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Alkali-Soluble Polysaccharides of *Rhizoclonium riparium* Alga Induce IL-1 Gene Expression via Protein Kinase Signaling Pathways

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Fortification of aquaculture foodstuff with various algae may improve the resistance of certain fish or shrimp to diseases and, as a routine procedure, has become ever more popular and, seemingly, important. Herein, we isolated certain alkali-soluble polysaccharides from a *Rhizoclonium riparium* alga (RASP), polysaccharides that can be separated into two different groups on the basis of the polysaccharide's molecular weight. Using gas chromatography−mass spectometry analysis, we found that the major monosaccharides constituting the higher molecular-weight group of RASP were galactose (41.99%), glucose (34.53%), xylose (20.24%), and mannose (3.24%). Using a murine-derived macrophage cell line J774A.1, we found that polysaccharide constituents of the higher molecular-weight group of RASP were able to induce interleukin-1beta (IL-1) gene expression via protein kinase-mediated signal transduction pathways. In essence, we found that *c*-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38), but not extracellular signal-regulated kinase (ERK), play an important role in the regulation of IL-1 gene expression in RASP-stimulated J774A.1 cells. To the best of our knowledge, this is the first occasion that polysaccharides from *R. riparium* have been demonstrated to exert immunomodulation properties by the induction of IL-1 within macrophages. Our current results provide support for the possible use of *R. riparium* as an additive to various food/foodstuff, to modulate the immune response of humans or certain animals.

KEYWORDS: Algae; polysaccharide; macrophage; IL-1; mitogen-activated protein kinases

INTRODUCTION

The interlukin-1beta (IL-1) cytokine, involved in various inflammatory and immunological processes, is produced by activated monocytes/macrophages, as well as by many other cell types (*1*). A precursor form of IL-1, referred to as prointerlukin-1beta (proIL-1), is translated from IL-1 mRNA under inflammatory conditions and cleaved into a 17 kDa mature secreted form of IL-1 by an interleukin-1 converting enzyme (*2*). IL-1 represents a potent inflammatory cytokine that features numerous different biological activities that regulate host defense and immune responses (*3*). Mice pretreated with recombinant IL-1 prior to infection with *Escherichia coli* typically reveal significantly reduced mortality rates as compared with mice that have not undergone such pretreatment (*4*). The potentially beneficial functions of IL-1 require exact control to avoid causing serious damage (5, *6*).

It would appear from our review of the literature that the numbers of reports describing the immunostimulatory properties of algal extracts upon aquacultured shrimp are ever increasing with the passage of time (e. g., immersion within or injection of shrimp with a hot water algal extract has been reported to have increased the shrimp's inherent immune ability as well as

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its resistance to pathogen infection (*7*, *8*)). In addition, polysaccharides extracted from certain algae have been shown to induce cytokine production within certain mammalian cells (*9*−*12*); however, the cellular signaling pathways involved in the regulation of cytokine gene expression within algal polysaccharide-stimulated macrophages would still appear to be somewhat unclear. It has often been reported that mitogen-activated protein kinases (MAPKs) play important roles in the regulation of a multitude of cellular responses, including cytokine gene expression (*13*−*15*). We have demonstrated previously that macrophages activated by polysaccharides from certain brown seaweed (*13*), fungus (*14*), and bacteria (*15*) are able to induce the activation of the MAPKs: extracellular signal-regulated kinase (ERK), *c*-Jun N-terminal kinase (JNK), and mitogen-activated protein kinase (p38) as well as relevant signal-transduction cascades, leading to nuclear translocation of certain transcription factors related to IL-1 gene expression. In a previous study, we reported that the filamentous alga *Rhizoclonium riparium* was able to be used as a partial substitute for the wood fiber that is typically used to make paper, due to the alga's fibrillar morphology and high cellulose content (*16*, *17*). By contrast, the potential immunological properties of polysaccharides derived from *R. riparium* have, to the best of our knowledge, never been reported previously.

Herein, we report on how we have isolated alkali-soluble polysaccharides from *R. riparium* (RASP) and investigated their immunomodulation functions, including the stimulation of IL-1 gene expression within murine macrophages. In essence, we found that RASP stimulates IL-1 mRNA expression, proIL-1 production, and IL-1 secretion. Using MAPK inhibitors, we have demonstrated that JNK and p38, but not ERK, related signaling pathways are involved in the regulation of RASP-mediated proIL-1 production. In addition, by conducting certain polymyxin-B inhibitory experiments and employing a Limulus amebocyte lysate (LAL) assay, we believe that we have ruled out the possibility of the activation of macrophages by RASP being due to lipopolysaccharide (LPS) contamination of the RASP sample we used. Furthermore, we have analyzed the chemical characteristics of certain RASP, including the distribution of their approximate molecular weights and gas chromatography−mass spectometry (GC−MS) analyses of the major monosaccharide composition of RASP. To the best of our knowledge, this is the first paper to demonstrate that certain polysaccharides from *R. riparium* reveal immunomodulation activity within certain macrophages.

MATERIALS AND METHODS

Materials and Cell Cultures. LPS (from *E. coli* 0111:B4), polymyxin B (PMB), monoclonal anti-MAP kinase, activated (diphosphorylated ERK) antibody, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) antibody, monoclonal anti-p38 MAP kinase, activated (diphosphorylated p38) antibody, and monoclonal anti-actin antibody were purchased from Sigma Co. (St. Louis, MO). The Limulus ameboctye lysate (LAL) PYROCHROME assay kit was purchased from CAPE COD, Inc. The mouse IL-1beta Enzyme-Linked Immunosorbent Assay Kit was purchased from R & D Systems, Inc. (Minneapolis, MN). RE$_{ZO}$l C&T was from PROtech Technology Co. (Taipei, Taiwan). Anti-IL-1beta, polyclonal antibody, anti-rabbit IgG-HRP, and anti-mouse IgG-HRP were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Protein kinase inhibitors: PD98059, SP600125, and SB203580 were purchased from Calbiochem-Novabiochem Corp. Oligonucleotides: primers for IL-1 and glyceraldehyde phosphate dehydrogenase (GAPDH) were synthesized from local MD Bio., Inc. (Taipei, Taiwan). Murine macrophage J774A.1 cells were obtained from ATCC (Rockville, MD), propagated in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mM l-glutamine (Life Technologies, Inc., MD), and cultured in a 37 °C, 5% CO_2 incubator.

Preparation of RASP. *R. riparium* was collected from the aquaculture ponds at the Taihsi Branch Station of the Taiwan Fishery Research Institute. Most of the algae were transported to the laboratory in Keelung, where these algae were first rinsed with sterile seawater, selectively cultured for at least 1 year to arrive at a purified strain, and then maintained under sterile conditions. After harvest from the culture tank, the alga was cleaned with distilled water and air-dried. Then whole cleaned and dried algal specimens were placed in a Wiley mill and ground into powder. The powder was sieved through a mechanical sieve sets to provide particles that were smaller than 40 mesh but larger than 100 mesh. Ten grams of *R. riparium* samples was extracted with 24% potassium hydrate and stirrred for 24 h at 20 °C. Then, the extracts were filtered by a G4 glass filter, neutralized with 20% acetic acid, and dialyzed with deionized water by using dialysis membrane tubing (M_w cutoff 2000, Spectrapor) at 4 °C. After dialysis, the extracts were vacuum-concentrated at 50 °C, giving a final volume of 100 mL to which four volumes of 95% ethanol was added slowly at 4 °C. Then, the mixture was centrifuged to give the precipitates (RASP) of ca. 350 mg (dry weight).

Gel-Filtration Chromatography of the RASP. One hundred milligrams of RASP was purified by gel-filtration chromatography using a Sephadex G-100 (Pharmacia Biotech) column (100 cm × 1.6 cm) with deionized water as the eluent. The flow rate was set at 0.3 mL/min, and 5.0 mL per tube was collected. Under the same conditions, the Shodex standard P-82 was used as an internal standard of molecular weight, and all chromatography processes were executed under 4 °C. After chromatography, each tubing sample was subjected to analysis to determinate its total carbohydrate content, which was performed using the phenol-sulfuric acid colormetric method (with glucose as the calibration) (*18*), and the absorption was directly determined to be UV 280 nm for protein.

GC−MS Analysis. The RASP (high molecular-weight group) was first hydrolyzed with 72% sulfuric acid, then the inositol (internal standard) was added, and the solutions were reduced with sodium borohydride to alditols (*16*). After acetylation, the concentrated dichloromethane solutions were injected into a GC−MS (HP 6890, with MS detector model HP 5973) instrument fitted with a SP-2330 column, 30 m in length, 0.25 mm i.d., and 0.2 μm film; an oven temperature of 220 °C; an injection temperature of 280 °C; a detector temperature of 300 °C; and helium as a carrier gas at a split ratio of 25:1. Carbohydrate analysis was done with inositol as the internal standard; its integrated peak area was used to establish the relative amounts of the constituents. The compounds were identified by comparing their mass spectrometric fragmentation patterns with those of authentic standards, and the quantity of compounds was obtained by integrating the peak area of the spectrograms.

Enzyme Linked Immunosorbent Assay (ELISA). In the time course study, J774A.1 cells (1×10^6/mL) were stimulated with RASP (100 μg/mL) for the indicated time points within 24 h. In the dose response study, J774A.1 cells (1×10^6/mL) were stimulated with RASP (0 to ∼100 μg/mL) for 24 h. The IL-1 concentration in the condition medium was analyzed by ELISA according to the protocol from the R & D mouse IL-1beta ELISA System (R & D Systems, Inc). In brief, 50 μL of biotinylated antibody reagent and 50 μL of supernatant were added to an anti-mouse IL-1 precoated stripwell plate and incubated at room temperature for 3 h. After washing the plate three times with washing buffer, 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, and 100 μL of a premixed TMB 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 to each well to stop the reaction, the absorbance of the plate was measured by a MRX microplate reader (Dynex Tech. Inc.) at a 450−550 nm wavelength.

Monitor of LPS Contamination in Experiments. We were aware of the problem of LPS contamination in studies of RASP-mediated reactions and signaling. Our reagents and utensils for preparation of RASP were either endotoxin-free grade or washed with PBS containing 50 μg/mL PMB and then rinsed with PBS. To rule out possible LPS contamination of RASP samples, J774A.1 cells with or without PMB (10 μg/mL) were preincubated for 30 min, followed by treatment with RASP (100 μg/mL) and LPS (1 or 2 μg/mL) for 6 h, respectively; proIL-1 expression was analyzed by Western blotting. In addition, the LPS concentration in the RASP sample was analyzed by a Limulus ameboctye lysate (LAL) PYROCHROME assay kit (CAPE COD, Inc.).

Western Blotting. Whole cell lysates were separated by 12% SDS−PAGE and electrotransferred to a PVDF membrane. The membrane was incubated in blocking solution (5% nonfat milk in PBS with 0.1% Tween 20) at room temperature for 1 h. The membrane was incubated with anti-IL-1beta antibody or anti-MAPKs antibody at room temperature for 2 h. After washing three times in PBS with 0.1% Tween 20, the membrane was incubated with an HRP-conjugated secondary antibody directed against the primary antibody. The membrane was developed by an enhanced chemiluminescence Western-blotting detection system (DuPont NEN Research Product Co, Boston, MA) according to the manufacturer's instructions.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated by the RE_{zo}l C&T method according to the manufacturer's instruction. For reverse transcription, the reaction was performed at 42 °C for 30 min, at 99 °C for 5 min, and then cooled to 4 °C. For future PCR, the PCR mixture was held at 94 °C for 2 min and then cycled 30 times at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 2 min followed by 10 min at 72 °C at the final cycle. The products from PCR reaction were examined by 1% agarose gel electrophoresis with ethidium bromide (EtBr) and normalized by comparison to RT-PCR of mRNA of GAPDH, a constitutively expressed gene. Each EtBr-stained band was quantified using ImageQuaNT software from a PhosphorImager from Molecular Dynamics (Sunnyvale, CA).

Statistical Analysis. Statistical differences between the experimental groups were examined by analysis of variance, and the statistical significance was determined at $p < 0.05$. The experiments were conducted three times or as indicated, and all data are expressed as mean ± SE.

RESULTS AND DISCUSSION

Chemical Compositions of RASP. The crude alkali-soluble polysaccharides of *R. riparium* (RASP) were obtained experimentally featuring a yield of 3.5% (based on dry algal weight). Gel-filtration chromatography using a Sephadex G-100 column was carried out to obtain preliminary molecular-weight information regarding the RASP evaluated in the immunological assays, including cytokine production. The RASP investigated herein revealed two obvious and different molecular-weight groups, details of which are shown in **Figure 1**. The higher molecular-weight group was comprised of monosaccharides that were shown to feature a size greater than 1000 kDa/mol, and the lower molecular-weight group was a size below 5000 Da/mol. Using a Bio-Rad protein assay method, we found that RASP contains about 18.3% protein (calculated based upon bovine serum albumin). The RASP revealed obvious absorption of UV at 280 nm, a result that indicated that the RASP might contain glycoproteins. Further, the polysaccharides were observed to be primarily composed of galactose (41.99%), glucose (34.53%), xylose (20.24%), and mannose (3.24%), as analyzed by GC−MS (**Table 1**). This result compared favorably with the results of our previous study that revealed that alkali-soluble polysaccharides of *R. riparium* resulted in the extraction of a substantially larger proportion of galactose and glucose than was the case for water extraction (*16*). It has been reported that analysis of the carbohydrate composition of *Ganoderma lucidum* polysaccharides indicated that glucose and mannose were present and existed as the major components together with smaller amounts of other sugars, including fucose, *N*-acetylglucosamine, xylose, and rhamnose, and in addition, the polysaccharides were also shown to contain 15.6% protein (*19*).

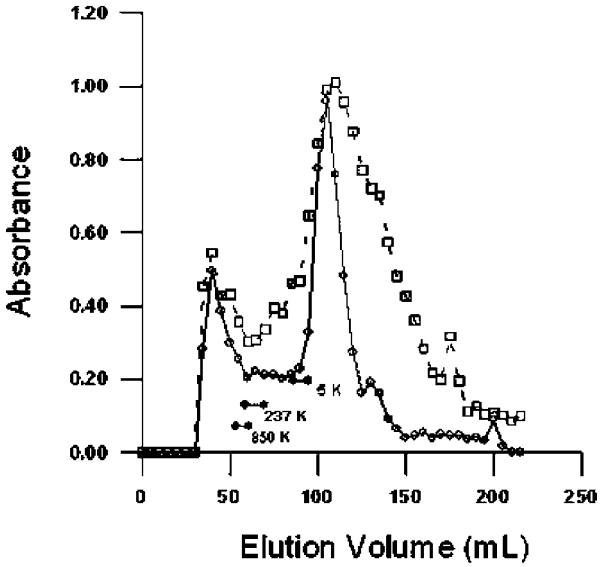

Figure 1. Gel-filtration chromatography elution profile for the RASP. The column was loaded with 100 mg of sample and then eluted with distilled water. The flow rate was 0.3 mL/min, 5.0 mL per tube was collectd, and each tubing was analyzed for total carbohydrate quantity (-○-) and direct absorption determination at UV 280 nm for protein quantity (-■-). An internal standard of molecular weight was presented with the figure (●-●), and all chromatography processes were executed under 4 °C.

RASP Stimulates IL-1 Gene Expression within Murine Macrophage J774A.1 Cells. IL-1 is one of the important pro-inflammatory cytokines and is produced mainly by activated macrophages, and this cytokine mediates multiple biological effects including the activation of the immune response for various species (*1*). Some details pertaining to the function of polysaccharides isolated from different algae as regards the stimulation of cytokine expression have been previously reported (*9*, *20*). To test whether RASP could stimulate IL-1 protein secretion within J774A.1 cells, we used an ELISA technique to characterize the kinetics and the dose−response characteristics of IL-1 secretion within RASP-stimulated cells. In the secretory kinetic study, J774A.1 cells were stimulated with RASP (100 μg/mL) and LPS (1 μg/mL) during a testing period of from 0 to 24 h, the concentration of released IL-1 in conditioned media being measured by an ELISA technique. The presence of IL-1 was able to be detected in conditioned media as early as 3 h post-RASP stimulation of J774A.1 cells, the levels of thus-released IL-1 remaining elevated for up to 24 h post-RASP stimulation **(Figure 2A)**. In essence, secreted IL-1 levels of around 20, 80, 110, 150, 230, and 300 pg/mL were detected in the conditioned medium harvested from a J774A.1 cell culture at, respectively, 3, 6, 9, 12, 18, and 24 h post-stimulation for RASP-stimulated cells, whereas the concentration of LPS-induced IL-1 for cultured J774A.1 cells was about 250 pg/mL at 6 h post-stimulation. For the dose−response study described herein, cells were stimulated with various concentrations of RASP (0 to ∼100 μg/mL) for a period of 24 h, the consequent IL-1 secretion from cells increasing in a dosage-dependent fashion, and the level increasing with an increase in the RASP concentration to which cells were exposed (**Figure 2B**). These

Table 1. Sugar Composition of Alkali-Soluble Polysaccharide of *R. riparium* Alga

retention time (min)	chemical compound	%	primary fragments in the mass spectra
9.55	1,2,3,4,5-penta-*o*-acetyl-6-deoxy-L-mannitol	3.32	43, 115, 128, 157, 170,[a] 187, 201, 231, 303
11.12	1,2,3,4,5-penta-*o*-acetyl-D-xylitol	28.91	43, 103, 115,[a] 127, 145, 187, 217, 289
15.48	D-mannitol, hexaacetate	5.54	43,[a] 103, 115, 139, 157, 187, 217, 259, 289
16.60	galactitol, hexaacetate	39.35	43,[a] 103, 115, 145, 187, 217, 259, 289
18.12	D-glucitol, hexaacetate	17.87	43, 85, 95, 103, 115,[a] 145, 187, 289

[a] Base peak with 100% intensity.

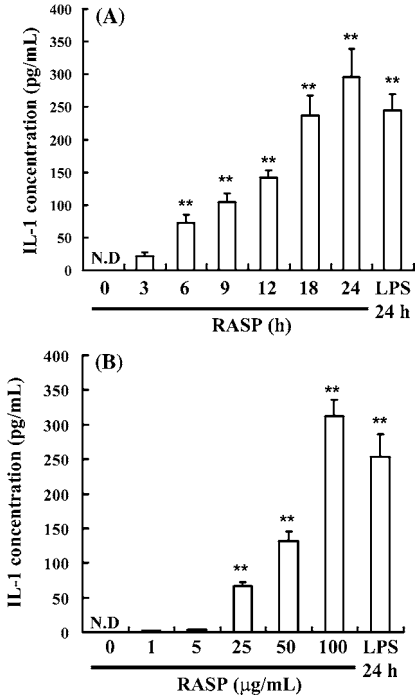

Figure 2. RASP induces IL-1 secretion in J774A.1 cells. (**A**) Cells (1×10^6/mL) were treated with RASP (100 μg/mL) or LPS (1 μg/mL) for the time indicated within 24 h. Condition media were harvested and analyzed for IL-1 concentration using IL-1 specific ELISA; one of four experiments is presented ($n = 4$). N.D. means nondetectable. (**B**) Dose response of ELISA analyses of IL-1 secretion in RASP-treated cells. Cells were treated with various concentrations of RASP as indicated (0 to ~100 μg/mL) or LPS (1 μg/mL) for 24 h. IL-1 concentration was measured by IL-1 specific ELISA; one of four experiments is presented ($n = 4$). N.D. means nondetectable.

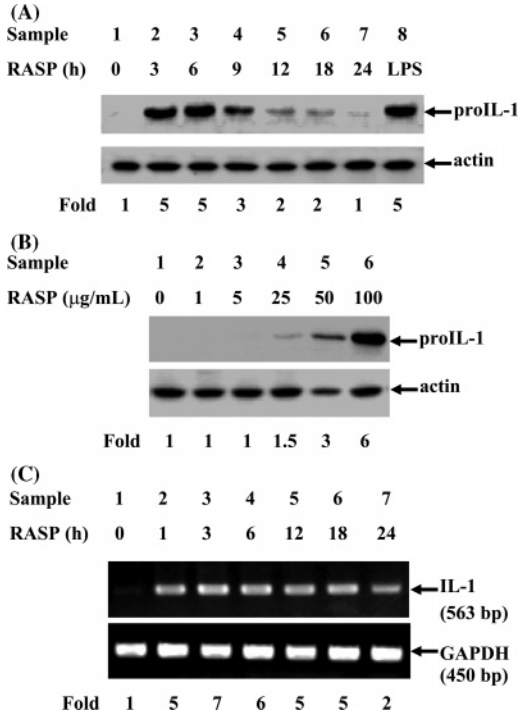

Figure 3. RASP induces proIL-1 protein and proIL-1 mRNA expression in J774A.1 cells. (**A**) Western-blotting analysis of proIL-1 protein expression in RASP-treated cells. Cells were treated with RASP (100 μg/mL) or LPS (1 μg/mL), and whole cell lysates were harvested at the time indicated within 24 h. ProIL-1 expression was analyzed by Western blotting. The proIL-1 and actin (as an internal control) are indicated as arrows on the right side, and one of four experiments is presented ($n = 4$). (**B**) Dose response of Western-blotting analyses of proIL-1 protein expression in RASP-treated cells. Cells were treated with various concentrations of RASP as indicated (0 to ~100 μg/mL) for 6 h, and whole cell lysates were analyzed by Western blotting. The proIL-1 and actin (as an internal control) are indicated as arrows on the right side, and one of four experiments is presented ($n = 4$). (**C**) RT-PCR analysis of proIL-1 mRNA expression in RASP-treated cells. Total RNA was isolated from cells treated with RASP between 1 and 24 h. Ethidium bromide-stained agarose gel with amplified proIL-1 mRNA at 563 bp and normalized by comparison to RT-PCR of GAPDH mRNA (450 bp) is indicated with arrows for proIL-1 and GAPDH. One of four experiments is presented ($n = 4$).

results were similar to the observation that an acidic polysaccharide isolated from the root of *Panax ginseng* (a medicinal herb) was able to induce IL-1 expression within murine macrophages (*21*) and within THP-1 human monocytes (*22*). Since IL-1 is able to stimulate a variety of pro-inflammatory responses, some of which are an important component of an individual's defense against pathogens and, further, since IL-1 also functions as an immunoadjuvant, our results relating to RASP-mediated IL-1 expression within cultured J774A.1 cells suggest that RASP could be used in the process of the modulation of immune responses.

We further investigated the molecular mechanism controlling the secretion of mature IL-1 from RASP-stimulated macrophages. A RASP-induced IL-1 precursor, proIL-1, was detected in the whole cell lysates after RASP stimulation by means of Western-blotting analysis. In this time course study, the expression of proIL-1 protein within RASP-stimulated cells was detected at between 3 and 18 h after RASP stimulation, the level peaking at 6 h post-stimulation. Further, the level of expression of proIL-1 protein was noted to commence its decline at around 9 h post-stimulation, with proIL-1 expression gradually returning to a basal level approximately 24 h post-RASP stimulation (**Figure 3A**). In addition, the expression of proIL-1 increased with increasing RASP concentrations in a dosage-dependent manner (**Figure 3B**), a relationship that was similar to that of IL-1 secretion in response to RASP stimulation (**Figure 2B**). Moreover, using an RT-PCR method, we demonstrated that IL-1 mRNA expression was detected within cultured J774A.1 cells 1 h after RASP stimulation, the level

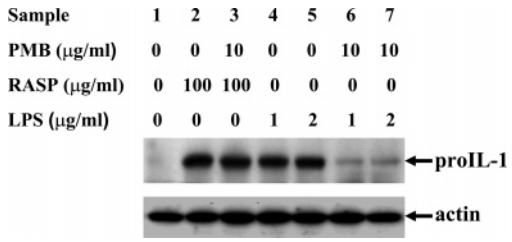

Figure 4. RASP is LPS-free. Polymyxin B sulfate (PMB) inhibited LPS-induced but not RASP-induced proIL-1 expression. Preincubation of J774A.1 cells with or without PMB (10 μg/mL) for 30 min, followed by treatment with RASP (100 μg/mL) or LPS (1–2 μg/mL) for 6 h. Whole cell lysates were analyzed by Western blotting. The proIL-1 and actin (as an internal control) are indicated as arrows on the right side, and one of four experiments is presented ($n = 4$).

peaking 3 h post-stimulation (at about 7-fold basal level). Further, 24 h post-RASP stimulation, IL-1 mRNA expression began to decline but still remained about 2-fold greater than that of corresponding control cells (**Figure 3C**). Such results demonstrate that RASP stimulates IL-1 expression both at a transcriptional and a translational level, a result that would appear to be similar to the ability of polysaccharides isolated from *Ganoderma lucidum* (a medical mushroom) to stimulate IL-1 expression within human and murine macrophages (*14*, *19*); however, the effect of RASP on IL-1 converting enzyme (an enzyme that is able to cleave 34 kDa proIL-1 into a 17 kDa mature form of IL-1) needs further investigation (*2*).

RASP Is LPS Free. Lipopolysaccharide is a cell-wall component of Gram-negative bacteria and is a potent stimulator of macrophages as regards IL-1 expression (*15*). To rule out the possibility of RASP-induced IL-1 expression being due to LPS contamination of RASP during sample preparation, we tested the effect of polymyxin B (PMB), an antibiotic that was used to neutralize the activity LPS (*14*), on RASP- and LPS-induced proIL-1 expression within J774A.1 cells. We found that PMB could be used to completely inhibit LPS-induced proIL-1 expression (**Figure 4**, samples 4 and 5 vs samples 6 and 7); yet, by contrast, PMB was not able to be used to inhibit RASP-induced proIL-1 expression (**Figure 4**, samples 2 vs 3). We have demonstrated that relatively low concentrations of LPS (10 ng/mL) are able to induce detectable proIL-1 and IL-1 protein expression within J774A.1 cells (*15*). By contrast, however, in this study, we found that RASP at a dosage of 5 μg/mL did not induce detectable 1L-1 release (**Figure 2B)** and/or proIL-1 expression (**Figure 3B**) within J774A.1 cells, indicating that the concentration of LPS in a RASP sample must be below 0.2% for the RASP to induce IL-1 gene expression. In addition, we determined the LPS content of RASP samples by means of a Limulus ameboctye lysate (LAL) assay, and we further observed an insignificant level of endotoxin (<1 EU mL^{-1}) in the tested RASP samples (data not shown). Taking these results together, we believe that the effect of RASP upon IL-1 gene expression was not due to LPS contamination of the RASP but to the presence of the RASP itself.

RASP Activates Mitogen-Activated Protein Kinase (MAPKs) within J774A.1 Cells. Activation of macrophages by extracellular stimuli (e.g., polysaccharides from brown seaweed or *G. lucidum*) transduces certain specific cellular responses through the activation of MAPK signaling pathways (*13*, *14*). There are three major groups of MAPKs in mammalian cells, including ERK, JNK, and p38. MAPKs are serine/threonine protein kinases that are able to phosphorylate both

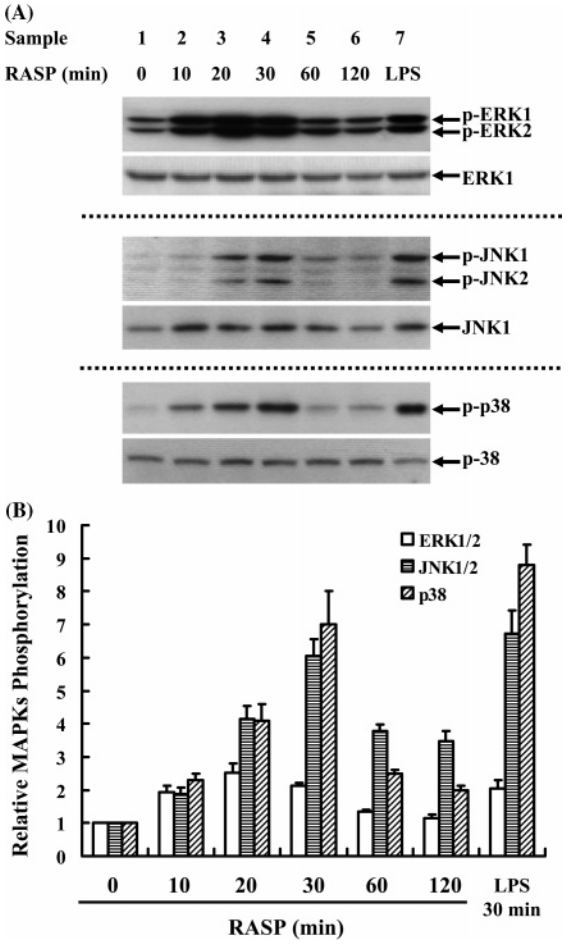

Figure 5. Time course of RASP-induced MAPKs phosphorylation. (**A**) Analysis of time course of ERK, JNK, and p38 phosphorylation in RASP-treated J774A.1 cells. Cells were stimulated with RASP (100 μg/mL) or LPS (1 μg/mL), and the cell lysates were collected after stimulation at the time indicated. Cell lysates were analyzed by Western blotting with anti-diphosphorylated ERK, anti-diphosphorylated JNK, or anti-diphosphorylated p38 monoclonal antibody. One of four experiments is presented ($n = 4$). (**B**) Histograms represent quantification by PhosphorImager of RASP-stimulated phospho-ERK, phospho-JNK, and phospho-p38 in J774A.1 cell samples using ImageQuaNT software from Molecular Dynamics. All data of relative folds are expressed as comparisons with untreated cells ($t = 0$, phosphorylation of control cells defined as 1-fold).

cytoplasmic and nuclear targets related to gene expression. The major function of the MAPK pathways is to control eukaryotic gene expression programs in response to extracellular signals. MAPKs directly control gene expression by phosphorylating transcription factors (*23*). To examine RASP-mediated signal-transduction pathways in the regulation of IL-1 gene expression, we tested whether RASP was able to stimulate MAPKs. Using Western-blotting analyses with an anti-phospho-ERK antibody, we detected an activated, Thr202/Tyr204-phosphorylated form of ERK in whole cell lysates upon RASP stimulation. The phosphorylated ERK within cultured J774A.1 cells was detected in cell lysate at around 10 min after RASP stimulation and peaked 20 min post-stimulation. Some 60 min post-stimulation, the level of ERK phosphorylation had gradually returned to the basal level (**Figure 5A**,**B**). ERK-related signaling pathways are reported to be associated with cell proliferation (*24*), suggesting that RASP would thus increase the level of J774A.1 cell proliferation (see the next section). The observed inflammatory

response of J774A.1 cells to RASP-induced IL-1 expression prompted us to investigate the possibility of RASP activation of the stress-related JNK pathway. J774A.1 cells incubated with RASP revealed some level of JNK phosphorylation, as determined by a Western-blotting analysis technique employing anti-phospho-JNK, an antibody that recognizes the activated, Thr183/Tyr185-phosphorylated form of JNK (*14*). We noted that the level of RASP-induced JNK phosphorylation gradually increased at about 10 min after RASP stimulation, reaching the maximal level (a 6-fold increase over control levels) at around 30 min post-stimulation, and 120 min after such stimulation, the level of induced JNK phosphorylation had dropped back to a level of 3-fold that of the untreated control sample (**Figure 5A**,**B**). To explore additional RASP-mediated signal-transduction pathways, we further examined whether RASP was able to induce the phosphorylation of p38, another important stress-related MAPK-involving event (*23*). Upon RASP stimulation, the level of p38 phosphorylation was observed to gradually increase, as detected by Western-blotting analyses using anti-phospho-p38, an antibody that specifically recognizes the activated, Thr180/Tyr182-phosphorylated form of p38 (*23*). The time course study of RASP-induced p38 phosphorylation indicated that at about 30 min after RASP stimulation of J774A.1 cells, p38 showed a 7-fold increase in the level of phosphorylation as compared to that observed for control cells, and 60 min post such stimulation, the level had returned to about 2-fold that of the control level (**Figure 5A**,**B**). Comparing such results with corresponding results of some of our previous studies, we found that RASP and *G. lucidum* polysaccharides exerted a similar effect upon MAPK phosphorylation, although these two entities revealed different reaction kinetics for such effects (*14*).

Role of RASP-Induced Protein Kinase in the Regulation of proIL-1 Expression. To elucidate the role of various RASP-induced protein kinase-mediated signaling pathways in the regulation of proIL-1 expression, we utilized certain specific pharmacological antagonists such as PD98059, SP600125, and SB203580 that inhibit the activation of, respectively, MEK1, JNK, and p38 (*25*−*27*). The dose−response for the impact of specific protein-kinase inhibitors upon proIL-1 expression for J774A.1 cells was monitored by directly assaying individual kinase activity, and the effective working concentrations of protein-kinase inhibitors were also determined (data not shown). Initially, we examined the signaling pathway of RASP-induced MEK1/ERK as regards the regulation of proIL-1 expression within J774A.1 cells. These cells were exposed to various concentrations of PD98059 as indicated previously, followed by incubation with RASP for an additional 6 h. The data derived from the Western-blotting analyses that we conducted indicated that PD98059 exerted no significant effect upon RASP-induced proIL-1 expression within cultured J774A.1 cells (**Figure 6A**). By contrast, however, PD98059 significantly inhibited LPS-induced proIL-1 expression (**Figure 6D**), such a result indicating that MEK1/ERK-related signaling pathways are not at all associated with RASP-induced proIL-1 expression. Subsequently, we used SP600125 (a JNK inhibitor) to investigate the role of JNK in the regulation of proIL-1 expression within RASP-stimulated cells. We observed that SP600125 (10 μM) significantly inhibited RASP-induced proIL-1 expression, indicating that JNK-related signaling pathways play important roles in RASP-mediated proIL-1 expression (**Figure 6B**). In our previous study, we found that PD98059, but not SP600125, significantly inhibited *G. lucidum* polysaccharide-induced proIL-1 expression (*14*), suggesting that ERK and JNK play different

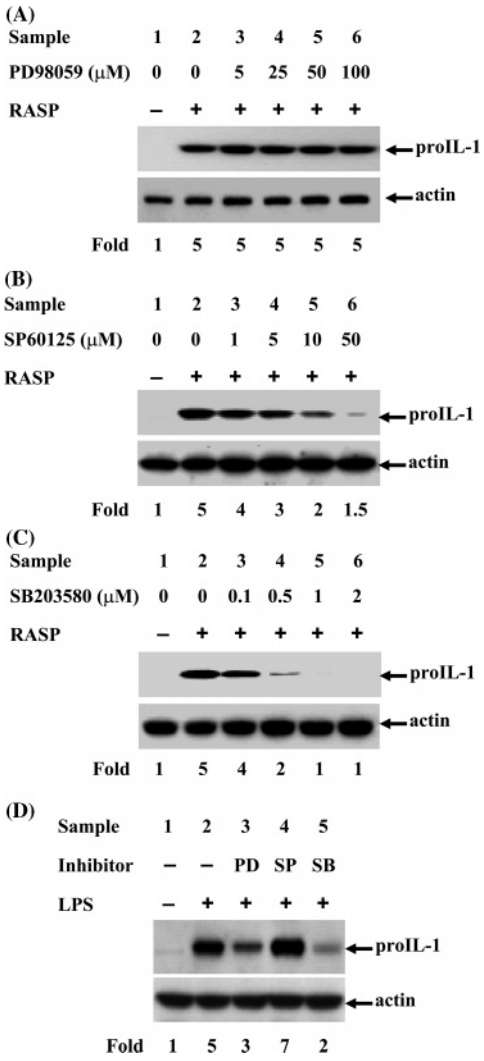

Figure 6. Effect of protein kinase inhibitors on RASP-induced proIL-1 protein expression in J774A.1 cells. Effect of (**A**) PD98059, (**B**) SP600125, and (**C**) SB203580 on RASP-induced proIL-1 protein expression. Cells were preincubated with inhibitors for 1 h followed by RASP stimulation (100 μg/mL) for an additional 6 h. The proIL-1 expression was analyzed by Western blotting. The proIL-1 and actin (as an internal control) are indicated as arrows on the right side, and one of four experiments is presented ($n = 4$). (**D**) Effect of protein kinase inhibitors on LPS-induced proIL-1 protein expression. Cells were preincubated with PD98059 (PD, 100 μM), SP600125 (SP, 50 μM), and SB203580 (SB, 2 μM) for 1 h followed by LPS (1 μg/mL) stimulation for an additional 6 h. The proIL-1 expression was analyzed by Western blotting. The proIL-1 and actin (as an internal control) are indicated as arrows on the right side, and one of four experiments is presented ($n = 4$).

roles as regards proIL-1 expression within RASP-stimulated and *G. lucidum* polysaccharide-stimulated J774A.1 cells. Moreover, SB203580 was observed to completely inhibit RASP-induced proIL-1 expression by J774A.1 cells, at a SB203580 concentration above 1 μM (**Figure 6C**), such a result being similar to that elicited by stimulation with *G. lucidum* polysaccharides (*14*). Our results suggest that JNK- and p38-related, but not ERK-related signaling pathways, are involved in RASP-induced proIL-1 expression within J774A.1 cells. Furthermore, we also observed that LPS-induced proIL-1 expression was inhibited by PD98059 and SB203580 but not SP600125 (**Figure D**). Taken together, our results have indicated that IL-1 expression is influenced via differential protein kinase-mediated signaling

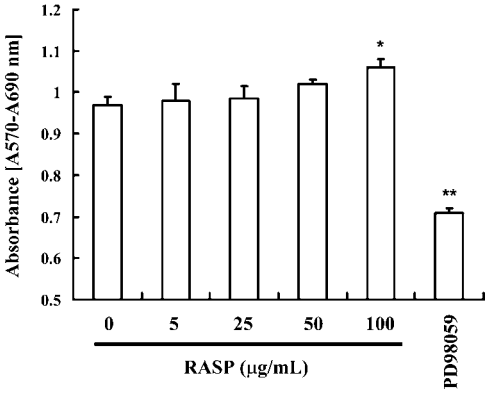

Figure 7. Effect of RASP on J774A.1 cell viability. Cells were either treated with RASP (0 to ~100 μg/mL), DMSO vehicle, or PD98059 (100 μM) for 24 h, followed by incubation with the MTT reagent and precipitate solubilized, and the absorbance (A550–A690) was measured by spectrophotometry.

pathways within RASP- and LPS-stimulated J774A.1 cells. Furthermore, no cytotoxic effect was observed after J7774A.1 cells were treated with various concentrations of RASP for a period of 24 h as measured by MTT assay (**Figure 7**). Further, RASP at the high concentrations (i.e., around 100 μg/mL) was observed to increase J774A.1 cell proliferation. By contrast, PD98059, a specific inhibitor of MEK1/ERK, significantly inhibited J774A.1 cell viability. We suggest that the RASP-mediated increase in J774A.1 cell proliferation may be due to the activation of ERK-related signaling pathways (**Figure 5**).

In summary, we have used a macrophage model to investigate, in vitro, the immunomodulating properties of alkali-soluble polysaccharides of *R. riparium* (RASP). We have found that RASP activates IL-1 mRNA expression, proIL-1 protein expression, and IL-1 secretion within a murine-derived macrophage cell line. Further, we also dissected RASP-mediated protein kinase signaling pathways involved in the regulation of IL-1 expression. In essence, JNK and p38, but not ERK, are involved in RASP-mediated IL-1 expression. Our current results provide an avenue whereby it remains possible to use RASP as a natural food/foodstuff additive to modulate the immune response of either humans or animals.

LITERATURE CITED

(1) Dinarello, C. A. Biologic basis for interleukin-1 in disease. *Blood* **1996**, *87*, 2095−2147.

(2) Cerretti, D. P.; Kozlosky, C. J.; Mosley, B.; Nelson, N.; Van Ness, K.; Greenstreet, T. A.; March, C. J.; Kronheim, S. R.; Druck, T.; Canniz, L. A. et al. Molecular cloning of the interleukin-1 beta converting enzyme. *Science* **1992**, *256*, 97−100.

(3) Dinarello, C. A. Interleukin-1 beta. *Crit. Care Med.* **2005**, *12*, S460−462.

(4) Joshi, V. D.; Kalvakolanu, D. V.; Hebel, J. R.; Hasday, J. D.; Cross, A. S. Role of caspase 1 in murine antibacterial host defenses and lethal endotoxemia. *Infect Immun*. **2002**, *70*, 6896−6903.

(5) Waage, A.; Espevik, T. Interleukin-1 potentiates the lethal effect of tumor necrosis factor alpha/cachectin in mice. *J. Exp. Med.* **1988**, *167*, 1987−1992.

(6) Kay, J.; Calabrese, L. The role of interleukin-1 in the pathogenesis of rheumatoid arthritis. *Rheumatology* **2004**, *4*, iii2−iii9.

(7) Yeh, S. T.; Lee, C. S.; Chen, J. C. Administration of hot water extract of brown seaweed *Sargassum duplicatum* via immersion and injection enhances the immune resistance of white shrimp *Litopenaeus vannamei*. *Fish Shellfish Immunol.* **2006**, *20*, 332−345.

(8) Hou, W. Y.; Chen, J. C. The immunostimulatory effect of hot water extract of *Gracilaria tenuistipitata* on the white shrimp *Litopenaeus vannamei* and its resistance against *Vibrio alginolyticus*. *Fish Shellfish Immunol.* **2005**, *19*, 127−138.

(9) Hasegawa, T.; Kimura, Y.; Hiromatsu, K.; Kobayashi, N.; Yamada, A.; Makino, M.; Okuda, M.; Sano, T.; Nomoto, K.; Yoshikai, Y. Effect of hot water extract of *Chlorella vulgaris* on cytokine expression patterns in mice with murine acquired immunodeficiency syndrome after infection with *Listeria monocytogens*. *Immunopharmacology* **1997**, *35*, 273−282.

(10) Dantas, D. C.; Kaneno, R.; Queiroz, M. L. The effects of *Chlorella vulgaris* in the protection of mice infected with *Listeria monocytogenes*. Role of natural killer cells. *Immunopharmacol. Immunotoxicol.* **1999**, *21*, 609−619.

(11) Zvyagintseva, T. N.; Shevchenko, N. M.; Nazarova, I. V.; Scobun, A. S.; Luk'yanov, P. A.; Elyakova, L. A. Inhibition of complement activation by water-soluble polysaccharide of some far-eastern brown seaweeds. *Comp. Biochem. Physiol.* **2000**, *126*, 209−215.

(12) Matsumoto, S.; Nagaoka, M.; Hara, T.; Kimura-Takagi, I.; Mistuyama, K.; Ueyama, S. Fucoidan derived from *Cladosiphon okamuranus* Tokida ameliorates murine chronic colitis through the down-regulation of interleukin-6 production on colonic epithelial cells. *Clin. Exp. Immunol*. **2004**, *136*, 432−439.

(13) Hsu, H. Y.; Chiu, S. L.; Wen, M. H.; Chen, K. Y.; Hua, K. F. Ligands of macrophage scavenger receptor induce cytokine expression via differential modulation of protein kinase signaling pathways. *J. Biol. Chem.* **2001**, *276*, 28719−28730.

(14) Hsu, H. Y.; Hua, K. F.; Lin, C. C.; Lin, C. H.; Hsu, J.; Wong, C. H. Extract of Reishi polysaccharides induces cytokine expression via TLR4-modulated protein kinase signaling pathways. *J. Immunol.* **2004**, *173*, 5989−5999.

(15) Hsu, H. Y.; Wen, M. H. Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. *J. Biol. Chem.* **2002**, *277*, 22131−22139.

(16) Chao, K. P.; Su, Y. C.; Chen, C. S. Chemical composition and potential for utilization of the marine alga *Rhizoclonium* sp. *J. Appl. Phycol.* **1999**, *11*, 525−533.

(17) Chao, K. P.; Su, Y. C.; Chen, C. S. Feasibility of utilizing *Rhizoclonium* in pulping and papermaking. *J. Appl. Phycol.* **2000**, *12*, 53−62.

(18) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* **1956**, *28*, 350−356.

(19) Wang, Y. Y.; Khoo, K. H.; Chen, S. T.; Lin, C. C.; Wong, C. H.; Lin, C. H. Studies on the immuno-modulating and antitumor activities of *Ganoderma lucidum* (Reishi) polysaccharides: functional and proteomic analyses of a fucose-containing glycoprotein fraction responsible for the activities. *Bioorg. Med. Chem.* **2002**, *10*, 1057−1062.

(20) Pugh, N.; Ross, S. A.; El Sohly, H. N.; El Sohly, M. A.; Pasco, D. S. Isolation of three high molecular weight polysaccharide preparations with potent immunostimulatory activity from *Spirulina platensis*, *Aphanizomenon flos-aquae*, and *Chlorella pyrenoidosa*. *Planta Med.* **2001**, *67*, 737−742.

(21) Song, J. Y.; Han, S. K.; Son, E. H.; Pyo, S. N.; Yun, Y. S.; Yi, S. Y. Induction of secretory and tumoricidal activities in peritoneal macrophages by ginsan. *Int. Immunopharmacol.* **2002**, *2*, 857−865.

(22) Sonoda, Y.; Kasahara, T.; Mukaida, N.; Shimizu, N.; Tomoda, M.; Takeda, T. Stimulation of interleukin-8 production by acidic polysaccharides from the root of *Panax ginseng*. *Immunopharmacol*. **1998**, *38*, 287−294.

(23) Yang, S. H.; Sharrocks, A. D.; Whitmarsh, A. J. Transcriptional regulation by the MAP kinase signaling cascades. *Gene* **2003**, *320*, 3−21.
(24) Yoon, S.; Seger, R. The extracellular signal-regulated kinase: Multiple substrates regulate diverse cellular functions. *Growth Factors* **2006**, *24*, 21−44.
(25) Alessi, D. R.; Cuenda, A.; Cohen, P.; Dudley, D. T.; Saltiel, A. R. PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase in vitro and in vivo. *J. Biol. Chem.* **1995**, *270*, 27489−27494.
(26) Bennett, B. L.; Sasaki, D. T.; Murray, B. W.; O'Leary, E. C.; Sakata, S. T.; Xu, W.; Leisten, J. C.; Motiwala, A.; Pierce, S.; Satoh, Y.; Bhagwat, S. S.; Manning, A. M.; Anderson, D. W. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci.* **2001**, *98*, 13681−13686.
(27) Hazzalin, C. A.; Cano, E.; Cuenda, A.; Barratt, M. J.; Cohen, P.; Mahadevan, L. C. p38/RK is essential for stress-induced nuclear responses: JNK/SAPKs and c-Jun/ATF-2 phosphorylation are insufficient. *Curr. Biol.* **1996**, *6*, 1028−1031.

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