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Polysaccharides from *Dioscorea batatas* Induce Tumor Necrosis Factor-α Secretion via Toll-like Receptor 4-Mediated Protein Kinase Signaling Pathways

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The bioactive polysaccharides (named ZPF1) from yam (*Dioscorea batatas*) were chemically determined, suggesting repeating β-1,4-mannan as mainly having a feature of acetylation on C_2−OH and C_3−OH, around 28%. The ZPF1 participated in the stimulation of murine wild-type macrophages predominantly in tumor necrosis factor-α (TNFα). Toll-like receptor 4 is proved to be one of the cellular receptors in ZPF1-mediated TNFα secretion. Reactive oxygen species transmission and PI3-kinase are found necessary for regulating TNFα secretion by ZPF1 stimulation. Moreover, we found that extracellular signal-regulated kinase 1/2, Jun N-terminal kinase 1/2, and p38 mitogen-activated protein kinase play important roles in the regulation of TNFα secretion in ZPF1-stimulated macrophages.

KEYWORDS: ***Discorea batatas*; mucopolysaccharides; macrophages; tumor necrosis factor-α; Toll-like receptor**

INTRODUCTION

Many herbal plants such as *Echinacea* are reputed to be beneficial in regulating immune functions (*1, 2*). In Asia, yam (*Dioscorea*) has been widely used to promote health and for the treatment of several illnesses in traditional medicine (*3, 4*). The major constituents of yam, including polysaccharides and proteins, were found to contain antioxidant, immunostimulatory, and angiotensin converting enzyme inhibitory activities (*5*−*8*).

Dioscorin is the major storage protein in yam and functions against angiotensin, which converts enzyme to cause hypertension (*7*). Diosgenin is used in making progesterone and other steroid drugs (*9*). Recently, the biological activities of the polysaccharides in yam have attracted increasing attention in the biochemical and medical fields because of their immunomodulatory and antitumor effects (*10*). Yam mucopolysaccharide (YMP) has been proven to possess immunostimulating bioactivities, for example, enhancement of INF-γ secretion of splenic lymphocytes, phagocytosis, and macrophages bioactivities (*6*−*8*).

It is well-known that macrophages play an important role in defending mechanisms against host infections and destroying tumor cells through the secretion of various cytokines (*11*). In immune response, the macrophages directly destroy foreign microorganisms and tumor cells by indirectly released mediators, such as interleukin-1, tumor necrosis factor-α (TNFα), etc., which activate other immune cells. The cytotoxicity against tumor cells is dependent on the level of the activation of macrophages. Lately, examining how modifiers of various biological responses modulate the antitumor properties of macrophages has become an active area in cancer chemotherapy research (*6, 12*).

In this study, bioactive polysaccharides (ZPF1) were isolated from yam (*Dioscorea batatas*) tubers, and the structure was determined by employing different chemical methods such as gas chromatography−mass spectrometry (GC-MS), NMR, and MS. Meanwhile, the signaling pathways involved in ZPF1-mediated TNF secretion and related cellular receptor were studied.

MATERIALS AND METHODS

Chemicals and Antibodies. Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), sulfuric acid, and *N*-acetyl-cysteine (NAC) were purchased from Sigma-Aldrich (St. Louis, MO). Phenol was purchased from RDH Chemical (CA). The reagent for the protein assay was bought from Bio-Rad (CA). A mouse TNF enzyme-linked immunosorbent assay (ELISA) kit was purchased from R&D Systems (Minneapolis, MN). For the antibodies, antimouse IgG-HRP was obtained from Santa Cruz Biotechnology (Santa Cruz, CA); monoclonal anti-MAPK, activated (diphosphorylated ERK1/2) antibody, monoclonal anti-JNK

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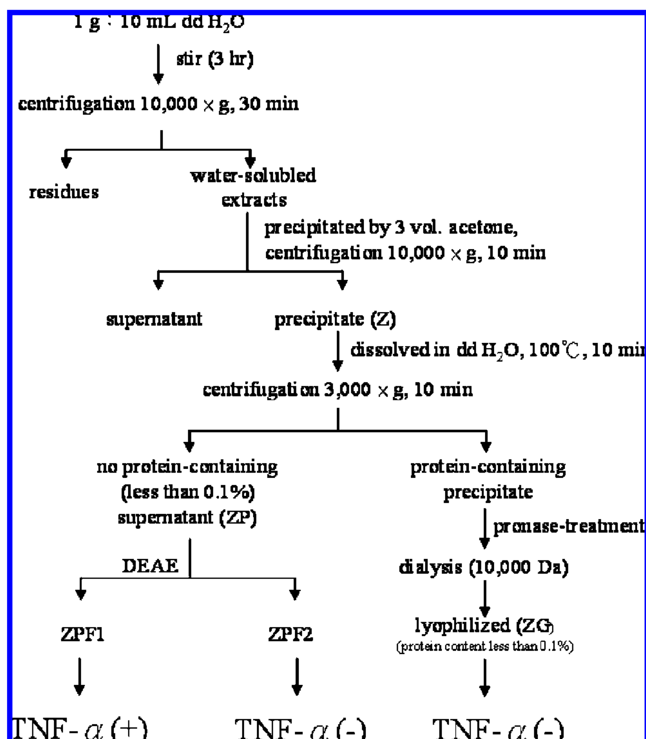

Figure 1. Scheme for the isolation and purification of yam's polysaccharides from *D. batatas*.

kinase, activated (diphosphorylated JNK1/2) antibody, monoclonal anti-p38 MAPK, and activated (diphosphorylated p38) antibody were purchased from Sigma-Aldrich. For the kinase assay kits, ERK1/2 and p38 MAPK assay kits were purchased from Cell Signaling Technology (Beverly, MA). Antimouse TLR4/MD2 neutralizing antibody was purchased from eBioscience, Inc. (CA). For protein kinase inhibitors, PD98059, SB203580, SP600125, and LY294002 were purchased from Calbiochem-Novabiochem (San Diego, CA).

Extraction and Purification of *D. batatas* Polysaccharides. Fresh yam (*D. batatas*) tubers were planted and identified by Assistant Researcher, Chi-Luan Wen, Seed Improvement and Propagation Station (Taiwan). Harvested tubers were cut into slices or strips, lyophilized, and then ground into a fine powder for further use. The lyophilized yam powder was suspended in 10 volumes of distilled water. After centrifugation at 10000*g* for 30 min, the supernatant was precipitated by three volumes of acetone at 4 °C. The obtained precipitate was named "the crude mucilage" (Z) (**Figure 1**). The crude mucilage was further dissolved in H_2O and heated to 100 °C for 10 min for denaturation of proteins or glycoproteins. After the mixture was cooled and centrifuged, the supernatant and precipitate were lyophilized and designated ZP (no proteins containing) and ZG (proteins or glycoproteins), respectively. The precipitate was treated further with Pronase (Roche) for removal of the peptide part and dialyzed against H_2O (molecular mass cut, 10 kDa), and the remaining carbohydrates part was named ZG and tested the TNF induction activity. The supernatant (ZP) was further purified by anionic DEAE chromatography [1.2 cm (D) × 7 cm (H)] in fast protein liquid chromatography (FPLC). The nonbound fractions of DEAE were ZPF1 and 1.2 N NaCl-eluted ZPF2. During procedures of purification, all fractions containing carbohydrates of the chromatography were detected by phenol−sulfuric acid and measured at 490 nm quantitatively.

Sugar Compositions and Linkages. The sugar composition analysis was determined by GC-MS. The GC-MS analysis of yam was performed by methanolysis with 0.5 M methanolic/HCl at 80 °C for 16 h and trimethylsilyation with Sylon HTP (HMDS/TMCS/pyridine, 3:1:9) trimethylsilyation reagent (Supelco, PA). The final trimethylsilyated (TMS) derivatives were kept in *n*-hexane for GC-MS analysis (*13*). For the carbohydrate linkage analysis, the Hakomori methylation analysis was carried out (*14*). The yam polysaccharide was per-*O*-methylated with methyl iodide and dimethylsulfoxide anion in dimethylsulfoxide and then hydrolyzed by 2 M trifluoroacetic acid at 120 °C for 2 h. The solvent was evaporated by compressed air, and the residue was reduced with 0.45 M $NaBD_4$ in 1 M NH_4OH for 2 h at room temperature. The reaction was quenched with 100% acetic acid and coevaporated with 10% acetic acid/methanol. The residue was peracetylated with acetic anhydride at 100 °C for 1 h. After $CHCl_3$/H_2O partition, the sample was suspended in *n*-hexane and finally analyzed by GC-MS. The analytical methods for GC-MS followed the parameters of a previous report (*13*).

Ratio of *O*-Acetylation in Yam Polysaccharide. The ratio of *O*-acetylation in yam polysaccharide was measured by Hestrin's method (*15*).

NMR. NMR spectra of polysaccharide in D_2O were recorded on a Bruker Avance 400 spectrometer (equipped with BBOZ gradient probes) at 300 K, with standard pulse sequences provided by Bruker. Two-dimensional (2D) $^1H-^{13}C$ heteronuclear single quantum coherence (HSQC) spectra were recorded with ^1J H−C coupling constants at 145 Hz. 2D $^1H-^{13}C$ heteronuclear multiple-quantum coherence (HMQC) spectra were recorded with PL12 = 120 dB to observed the $^1J_{H-C}$ coupling constants of anomeric positions.

Cell Cultures. Murine J774A.1 macrophages were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Murine macrophage HeNC2 (TLR4+) and GG2EE (TLR4−) cells were provided kindly by Dr. Danuta Radzioch, McGill University (Montreal, Canada). All cells were propagated in RPMI 1640 medium supplemented with 10% heated-inactivated fetal bovine serum (FBS, HyClone, Logan, UT) and 2 mM L-glutamine (Invitrogen Life Technologies, Carlsbad, CA) and cultured at 37 °C in a 5% CO_2 incubator.

Measurement of Intracellular H_2O_2 Production. Intracellular H_2O_2 production stimulated by ZPF1 was measured by detecting the fluorescent intensity of carboxyl-DCFH (CM-DCFH) oxidized product, CM-DCF (Molecular Probes, Inc., Eugene, OR). Briefly, J774A.1 (1 × 10^6 cells/mL) cells were grown in serum and phenol red-free RPMI medium for 24 h and then were preincubated with 2 μM CM-DCFH and NAC at 37 °C for 30 min in the dark. Then, the fresh starvation medium containing ZPF1 was added for an additional 1 h. The relative fluorescent intensity of fluorophore CM-DCF, which was formed by peroxide oxidation of the nonfluorescent precursor, was detected at an excitation wavelength of 485 nm and an emission wavelength of 530 nm with a fluorometer, Cytofluor 2300 (Millipore Inc., Bedford, MA).

Antioxidant NAC Inhibited TNF Secretion from ZPF-1-Stimulated J774A.1 Macrophages. Cells (1.0 × 10^6/mL) were grown in serum and phenol red-free RPMI medium (starvation medium) for 24 h and then preincubated in a different concentration of NAC at 37 °C for 30 min in the dark. The fresh starvation medium containing ZPF1 was added for an additional 6 h. After incubation, the released TNF in conditioned media was measured by ELISA.

Western Blot for Detection of Phosphorylation Level of Mitogen-Activated Protein Kinases (MAPKs). To investigate the inhibitory effect of ZPF1 on the phosphorylation level of MAPKs in ZPF1-stimulated J774A.1 cells, the cells were stimulated with ZPF1 (10 μg/mL) for 0−60 min at 37 °C. The whole cell lysates were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis and electrotransferred to a polyvinylidene fluoride filter. Filters were incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20). These blocking steps were performed at room temperature for 1 h. Filters were incubated with specific primary antibody for 2 h. After they were washed, the filters were incubated with a horse radish peroxidase (HRP)-conjugated secondary antibody directed against primary antibody. Filters were developed by an enhanced chemiluminescence Western blot detection system.

Kinase Activities of ERK1/2 and p38 Induced by Yam's Polysaccharides. Methods for assaying these activated protein kinases were followed by Hsu et al. (*16*). The immunocomplexes were incubated with Elk fusion protein (for ERK1/2) and ATF-2 fusion protein (for p38) with kinase assay buffer in the presence of cold ATP at 30 °C for 30 min. The phosphorylation levels of Elk fusion protein and ATF-2 fusion protein were analyzed by Western blot.

ELISA. Fifty microliters of biotinylated antibodies reagent was added to antimouse TNF precoated stripwell plates. Fifty microliters

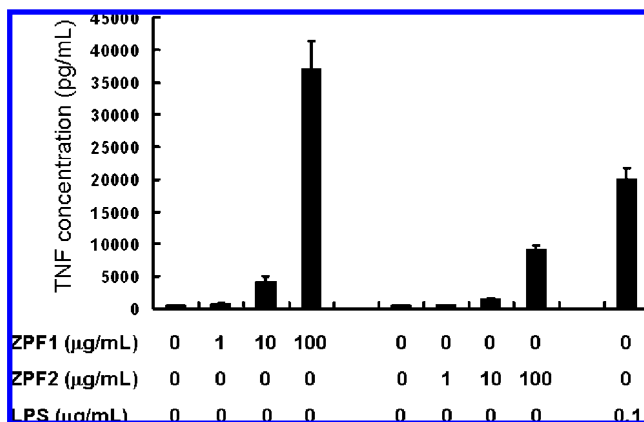

Figure 2. Effects of ZPF1, ZPF2, and LPS on TNF secretion from J774A.1 macrophages. Cells (1×10^6/mL) were incubated with various concentration of ZPF1, ZPF2 (concentration, 1−100 μg/mL), or LPS (0.1 μg/mL) for 6 h, and the released TNF in conditioned media was measured by ELISA. The data were expressed as the means ± SE with three separate experiments.

of the supernatant concentrate from tested samples was added and incubated at room temperature for 2 h. After the plate was washed three times with the washing buffer provided in kit components, 100 μL of diluted streptavidin−HRP concentrate was added to each well and incubated at room temperature for 30 min. The washing process was repeated. One hundred microliters of premixed tetramethyl benzidine substrate solution was added to each well and developed at room temperature in the dark for 30 min. Following the addition of 100 μL of provided stop solution (2 N H_2SO_4) to each well to stop the reaction, the absorbance of the plate was measured at 450−550 nm wavelengths by MRX microplate reader (Dynex Tech. Inc.).

Statistical Analysis. All values are given as means ± standard deviations (SDs). Data analysis involved one-way analysis of variance (ANOVA) with subsequent Scheffe's test.

RESULTS

The immunological active carbohydrates of yam were obtained by a series of purification procedures (**Figure 1**). The sugar and protein contents of crude mucilage (Z, 7.82% w/w) determined by phenol−sulfuric acid and Bio-Rad protein assays were 15.9 and 28.3%, respectively. The preliminary data showed that the polysaccharide part of hot water extracts of crude mucilage, which is designated ZP, had significant stimulation on TNF in J774A.1 macrophages. However, the carbohydrate part of ZG was negative in TNF expression (data not shown). ZP was further separated by anionic DEAE chromatography (1.2 cm D × 7 cm H) and divided into two fractions, ZPF1 and ZPF2. ZPF1 was obtained from a non-DEAE-bounded fraction and ZPF2 from a 1.2 N NaCl-eluted fraction. Comparing the action of both fractions on TNF stimulation, ZPF1 was more effective (**Figure 2**). The chemical structure of ZPF1 was determined by a series of experiments, including sugar analyses, mass, and NMR spectra. The major sugar composition of ZPF1 was mannose, in addition to a small proportion of glucose and galactose in a ratio of 76.7, 16.9, and 6.3%, respectively. The sugar linkage of the ZPF1 was mainly 1,4-mannosidic, and the anomeric configuration was assigned as β-form due to the ${}^1J_{H-C}$ = 160 Hz in the ^{1}H NMR spectrum (**Figure 3a**,**b**). In addition, the acetyl groups were found in ZPF1, and the ratio of *O*-acetylation was estimated as 28% by Hestrin's method (*15*). The HSQC data showed the *O*-acetylation of C-2 and C-3 positions (**Figure 3c**).

Generally, one of the induced immunological events is known to be the oxidative burst when cells meet foreign components.

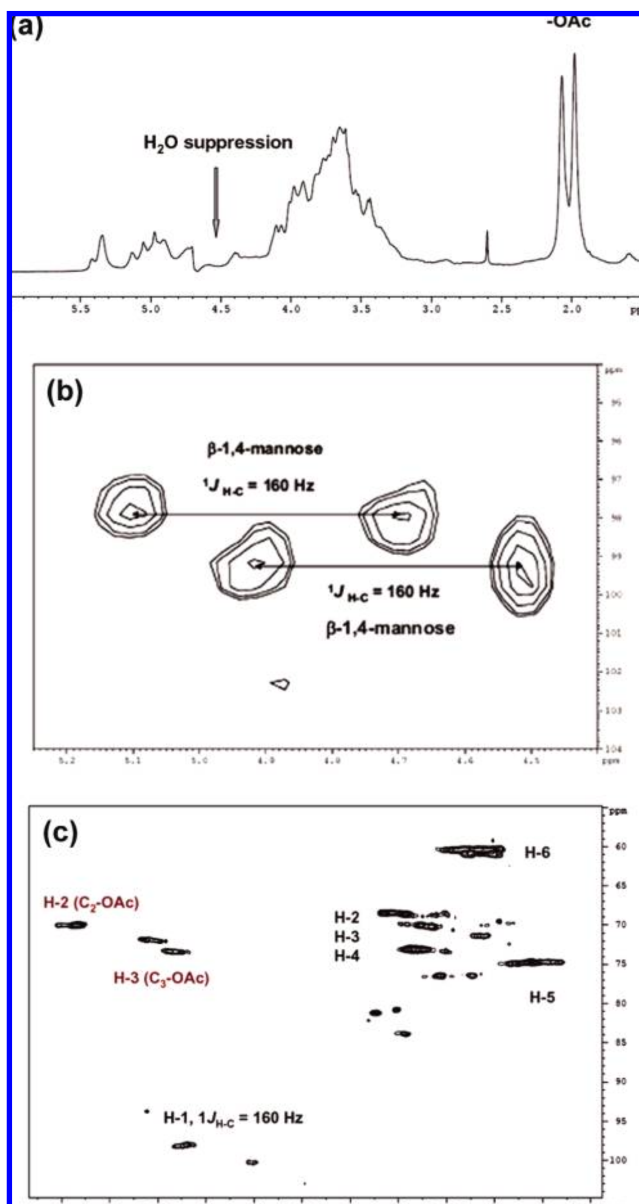

Figure 3. NMR spectra of yam's polysaccharide ZPF1. (**a**) The 400 MHz ^{1}H NMR spectrum in D_2O at 300 K. (**b**) 2D ^{1}H−^{13}C HMQC spectrum, recorded with PL12 = 120 dB to observe the ${}^1J_{H-C}$ coupling constants of anomeric positions. (**c**) 2D ^{1}H−^{13}C HSQC spectrum, recorded with ${}^1J$ H−C coupling constants at 145 Hz.

The oxidative burst is characterized by the production of strong oxidizing molecules, superoxide and nitric oxide primarily, which are known as reactive oxygen species (ROS) (*17*). To examine whether ROS involved in TNF secretion in ZPF1-stimulated J774A.1 macrophages, initially, we examined whether ZPF1 could induce H_2O_2 production in J774A.1 macrophages. We found that ZPF1 rapidly induced significant H_2O_2 production in J774A.1 macrophages as compared to the case for untreated control cells (**Figure 4a**). By contrast, pretreatment of J774A.1 macrophages with NAC, a potent antioxidant, for 30 min, rapidly reduced ZPF1-induced H_2O_2 production (**Figure 4a**). Furthermore, we found that NAC was able to elicit a dose-dependent inhibition in ZPF1-induced TNF secretion, indicating that H_2O_2 is involved in TNF secretion in ZPF1-stimulated J774A.1 macrophages (**Figure 4b**).

Because TNF is one of the important pro-inflammatory cytokines, it is produced mainly by activated macrophages. Activation of macrophages by extracellular stimuli transduces

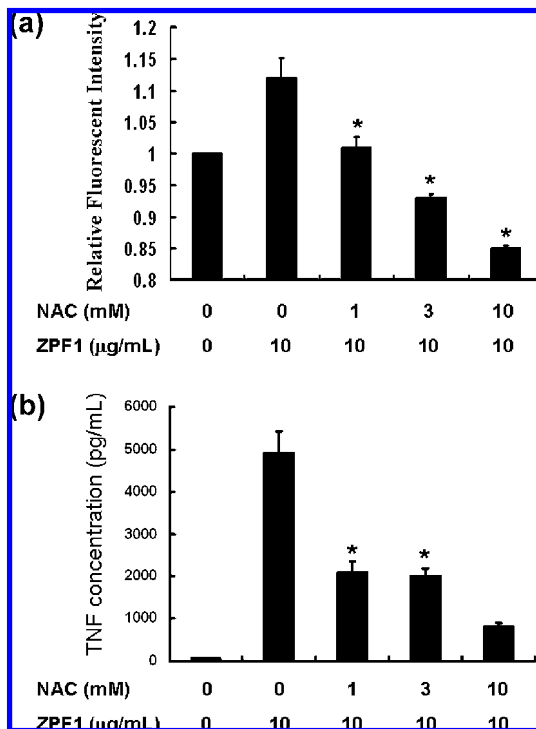

Figure 4. Effect of NAC on TNF secretion from ZPF1-stimulated J774A.1 macrophages. (**a**) Cells (1×10^6/mL) were preincubated with various concentrations of NAC for 30 min, followed by ZPF1 stimulation (10 μg/mL) for an additional 1 h. The H_2O_2 expression level was determined by measuring the relative fluorescence intensity of fluorophore CM-DCF. The data were expressed as the means ± SE with three separate experiments. The asterisk indicated significant differences at the level of $p < 0.05$ vs untreated control. (**b**) Cells (1×10^6/mL) were preincubated with various concentrations of NAC for 30 min, followed by ZPF1 stimulation (10 μg/mL) for an additional 6 h, and the released TNF in conditioned media was measured by ELISA. The data were expressed as the means ± SE with three separate experiments. The asterisk indicated significant differences at the level of $p < 0.05$ vs NAC untreated control.

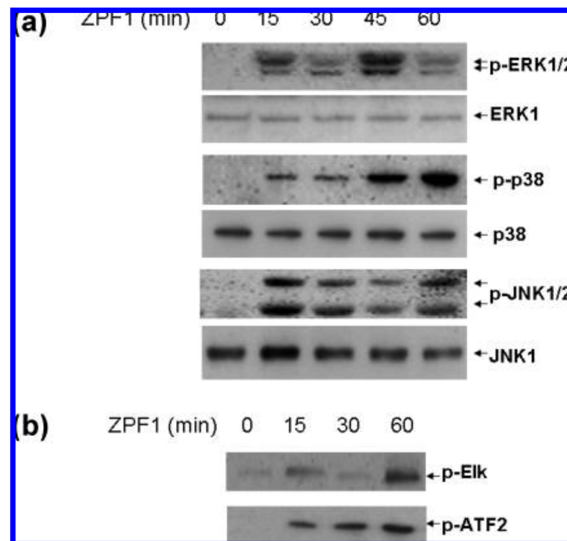

Figure 5. ZPF1-induced phosphorylation and kinase activity of MAPKs. J774A.1 cells were incubated with ZPF1 (10 μg/mL), and the cell lysates were collected at different periods of time. (**a**) Cell lysates were analyzed by Western blot with antidiphosphorylated ERK1/2, antidiphosphorylated p38, and antidiphosohorylated JNK1/2 monoclonal antibody, respectively. (**b**) ZPF1-induced ERK1/2 and p38 kinase activities were monitored by the phosphorylation level of Elk-1 and ATF-2.

certain specific cellular responses through the activation of MAPKs signaling pathways (*16, 18*). There are three major groups of MAPKs in mammalian cells, including ERK1/2, JNK1/2, and p38. MAPKs are serine/threonine protein kinases that are able to phosphorylate both cytoplasmic and nuclear targets related to gene expression. To examine ZPF1-mediated signal transduction pathways in the regulation of TNF expression, the phosphorylation of ERK1/2, JNK1/2, and p38 was determined individually. First, the phosphorylated ERK1/2 within cultured J774A.1 cells was detected in cell lysate at around 15 min after ZPF1 stimulation. The level of ERK1/2 phosphorylation reached its maximum after 45 min and gradually returned to the basal level after 60 min (**Figure 5a**). The ZPF1-stimulated phosphorylation of ERK1/2 substrate, Elk, was further proven in the downstream (**Figure 5b**). To explore ZPF1-mediated additional signal transduction pathways, we further examined whether ZPF1 induces p38 phosphorylation, another important stress-related MAPK member. The time-course study of ZPF1-induced p38 phosphorylation showed that the level was gradually increased during the 60 min poststimulation (**Figure 5a**). The phosphorylated p38 performed further phosphorylated the activating transcription factor-2 (ATF-2), a downstream substrate of p38. It was detected at 15 min poststimulation and reached the maximal level at 60 min (**Figure 5b**). In addition, the level of JNK1/2 phosphorylation by ZPF1 induction reached the maximum at 15 min and returned to the basal level after 45 min (**Figure 5a**).

To elucidate the role of ZPF1-induced protein kinase-mediated signaling pathways in the regulation of TNF expression, certain specific pharmacological antagonists such as PD98059, SB203580, and SP600125 were applied to inhibit the activation of MEK1, p38, and JNK1/2, respectively. The dose response to the impact of specific protein-kinase inhibitor upon MAPK kinase activity for J774A.1 macrophages was monitored by direct assay of individual kinase activity. Before the assay, the effective working concentrations of protein kinase inhibitors were determined as previously reported (*16*). Initially, while examining the signaling pathway of the ZPF1-induced MEK1/ERK1/2 regarding the regulation of TNF expression within J774A.1 macrophages, cells were exposed to different concentrations of inhibitor, as indicated by PD98059 (1−20 μM), followed by incubation with ZPF1 for an additional 6 h. The data deriving from the ELISA analyses indicated that PD98059 inhibited TNF production in a dose-dependent manner (**Figure 6**), which means that the MEK1/ERK1/2-related signaling pathway is important for TNF expression stimulated by ZPF1.

Subsequently, SB203580 concentration, a specific p38 inhibitor, from 0.1−3 μM, was observed to moderately inhibit the ZPF1-induced TNF production by J774A.1 macrophages (**Figure 6**). Moreover, another JNK1/2 inhibitor, SP600125, was used to investigate the role of JNK1/2 in the regulation of TNF production within ZPF1 stimulated cells. As a result, SP600125 (5−20 μM) could significantly inhibit the ZPF1-induced TNF production, indicating that JNK1/2-related signaling pathways play important roles in the ZPF1-mediated TNF production (**Figure 6**). However, in a lower concentration of SP600125 (1 μM), the same inhibition was not replicated. Overall, our results suggest that ERK1/2, JNK1/2, and p38-related signaling pathways are all involved in the ZPF1-induced TNF production within J774A.1 macrophages.

Toll-like receptor 4 (TLR4) was proven to mediate cytokines expression in response to polysaccharides from fungus *Ganoderma lucidum* within macrophages (*16, 19*). To investigate

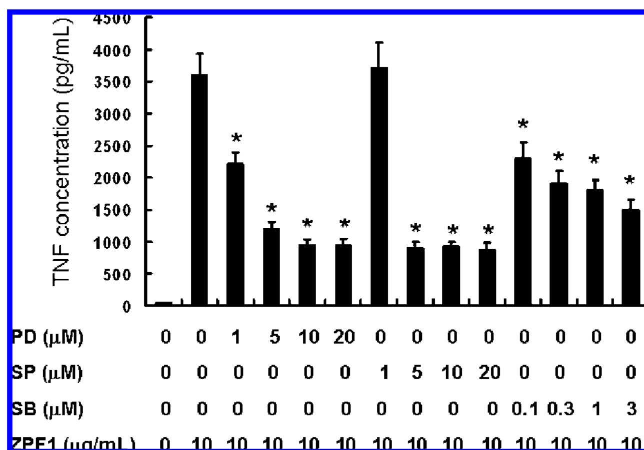

Figure 6. Effect of protein kinase inhibitors on TNF secretion from ZPF1-stimulated J774A.1 macrophages. Cells (1×10^6/mL) were preincubated with various concentrations of inhibitors or DMSO (vesicle) for 30 min, followed by ZPF1 stimulation (10 μg/mL) for an additional 6 h. After incubation, the released TNF in conditioned media was measured by ELISA. The data were expressed as the means ± SE with three separate experiments. The asterisk indicated significant differences at the level of $p < 0.05$ vs DMSO.

whether TLR4 participates in the host response to yam's polysaccharides, two murine macrophage cell lines HeNC2 (with functional TLR4) and GG2EE (lacking functional TLR4) were used to examine it (*20*). HeNC2 cells produced TNF in a dosage-dependent fashion upon stimulation by ZPF1, whereas no significant production in TNF secretion from ZPF1-treated GG2EE cells was observed (**Figure 7a**). In addition, to investigate the potential interaction of TLR4 and ZPF1 leading to the induction of TNF secretion, experiments were conducted via preincubation of J774A.1 macrophages with the TLR4/MD2 neutralizing antibody, which was known to specifically inhibit LPS-induced signaling through TLR4. As expected, TLR4/MD2 neutralizing antibody inhibited both ZPF1- and LPS-induced TNF secretion (**Figure 7b**). These results indicated that TLR4 might be a major cellular receptor for yam's polysaccharides during TNF secretion.

It has been reported that PI3-kinase is an important regulator of Toll-like receptors, especially TLR4-mediated signaling in macrophages (*21*). To explore the potential role of PI3-kinase in the regulation of TNF secretion from ZPF1-stimulated J774A.1 macrophages, a specific inhibitor of PI3-kinase, LY294002, was used. J774A.1 cells were incubated with and without LY294002 for 30 min and then treated with ZPF1 for an additional 6 h. We found that LY294002 inhibited ZPF1-mediated TNF secretion from J774A.1 macrophages in a dose-dependent manner (**Figure 8**).

To dissect the role of PI3-kinase in ZPF1-mediated signaling in the regulation of TNF secretion, LY294002 was further used to examine the role of PI3-kinase in the activation of ERK1/2, p38, and JNK1/2 within ZPF1-stimulated J774A.1 macrophages. J774A.1 cells were preincubated with and without LY294002 for 30 min and treated with ZPF1 (10 μg/mL) for an additional 30 min. Cell lysates were analyzed by Western blot with antidiphosphorylated ERK1/2, antiphosphorylated p38, or antidiphosohorylated JNK1/2 mAb. In the absence of LY294002, ZPF1 induced phosphorylation of ERK1/2, p38, and JNK1/2 as compared with the control cells (**Figure 9**). Surprisingly, the activation of ERK1/2, p38, and JNK1/2 phosphorylation showed no change even when the cells were preincubated with high concentrations of LY294002 (20 μM) (**Figure 9**). It indicated that PI3-kinase did not lay upstream of ERK1/2, p38, and JNK1/2 within ZPF1-stimulated J774A.1 macrophages.

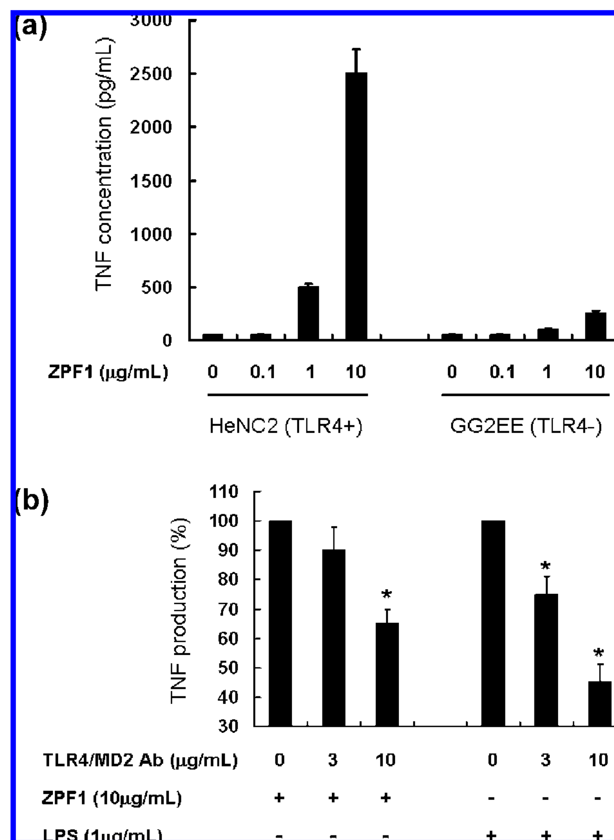

Figure 7. TLR4 is the putative receptor for ZPF1-mediated TNF secretion. (**a**) HeNC2 and GG2EE (1×10^6/mL) were incubated with yam's polysaccharide ZPF1 for 6 h. The TNF concentration in media was determined by ELISA. The data were expressed as the means ± SE with three separate experiments. (**b**) J774A.1 macrophages were incubated with TLR4/ND2 neutralizing antibody for 30 min, followed by ZPF1 and LPS stimulation for an additional 6 h. The TNF concentration in media was determined by ELISA. The data were expressed as the means ± SE with three separate experiments. The asterisk indicates significant differences at the level of $p < 0.05$ vs unpretreated control.

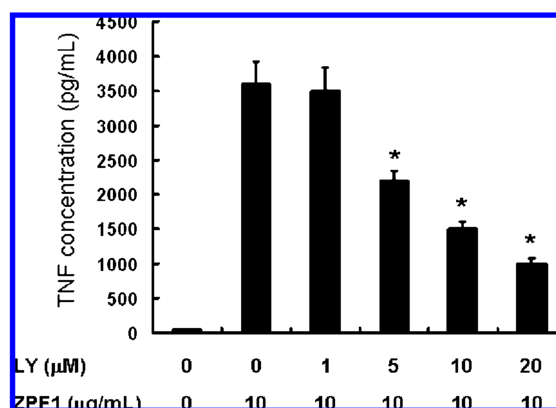

Figure 8. Effect of LY294002 on TNF secretion from ZPF1-stimulated J774A.1 macrophages. Cells (1×10^6/mL) were preincubated with various concentrations of LY294002 or DMSO for 30 min, followed by ZPF1 stimulation (10 μg/mL) for an additional 6 h. After incubation, the released TNF in conditioned media was measured by ELISA. The data were expressed as the means ± SE with three separate experiments. The asterisk indicates significant differences at the level of $p < 0.05$ vs DMSO.

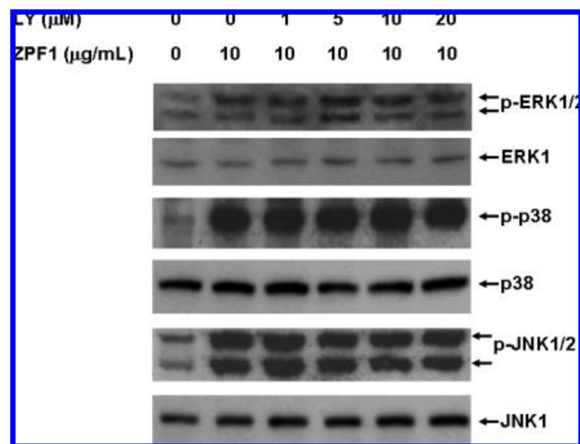

Figure 9. Effect of LY294002 on ZPF1-induced phosphorylation of ERK1/2, p38, and JNK1/2. J774A.1 macrophages were pretreated with various concentrations of LY294002 (1, 5, 10, and 20 μM) for 30 min before stimulation with ZPF1 (10 μg/mL) for an additional 30 min. The cell lysates were collected at different periods of time and analyzed by Western blot with antidiphosphorylated ERK1/2, antidiphosphorylated p38, or antidiphosohorylated JNK1/2 monoclonal antibody, respectively.

DISCUSSION

Immunologically active polysaccharides occur as the primary component in the cell walls of fungi, plants, and bacteria, for example, β-(1,3)-linked glucans of Japanese mushroom *Lentinus edodes* or *Ganoderma lucidum* (*22, 23*), fucogalactoxyloglucan, and arabinogalactan of *Echinacea purpurea* (*24*). The polysaccharide fractions from *G. lucidum* induce cytokines expression, including IL-1, IL-6, IL-12, INF-γ, TNF, GM-CSF, G-CSF, and M-CSF in fresh spleen cells from BALB/c mice (*25*). Zhao et al. pointed out that the presence of the polysaccharides from *Dioscorea opposita* Thunb root could make spleen T lymphocytes proliferation significantly increase (*26*).

A polydispersed β-(1,4)-linked acetylated mannan, CARN750, isolated from the *Aloe vera* plant, has been shown to have activity in wound repair and induction of cytokines releasing, to function as a antineoplastic, and to active macrophages (*27, 28*). In this study, we analyzed the inflammatory cytokine, TNF, stimulated by ZPF1 of *D. batatas* in murine macrophages, and determined the chemical structure of ZPF1 as β-1,4-mannan with acetylation. It suggests that ZPF1 of *D. batatas* possibly has multiple functions as does CARN750.

It has been suggested that acetylated glucomannans and glucans have the ability to form hydrogen bonds between several sugar chains (*29*). The acetyl groups of ZPF1 would be a sticking part in the structure. An overall conformation would bind or induce cellular receptors involved in the biological systems. Recently, the polysaccharide fractions from *Dioscorea alata* were reported to induce signal transduction pathways in RAW 264.7 cells (*30*). Our current results demonstrated that incubation of J774A.1 macrophages with ZPF1 quickly leads to activation of various phosphorylated kinases by TLR4 cellular receptor, including ERK1/2, JNK1/2, and p38, and further stimulation of downstream TNF expression by specific pharmacological antagonists and enzymes against various phosphorylated proteins. Also, H_2O_2 played an important role in ZPF1-mediated TNF expression within J774A.1 macrophages. We have demonstrated the differences in the activation time and the extents of various activities for ZPF1-stimulated MAPKs (ERK1/2, p38, and JNK1/2). PI3-kinase inhibitor (LY294002) did not alter ZPF1-induced ERK1/2, JNK1/2, and p38 phosphorylation (**Figure 9**) but inhibited TNF secretion (**Figure 8**). These results suggest that neither endogenous PI3-kinase nor PI3-kinase-related downstream signaling involve ZPF1-mediated activation of ERK1/2, JNK1/2, and p38. It is likely that other PI3-kinase-mediated pathway(s) participate in ZPF1-induced TNF expression (*21*). As compared to LPS-mediated TNF expression, this study indicates that ZPF1-mediated signaling in the regulation of TNF expression is different from those of LPS-mediated activation of PI3-kinase, which mediated only by the downstream signaling of JNK1/2 and p38 (*19*).

Our current results illustrate that MAPK pathways are simultaneously activated, and the dramatic induction of TNF expression suggests a cooperative effect comes along in the ZPF1-mediated regulation of TNF among these kinases. For a long time, polysaccharides have been thought to be important in producing the effect seen when curing different types of diseases treated by herbs used in traditional medicine in different parts of the world. It was especially thought that the polysaccharides play a role in combating acute inflammation by chemical stimuli (*29*). An important advantage of carbohydrate-based drugs is their low toxicity. No toxic effects have so far been reported. Polysaccharides from different medical plants exhibit biological activities that are substantial in treating illnesses among mankind in the future. The activity in the immune system is also speculated to lead to the production of more effective medicines as supplements in cancer treatment.

In summary, we have structurally determined and used an in vitro macrophage model to study the effect of *D. batatas* polysaccharides ZPF1 on cytokine expression. We have further systematically dissected the molecular mechanisms of ZPF1-mediated signals in regulation of TNF expression through TLR4 cellular receptor. Specifically, we have established the signal transduction pathways of ERK1/2, JNK1/2, and p38 as well as PI3-kinase and ROS in the cooperative regulation of TNF secretion. Taken together, the current findings suggest that ZPF1-mediated signal transduction in regulation of TNF expression will contribute to our understanding of polysaccharides of *D. batatas*-mediated immunomodulatory activities.

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