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Full Length Research Paper

Anti-inflammatory effect of *Papenfussiella kuromo* in lipopolysaccharide or peptidoglycan-induced macrophage cells

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Marine bioresources are known to be attractive as they sometimes contain new compounds showing several kinds of different bioactivities which are not possible in land plants. Brown algae, *Papenfussiella kuromo* is widely distributed in Korea and Japan. However, no studies have been conducted to evaluate the anti-inflammatory activities of the extract of *P. kuromo*. We studied its anti-inflammatory effect in Peptidoglycan (PGN)- and lipopolysaccharide (LPS)-induced RAW 264.7 macrophage cells activation. In the present study, interleukin (IL)-6 productions was measured using the enzyme-linked immunosorbent assay (ELISA), prostaglandin (PG) E_2 production was measured using the enzyme immunosorbent assay (EIA) kit and mitogen-activated protein kinase (MAPK) activation, as determined by western blot analysis and reverse transcription-polymerase chain reaction (RT-PCR). *P. kuromo* inhibited PGN-induced IL-6 production in a dose-dependent manner and also inhibited LPS-induced IL-6 production. Additionally, *P. kuromo* caused significant inhibition of both PGN- and LPS-induced PGE_2 expression. *P. kuromo* significantly inhibited PGN-induced extracellular signal-regulated kinase (ERK) 1/2 phosphorylation. Conclusively, *P. kuromo* inhibited nitrite oxide (NO), PGE_2 secretion and inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) expression by blocking ERK 1/2 phosphorylation in LPS- or PGN-induced RAW 264.7 cells. The results clarify the similarity and difference between PGN- and LPS-mediated signal transduction and induction of inflammatory cytokine in macrophages. Taken together, these findings may help to elucidate the mechanism by which *P. kuromo* modulates RAW 264.7 cell activation under inflammatory conditions.

Key words: *Papenfussiella kuromo*, peptidoglycan (PGN), lipopolysaccharide (LPS), macrophages.

INTRODUCTION

The ocean, which is called 'the mother of origin of life', is the source of structurally unique natural products that are mainly accumulated in living organisms. Several of these compounds show pharmacological activities and are helpful in the invention and discovery of biologically active compounds. Especially, algal are known to produce a lot of secondary metabolites including bioactive compounds with different activities (González et al., 2001). Now, the screening of algal extracts for biologically active compounds makes rapid progress with

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simple antibiotic assays and expanded to include testing for biologically active products. Compounds with antiviral, antibacterial, antifungal, anti-mitotic, and antitumorigenic activities have been detected in green, brown and red algae (Zhu et al., 2006; Oh et al., 2008; Bennamara et al., 1999). *Papenfussiella kuromo* is a brown alga belonging to the family Chordariaceae (order Ectocarpales, genus *Papenfussiella*). It is widely distributed in Japan and the eastern seaboard, southern coast, Jeju island in Korea. However, there is yet no report regarding *P. kuromo* inducing anti-inflammatory action.

Bacteria stimulate the innate immune system of a host and the release of inflammatory molecules such as cytokine and chemokines as a response to infections (Medzhitov and Janeway, 1998; Hoffmann et al., 1999). Lipopolysaccharide (LPS) is a well-known activator of the innate immune system in Gram-negative infections (Dziarski et al., 2000). Peptidoglycan (PGN), a cell wall component of Gram-positive bacteria, is an alternating β-linked N-acetylmuramyl and N-acetylglucosaminyl glycan whose residues are cross-linked by short peptides (Bone, 1994; Ulevitch, 1995). Like LPS as a cell wall component of Gram-negative bacteria, PGN induces most of the clinical manifestations of bacterial infections, including inflammation, fever, septic shock, etc (Schleifer and Kandler, 1972). Most of these effects are due to the activation of macrophages and generation of pro-inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin (IL)-6, and IL-8 (Wang et al., 2001; Xu et al., 2001; Bhakdi et al., 1991; Mattson et al., 1993). An inflammatory response implicates macrophages, which secrete a number of mediators (oxidants, cytokine, lytic enzymes, etc.) responsible for initiation, progression and persistence of acute or chronic state of inflammation (Kim et al., 2004). These inflammatory mediators are involved in the pathogenesis of many inflammation-associated human diseases (Simons et al., 1996; Glombitza and Koch, 1989; Ritchlin et al., 2003).

The aim of this study was to examine *P. kuromo* anti-inflammatory effect in PGN-induced or LPS-induced RAW 264.7 macrophage cells activation. Understanding the underlying mechanism related to the inhibition of inflammatory reaction by *P. kuromo* will be of benefit by adding to the potential new source of drug for the treatment of inflammatory diseases.

MATERIALS AND METHODS

Plant and extract

P. kuromo was collected by Dr. H.G. Choi. *P. kuromo* was air-dried in the dark at room temperature and then ground into a powder using a mechanical grinder. Approximately 500 g of the powdered materials was then extracted in 1500 ml of ethanol for 7 day (room temperature). EtOH extract yield is 42.9858 g. The extract was filtered (pore size, 0.45 µm), lyophilized, and kept at 4°C. The dried extract was then dissolved in phosphate buffered saline (PBS) in preparation for use. A sample of the *P. kuromo* has been deposited at the Herbarium of the College of Pharmacy, Wonkwang University, Iksan.

Reagents

PGN (derived from *Staphylococcus aureus*) was purchased from Fluka (Buchs, Switzerland). Roswell Park Memorial Institute (RPMI) 1640, penicillin, and streptomycin were obtained from Hyclone (Logan, UT, USA). Bovine serum albumin, LPS, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were purchased from Sigma (St. Louis, MO, USA). iNOS, p38, phosphorylated p38, extracellular signal-regulated kinase (ERK), phosphorylated ERK, Jun N-terminal kinases (JNK), phosphorylated JNK, β-actin, and peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-mouse IL-6 antibody and biotinylated anti-mouse IL-6 antibodies were purchased from BD Biosciences (San Jose, CA, USA). In addition, RNeasy Mini kit and QuantiTect Reverse Transcription kit were purchased from Qiagen (Hilden, Germany). The absorbance was read using the Spectra Max 190 UV-Vis Micro plate Reader (Molecular Devices). Finally, IL-6, COX-2, iNOS and β-actin oligonucleotide primers were purchased from Bioneer Corp. (Daejung, Republic of Korea).

Cell culture

The murine macrophage cell line, RAW 264.7, was obtained from the Korea Research Institute of Bioscience and Biotechnology (Seoul, Republic of Korea) and grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and 100 U/ml of penicillin/streptomycin sulfate. The cells were incubated in a humidified 5% CO_2 atmosphere at 37°C. To stimulate the cells, the medium was replaced with fresh RPMI 1640 medium, LPS or PGN were added in the presence or absence of *P. kuromo* for the indicated periods.

MTS assay for cell viability

The cell viability was determined by MTS assay. RAW 264.7 cells were plated at a density of 5×10^4 cells/well in 96-well plates (Nunc, Denmark). Each experiment included a non-treated group as control. *P. kuromo* (10, 50 and 100 µg/ml) was then added to each well, after which the plates were incubated for 24 h at 37°C under 5% CO_2. MTS solutions (5 mg/ml) were added to each well and the cells were cultured for another 2 h, after which the optical density was read at 490 nm. Cytotoxicity was then calculated using the formula: 1 - (mean absorbance value of treated cells / mean absorbance value of untreated cells).

Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded at 5×10^5 ml^{-1} per well in 24-well tissue culture plates and pretreated with various concentrations of *P. kuromo* (100 or 50 µg/ml) for 30 min before LPS (200 ng/ml) or PGN (30 µg/ml) stimulation for 24 h. ELISA plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ) were coated overnight at 4°C with anti-mouse IL-6 antibody diluted in coating buffer (0.1 M carbonate, pH 9.5) and were then washed three times with PBS containing 0.05% Tween 20. The nonspecific protein binding sites were blocked with assay diluent (PBS containing 10% FBS, pH 7.0) for at least 1 h. Immediately, each sample and IL-6 standard were added to the wells. After incubation for 2 h, a working detector (biotinylated anti-mouse IL-6 monoclonal antibody and streptavidin-horseradish peroxidase reagent) was added and incubated for 1 h. Accordingly,

Table 1. Sequences of oligonucleotide primers designed for RT-PCR.

cDNA	Primer sequence
IL-6	Forward 5-CATGTTCTCTGGGAAATCGTGG-3 Reverse 5-AACGCACTAGGTTTGCCGAGTA-3
COX-2	Forward 5-CACTCAGTTTGTTGAGTCATTC-3 Reverse 5-GATTAGTACTGTAGGGTTAATG-3
iNOS	Forward 5-AGCCCAACAATACAAATGACCCTA-3 Reverse 5-TTCCTGTTGTTTCTATTTCCTTTGT-3
β-actin	Forward 5-ATGAAGATCCTGACCGAGCGT-3 Reverse 5-AACGCAGCTCAGTAACAGTCCG-3

substrate solution (tetramethylbenzidine, TMB) was added to the wells and incubated for 30 min in the dark before the reaction was stopped with stop solution (2 N H_3PO_4). The absorbance was read at 450 nm using the Spectra Max 190 UV-Vis Micro Plate Reader (Molecular Devices). All subsequent steps took place at room temperature, and all standards and samples were assayed in duplicate.

Measurement of PGE_2 production

The RAW 264.7 cells were cultured in 24-well culture plates (5×10^5 ml^{-1}). *P. kuromo* (100 or 50 µg/ml), LPS (200 ng/ml) and PGN (30 µg/ml) were added to the culture medium and incubated at 37°C for 24 h. The medium was collected in a microcentrifuge tube and was centrifuged. The supernatant was decanted into a new microcentrifuge tube, and the amount of PGE_2 was determined by a PGE_2 Enzyme Immuno-Assay Kit (Amersham Biosciences, Little Chalfont, UK) according to the procedure described by the manufacturer.

Measurement of nitrite oxide (NO) production

NO production was assayed by measuring the nitrite in the supernatants of cultured RAW 264.7 cells. The cells were seeded at 1×10^5 ml^{-1} in 96-well culture plates. After preincubation of the RAW 264.7 cells for 18 h, the cells were pretreated with *P. kuromo* (100 or 50 µg/ ml) and stimulated with LPS (200 ng/ml) or PGN (30 µg/ml) for 24 h. The supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) and it was incubated at room temperature for 5 min. The concentrations of nitrite were measured by reading at 570 nm. Sodium nitrite ($NaNO_2$) was used to generate a standard curve.

Western blot analysis

Protein expression was assessed by western blot analysis according to standard procedures. The RAW 264.7 cells were cultured in 60-mm-diameter culture dishes (3×10^6 ml^{-1}) and were pretreated with various concentrations of *P. kuromo* (100 or 50 µg/ml). After 30 min or 1 h, LPS (200 ng/ml) or PGN (30 µg/ml) was added to the culture medium, and the cells were incubated at 37°C. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). The cell pellets were re-suspended in lysis buffer on ice for 15 min, and the cell debris was removed by centrifugation. The protein concentrations were determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Equal amounts of protein (20 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were then transferred onto a polyvinylidene membrane (Millipore, Bedford, MA).

The membrane was blocked with 5% nonfat milk in Tris buffered saline with Tween 20 buffer (150 mM NaCl, 20 mM Tris-HCl, and 0.05% Tween 20, pH 7.4). After blocking, the membrane was incubated with primary antibodies for 18 h. The membrane was then washed with Tris buffered saline with Tween 20 and was incubated with anti-mouse or anti-rabbit immunoglobulin G horseradish peroxidase-conjugated secondary antibodies. Immuno-reactivity was detected using enhanced chemiluminescence (Amersham, Milan, Italy).

RNA extraction and reverse-transcription PCR (RT-PCR)

The RAW 264.7 cells were cultured in 30-mm-diameter culture dishes (3×10^6 ml^{-1}) and were pretreated with various concentrations of *P. kuromo* (100 or 50 µg/ml). After 30 min, LPS (200 ng/ml) or PGN (30 µg/ml) was added to the culture medium, and the cells were incubated at 37°C. After incubation, the cells were washed twice in ice-cold PBS (pH 7.4). Total cellular RNA was isolated using RNeasy Mini kit (Qiagen, Hilden, Germany) and 1 µg of total RNA was reverse-transcribed using QuantiTect Reverse Transcription kit (Qiagen, Hilden, Germany). According to the manufacturer's instructions, the total RNA (2 µg) was converted to cDNA by treating it with 200 units of reverse transcriptase and 500 ng of oligo (dT) primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM deoxynucleotide triphosphates at 42°C for 1 h. The reaction was stopped by heating at 70°C for 15 min, and the cDNA mixture (3 µl) was used for enzymatic amplification. PCR was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphates, 2.5 units of Taq DNA polymerase, and 0.1 µM each IL-6, COX-2, iNOS and β-actin primers, respectively.

The conditions for amplification were as follows: denaturation at 94°C for 3 min for the first cycle and for 30 s starting from the second cycle, annealing of IL-6 at 57°C for 45 s, annealing of COX-2 at 53°C for 30 s and annealing of iNOS at 56°C for 30 s, and extension at 72°C for 90 s for 35 cycles. Final extension was performed at 72°C for 7 min. PCR products were electrophoresed on 2% agarose gel and were stained with ethidium bromide. The primers used are tabulated in Table 1.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA), followed by Dunnett's t-test for multiple comparisons (Lee et al., 2006). The data from the experiments are presented as means ± standard error of mean (SEM) (n=5). The numbers of independent experiments assessed are given in the figure legends.

RESULTS

Effects of *P. kuromo* on cytotoxicity in RAW 264.7 cells

The cytotoxicity of *P. kuromo* was evaluated in the RAW264.7 cells by an MTT assay. The results of *P.*

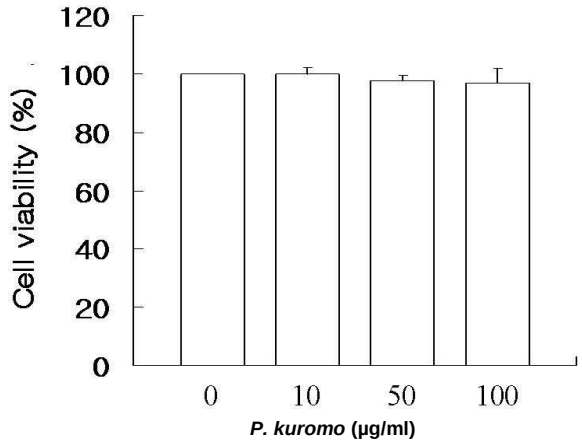

Figure 1. Effects of *P. kuromo* on cell viability in RAW 264.7 cells. Cell viability was evaluated using the MTS assay. Data represent the mean (SE) of duplicate measurements from 5 separate experiments (n = 5).

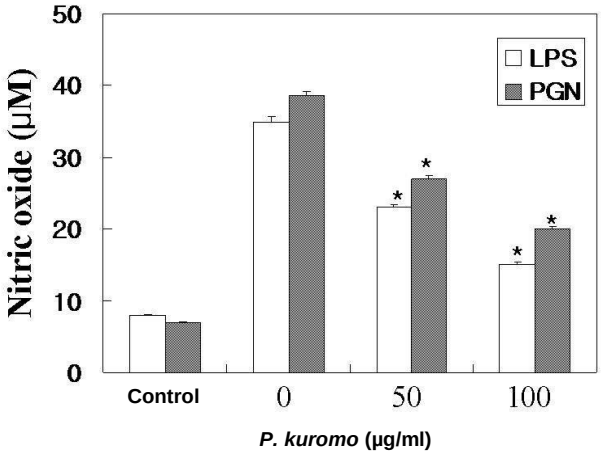

Figure 2. Effects of *P. kuromo* on LPS-induced or PGN-induced NO production in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of PK for 1 h prior to being incubated with LPS (200 ng/ml) or PGN (30 μg/ml) for 24 h. The culture supernatants were subsequently isolated and analyzed for nitrite levels. The experiment was repeated three times and similar results were obtained. *P < 0.05, compared with the LPS or PGN treated group. Data shown were representative of a total of six experiments (n = 5).

kuromo were found not to affect viability of the RAW 264.7 cells for 24 h (Figure 1).

Effects of *P. kuromo* on LPS-induced or PGN-induced NO production

To assess the effects of *P. kuromo* on the LPS-induced or PGN-induced NO production in RAW 264.7 cells, cell culture media were harvested and NO levels were measured. *P. kuromo* inhibited NO production dose-dependently in LPS stimulated RAW 264.7 cells. Also, *P. kuromo* inhibited NO production in PGN-stimulated RAW 264.7 cells in a dose-dependent (Figure 2).

Effects of *P. kuromo* on LPS-induced or PGN-induced PGE_2 production

To assess the effects of *P. kuromo* on the LPS- or PGN-induced PGE_2 production in RAW 264.7 cells, cell culture media were harvested and PGE_2 levels were measured. To examine whether *P. kuromo* inhibits PGE_2 production, cells were pre-incubated with *P. kuromo* for 1 h and then activated with LPS (200 ng/ml) or PGN (30 μg/ml) for 24 h. As shown in Figure 3, *P. kuromo* inhibited the production of PGE_2 in a dose-dependent manner.

Effects of *P. kuromo* on LPS-induced or PGN-induced iNOS mRNA and COX-2 mRNA expression

To assess the effects of *P. kuromo* on LPS-induced or PGN-induced mRNA expression of iNOS and COX-2, RAW 264.7 cells were evaluated to identify the anti-inflammatory mechanism. iNOS was strongly expressed in cells that were treated by LPS or PGN, and RT-PCR analysis showed that iNOS mRNA expression was related to the nitrite levels (Figure 4A). Also, as shown in Figure 4B, COX-2 mRNA expression was undetectable in unstimulated RAW 264.7 cells (control). However, in response to LPS or PGN, COX-2 is strongly expressed and *P. kuromo* significantly inhibited COX-2 expression in a dose-dependent manner.

Effects of *P. kuromo* on LPS-induced or PGN-induced IL-6 production and IL-6 mRNA expression

Since *P. kuromo* was found to inhibit the pro-inflammatory mediators, we investigated the effects of *P. kuromo* on LPS- or PGN-induced IL-6 production by ELISA. Pretreatment of *P. kuromo* reduced IL-6 production in a dose-dependent manner (Figure 5A). Since *P. kuromo* was found to inhibit the pro-inflammatory mediators, we investigated the effects of *P. kuromo* on LPS- or PGN-induced IL-6 expression by RT-PCR. Pretreatment of *P. kuromo* reduced IL-6 mRNA expression in a dose-dependent manner (Figure 5B).

Effects of *P. kuromo* on the phosphorylation of mitogen-activated protein kinase (MAPKs) in LPS-induced or PGN-stimulated RAW 264.7 cells

MAPKs are essential for LPS-induced iNOS expression to occur in RAW 264.7 cells. Therefore, the effects of *P.*

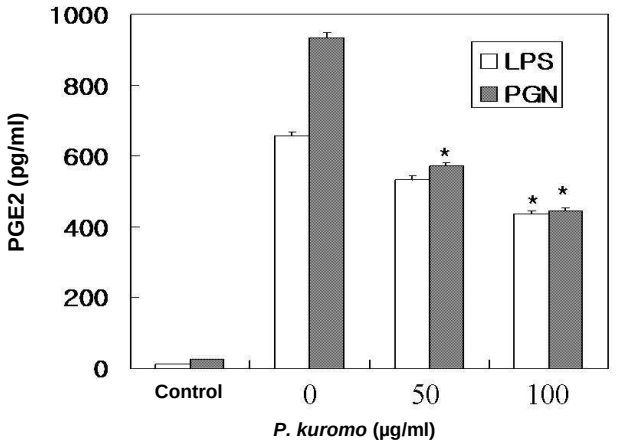

Figure 3. Effects of *P. kuromo* on LPS-induced or PGN-induced PGE2 production in RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentration of PK for 1 h before being incubated with LPS (200 ng/ml) or PGN (30 μg/ml) for 24 h. Control cells were incubated with the vehicle alone. *P < 0.05 compared with the LPS or PGN treated group. Data shown were representative of a total of six experiments (n = 5).

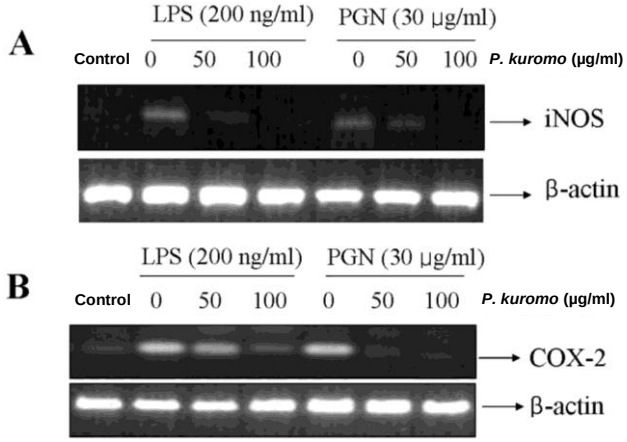

Figure 4. Effects of *P. kuromo* on LPS-induced or PGN-induced iNOS mRNA and COX-2 mRNA expressions in RAW 264.7 cells. (A) The iNOS mRNA and (B) COX-2 mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of PK for 1 h before being incubated with LPS (200 ng/ml) or PGN (30 μg/ml) for 24 h. The β-actin mRNA was carried out in parallel to confirm equivalency of cDNA preparation. The experiment was repeated five times and similar results were obtained (n = 5).

kuromo on the activation of MAPKs in LPS-or PGN-stimulated RAW 264.7 cells were evaluated. As shown in Figure 6, *P. kuromo* markedly inhibited the activating phosphorylation of ERK 1/2, whereas phosphorylation of JNK 1/2 and p38 MAPK was unaffected by treatment with *P. kuromo* (data not shown). Taken together, these results indicate that ERK 1/2 phosphorylation was inhibited by *P. kuromo* pretreatment.

DISCUSSION

Recently, many studies have evaluated the inhibitory effects of plant-derived anti-inflammatory agents *in vitro*. However, marine bio-resources are not sufficiently investigated in term of their full therapeutical option. Marine algae produce various metabolites and have been recognized as promising targets in the search for bio-logically active compounds. So far, we have performed the screening studies on marine edible algae which could serve as important resources in the discovery of anti-inflammatory activities. *P. kuromo* is a brown alga belonging to Chordariaceae, and is widely distributed along the shore of Korea, Japan. However, no studies conducted to date have reported the bioactivity by which the anti-inflammatory action of ethanol extract of *P. kuromo* occurs. In this study, we evaluated the pharmacological basis for *P. kuromo* for the treatment of various inflammatory diseases. The effects of *P. kuromo* on macrophage functions related to inflammation were investigated to verify possible mechanisms underlying its beneficial effects.

LPS and PGN, the toxicants from bacteria, are potent inducers of inflammatory cytokines, such as TNF-α, IL-6 and IL-8. Although, PGN is much less investigated in comparison to LPS, PGN is regarded as the Gram-positive equivalent to LPS in some aspects. We investigated PGN-induced signal transduction and bio-logical effects, as well as compare the effect of PGN with that of LPS. We demonstrated that *P. kuromo* inhibited LPS- or PGN-induced pro-inflammatory mediators, including NO and PGE_2. Especially, NO is an important mediator in the inflammatory process and is produced at inflamed sites by iNOS. High levels of NO have been reported in a variety of pathological processes including various forms of inflammation, circulatory shock, and carcinogenesis (MacMicking et al., 1997; Ohshima and Bartsch, 1994; Szabó, 1995). The activity of iNOS can be controlled by regulating its synthesis via activating intracellular signaling transduction. The effect of LPS sig-naling on iNOS gene expression has been investigated (Vallance and Leiper, 2002; Bronte and Zanovello, 2005). Therefore, an inhibitor of iNOS might be effective as a therapeutic agent for inflammatory diseases (Koo et al., 2001). The results of this study showed that *P. kuromo* inhibited LPS- or PGN-induced NO production in RAW 264.7 macrophages. To further investigate the mecha-nism underlying these inhibitions by *P. kuromo*, the expression of iNOS mRNA levels was examined by RT-PCR, respectively, which revealed that *P. kuromo* reduced iNOS mRNA expression. Taken together, these results indicate that *P. kuromo* has a potent anti-inflammatory effect that occurs through the inhibition of the expression of iNOS and NO production. To explore the mechanism of inhibition of NO and PGE_2 production

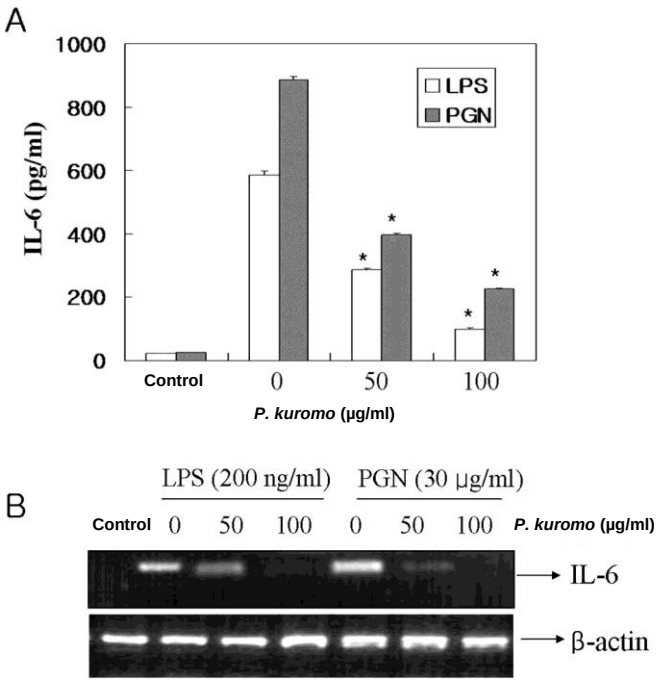

Figure 5. Effects of *P. kuromo* on LPS-induced or PGN-induced IL-6 production and IL-6 mRNA expression. (A) RAW 264.7 cells were pretreated with the indicated concentrations of PK for 1 h before being incubated with LPS (200 ng/ml) or PGN (30 μg/ml) for 24 h. Production of IL-6 was measured by ELISA. The experiment was repeated three times and similar results were obtained. Results are mean ± SE. Statistical significance: *P < 0.05 compared with the LPS or PGN treated group. (B) IL-6 mRNA was assessed by RT-PCR in RAW 264.7 cells. Cells were pretreated with the indicated concentrations of *P. kuromo* for 1 h before being incubated with LPS (200 ng/ml) or PGN (30 μg/ml) for 24 h. The β–actin mRNA was carried out in parallel to confirm equivalency of cDNA preparation. The experiment was repeated five times and similar results were obtained (n = 5).

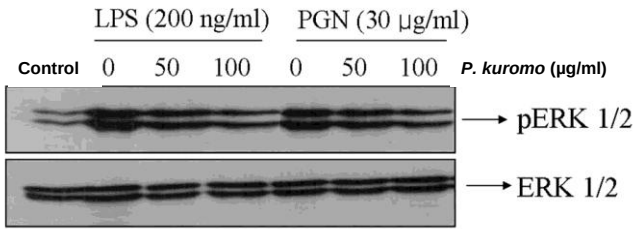

Figure 6. Effects of P. kuromo on phosphorylation of MAPKs in LPS- or PGN-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of PK for 30 min before being incubated with LPS (200 ng/ml) or PGN (30 μg/ml) for 30 min. Whole cell lysates were analyzed by Western blot analysis. The experiment was repeated five times and similar results were obtained (n = 5).

production in RAW 264.7 cells, the effects of *P. kuromo* on the iNOS and COX-2 gene were examined. *P. kuromo* inhibited the expression of COX-2 and iNOS mRNA in a dose-dependent manner, as assessed by RT-PCR, respectively. These results imply that *P. kuromo* exerts its effects through the inhibition of the iNOS and COX-2 transcription.

Pharmacological studies using MAPK-specific inhibitors experimented to show that the MAPKs (ERK, p38 and ERK) pathway is required for LPS-induced NO production and iNOS expression in RAW 264.7 macrophages. According to published reports, MAPKs have been implicated in the transcriptional regulation of the iNOS gene and specific MAPK inhibitors suppress the expression of the iNOS gene (Chen et al., 1999; Chan and Riches, 1998). Also, MAPKs play a critical role in the regulation of cell growth and differentiation, particularly in response to cytokine and stress (Johnson and Lapadat, 2002). Several studies have demonstrated that MAPKs are involved in LPS-induced iNOS expression (Kang et al., 2007; Chen et al., 1999; Kim et al., 2004). However, the role of MAPK activation in PGN-induced iNOS/NO production is still unclear. So, the effects of *P. kuromo* on the LPS- and PGN-induced phosphorylation of MAPKs were evaluated in this study. Interestingly, PK-pretreatment macrophages inhibited ERK 1/2 phosphorylation, but not JNK 1/2 and p38 MAPK phosphorylation in both LPS- and PGN-stimulation. This suggests that *P. kuromo* inhibition of iNOS/NO production might not be mediated by blocking ERK signals for iNOS gene expression in RAW 264.7 cells. In addition, the results show similarity and difference between Gram-negative bacteria and Gram-positive bacteria, and mediated signal transduction and induction of inflammatory cytokines in macrophages.

Conclusion

To conclude, we have shown that *P. kuromo* inhibited LPS- or PGN-induced IL-6, NO and PGE_2 productions, as well as iNOS and COX-2 expressions in macrophages and inhibited phosphorylation of ERK 1/2. Thus, from this study, data have been generated to show that the anti-inflammatory effect of *P. kuromo* can possibly be used as a therapeutic agent against Gram-negative and Gram-positive bacteria. The fact that *P. kuromo* has better effect on PGN, its anti-inflammatory activity will be greater against pathogens like *methicillin-resistant Staphylococcus aureus* (MRSA). Therefore, it is possible after further research, that *P. kuromo* could be used against septicemia. Taken together, these findings indicate that *P. kuromo* may represent a potential new source of drugs for the treatment of inflammatory disease.

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