) RAW264.7 cells (1×10^6 cells/mL) were pre-treated by MAPK inhibitors [U0126 (20 μM), SB203580 (SB, 10 μM) and SP600125 (SP, 10 μM)], PI3K/Akt inhibitors [wortmannin (50 μM) and LY294002 (50 μM)] or NF-κB inhibitors [hydroquinone (HQ, 100 μM) and BAY 11-7082 (Bay, 20 μM)] in the presence of Sc-SG (500 μg/mL) for 24 h. NO level from the supernatants was determined by Griess assay. (**B**) Effect of inhibitors on the viability of RAW264.7 cells was determined by MTT assay. Data represent mean ± S.E.M. of three independent observations performed in triplicate. *$p < 0.05$ and **$p < 0.01$ compared to control.

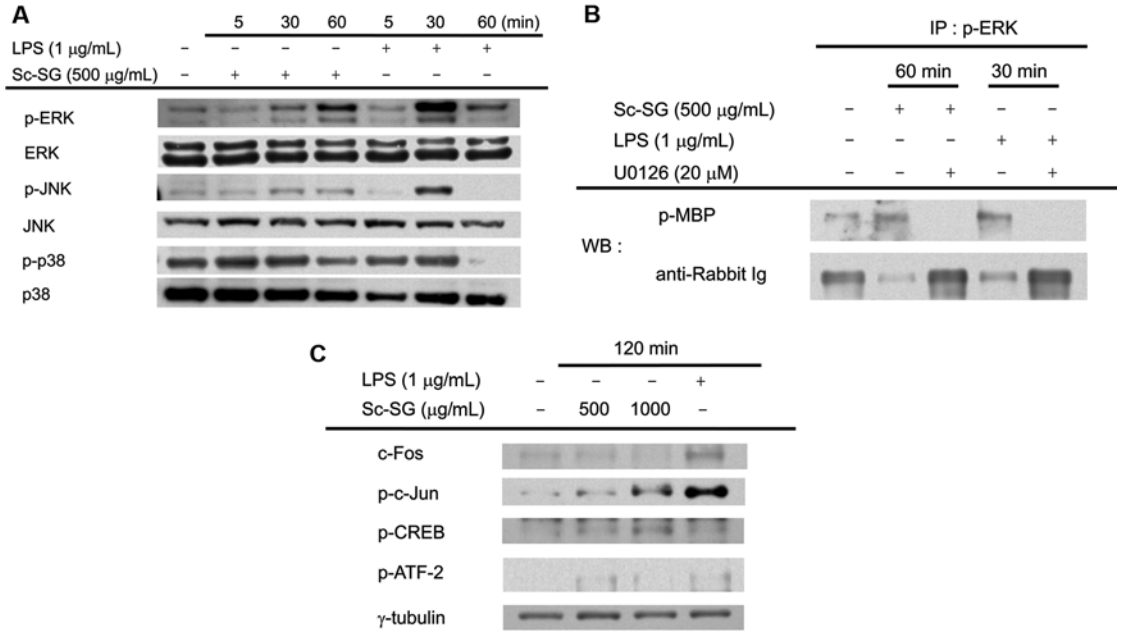

Fig. 4. Effect of Sc-SG on the phosphorylation of MAPK and translocation of AP-1. (**A**) RAW264.7 cells (5×10^6 cells/mL) were stimulated by Sc-SG (500 μg/mL) or LPS (1 μg/mL) for indicated times. After immunoblotting, the levels of phospho- or total ERK, p38, and JNK were identified by their antibodies. (**B**) The inhibitory effects of cafestol on immunoprecipitated ERK prepared from LPS-treated RAW264.7 cells were determined by measuring the level of phospho-MBP, as described in Materials and Methods. (**C**) The phospho- or total protein levels of p65, c-Jun, c-Fos, ATF-2, CREB, and γ-tubulin in nuclear fractions were determined by immunoblotting analysis with their phospho- or total protein antibodies. The results show one experiment out of three.

As MAPK activation is known to induce the translocation of AP-1, ATF-2, and CREB to the nucleus (Maynard and Ohh, 2007), nuclear levels of total or phospho-forms of c-Jun, c-Fos, CREB and ATF-2 were determined. As shown in Fig. 4C, phospho-forms of ATF-2, CREB, and c-Jun but not c-Fos were seen at 2 h, although the phosphorylation levels of these proteins were not remarkable when compared to LPS treatment. The lowered levels of these activation signals and transcription factor translocation seem to explain the reason for Sc-SG's ability to marginally increase (20%) the extent of NO production compared by LPS.

It was revealed that MAPK was not important in NO production from LPS-activated RAW264.7 cells (Fig. 3B), unlike Sc-SG stimulation (Fig. 3A). Most of evidence strongly supported different mechanisms involved in activation of macrophages between Sc-SG and LPS. Thus, LPS but not Sc-SG strongly altered the morphology of RAW264.7 cells (Fig. 1D) and the NO induction levels were distinct between these two stimuli. The expressional pattern of COX-2 was also different between LPS and Sc-SG (Fig. 2). These activation patterns seem to be as a result of interaction with different surface receptors, LPS to TLR4 or β-glucan to TLR2/dectin-1 (Vogel et al., 2001; Yadav and Schorey, 2006). It has been reported that MAPK is lined to TLR4 by interacting with TRAF6, MyD88, IRAK4, and TAK1 (Denkers et al., 2004), while the adaptor proteins associated with MAPK and TLR2/dectin1 are not clear yet. Therefore, further studies will be focused on dissecting the molecular activation mechanism for MAPK activity under Sc-SG treatment.

It is generally agreed that development of immunostimulatory biomaterials is essential for protecting the human body from various pathogenic infections and cancer formation (Indar and Maxwell-Armstrong, 2003). Appearance of new pathogens such as superbacteria that are antibiotic resistant and flu viruses evoke us to develop promising immunostimulators or adjuvant-functioning biomaterials for vaccination. In terms of these, β-glucans from various mushrooms could be considered as a good candidate for countering these inflammatory responses (Niu et al., 2009). Although the mechanisms by which these polysaccharides are able to up-regulate immune responses and ways by which MAPK is activated by Sc-SG were not fully investigated, the results of this study suggest a potentially important role of Sc-SG in MAPK-mediated activation of macrophages and its subsequent use could be applied as functional foods with immunostimulatory purpose. Additional role of Sc-SG as a strong immunoadjuvant without observable side effects should

further be examined using appropriate *in vivo* models.

In conclusion, this study showed that Sc-SG was able to increase NO production by up-regulating iNOS gene expression in macrophages. Sc-SG-induced macrophage activation was distinct from those by LPS as shown by morphological changes, induction levels and surface target molecules. Sc-SG-induced NO production was strikingly accompanied by MAPK (ERK, JNK, and p38) activation. Thus, inhibitors of ERK, p38, and JNK displayed significant inhibition of NO production and the phosphorylation of these proteins was enhanced by Sc-SG exposure. In agreement, phosphoforms of transcription factors such as c-Jun, c-Fos, and ATF-2 activated with MAPK were found to be translocated to the nucleus. Thus, the results of this study suggest that β-glucans from *Sparassis crispa* can be developed as a promising immunostimulatory principle, applicable to cancer patients, elderly people and children with lowered immuno-regulatory capability.

ACKNOWLEDGEMENTS

This work was supported by a grant (2010) from Kangwon National University, Korea.

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