International Immunopharmacology 10 (2010) 883–891

Contents lists available at ScienceDirect

International Immunopharmacology

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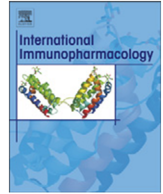

Peptidoglycan enhances proinflammatory cytokine expression through the TLR2 receptor, MyD88, phosphatidylinositol 3-kinase/AKT and NF-kappaB pathways in BV-2 microglia

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ARTICLE INFO

Article history:
Received 4 January 2010
Received in revised form 19 April 2010
Accepted 27 April 2010

Keywords:
Peptidoglycan
Microglia
iNOS
COX-2
NF-κB

ABSTRACT

In this study, we investigated the signaling pathways involved in inflammatory production caused by peptidoglycan (PGN), a cell wall component of the gram-positive bacterium, in BV-2 microglia. PGN caused a concentration- and time-dependent increase in inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) mRNA and protein levels. In addition, PGN also induced IL-1β, TNF-α and IL-6 mRNA up-regulation in a concentration-dependent manner. Moreover, PGN also increased Toll-like receptor 2 (TLR2) expression in BV-2 microglia. Administration of TLR2 neutralizing antibody effectively inhibited PGN-induced iNOS and COX-2 expression. On the other hand, PGN-induced iNOS and COX-2 up-regulation were attenuated by PI3-kinase inhibitors (LY294002 and wortmannin), and an AKT inhibitor. Treatment of BV-2 microglia with PGN caused a time-dependent activation of PI3-kinase (p85) and AKT. PGN-induced PI3-kinase/AKT activation, iNOS and COX-2 expression were also inhibited by MyD88 inhibitory peptide. Treatment of cells with NF-κB inhibitor (pyrrolidine dithiocarbamate), IκBα phosphorylation inhibitor (Bay 117082), or IκB protease inhibitor (l-1-tosylamido-2-phenylethyl chloromethyl ketone) inhibited PGN-induced iNOS and COX-2 expression. Furthermore, stimulation of cells with PGN also activated IKKα/β, IκBα phosphorylation, IκBα degradation, p65 phosphorylation at Ser^{536}, and increased κB-luciferase activity. PGN-induced IKKα/β phosphorylation, IκBα phosphorylation, and IκBα degradation were further inhibited by pre-treatment with PI3-kinase inhibitors. Moreover, PGN-mediated increase of κB-luciferase activity was also inhibited by pre-transfection with dominant-negative mutants of p85, AKT, IKKα or IKKβ. Our data demonstrate that PGN-induced iNOS, COX-2 and proinflammatory cytokine expression was mediated through the TLR2/MyD88/PI3-kinase/AKT pathway, which in turn initiates IKKα/β and NF-κB activation in BV-2 microglia.

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1. Introduction

Bacteria stimulate the innate immune system of a host and the release of inflammatory molecules such as cytokines and chemokines as a response to infection [1,2]. Lipopolysaccharide (LPS) is a well-known activator of the innate immune system in Gram negative infections. During Gram-positive infection, when no endotoxin is present, PGN, the major component of the cell wall of Gram-positive bacteria, activates the innate immune system of the host and induces the release of chemokines and cytokines [3–5]. These inflammatory molecules are the major cause of the various signs and symptoms that occur during bacterial infection, including fever, inflammation, and acute phase responses [3–5]. Previous reports showed that PGN binds CD14 and TLR2, which activates transcription factors and induces gene expression [6,7]. Even though it is well known that bacterial products have multiple and various effects on the regulation of host defense and immune responses by macrophages [8], little is known about how PGN regulates induction of the inflammatory mediators in microglia.

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doi:10.1016/j.intimp.2010.04.026

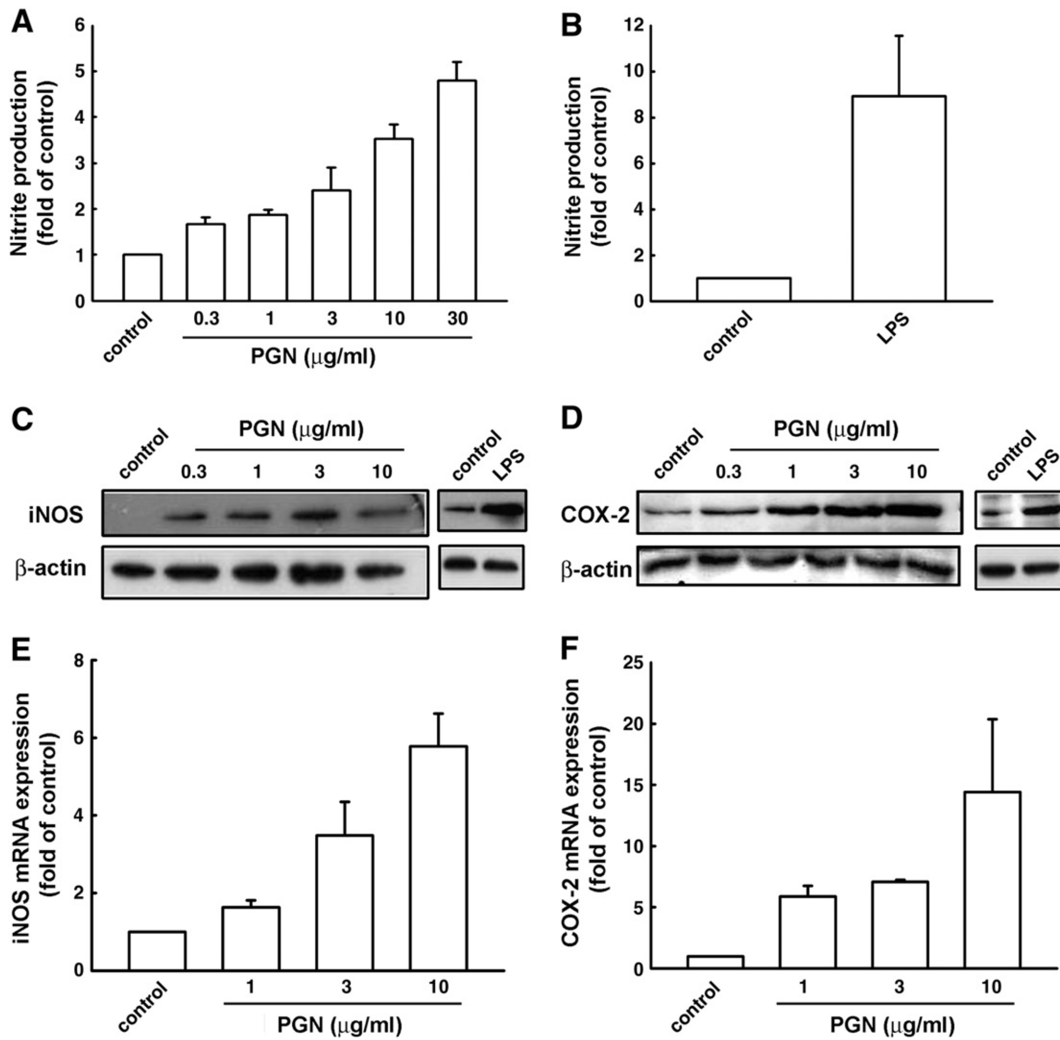

Fig. 1. PGN increases iNOS and COX-2 expression in BV-2 microglia. BV-2 microglial were incubated with various concentrations of PGN (A) or 100 ng/ml of LPS (B) for 24 h. The culture medium was collected and analyzed by Griess reaction. Whole cell lysis proteins were extracted and subjected to Western blot for detection of iNOS (C) and COX-2 (D) after 24 h incubation with PGN or LPS. Note that PGN increases iNOS or COX-2 protein expression in a concentration-dependent manner. Cells were stimulated with PGN at various concentrations for 6 h and the mRNA levels of iNOS (E) and COX-2 (F) were then analyzed by real-time PCR. Results are expressed as the mean ± S.E.M. of four independent experiments.

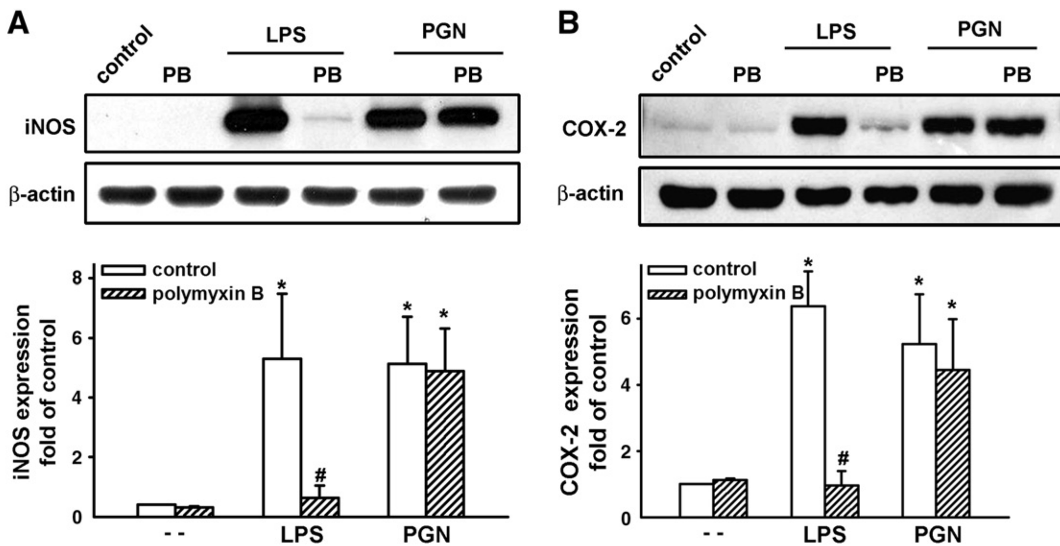

Fig. 2. Effects of polymyxin B on LPS- and PGN-induced iNOS and COX-2 production. Cells were pretreated with polymyxin B (PB, 1 μM) for 30 min before incubation with 100 ng/ml LPS or 10 μg/ml PGN for 24 h. Whole cell lysis proteins were subjected to Western blot for detection of iNOS (A) and COX-2 (B). The quantitative data are showed in lower panel. Results are expressed as the mean ± S.E.M. of four independent experiments. *, $p<0.05$ as compared with vehicle treatment group. #, $p<0.05$ as compared with the LPS or PGN treatment group, respectively.

Microglia have been proposed to play a role in host defense and tissue repair in the CNS. Under pathological conditions, activated microglia have been implicated as the predominant cell type governing inflammation-mediated neuronal damage. Once activated chronically, microglia are capable of producing a variety of inflammatory mediators and potentially neurotoxic compounds. The microglia response must be tightly regulated to avoid over activation and disastrous neurotoxic consequences [9]. Over activation of microglial cells may cause severe brain tissue damage in various neurodegenerative diseases [10]. Glial activation involves change in cell phenotype and the expression of new protein, such as iNOS and COX-2. The mechanism by which activated glial cells induce neuronal cell death has been shown to involve nitric oxide [11,12], reactive oxygen species and proinflammatory cytokines [13,14].

Although the signaling mechanisms related to the effects of LPS have been extensively studied in several cell types including macrophages and microglia, the signaling mechanisms related to the effects of PGN-induced inflammatory cytokine expression have not been investigated in microglia. In the present study, we explored the signaling pathways involved in PGN-induced iNOS, COX-2 and proinflammatory cytokine production in BV-2 microglia. Our results show that PGN stimulation causes the activation of TLR2/MyD88/PI3-kinase/AKT, and NF-κB, leading to up-regulation of inflammatory cytokine expression.

2. Materials and methods

2.1. Materials

PGN (derived from *Staphylococcus aureus*) was purchased from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and OPTI-MEM were purchased from Invitrogen (Carlsbad, CA). Goat anti-mouse and anti-rabbit horseradish peroxidase-conjugated IgG, normal rabbit IgG, primary antibodies against α-tubulin, AKT, IκBα, IKKα/β, TLR2 and phospho-AKT (Ser473) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antibodies against IKKα/β phosphorylated at $Ser^{180/181}$, p65 phosphorylated at Ser^{536} and PI3-kinase (p85) phosphorylated at Tyr^{458} were purchased from Cell Signaling and Neuroscience (Danvers, MA). TPCK, PDTC and AKT inhibitor (1 L-6-hydroxymethyl-chiro-inositol-2-[(*R*)-2-*O*-methyl-3-*O*-octadecylcarbonate]) were purchased from Calbiochem (San Diego, CA). NF-κB luciferase plasmid was purchased from Stratagene (La Jolla, CA). MyD88 and Control inhibitory peptide was purchased from Imgenex (San Diego, CA). The dominant-negative mutants of p85 (DN-p85; Δp85; deletion of 35 amino acids from residues 479 to 513 of p85) and AKT (DN-AKT; AKT K179A) were gifts from Dr. W. M. Fu (Department of Pharmacology, National Taiwan University, Taipei, Taiwan). The dominant-negative kinase inactive mutants (KM) of IKKα and IKKβ were gifts from Dr. H. Nakano (Juntendo University, Tokyo, Japan). The pSV-β-galactosidase vector, luciferase assay kit was purchased from Promega (Madison, MA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO).

2.2. Cell culture

The murine BV-2 cell line was cultured in DMEM with 10% FBS at 37 °C in a humidified incubator under 5% CO_2 and 95% air. Confluent cultures were passaged by trypsinization.

2.3. Assay of nitric oxide

Production of nitric oxide was assayed by measuring the nitrite levels of the stable nitric oxide metabolite in culture medium which was prepared as described previously [15]. Briefly, accumulation of nitrite in the medium was determined by colorimetric assay with Griess reagent. Cells (2×10^5 cells per well) in 24-well plates in 500 µl culture medium were stimulated with PGN for 24 h. One hundred µl of culture supernatant reacted with an equal volume of Griess reagent (one part 0.1% naphthylethylenediamine and one part 1% sulfanilamide in 5% H_3PO_4) in 96-well culture plates for 10 min at room temperature in the dark. The absorbance at 550 nm was determined using a microplate reader (Bio-Tek, Winooski, VT). Each experiment was performed in triplicate.

2.4. Western blot analysis

Cells were treated with PGN for indicated time periods and then washed with cold PBS, lysed for 30 min on ice with radioimmunoprecipitation assay buffer. Protein samples were separated by sodium dodecyl sulphate-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with

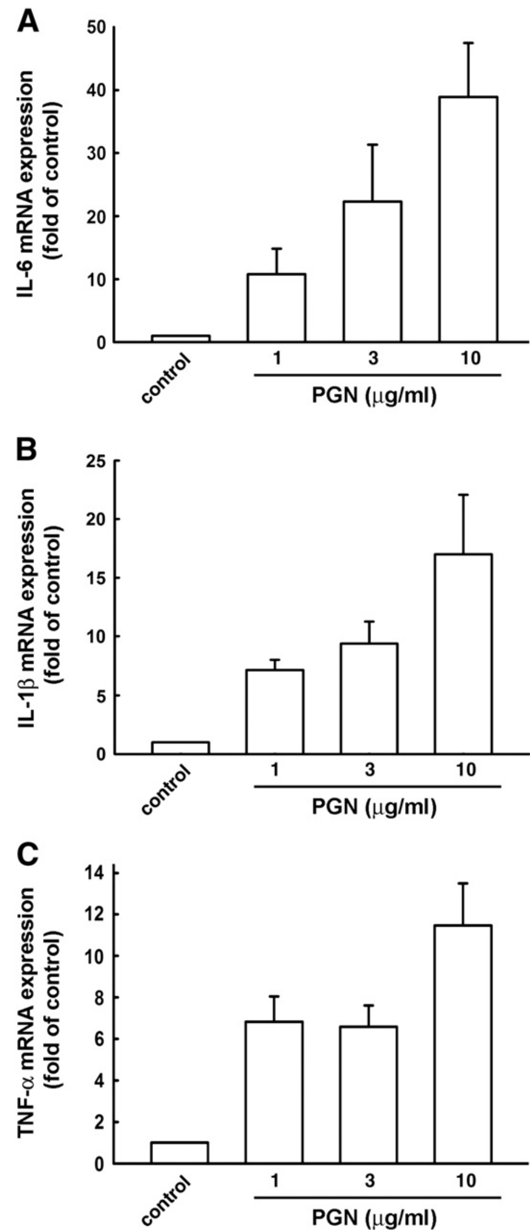

Fig. 3. PGN induces proinflammatory cytokine expression in BV-2 microglia. Cells were stimulated with PGN (10 µg/ml) for 6 h and the mRNA levels of IL-6 (A), IL-1β (B) and TNF-α (C) were then analyzed by real time-PCR. Note that PGN increases proinflammatory cytokine mRNA expression concentration-dependently. Results are expressed as the mean ± S.E.M. of four independent experiments.

5% BSA for 1 h at room temperature and then probed with primary antibodies. After undergoing three PBS washes, the membranes were incubated with secondary antibodies. The blots were visualized by enhanced chemiluminescence using Kodak X-OMAT LS film (Eastman Kodak, Rochester, NY). Quantitative data were obtained using a computing densitometer and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

2.5. Reverse transcriptase-PCR and quantitative real time-PCR

Total RNA was extracted from BV-2 cells using a TRIzol kit (MDBio Inc., Taipei, Taiwan). The reverse transcription reaction was performed using 2 μg total RNA that was reverse transcribed into cDNA using oligo(dT) primer, then amplified using oligonucleotide primers:

CD11b 5′-GTGAAGCCCAAGATCGTC-3′ and 5′-AGCAATCTCAGCACAGTAAG-3′;

GAPDH 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′;

Each PCR cycle was carried out for 30 s at 94 °C, 1 min at 55 °C, and 1 min at 72 °C.

PCR products were then separated electrophoretically in a 2% agarose gel and stained with ethidium bromide. The band intensity was quantified with a densitometric scanner and presented as relative level of GAPDH.

Quantitative real time-PCR was detected using SYBR Green I Master Mix and analyzed with a model 7900 Sequence Detector System (Applied Biosystems, Foster City, CA). After preincubation at 50 °C for 2 min and 95 °C for 10 min, the PCR was performed as 40 cycles of 95 °C for 10 s and 60 °C for 1 min. The threshold was set above the non-template control background and within the linear phase of target gene amplification to calculate the cycle number at which the transcript was detected (denoted as C_T). The oligonucleotide primers were iNOS: 5′-CCCAGAGTTCCAGCTTCTGG-3′ and

5′-CCAAGCCCCTCACCATTATCT-3′;

COX-2: 5′-5′-TGGGGTGATGAGCAACTATT-3′ and 5′-AAGGAGCTCTGGGTCAAACT-3′;

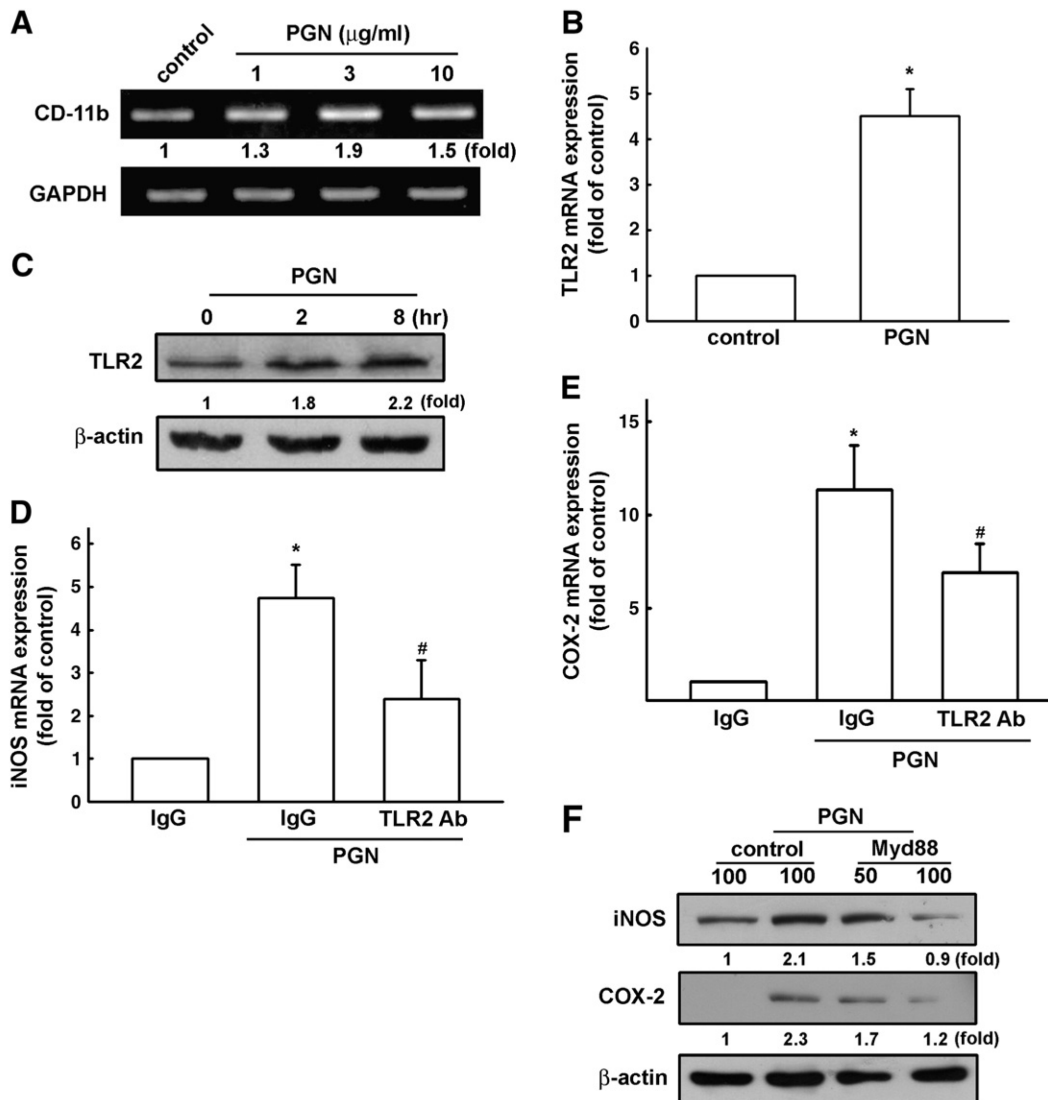

Fig. 4. Involvement of TLR2 receptor/MyD88 in PGN-mediated iNOS and COX-2 production in BV-2 microglia. (A) BV-2 cells were treated with various concentrations of PGN. Total RNA was extracted and subjected to RT-PCR for the β-integin marker of microglia, CD11b. Cells were stimulated with PGN (10 μg/ml) for indicated time periods and the mRNA and protein levels of TLR2 were determined by real-time PCR (B; 0 and 6 h) and Western blot (C; 0, 2 and 8 h). Note that CD11b and TLR2 expression are increased in response to PGN application. Cells were preincubated with anti-TLR2 antibody (TLR2 Ab) (5 μg) or control IgG for 30 min, and then stimulated with 10 μg/ml PGN for 6 h and subjected to real time-PCR for iNOS (D) or COX-2 (E). Results are expressed as the mean ± S.E.M. of four independent experiments. *, $p<0.05$ as compared with control group. #, $p<0.05$ as compared with the PGN treatment group. (F) Cells were pretreated with MyD88 inhibitory peptide (50 or 100 μM) for 24 h followed by stimulation with PGN for another 24 h, and protein levels of iNOS and COX-2 were determined by Western blot. Relative RNA or protein expression was normalized by GAPDH or β-actin, respectively.

IL-1β: 5′-TGGGGGAGATTCTCACTTTG-3′ and 5′-CCATCAGCGTTCC-CATACTT-3′; IL-6: 5′-CCAGTTGCCTTCTTGGGACTG-3′ and 5′-CAG-GTCTGTTGGGAGTGGTATCC-3′;

TNF-α: 5′-AAAATTCGAGTGACAAGCCTGTAG-3′ and 5′-CCCTTGAA-GAGAACCTGGGAGTAG-3′;

TLR2: 5′-AATTGCATCACCGGTCAGAAA-3′ and 5′-GTTTGCTGAA-GAGGACTGTTATGG-3′

GAPDH: 5′-CTCAACTACATGGTCTACATGTTCCA-3′ and 5′-CTTCCC-ATTCTCAGCCTTGACT-3′

2.6. Transfection and reporter gene assay

BV-2 microglia were co-transfected with 0.8 μg pκB-luciferase plasmid, 0.4 μg β-galactosidase expression vector and either control vector (pcDNA3.1), or dominant-negative mutants (DN) DN-p85 or DN-AKT, or kinase-inactivate mutants (KM) KM-IKKα or KM-IKKβ (0.4 μg). The microglia were grown to 80% confluence in 6-well plates and were transfected with Lipofectamine 2000 (LF2000; Invitrogen) on the following day. Plasmid DNA and LF2000 were premixed in OPTI-medium for 20 min and then applied to the cells (0.8 ml/well). Medium containing 20% FCS (0.8 ml) was added 4 h later. After 24 h transfection, medium containing LF2000 was replaced with fresh serum-free DMEM and treated with PGN for another 24 h. To prepare lysates, 100 μl reporter lysis buffer (Promega, Madison, WI) was added to each well, and cells were scraped from plates. The supernatant was collected after centrifugation at 10,000 *g* for 2 min. Aliquots of cell lysates (20 μl) containing equal amounts of protein (30 μg) were then placed into wells of an opaque black 96-well microplate. The luciferase activity was determined by using a dual-luciferase reporter assay system (Promega) and activity value was normalized by β-galactosidase expression vector.

2.7. Statistics

The values given are means ± S.E.M. The significance of difference between the experimental group and control was assessed by Student's *t* test. The difference was considered to be significant if the *p* value was <0.05.

3. Results

3.1. PGN increases the expression of iNOS and COX-2 in microglia

As shown in Fig. 1A, BV-2 microglia treated with PGN (1–30 μg/ml) for 24 h increased the secretion of nitric oxide in a concentration-dependent manner. After 24 h of treatment with 10 μg/ml PGN, the nitric oxide production had increased by approximately 3-fold over basal levels ($n = 5$). Exposure of BV-2 cells to LPS (100 ng/ml) increased nitrite production approximately 9-fold over basal levels (Fig. 1B). In addition, PGN or LPS-induced iNOS and COX-2 protein overexpression was analyzed by Western blotting (Fig. 1C and D). PGN-induced iNOS and COX-2 mRNA up-regulation was analyzed by real-time PCR (Fig. 1E and F). We also examined whether the up-regulation of iNOS and COX-2 resulted from the increased proliferation of BV-2 following PGN treatment. However, PGN did not significantly affect cell viability at concentrations between 1 and 10 μg/ml using MTT assay (data not shown). To further confirm this stimulation-specific mediation by PGN without LPS contamination,

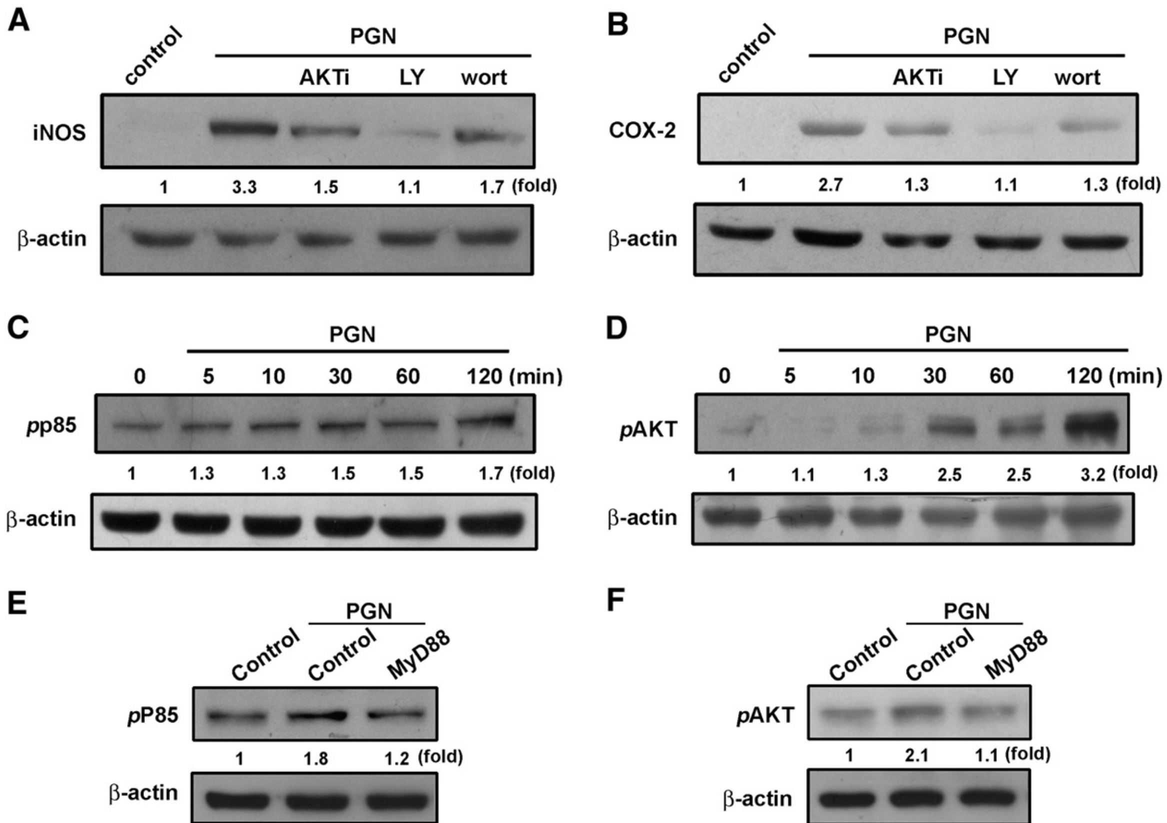

Fig. 5. The PI3-kinase/AKT pathway is involved in PGN-induced iNOS and COX-2 production in BV-2 cells. BV-2 microglia were pretreated with AKT inhibitor (AKTi, 10 μM), LY294002 (LY, 10 μM) or wortmannin (wort, 1 μM) for 30 min and then stimulated with PGN (10 μg/ml) for 24 h. Whole cell lysis proteins were subjected to Western blot for detection of iNOS (A) and COX-2 (B). BV-2 microglia were incubated with PGN (10 μg/ml) for the indicated time periods, and cell lysates were separated by SDS-PAGE and immunoblotted with anti-phosphoPI3-kinase (pp85; C) or phosphoAKT (pAKT; D). Note that PGN-induced iNOS and COX-2 expression are significantly antagonized by AKT inhibitor, LY294002 or wortmannin, and PGN induced PI3-kinase (p85) or AKT phosphorylation in a time-dependent manner. Cells were pretreated with MyD88 inhibitory peptide for 24 h followed by stimulation with PGN for 1 h, and phosphorylation level of PI3-kinase (p85) (E) and AKT (F) were determined by Western blot. Results are the representative at least three independent experiments. Relative protein expression or phosphorylation levels were normalized by β-actin.

polymyxin B, an LPS inhibitor was tested. We found that polymyxin B (1 μM) completely inhibited LPS (100 ng/ml)-induced iNOS and COX-2 expression. However, it had no effect on PGN (10 μg/ml)-induced iNOS and COX-2 expression (Fig. 2A and B).

3.2. PGN increases the expression of proinflammatory cytokines in BV-2 microglia

We further examined the proinflammatory cytokine expression after PGN treatment. PGN-induced proinflammatory cytokines mRNA up-regulation was analyzed by real-time PCR. As shown in Fig. 3, PGN also induced IL-6, IL-1βand TNF-α mRNA up-regulation in a concentration-dependent manner. Additionally, increased expression of CD11b, the β-integrin marker of microglia, represents microglial activation during neurodegenerative inflammation. After PGN treatment, CD11b was also overexpressed in BV-2 microglial cells (Fig. 4A).

3.3. PGN-stimulated iNOS and COX-2 expression is mediated through TLR2 receptor/MyD88

Toll-like receptors play an important role in host detection and recognition of pathogens and initiation of a rapid defensive response [16,17]. We therefore evaluated the expression of TLR receptor genes in BV-2 microglial cells using real-time PCR. As shown in Fig. 4B, the TLR2 receptor was overexpression after PGN treatment. In addition, we also confirmed the effect of PGN-induced the TLR2 receptor up-regulation by Western blot (Fig. 4C). To further investigate whether the PGN-induced iNOS and COX-2 expression was mediated through the TLR2 receptor, BV-2 cells were pretreated with TLR2 receptor-neutralizing antibody (4 μg) or control IgG antibody and then stimulated with PGN. The results shown in Fig. 4D and E indicate that treatment with TLR2 receptor-neutralizing antibody attenuated the PGN-evoked iNOS and COX-2 expression ($p<0.05$, $n=5$). Furthermore, MyD88 is well known as an adaptor protein mediates TLR2 signal transduction. Cells were pretreated with the Control or MyD88 inhibitory peptide (50 or 100 μM) for 24 h followed by stimulation with PGN for another 24 h, and protein levels of iNOS and COX-2 were determined. Pretreatment with MyD88 inhibitory peptide inhibited PGN-induced iNOS and COX-2 expression (Fig. 4F). These results suggest that the stimulatory effect of PGN was mediated through TLR2 receptor/MyD88 in BV-2 microglial cells.

3.4. Involvement of the PI3-kinase/AKT signaling pathway in PGN-mediated iNOS and COX-2 up-regulation

We then investigated the signaling pathway involved in the enhancement of iNOS and COX-2 expression stimulated by PGN. As shown in Fig. 5A and B, PGN-induced increase of iNOS and COX-2 expression was antagonized by treatment with the PI3-kinase inhibitors LY294002 (10 μM), wortmannin (1 μM), and an AKT inhibitor (10 μM). At the indicated concentrations, none of these inhibitors affected cell viability. In addition, PGN activated PI3-kinase (p85) and AKT in a time-dependent manner, as evidenced by the increase in phosphorylated p85 (Tyr^{458}) and phosphorylated AKT (Ser^{473}), respectively (Fig. 5C and D). In addition, cells were pretreated with the Control or MyD88 inhibitory peptide (100 μM) for 24 h followed by stimulation with PGN for 1 h, and phosphorylation of PI3-kinase (p85) and AKT were determined. Pretreatment with MyD88 inhibitory peptide inhibited PGN-induced PI3-kinase (p85) and AKT expression (Fig. 5E and F). Taken together, these data suggest that the activation of PI3-kinase/AKT pathway is required for the iNOS and COX-2 up-regulation induced by PGN in BV-2 microglia.

3.5. Involvement of NF-κB in PGN-induced iNOS and COX-2 production

It has been reported that NF-κB activation is necessary for iNOS and COX-2 induction in micorglia [15,18–22]. We therefore examine whether NF-κB pathway is related to the increase of iNOS and COX-2 expression induced by PGN. As shown in Fig. 6A and B, a NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC, 30 μM) effectively antagonized the enhancement of iNOS and COX-2 expression induced by PGN. Furthermore, pretreatment with an IκB protease inhibitor (L-1-tosylamido-2-phenylethyl chloromethyl ketone; TPCK, 3 μM), or an IκBα phosphorylation inhibitor (Bay 117082, 3 μM) also reversed the potentiating action of *PGN*. However, treatment of cells with PDTC (30 μM), TPCK (3 μM), or Bay 117082 (3 μM) did not affect cell viability, which was assessed by MTT assay (data not shown). To directly determine NF-κB activation after PGN treatment, cells were transiently transfected with κB-luciferase plasmid as an indicator of NF-κB activation. As shown in Fig. 6C, PGN treatment of BV-2 cells for 24 h increased κB-luciferase activity. Co-transfection of cells with DN-p85α, DN-AKT, KM-IKKα, or KM-IKKβ mutant effectively reduced PGN-induced κB-luciferase activity. We then further examined

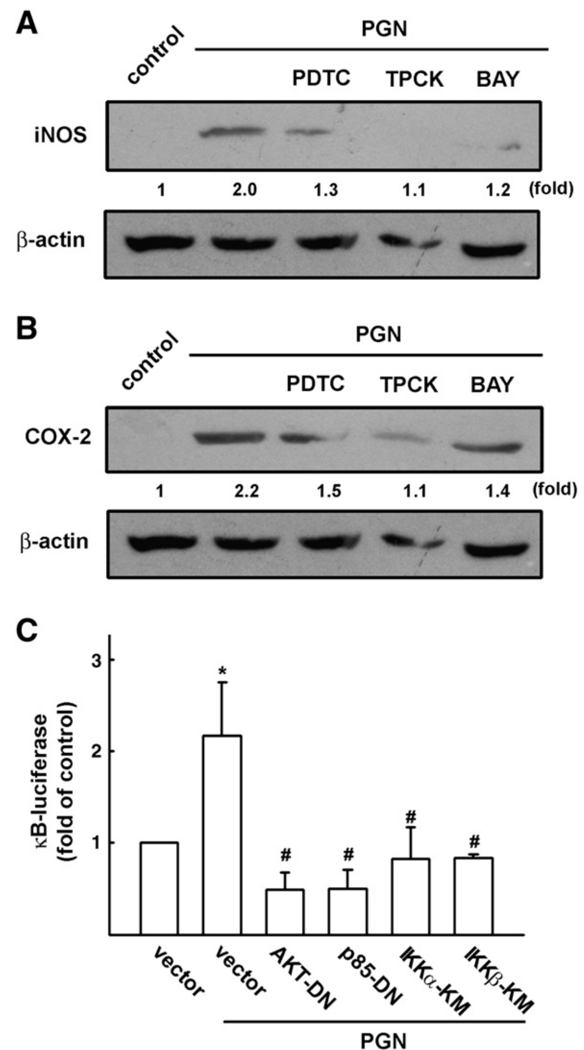

Fig. 6. NF-κB is involved in the potentiation of iNOS and COX-2 production by PGN. BV-2 cells were pretreated with PDTC (30 μM), TPCK (3 μM) or Bay 11-7082 (3 μM) followed by stimulation with PGN (10 μg/ml) for 24 h. Whole cell lysis proteins were subjected to Western blot for detection of iNOS (A) and COX-2 (B). (C) Cells were co-transfected with κB-luciferase expression plasmid, and p85α or AKT dominant negative mutant for 24 h. Luciferase activity was then assayed after PGN stimulation for another 24 h. Relative protein expression were normalized by β-actin. Results are expressed as the mean ± S.E.M. of four independent experiments. *, $p<0.05$ as compared with control group. #, $p<0.05$ as compared with the PGN treatment group.

the upstream molecules involved in *PGN*-induced NF-κB activation. Treatment of BV-2 microglial cells with *PGN* increased *IKK*α/β phosphorylation, *IκB*α phosphorylation and *IκB*α degradation time-dependently (Fig. 7A, B and C). Moreover, treatment of BV-2 cells with *PGN* also induced p65 phosphorylation at Ser^{536} in a time-dependent manner (Fig. 7D). Furthermore, pretreatment of cells with LY294002 or wortmannin attenuated PGN-induced *IκB*α phosphorylation and *IκB*α degradation (Fig. 7E and F). Taken together, these results suggesting that the NF-κB activation are regulated by PI3-kinase/AKT signaling pathway, and the NF-κB activation is required for PGN-induced iNOS and COX-2 expression in BV-2 microglia (Fig. 8).

4. Discussion

Microglial cells are the resident immune cells of the brain. In response to injury or infection, microglial cells readily become activated and consequently release proinflammatory cytokines, free radicals and eicosanoids. These factors are believed to contribute to microglia-mediated neurodegeneration [23–25]. In addition, it has been reported that iNOS and COX-2 are induced in various types of CNS injuries and diseases [26,27]. These two enzymes are often co-expressed in disease states associated with gliosis. Moreover, it was also found that iNOS and COX-2 were expressed in glial cells of substantia nigra of post-mortem Parkinson's patients [28]. In previous reports, the expression of iNOS and COX-2 has been identified in microglial cells in rodent brain and microglia cell culture after LPS treatment [15,29,30]. Gram-positive pathogens are etiologic agent of CNS infectious diseases and are associated with brain abscess [31,32]. In addition, it has recently been reported that exposure of microglia to *S. aureus*, leads to the elaboration of a wide array of inflammatory mediators and enhanced expression of surface receptors that play a pivotal role in bacterial recognition and antigen presentation [33–35]. However, the signaling pathways involved in PGN-induced inflammatory mediators expression in microglia are still unclear. The present study reveals that *S. aureus*-derived PGN is able to induce the expression of iNOS, COX-2 as well as proinflammatory cytokines in microglia. Thus, PGN also induces microglial surface markers, CD11b up-regulation in BV-2 microglia cell line.

NF-κB and AP-1 are prerequisite and ubiquitous transcription factors for the expression of many inflammation-related genes, including iNOS, COX-2, TNF-α, IL-1β and IL-6. For example, PGN-induced activation of the Rac1/PI3-kinase/AKT cascade results in an increase in NF-κB activation, and causes COX-2 expression in RAW 264.7 macrophages [8]. In addition, PGN has also been reported to activate NF-κB in microglia [36,37]. The results of the present study show that NF-κB is involved in the action of PGN-induced iNOS and COX-2 production in microglia, and that the inhibitors of the NF-κB-dependent signaling pathway, including PDTC, TPCK or Bay 117082 inhibited the potentiating effects. In addition, treatment of BV-2 microglial with PGN resulted in increase in IKKα/β phosphorylation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, and NF-κB activity. On the other hand, it has also been reported that PGN-

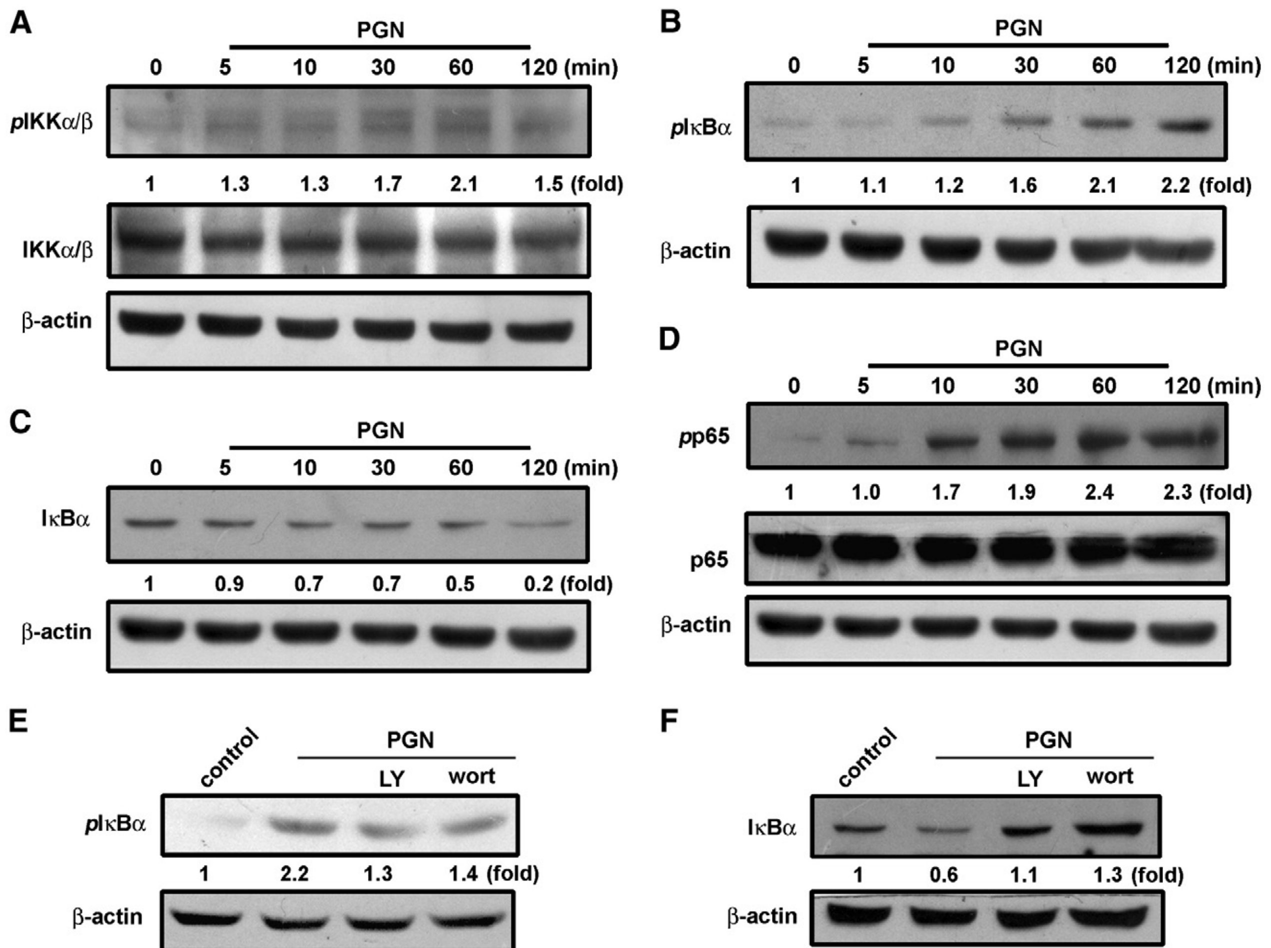

Fig. 7. PGN induces IKKα/β activation, IκBα phosphorylation, IκBα degradation and p65 Ser^{536} phosphorylation in BV-2 microglia. BV-2 microglia were incubated with PGN (10 μg/ml) for the indicated time intervals, and cell lysates were separated by SDS-PAGE and immunoblotted with anti-phosphoIKKα/β (A), phosphoIκBα (B), IκBα (C), and phosphop65 Ser^{536} (D). Note that PGN induces IKKα/β phosphorylation, IκBα phosphorylation, IκBα degradation, and p65 Ser^{536} phosphorylation. Cells were pretreated with LY294002 or wortmannin for 30 min followed by stimulation with PGN for 60 min. The cell lysates were then evaluated using immunoblotting with antibody specific for phosphoIκBα (E), and IκBα (F). Results are the representative at least three independent experiments. Relative phosphorylation levels of NF-κB protein were normalized by each total protein or β-actin.

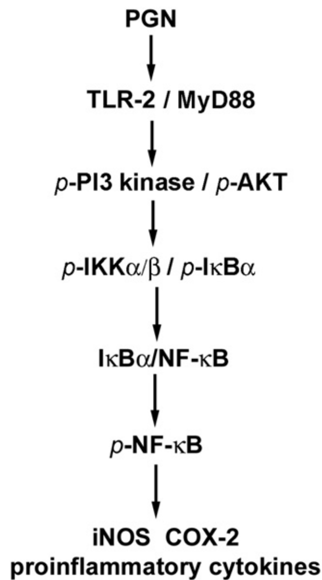

Fig. 8. Schematic diagram of the signaling pathways involved in PGN-induced inflammatory mediator expression in microglia. PGN binds with TLR2 receptor/MyD88, and activates PI3-kinase/AKT pathway, which in turn induces IKKα/β phosphorylation, IκBα phosphorylation, IκBα degradation, p65 Ser^{536} phosphorylation, leading to the NF-κB activation, and iNOS and COX-2 production.

increased FAK, PI3-kinase, AKT activation, which enhances binding of c-Jun to the AP-1 site, resulting in the IL-6 expression in synovial fibroblasts [38]. However, PGN-activated AP-1 expression to increase cytokine expression in microglia needs more experiments to investigation. Chen et al. [8], and Chiu et al. [38], and our results also demonstrate that PI3-kinase activation leads to phosphorylation of phosphatidyl inositides, which then activates the downstream main target, AKT. In this study, we also found that PI3-kinase inhibitors (LY 294002 and wortmannin), or an AKT inhibitor all inhibited PGN-induced iNOS and COX-2 expression. We also found that PGN-induced IκBα phosphorylation, and IκBα degradation were antagonized by PI3-kinase/AKT inhibitors. Furthermore, the PGN-mediated increase in κB-luciferase activity was also inhibited by DN-p85α or DN-AKT. These results suggest that the PI3-kinase/AKT signal pathway might be very important for cytokines induction caused by PGN.

The Toll-like receptor (TLR) family consists of at least 10 different receptors, but little is known about their biological and pathological functions in the CNS. The many different TLRs expressed on microglia are likely the most important first line of defense against pathogens. As is general for microglial responses, TLR-mediated responses can work in either beneficial or detrimental ways, depending on the strength and timing of the activating signal. Other cells in the CNS, including astrocytes, neurons, and oligodendrocytes, can also express multiple functional TLRs upon activation. They play important roles in cellular migration, differentiation, inflammation, and in mounting repair processes following trauma. During bacterial infection, the mammalian innate immune system can recognize bacteria and their cell wall components through two distinct receptors, CD14 and TLRs that initiate inflammatory responses [16,39,40]. It has been reported that TLR2 acts as the receptor for PGN [8,38]. Although microglia have recently been shown to express TLR2, the functional significance of this receptor in mediating microglial activation remains unclear. A recent study has shown that PGN derived from Gram-positive bacterium *S. aureus* promotes microglial cells uptake of Alzheimer disease-associated amyloid beta peptide through the TLR2 signaling pathway [37]. Previous report also demonstrated that TLR2 is pivotal for recognition of *S. aureus*-derived PGN but not intact bacteria by microglia [41]. In our study, we found that PGN induces the TLR2 receptor overexpression in BV-2 microlgia (Fig. 4B and C). Furthermore, PGN-mediated induction of iNOS and COX-2 expression were inhibited by pretreatment with TLR2 receptor-neutralizing antibody (Fig. 4D and E), which was consistent with the results that PGN induced activation of signaling transduction pathways mediated through TLR2 in various cell types [6,8,37,38,41].

MyD88 is well known as an adaptor protein which mediates TLR2 signal transduction. For example, Der p2 induces asthma through the TLR2/MyD88-dependent signaling pathway in airway smooth muscle [42]. It has also been reported that TLR2 utilizes adaptor protein, MyD88, to induce inflammatory responses in microglia [43,44]. Moreover, TLR2 utilizes MyD88 to activate IL-1 receptor-associated kinase, followed by trigger activation of NF-κB, which is required for induction of gene expression [45]. Here, we also showed that PGN-induced PI3-kinase/AKT activation (Fig. 5E and F), and iNOS and COX-2 expression (Fig. 3F) are reduced by MyD88 inhibitory peptide. In conclusion, our study is the first to provide fundamental information on the molecular mechanism by which PGN/TLR2 increases iNOS and COX-2 expression in microglia. PGN increases iNOS and COX-2 expression by binding to the TLR2 receptor/MyD88 which in turn activates PI3-kinase/AKT signaling pathway leading to IKKα/β phosphorylation, IκBα phosphorylation, IκBα degradation and activation of the NF-κB pathway (Fig. 8). Our results provide a mechanism linking PGN and proinflammatory cytokine expression, which indicate that PGN plays a regulatory role in microglia activation. We also suggest that PGN-induced neuroinflammation might involve other signaling molecules. The possibility might require further experiments to investigate. With a better understanding of these signal transduction pathways, we can develop novel therapeutic strategies to reduce neuroinflammation caused by Gram-positive organisms.

Acknowledgements

This work was supported by grants from the National Science Council of Taiwan (NSC98-2320-B-039-009-MY2 and NSC98-2627-B-039-005-) and China Medical University (CMU97-229, CMU98-N1-29 and CMU98-C-14).

We thank Dr. H. Nakano (Juntendo University, Tokyo, Japan) for providing IKKα and IKKβ kinase-inactive mutants; Dr. W. M. Fu (National Taiwan University, Taipei, Taiwan) for providing p85 and AKT dominant-negative mutants.

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