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International Immunopharmacology 3 (2003) 1873–1882

International Immunopharmacology

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Toll-like receptor 4-dependent activation of macrophages by polysaccharide isolated from the radix of *Platycodon grandiflorum*

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Received 22 August 2003; received in revised form 3 September 2003; accepted 22 September 2003

Abstract

Platycodon grandiflorum, a traditional oriental herbal medicine, is known to have immunostimulatory and antitumor effects. PG, a polysaccharide isolated from *P. grandiflorum*, has been reported to activate macrophages and B cells. Here, we investigated the membrane receptor and intracellular signaling responsible for the activation of macrophages by PG. PG induced the production of nitric oxide (NO) and the mRNA expression of iNOS in RAW 264.7 cells. To investigate the membrane receptor involved in the activation of NO production, we examined the effect of PG on the production of NO in mouse peritoneal macrophages isolated from wild type C3H/HeN and functional Toll-like receptor 4 (TLR4)-deficient C3H/HeJ mice. PG induced NO production by macrophages isolated from C3H/HeN mice, but had no effect on NO production by macrophages isolated from C3H/HeJ mice. Moreover, monoclonal antibodies directed to TLR4 blocked PG-mediated induction of NO production. In addition, LBP and sCD14 was also found to be involved in the activation of NO production by PG. To further investigate, we examined the effect of PG on the activation of DNA binding of NF-κB, which is a downstream transcriptional regulator of TLR4. PG caused degradation of IκB and activation of DNA binding of NF-κB. In addition, TPCK, a specific NF-κB inhibitor, abolished PG-mediated induction of DNA binding of NF-κB, production of NO and mRNA expression of iNOS, demonstrating the involvement of NF-κB in PG-mediated macrophage activation. Taken together, these results suggest that PG-mediated induction of NO production and iNOS mRNA expression in macrophages is mediated, at least in part, by TLR4/NF-κB signaling pathway.

Keywords: *Platycodon grandiflorum*; Macrophage; Toll-like receptor; Nitric oxide; NF-κB

1. Introduction

Polysaccharides obtained from many natural sources represent a structurally diverse class of macromolecules, and are known to affect a variety of biological responses, especially the immune response. Many polysaccharides were studied in the biomedical

Abbreviations: PG, A polysaccharide isolated from the radix of *Platycodon grandiflorum*; iNOS, Inducible nitric oxide synthase; LPS, Lipopolysaccharide; LBP, LPS-binding protein; sCD14, Soluble CD14; NO, Nitric oxide; TLR, Toll-like receptor; NF-κB, Nuclear factor for immunoglobulin κ chain in B cells.

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doi:10.1016/j.intimp.2003.09.005

area because of their immunomodulating activity. The enhancement of host immune responses has been recognized as a possible means for inhibiting tumor growth without harming the host, and extensive studies have been undertaken to find immunostimulatory materials from a variety of sources. Several polysaccharides isolated from *Lentinus edodes*, *Schizophyllum commune*, *Angelica gigas* and *Phellinus linteus* have been shown to have immunostimulatory activities [1–4]. The root of *Platycodon grandiflorum* (Campanulaceae), commonly known as Doraji in Korea, has been used traditionally as a folk remedy for adult diseases such as bronchitis, asthma and pulmonary tuberculosis. Moreover, it has been reported that the root of *P. grandiflorum* prevents obesity, hypecholesterolemia, hypertension, diabetes and hyperlipidemia [5–8]. In the previous study, we demonstrated that a polysaccharide isolated from *P. grandiflorum* selectively activates B cells and macrophages, but not T cells [9].

Macrophages play a major role in host defense against infection and cancer. Stimulation of macrophages by lipopolysaccharide (LPS) enhances the production of inflammatory mediators, such as nitric oxide (NO), tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β). Recently, Toll-like receptors (TLRs) were identified as important membrane receptors involved in the activation of macrophages by a variety of pathogens [10]. CD14, another membrane receptor of macrophages, acts as a pattern recognition receptor and enhances signaling via TLRs by immobilizing the ligands at high local concentration [11]. Binding of ligands to TLR leads to the formation of a complex between cytoplasmic region of TLR and the adaptor protein Myd88 and the interleukin-1 receptor associated kinase (IRAK). This is followed by the activation of tumor necrosis factor-associated factor 6 (TRAF6) and TRAF6 in turn activates IκB kinase (IKK) complex. The activation of IKK complex leads to the degradation of IκB and the activation of NF-κB, which regulates a wide spectrum of target genes [12].

NF-κB is a pleiotropic regulator of many genes involved in immune response, including iNOS [13]. NF-κB is a dimeric transcription factor formed by the homo- or hetero-dimerization of Rel family proteins, including p50 and p65 [14]. The activity of NF-κB is primarily controlled at the post-transcriptional level [15]. In unstimulated cells, NF-κB exists in an inactive state, in the cytoplasm, complexed with an inhibitory protein, called IκB. Upon stimulation, IκB undergoes phosphorylation and degradation, and NF-κB is translocated into the nucleus, where it binds to DNA and activates transcription of a variety of genes [16]. De novo synthesis of new IκB protein occurs after its phosphorylation and degradation by IKK complexes and then IκB protein enters the nucleus, dissociates NF-κB from DNA, and again inactivate NF-κB [17].

The objective of the present study was to investigate the molecular mechanism responsible for the activation of macrophages by PG and we used nitric oxide production and iNOS gene expression as markers of macrophage activation. The current data suggest that the activation of macrophages by PG is mediated by activation of the membrane receptor, TLR4.

2. Material and methods

2.1. Materials

All reagents were purchased from Sigma (St. Louis, MO, USA) unless otherwise stated. LPS used in this study is from *Salmonella typhosa* (Sigma). Immunoblotting antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Neutralizing antibodies to surface receptors were purchased Santa Cruz Biotechnology (Santa Cruz, CA, USA) and LBP and sCD14 were purchased from R&D Systems (Minneapolis, MN, USA). Fetal bovine serum was form Gibco BRL Life Technology (Rockville, MD). The roots of *P. grandiflorum*, which had been cultivated for 20 years, were kindly provided by Sungho Jang Saeng Doragi, Korea. The dried root of *P. grandiflorum* (250 g) was extracted with hot water for 4 h, and the extract filtered, and subjected to Diaion HP-20 chromatography (Mitsubishi Chemical, Japan). The eluant obtained was lyophilized, treated with a solvent mixture of H_2O/acetone (1/5), and centrifuged to obtain the H_2O/acetone-insoluble fraction (64 g). This light yellow powder was identified as an inulin-type polysaccharide, which was named PG. It has previously been reported that the polysaccharide isolated from the

aqueous extracts of the radix of *P. grandiflorum* is an inulin-type polyfructose with a (2 → 1) linked D-fructose [8,18]. PG will be made available to qualified investigators who may be interested in utilizing this material.

2.2. Animals and cell culture

C3H/HeN and C3H/HeJ mice were obtained from Daehan Biolink (Chungbuk, Korea), and maintained under SPF conditions until used. RAW 264.7 cells (murine macrophage line) were purchased from American Type Culture Collection (Bethesda, MD, USA). Cells were grown in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Peritoneal macrophages were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 mM 2-mercaptoethanol. Nonadherent cells were removed by repeated washing after a 2-h incubation at 37 °C. Cells were plated and incubated in the presence of 5% CO_2 at 37 °C.

2.3. Nitrite quantification

Nitrite accumulation was used as an indicator of NO production in the medium as previously described [9]. Cells were plated at 5×10^5 cells/ml in 96-well culture plates and stimulated with PG or LPS for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2% phosphoric acid) and incubated at room temperature for 10 min. Using $NaNO_2$ to generate a standard curve, the concentration of nitrite was measured by O.D. reading at 540 nm.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIZOL™ Reagent (Molecular Researh Center, Cincinnati, OH, USA). The sequences used were as follows: iNOS, sense: 5′-CTGCAG-CACTTGGATCAGGAACCTG-3′, antisense: 5′-GGGAGTAGCCTGTGTGCACCTG-GAA-3′, β-actin, sense: 5′-TGGAATCCTGTGG-CATCCATGAAAC-3′, antisense: 5′-T-AAAACG-CAGCTCAGTAACAGTCCG-3′. Equal amounts of RNA were reverse-transcribed into cDNA using oligo$(dT)_{16}$ primers. For PCR, samples were heated to 94 °C for 3 min and cycled 30 times at 94 °C for 30 s, 55 °C for 30 s, and 94 °C for 1 min, after which an additional extension step at 72 °C for 5 min was included. PCR products were electrophoresed in 3% NuSieve 3:1 gels (FMC Bioproducts, Rockland, ME, USA) followed by staining with ethidium bromide. The iNOS and β-actin primers produce amplified products at 496 and 430 bp, respectively.

2.5. Immunoblot analysis

Cells were initially incubated for 4 h at 37 °C and then stimulated with PG for 5–360 min, after which the reactions were terminated with ice-cold PBS. Cytosolic extract (20 μg) were separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes (Amersham International, Buckinghamshire, UK). The membranes were pre-incubated for 1 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% Tween-20 and 3% bovine serum albumin. The nitrocellulose membranes were incubated with IκB-α and IκB-β specific antibodies purchased Santa Cruz Biotechnology. Immunoreactive bands were then detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham).

2.6. Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared as previously described [19]. The oligonucleotide sequences for NF-κB were: 5′-GATCTCAGAGGGGACTTTCCGAG-AGA-3′. The double-stranded deoxyoligonucleotides were end-labeled with [γ-^{32}P]-ATP. Nuclear extracts (5 μg) were incubated with poly (dI-dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM KCl, 30 mM HEPES, 1.5 mM MgCl2, 0.3 mM EDTA, 10% glycerol, 1 mM DTT, 1 mM PMSF, 1 μg/ml of aprotinin and 1 μg/ml of leupeptin) for 10 min. DNA binding activity was separated from the free probe using a 4.8% polyacrylamide gel in 0.5 X TBE buffer (44.5 mM Tris, 44.5 mM boric acid and 1 mM EDTA). Following electrophoresis, the gel was dried and subjected to autoradiography.

3. Results

3.1. Activation of macrophages by PG

PG was reported to activate macrophages and induce NO production in mouse peritoneal macrophages [9]. In the present study, we confirmed the effect of PG on the activation of macrophages using RAW 264.7 cells, a murine macrophage cell line. The basal level of nitrite in unstimulated cells was less than 2 nmol/10^6 cells (Fig. 1A). Upon stimulation with PG (1, 3, 10, 30 or 100 μg/ml), the nitrite generation by RAW 264.7 cells was increased in a dose-dependent manner (2-, 9-, 25-, 50- and 65-fold, respectively) (Fig. 1A). The production of IL-1β, TNF-α and IL-6 was also enhanced by PG in RAW 264.7 cells (data not shown). To investigate whether the increase in the production of NO was due to the induction of iNOS gene expression, we examined the effect of PG on mRNA expression of iNOS using RT-PCR. As shown in Fig. 1B, the mRNA expression of

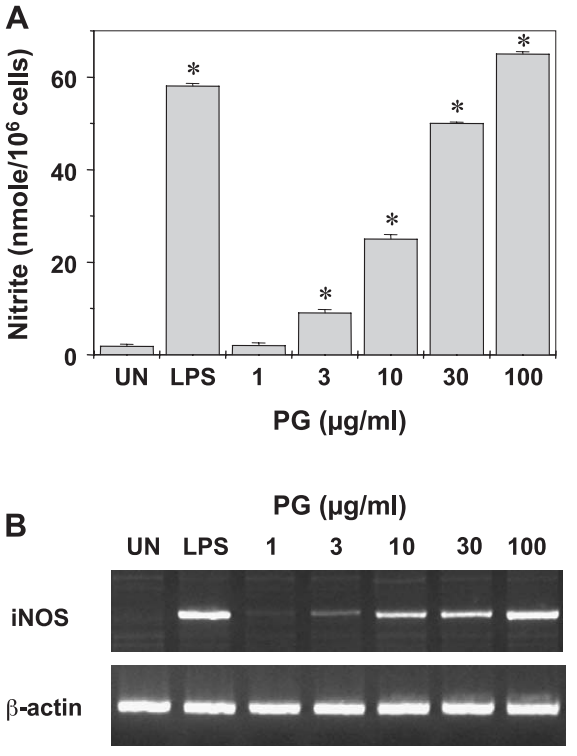

Fig. 1. Effect of PG on NO production and iNOS gene expression in RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/ml) were incubated with PG (1, 3, 10, 30 and 100 μg/ml) or LPS (1 μg/ml) for 24 h. The supernatant nitrite levels were determined using Griess reagent (A). Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using the Student's *t*-test versus the control group (*$p < 0.01$). Total RNA was then isolated and analyzed for the magnitude of mRNA expression of iNOS and β-actin using RT-PCR (B). Results are representative of more than three independent experiments.

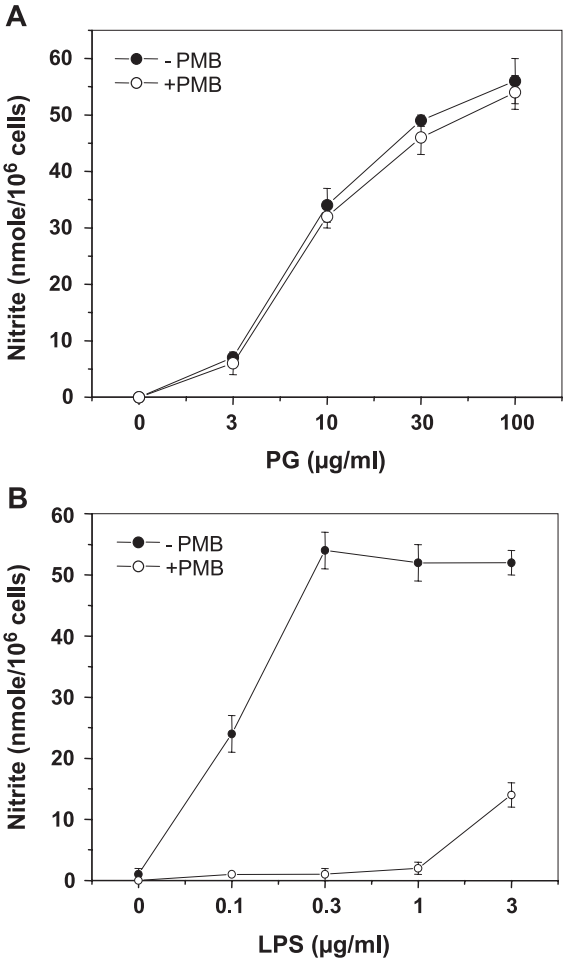

Fig. 2. Effect of polymyxin B on NO release by PG or LPS. PG or LPS was pre-incubated with polymyxin B (1 U/ml) for 1 h, and then used to activate macrophages. RAW 264.7 cells (5×10^5 cells/ml) were treated with indicated concentrations of PG (A) or LPS (B) for 24 h. The supernatant nitrite levels were determined using Griess reagent. Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using the Student's *t*-test versus the corresponding group without PMB treatment (*$p < 0.01$). One of two representative experiments is shown.

iNOS was dose-dependently increased. PG also induced the mRNA expression of IL-1β, TNF-α and IL-6 in a similar pattern (data not shown). The mRNA expression of β-actin was unaffected by PG. These results demonstrate that PG activates macrophages and PG-mediated induction of cytokine expression occurs at the level of transcription.

To test for a possible contamination of bacterial LPS in PG, we examined the effect of polymyxin B (PMB) on PG-induced production of NO. PMB is known to inhibit LPS activity by binding to the lipid A moiety. As shown in Fig. 2, PMB (1 U/ml) abolished LPS-induced NO production in RAW 264.7 cells, whereas it did not inhibit PG-induced NO production. These results demonstrate that the activation of macrophages by PG was not due to the contamination of LPS in PG.

3.2. TLR4-dependent activation of macrophages by PG

To examine the involvement of TLR4 in the PG-mediated activation of macrophages, we compared the effect of PG on NO production by peritoneal macrophages from wild-type C3H/HeN mice and functional TLR4-deficient C3H/HeJ mice. Macrophages from C3H/HeJ mice were found to be hyporesponsive to both PG and LPS, demonstrating that TLR4 plays a pivotal role in both PG- and LPS-induced production of NO (Fig. 3A and B). To further confirm, we

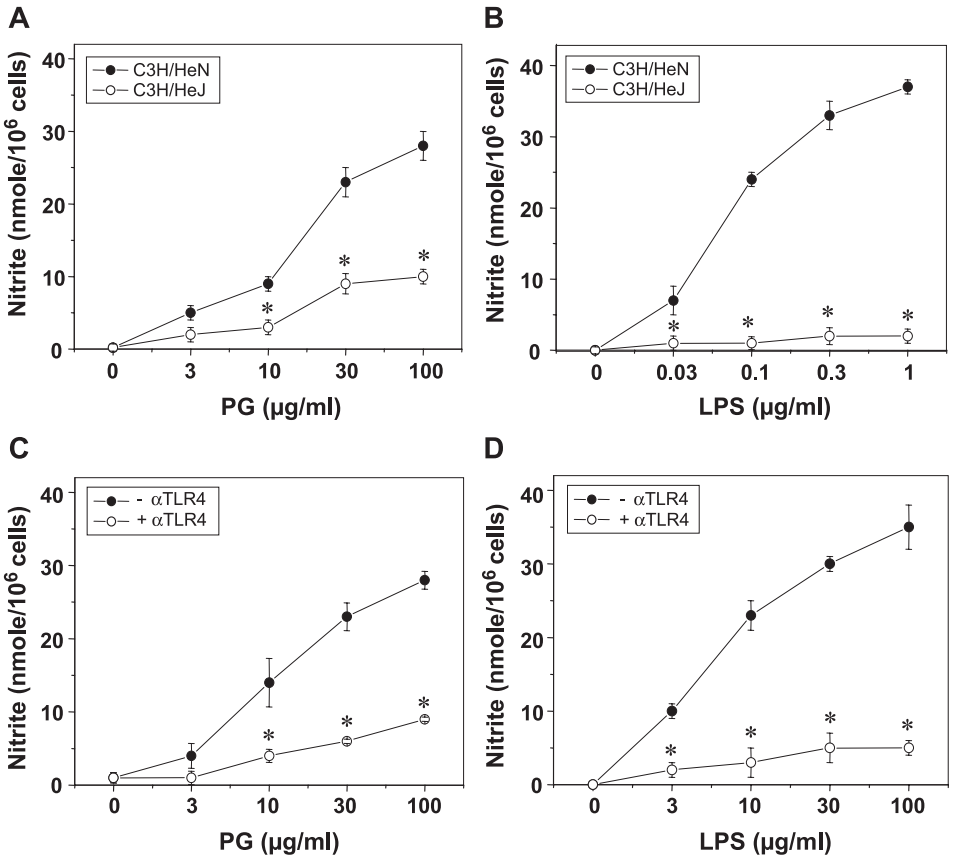

Fig. 3. Involvement of TLR4 in the activation of NO release by PG. Peritoneal macrophages (5×10^5 cells/ml) from C3H/HeN and C3H/HeJ mice were stimulated with indicated concentrations of PG (A) or LPS (B) for 24 h. RAW 264.7 cells (5×10^5 cells/ml) were pre-incubated with anti-TLR4 (αTLR4) antibodies for 1 h, and then treated with indicated concentrations of PG (C) or LPS (D) for 24 h. Supernatant nitrite levels were determined using Griess reagent. Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using the Student's *t*-test versus the corresponding group without antibody treatment (*$p < 0.01$). One of two representative experiments is shown.

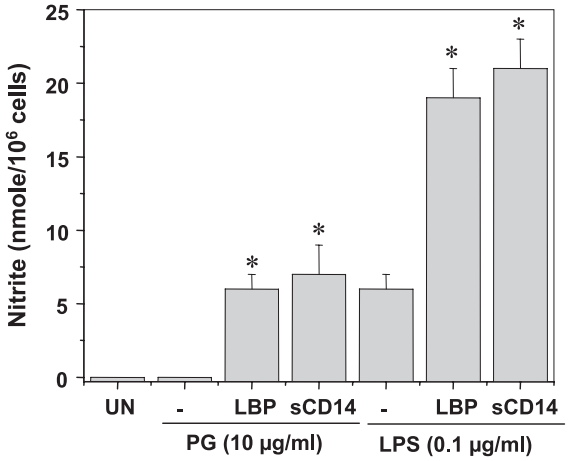

Fig. 4. Effect of LBP and sCD14 on PG-mediated NO release in RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/ml) were washed three times with serum-free medium, treated with PG (10 μg/ml) or LPS (0.1 μg/ml) in the presence or absence of LBP (1 μg/ml) or sCD14 (10 μg/ml) and incubated for 24 h in serum-free medium. The supernatant nitrite levels were determined using Griess reagent. Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using the Student's *t*-test versus the corresponding group without antibody treatment (*$p < 0.01$). One of two representative experiments is shown.

assessed the effect of anti-TLR4 antibody on PG- and LPS-induced NO production by RAW 264.7 cells. Anti-TLR4 antibody significantly blocked PG-mediated production of NO in RAW 264.7 cells (Fig. 3C). LPS-induced NO production was also suppressed by pretreatment of RAW 264.7 cells with anti-TLR4 antibody (Fig. 3D). These results suggest that the PG-mediated activation of macrophages is TLR4-dependent and PG shares a common membrane rceptor with LPS.

3.3. Effect of LBP and sCD14 on PG-mediated activation of macrophages

To further investigate whether the activation of RAW 264.7 cells by PG is dependent on the presence of free LBP and sCD14 in the serum, we examined PG-induced NO production by RAW 264.7 cells in the presence and absence of LBP and sCD14. Cells were washed three times with serum-free medium to remove LBP and sCD14 as completely as possible. PG could not induce NO production in the absence of LBP and sCD14. However, treatment with LBP (1 μg/ml) or sCD14 (10 μg/ml) significantly potentiated the PG-mediated production of NO in RAW 264.7 cells (Fig. 4). LPS-induced nitrite generation was also significantly increased by treatment with LBP (1 μg/ml) or sCD14 (10 μg/ml). These results suggest that PG requires LBP and sCD14 to activate macrophages.

3.4. Induction of NF-κB by PG in macrophages

As described previously, signals originated from TLR are known to activate NF-κB. Therefore, we examined the effect of PG on IκB degradation and NF-κB activation in macrophages. Both IκBα and IκBβ were significantly reduced by PG within 15 min. The amount of IκBα protein returned to normal

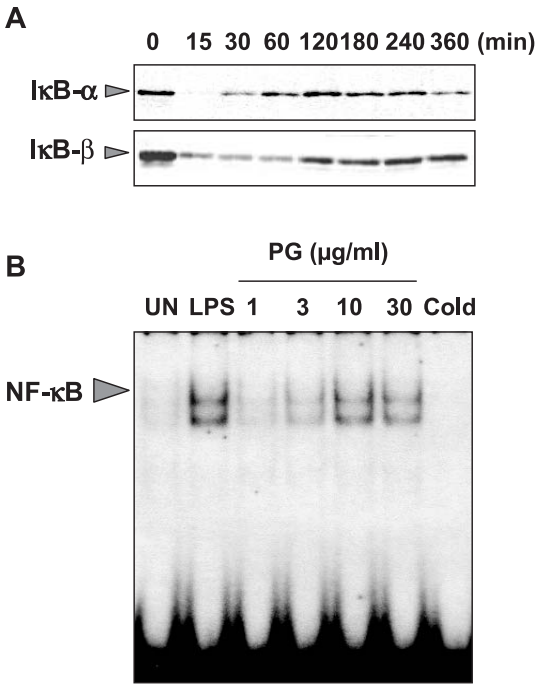

Fig. 5. Effect of PG on IκB degradation and NF-κB activation in RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/ml) were stimulated with indicated concentrations of PG for indicated times. Cytosolic extracts (10 μg) were then prepared and subjected to Western immunoblotting using antibodies specific for phosphorylated form of IκBα and IκBβ (A). Nuclear extracts (5 μg/ml) were prepared and analyzed for DNA binding activity of NF-κB using EMSA (B). Results are representative of three independent experiments.

level within 60 min (Fig. 5A). However, the recovery of IκBβ protein was slightly delayed compared to IκBα (Fig. 5A). We also assessed the effect of PG on the DNA binding activity of NF-κB using EMSA. Treatment of RAW 264.7 cells with PG caused a marked increase in NF-κB DNA binding to its cognate site (Fig. 5B). The specificity of the retarded bands was confirmed by the addition of an excess amount of unlabeled double-stranded NF-κB oligonucleotide. To convincingly demonstrate the specificity of NF-κB activation by PG, the effects of TPCK, an inhibitor of NF-κB, was investigated using EMSA. TPCK was reported to inhibit the activation of NF-κB by stabilizing the inhibitory subunit, IκBα [20]. In the presence of TPCK, PG-induced DNA binding of NF-κB was noticeably inhibited (Fig. 6A). TPCK also inhibited the NO production and iNOS mRNA expression induced by PG in RAW 264.7 cells, confirming the involvement of NF-κB in PG-induced production of NO (Fig. 6B,C). These results indicate that activation of macrophages by PG is mediated by the TLR4-dependent activation of transcription factor NF-κB, an important downstream regulator of TLR4.

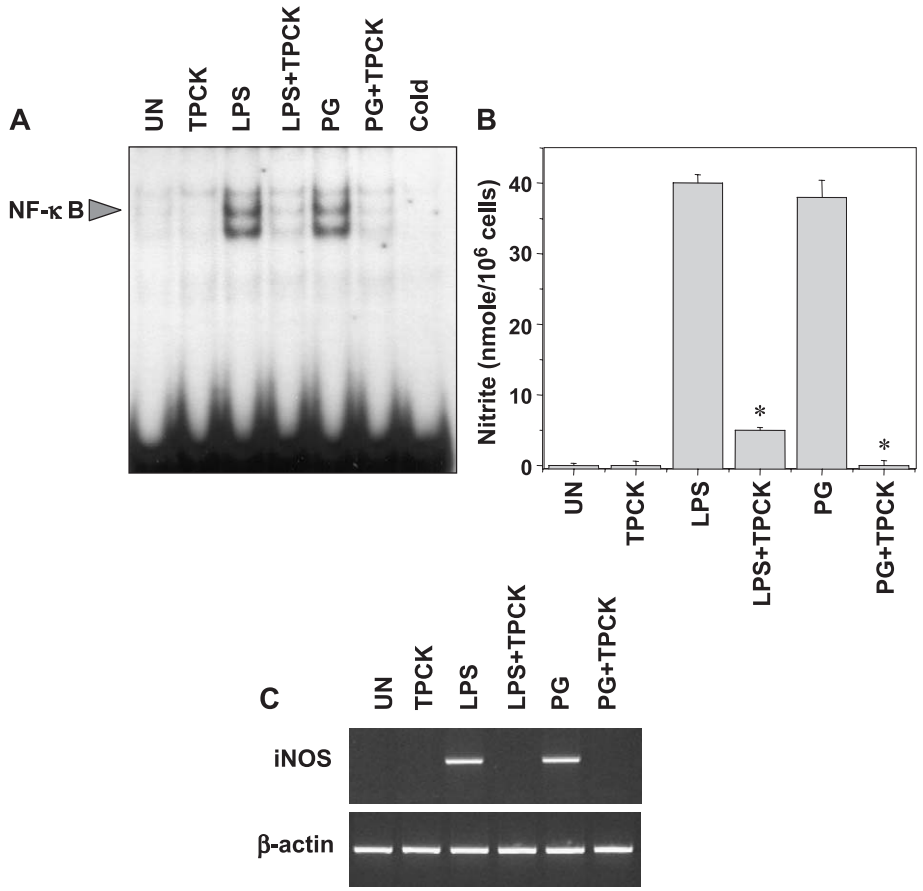

Fig. 6. Effect of TPCK on PG-mediated induction of NF-κB, NO production and iNOS mRNA expression in RAW 264.7 cells. RAW 264.7 cells (5×10^5 cells/ml) were incubated with TPCK (10 μM) in the presence or absence of PG for 6 h (A) or 24 h (B and C). Nuclear extracts (5 μg/ml) were then prepared and analyzed for DNA binding activity of NF-κB using EMSA (A). The supernatant nitrite levels were determined using Griess reagent (B). Each column shows the mean ± S.D. of triplicate determinations. Significance was determined using the Student's *t*-test versus the corresponding group without antibody treatment (*$p < 0.01$). Total RNA was then isolated and analyzed for the magnitude of mRNA expression of iNOS and β-actin using RT-PCR (C). One of two representative experiments is shown.

4. Discussion

P. grandiflorum is claimed to have a variety of biological activities, including immunostimulation and antitumor effects. PG, a polysaccharide isolated from *P. grandiflorum*, is known to selectively activate macrophages and B cells, but not T cells [9]. However, the molecular mechanism responsible for immunostimulatory activity of this polysaccharide is not fully understood. In the present study, we attempted to characterize membrane receptors and subsequent intracellular signaling pathways involved in the activation of macrophages by PG.

Toll-like receptors are a family of membrane receptors that recognize components of bacteria. Several TLRs differing in their specificity have been identified. They respond to various components of bacteria, including LPS of Gram-negative bacteria, lipoproteins of Gram-positive bacteria, bacterial DNA and flagella (reviewed in Ref. [10]). Although TLRs have been well known as pattern recognition receptors for bacterial components, a variety of molecules from various sources were found to be TLR ligands. One of the most famous TLR4 ligands other than those from bacterial sources is paclitaxel. Paclitaxel, isolated from the bark of the Pacific yew tree, is a promising anti-cancer agent and is shown to block cells at the G_2/M junction of the cell cycle [21]. In addition to the blockage of mitosis, paclitaxel activates the innate immune response by TLR4-dependent activation of NF-κB and induction of gene expression of inflammatory mediators [22–24]. TLR4 is also involved in viral recognition. The F protein of respiratory syncytial virus induces proinflammatory cytokines by binding to TLR4 and CD14 [25,26]. Recently, there have been several reports demonstrating TLR-dependent activation of macrophages by polysaccharides. Ando and coworkers reported that safflower polysaccharides activate the transcription factor NF-κB via TLR4 and induce cytokine production by macrophages [27]. We previously demonstrated that polysaccharides isolated from the cell culture of the seeds of *Acanthopanax senticosus* activate B cells and macrophages through TLR4 [28]. Immunostimulating activity of polysaccharides isolated from the root of *Acanthopanax koreanum* was also reported to be mediated by TLR4 in B cells [29]. In this report, various membrane receptors other than TLR4, including TLR2, CD19 and CD79b, were also shown to be involved in the activation of B cells by polysaccharides from *A. koreanum*, demonstrating a broader receptor profile of these polysaccharides than LPS in B cells. In the present study, we clearly demonstrated that the activation of macrophages by PG is also mediated by TLR4. Our previous study also showed that CD14 and CD11b are also involved in the activation of macrophages by PG [9]. These reports suggest that TLR has a relatively broad specificity to polysaccharides isolated from a variety of sources.

NO, a short-lived free radical synthesized from L-arginine by nitric oxide synthase (NOS), contributes to the killing of microorganisms and tumor cells by activated macrophages and mediates a variety of biological functions as an intracellular messenger molecule [30]. Activated macrophages also produce a variety of cytokines, including TNF-α and IL-1β. In turn, TNF-α and IL-1β induce the proliferation of other cells and the expression of various genes via the autocrine and paracrine system. TNF-α has also been recognized as an important host defense molecule that has tumor-selective cytotoxicity. Moreover, the induction of NO and TNF-α production and gene expression by activated macrophages can lead to cytostatic and cytotoxic activities on malignant cells [31–33]. Therefore, PG-mediated activation of macrophages and production of various cytokines may contribute to its antitumor activity. There have been numerous reports demonstrating antitumor activity of polysaccharides isolated from various sources via stimulation of the immune system. Song et al. [34] reported the immunostimulatory and antitumor effect of ginsan, a polysaccharide isolated from *Panax ginseng*. We also previously demonstrated that angelan, a polysaccharide isolated from *A. gigas* Nakai, potentiated in vivo immune functions and increased survival times of mice implanted with B16F10 melanoma cells [3]. The antitumor and immunomodulatory effect of the extract of *P. grandiflorum* was also reported [35]. These reports suggest that immunostimulatory polysaccharides isolated from various natural sources are good candidates for development of antitumor agents.

In summary, the results presented in this report suggest that PG induces macrophage activation through TLR4/NF-κB signaling pathway. However, further studies should be undertaken to fully under-

stand overall intracellular process related to the macrophage activation by PG. It is likely that PG may be used clinically as a biological response modifier to activate macrophages. The immunostimulatory effect of PG also suggests a possible application of PG as a supportive agent in anticancer therapy.

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