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Macrophage activation by glycoprotein isolated from *Dioscorea batatas*

Pham Thi Thu Huong[1,2] and Young Jin Jeon[1]

[1]*Department of Pharmacology, Chosun University College of Medicine, Gwangju 501-759*
[2]*Chemical Biology Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Cheongwongun 363-883 Korea*

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We demonstrate that glycoprotein isolated from *Dioscorea batatas* (GDB) activates macrophage function. Analysis of the infiltration of macrophages into peritoneal cavity showed GDB treatment significantly increased the recruitment of macrophages into the peritoneal cavity. In order to further confirm and investigate the mechanism of GDB on macrophage activation, we analyzed the effects of GDB on the cytokine expression including IL-1β, TNF-α, and IL-6 in mouse peritoneal macrophages. GDB increased the expression of IL-1β, TNF-α, and IL-6. Cytokine induction by GDB was further confirmed by RT-PCR and ELISA in mouse macrophage cell line, RAW264.7 cells. Treatment of RAW264.7 cells with GDB produced strong induction of NF-κB DNA binding and MAPK phosphorylation, markers for macrophage activation and important factors for cytokine gene expression. Collectively, this series of experiments indicates that GDB stimulates macrophage activation.

***Key words*:** Glycoprotein, Macrophages, IFN-γ, TNF-α, IL-1β, NF-κB

INTRODUCTION

Dioscorea species have been used not only in traditional Chinese medicine, but also in medicine as a major source of steroid precursors. The plants of this genus possess immunomodulatory activity *in vitro* on the viability, cell-mediated cytotoxicity and IFN-γ secretion of splenic lymphocytes (Jin *et al*., 2006). Phytochemical investigations have demonstrated that steroid saponins are active principles of *Dioscorea* species (Chen *et al*., 2003). Among these, *Dioscorea japonica*, has long been used in ethnomedicine for the treatment of poor appetite, chronic diarrhea, asthma, dry coughs, frequent or uncontrollable urination, diabetes, and emotional instability (Chen *et al*., 2003). The common name for the plants of the genus Dioscorea of the *Dioscoreaceae* family is Yam.

Macrophages can be stimulated by cytokines, such as IFN-γ, or microbial components LPS (Stuehr and Nathan, 1989; Higuchi *et al*., 1990) and play important roles in both innate and acquired immunity. For innate immunity, macrophages phagocytise and kill microbes, release inflammatory mediators such as NO. For acquired immunity, macrophages serve as antigen presenting cells and release cytokines including tumor necrosis factor (TNF-α), IL-1, IL-6, and IL-12 to regulate the functions of helper T cells (Billack, 2006). When activated, macrophages suppress the growth of a variety of tumor cells and microorganisms (Lowenstein *et al*., 1993). Due to the critical role that macrophage activation plays in innate immune response, immunomodulating compounds targets macrophages (Jeon *et al*., 2000; Lee and Jeon, 2003; Li *et al*., 2010).

Most cellular responses to extracellular stimuli are mediated by cascades of kinase and phosphatase. One of the most important kinase families in inflammatory cells is mitogen activated protein kinase (MAPK), which are strongly conserved through evolution, suggesting their vital role in intracellular signaling. To date, several distinct MAPKs expressed in vertebrates have been identified, including ERK1/2, stress-activated protein kinases (SAPK)/c-Jun NH2-terminal kinase (JNK), and p38 MAPK (Su and Karin, 1996). p38, ERK1/2, and SAPK/JNK are serine threonine kinases that are located in the cytoplasm until activates by dual phosphorylation on both Tyr and Thr residues at Thr-Gly-Tyr, Thr-Glu-Tyr, and Thr-Pro-Tyr, respectively (Raingeaud *et al*., 1995). Macrophage activation by certain external stimuli including lipopolysaccharide, a component of gram-negative bacterial cell wall, results in the phosphorylation of MAPK, and activated MAPK stimulates tran-

Correspondence to: Young Jin Jeon, Department of Pharmacology, College of Medicine, Chosun University, 375, Susuk-dong, Dong-gu, Gwangju 501-759, Korea
E-mail: yjjeon@chosun.ac.kr

ABBREVIATIONS: interferon-γ, IFN-γ; interleukin-1β, IL-1β; tumor necrosis factor 1α, TNFα; nuclear factor kB, NF-κB, mitogen activated protein kinase; MAPK

scription factors such as NF-κB and AP-1 (Weinstein *et al.*, 1992; Whitmarsh and Davis, 1996).

In the present study we investigated the effect of glycoprotein (GDB) isolated from *Dioscorea batatas* on macrophages in the animal models and *in vitro* effects on macrophage activation. We further analyzed the effects of GDB on the production of cytokine and the activation of NF-κB and MAPK in RAW264.7 cells, mouse macrophage cell line.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice weighing 21~23 g were housed under a 12-h light/dark cycle in a temperature-controlled room (22~24°C). Mice were given access to standard chow and water *ad libitum*.

Preparation of Glycoprotein (GDB) from Dioscorea batatas. The tubers of *Dioscorea batatas* were harvested in May at the Okcheon County in North Chungcheong Province, South Korea, and were stored at 4°C until used. For isolation of the proteins, whole tubers were used. Glycoproteins were isolated from *Dioscorea batatas* according to the procedure of previously reported (Huong *et al*., 2011; Gaidamashvili *et al*., 2004).

Isolation of inflammatory peritoneal macrophages. Thioglycollate-elicited peritoneal exudate cells were obtained from C57BL/6 mice following intraperitoneal injection of 1 m*l* Brewer Thioglycollate broth (4.05 g/100 m*l*) (Difco Laboratories, Detroit, MI, USA) and lavage of the peritoneal cavity with 5 m*l* of medium 3~4 days later. The cells were washed twice and resuspended in RPMI-1640 containing 10% heat-inactivated FBS, penicillin (100 IU/m*l*) and streptomycin (100 mg/m*l*) (RPMI-FBS). Macrophages were isolated from peritoneal exudate cells as described by Klimetzek and Remold (Klimetzek and Remold, 1980). Peritoneal exudate cells were seeded on Teflon-coated petri dishes and allowed to adhere for 2~3 h in a 5% CO_2 humidified atmosphere. The non-adherent cells were removed by washing the dishes twice with 10 m*l* of pre-warmed medium and PBS. The plates were incubated for 15 min at room temperature and the macrophages were removed by rinsing 10 times using a 10-m*l* syringe. The viability of the detached cells was assessed by trypan blue exclusion. The proportion of macrophages was determined by acridine orange staining of cytoplasm. Cell preparations were greater than 95% viable and contained greater than 95% macrophages.

Cytokine determination. Peritoneal macrophages and RAW 264.7 cells were stimulated with GDB for 24 h. Cells were harvested and subjected to measure IL-1β using ELISA kit (BD Biosciences, San Dieog, CA) according to the manufacturer's instructions. Culture supernatants were collected and determined the concentrations of each cytokine (IL-6 and TNF-α). Accumulation of NO_2^- in culture supernatants was measured using the assay system described by Green *et al*. (Green *et al.*, 1982).

RT-PCR. Total RNA was isolated with Tri Reagent (Molecular Researh Center, Cincinnati, OH, USA). The forward and reverse primer sequences are as follows: IL-1β: 5'-TGC AGA GTT CCC CAA CTG GTA CAT C-3', 5'-GTG CTG CCT AAT GTC CCC TTG AAT C-3'; TNF-α: 5'-CCT GTA GCC CAC GTC GTA GC-3', 5'-TTG ACC TCA GCG CTG AGT TG-3'; and β-actin: 5'-TGG AAT CCT GTG GCA TCC ATG AAA C-3', 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'. Equal amounts of RNA were reverse transcribed into cDNA with oligo(dT)15 primers. PCR was performed with cDNA and each primer. Samples were heated to 94°C for 5 min and cycled 30 times at 94°C for 1 min, 55°C for 1.5 min, and 94° for 1 min, after which an additional extension step at 72°C for 5 min was included. PCR products were electrophoresed in 8% polyacrylamide gel followed by staining in ethidium bromide. The IL-1β, TNF-α, and β-actin primers produce amplified products at 387, 374, and 349 bp, respectively.

EMSA. RAW 264.7 cells were grown at 5×10^5 cells/m*l* in petri-dish and then treated with GDB for 2 h. Nuclear extracts were prepared as earlier described (Xie *et al.*, 1993). Briefly, cells were lysed with hypotonic buffer (10 mM HEPES, 1.5 mM $MgCl_2$, pH 7.5) and nuclei were pelleted by centrifugation at 3000 ×g for 5 min. Nuclear lysis was performed using a hypertonic buffer (30 mM HEPES, 1.5 mM $MgCl_2$, 450 mM KCl, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/m*l* of aprotinin, and 1 μg/m*l* of leupeptin). Following lysis, the samples were centrifuged at 14,500 ×g for 15 min, and supernatant was retained for use in the DNA binding assay. The double-stranded oligonucleotides were end-labeled with [γ-^{32}P]-ATP. Nuclear extracts (5 μg) were incubated with poly (dI-dC) and the [^{32}P]-labeled DNA probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/m*l* of aprotinin, and 1 μg/m*l* of leupeptin) for 10 min. DNA binding activity was separated from free probe using a 5% polyacrylamide gel in 0.5X TBE buffer. Following electrophoresis, the gel was dried and subjected to autoradiography.

Western immunoblot analysis. Whole cell lysates were separated by 10% SDS-PAGE, then electro-transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were preincubated for 1 hr at room temperature in Tris-buffered saline, pH 7.6 containing 0.05% Tween-20 and 3% bovine serum albumin. The nitrocellulose membranes were incubated with specific antibodies. Immunoreactive bands were then detected

by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham). Antibodies against, p38, phospho-p38 (Thr180/Tyr182), ERK1/2, phospho-ERK1/2 (Thr202/Tyr204), JNK, phospho-SAPK/JNK (Thr183/Tyr185), and β-actin (45 kDa) were obtained from Cell Signaling Biotechnology, Inc (MA, USA). Goat anti rabbit IgG-HRP antibody was from purchased from Santa Cruz Biotechnology, Inc (CA, USA).

Statistical analysis. The mean ± SD was determined for each treatment group in a given experiment. When significant differences occurred, treatment groups were compared to the vehicle control using Student's *t* test.

RESULTS

Effects of glycoprotein (GDB) on macrophage- recruitment into the peritoneal cavity. Isolation and characterization of GDB were performed by anion-exhange chromatography and SDS-PAGE (Huong *et al*., 2011). In order to examine the immunostimulatory effects of GDB, we analyzed the effects of GDB on the infiltration of macrophage into the peritoneal cavity using an animal model. After treating mice with starch, differential counting of macrophages was performed. Two weeks of GDB treatment significantly increased the recruitment of macrophages (Fig. 1A). Administration of 200 mg/kg GDB for 2 weeks showed no significant change including body weight from vehicle treated group (Fig. 1B).

Effects of GDB on the cytokine production of macrophages. To investigate macrophage activation by GDB, we analyzed the effects of GDB on the cytokine expression including IL-1β, TNF-α and IL-6 in mouse peritoneal macrophages. Peritoneal macrophages were isolated and treated with GDB (5, 20 and 100 μg/m*l*) for 24 h. ELISA showed that GDB significantly induced the production of IL-1β, TNF-α, and IL-6 in a dose-dependent manner (Fig. 2A, B, C).

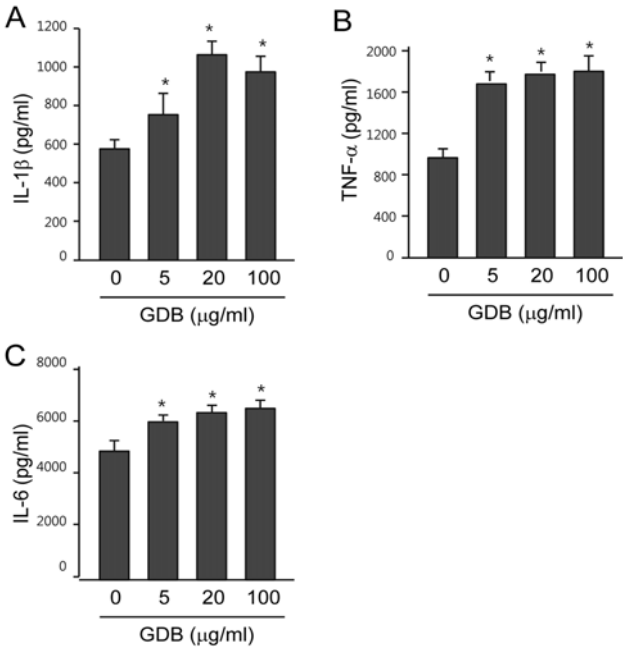

Fig. 2. Effects of GDB on the cytokine production of peritoneal macrophages. Peritoneal macrophages were isolated and treated with GDB (5, 20 and 100 μg/m*l*) for 24 h. Cell lysates were then prepared and analyzed for the (A) IL-1β, (B) TNF-α, and (C) IL-6. The results are presented as the mean ± SD. *Denotes significant differences ($P < 0.05$) from the control group. One representative of three experiments is shown.

In order to further confirm and investigate the mechanism of GDB on macrophage activation, we analyzed the

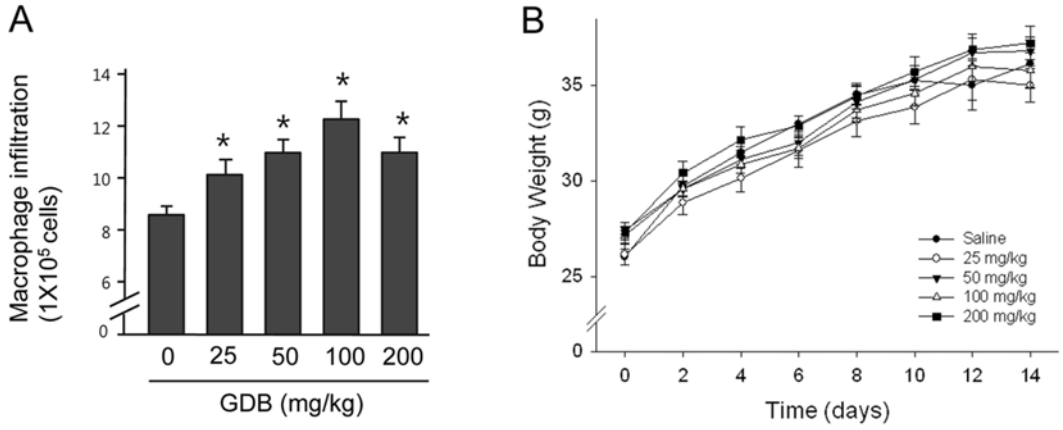

Fig. 1. Recruitment of macrophages by GDB. Animals were treated with GDB (25, 50, 100 or 200 mg/kg) for 2 weeks. (A) Peritoneal cells were isolated and differential counting for macrophages was performed. The results are presented as the mean ± SD of 10 mice per group *Denotes the significant differences ($P < 0.05$) from the control group. (B) Body weights were measured every consecutive day for 2 weeks.

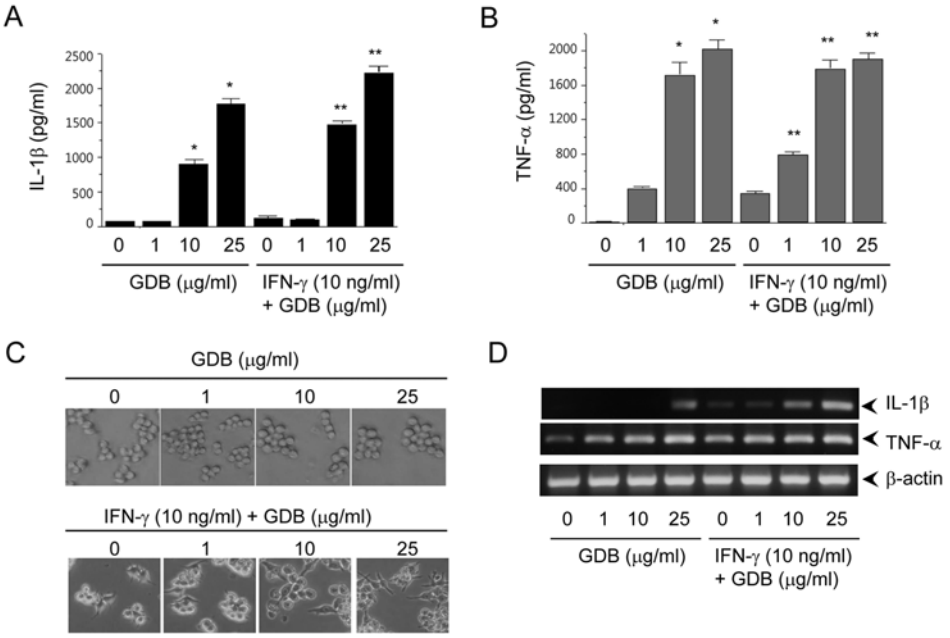

Fig. 3. Effects of GDB on the cytokine production of RAW264.7 cells. RAW264.7 cells were treated with GDB (1, 10 and 25 μg/m*l*) in the presence or absence of IFN-γ for 24 h. Cell lysates were then prepared and analyzed for the (A) IL-1β and (B) TNF-α. (C) Morphological change of RAW 264.7 cells were analyzed using inverted microscope. (D) For RT-PCR analysis cells were treated for 8 h. Total RNA was then isolated and analyzed for IL-1β and TNF-α using RT-PCR. The results are presented as the mean ± SD. *Denotes significant differences ($P < 0.05$) from the control group. One representative of three experiments is shown.

effects of GDB on the cytokine expression including IL-1β and TNF-α in macrophage cell line, RAW 264.7 cells. ELISA showed that GDB increased the production of IL-1β and TNF-α in a dose-related manner (Fig. 3A, B). In the presence of IFN-γ, GDB synergistically induced IL-1β production. GDB itself strongly induced TNF-α production, and the synergism between IFN-γ and GDB was shown only in low dose (1 μg/m*l*) of GDB. Morphological change further confirmed the synergistic activation of macrophage by IFN-γ and GDB (Fig. 3C). RT-PCR analysis showed that GDB increased the expression of IL-1β and TNF-α in the presence or absence of IFN-γ (Fig. 3D). These results suggest that GDB strongly expressed the cytokine markers of macrophage activation including IL-1β and TNF-α.

Effects of GDB on the activation of NF-κB in RAW264.7 cells. To investigate the mechanism by which GDB activated macrophages, we analyzed the effect of GDB on NF-κB, AP-1, and Oct. EMSA showed that GDB treatment of RAW264.7 cells for 2 h induced NF-κB DNA binding activity in a dose-related manner (Fig. 4A), while both AP-1 and Oct had moderate basal binding activities and were not significantly influenced by GDB (Fig. 4B). Kinetic study further showed that GDB induced NF-κB DNA binding activity at 30 min, peaked at 1 h and reduced (Fig. 4C). These results suggest that GDB activates NF-κB, which is important in the activation and production of cytokines in macrophages. The specificity of the bindings was demonstrated by competition assays with ^{32}P-unlabeled κB or mutant κB probes (Fig. 4D).

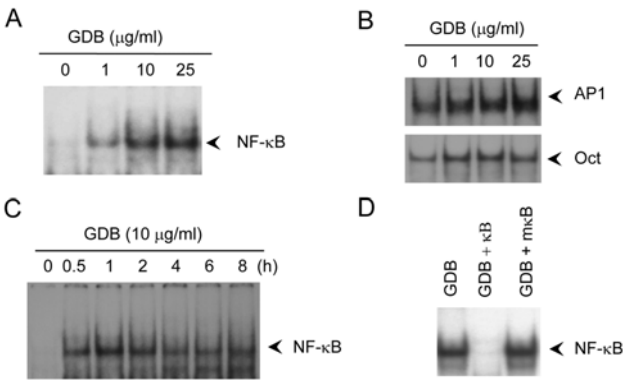

Fig. 4. Effects of GDB on the activation of NF-κB in RAW264.7 cells. RAW264.7 cells were treated with GDB (1, 10 and 25 μg/m*l*) for 2 h. Nuclear extracts were isolated and analyzed for the determination of (A) NF-κB and (B) AP1 and Oct. (C) Cells were treated with GDB (10 μg/m*l*) for the indicated times, nuclear extracts were isolated and subjected to EMSA. (D) In competition studies, 1 pmole of unlabeled κB (κB) or mutant κB (mκB) was added to the reaction mixture. Reaction products were electrophoresed, and the gels were dried and autoradiographed. One representative of three experiments is shown.

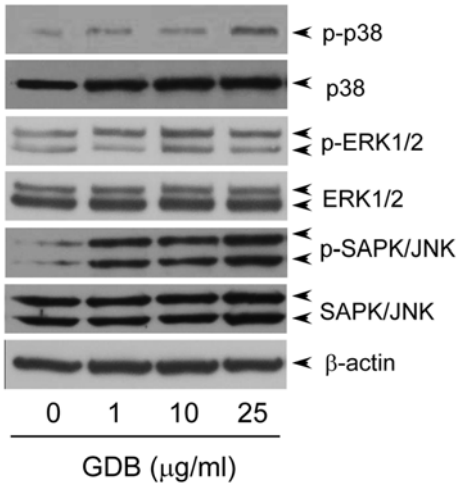

Fig. 5. Effects of GDB on the activation of MAPK in RAW264.7 cells. RAW264.7 cells were treated with GDB (1, 10 and 25 μg/m*l*) for 30 min. Cell lysates were prepared and subjected to immunoblot assay using antibodies against phospho- and non-phospho-p38, ERK1/2 and SAPK/JNK proteins. β-actin was used as a control. One representative of three experiments is shown.

Effects of GDB on the activation of MAPK in RAW264.7 cells. Most cellular responses to extracellular stimuli are mediated by cascades of kinase and phosphatase. One of the most important kinase families in inflammatory cells is MAPKs, which are strongly conserved through evolution, suggesting their vital role in intracellular signaling. And since MAPK plays an important role in macrophage activation, we analyzed the effects of GDB on MAPKs activity by immunoblot analysis. When cells were treated with GDB (1, 10, and 25 μg/m*l*) for 30 min, we found that phosphorylation of p38, ERK1/2 and especially SAPK/JNK was markedly induced (Fig. 5). These results suggest that GDB activates macrophages through the activation of signal transduction pathways including, p38, ERK1/2 and SAPK/JNK.

DISCUSSION

We demonstrate that GDB has significant immunomodulatory effects on immune responses mediated by macrophages. Treatment of C57BL/6 mice with GDB caused a significant increase in macrophages numbers in the peritoneal cavity. Activity analysis of GDB in cell culture system further confirmed the immunostimulatory activity of GDB. Macrophages play a critical role in the first line of defense against tumor cells. Once activated, macrophages produce a number of cytotoxic molecules (Stuehr and Nathan, 1989). Because GDB treatment of RAW264.7 cells induced a significant increase in IL-1β and TNF-α, it is possible that the tumoricidal activity of macrophages could be mediated by cytotoxic. The GDB stimulation on the function of macrophage suggests that phagocytic cells might be one of the targets for the immunopotentiating action of GDB. However, the mechanisms by which GDB exerts those effects on macrophages are not fully elucidated.

In the present study, we also showed that NF-κB is positively regulated by GDB for macrophage activation. The NF-κB is a pleiotropic regulator of many genes involved in immune and inflammatory responses (Pahl, 1999). NF-κB exists in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, IκB. Macrophage activation by certain external stimuli results in the phosphorylation of IκB, thus releasing the active DNA-binding form of NF-κB. The active NF-κB translocates to the nucleus and binds to κB motifs in the regulatory region of a variety of genes.

We also showed GDB activates MAPKs, especially SAPK/JNK. A major role for MAPK is to transmit extracellular signals to the nucleus, where the transcription of specific genes is induced by phosphorylation and activation of transcription factors. These signaling are coupled to different environmental stimuli. For example, the ERK MAPKs are activated by a Ras-dependent pathway in response to many growth factors and hormones (Robinson and Cobb, 1997). In contrast, the SAPK/JNK and p38 MAPKs are activated by environmental stresses, such as UV radiation, osmotic shock, heat shock, protein synthesis inhibitors, and LPS (Whitmarsh and Davis, 1996; Ip and Davis, 1998). The SAPK/JNK and p38 MAP kinases also are activated by treatment of cells with proinflammatory cytokines, including IL-1 and TNF (Whitmarsh and Davis, 1996; Ip and Davis, 1998).

Some membrane proteins are assumed to act as receptors in macrophages, even though the membrane receptor of GDB is not determined yet. Possible candidates are CR3, CD14, and Toll-like receptors (TLRs). Complement receptor CR3 (also called CD11b/CD18, Mac-1, and $\alpha_M\beta_2$-integrin) is identified as the membrane receptor for β-glucans (Thornton *et al*., 1996). CD14 is a 55-kDa glycosylphosphatidylinositol-anchored protein expressed on the surface of monocytes and neutrophils and binds to LPS with high affinity (Goyert *et al*., 1988). They are expressed on the surface of neutrophils, monocytes, macrophages, and NK cells and are involved in numerous cell-cell and cell-substrate interactions (Hynes, 1992). TLRs, a mammalian transmembrane protein family, play important roles in innate immune recognition (Kopp and Medzhitov, 1999).

In summary, these experiments demonstrate that GDB is a potent enhancer of macrophage activation. Based on our findings, the most likely mechanism that can account for this biological effect involves the activation of NF-κB and MAPK. Due to the critical role that macrophage activation plays in innate immune response, the activation effects of GDB on macrophages suggest that this compound may represent useful immunopotentiating agents.

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