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Inflexin attenuates proinflammatory responses and nuclear factor-κB activation in LPS-treated microglia

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ABSTRACT

Activated microglia participate in neuroinflammation which contribute to neuronal damage. Suppression of microglial activation would have therapeutic benefits, which lead to alleviation of the progression of neurodegeneration. In this study, the inhibitory effects of inflexin, a putative antiinflammatory agent isolated from *Isodon excisus* (Max.) Kudo (Labiateae), on the production of proinflammatory mediators were investigated in the lipopolysaccharide (LPS)-stimulated microglia. Inflexin significantly inhibited the release of nitric oxide (NO). Consistently, both the mRNA and the protein levels for the inducible NO synthase were decreased by inflexin in a concentration-dependent manner. Inflexin also inhibited the expression of cyclooxygenase (COX)-2, but not the COX-1 and effectively reduced the LPS-induced expression of proinflammatory cytokines in a dose-dependent manner. Furthermore, inflexin inhibited the degradation of IκB-α and the activation of NF-κB, p65 and Akt, while the MAPKs signal pathway was not affected. Our data suggest that inflexin was able to suppress neuroinflammation via inhibition of NF-κB activation and Akt pathway indicating that inflexin may be developed as a potent therapeutic agent in treating neuroinflammatory diseases.

1. Introduction

Inflammatory responses in the brain are thought to be mainly associated with activity of glial cells. Microglia are the resident macrophages and immune surveillance cells of the central nervous system and play an active role in brain inflammatory, immune, and degenerative processes (Xiong et al., 2003). Especially activated microglia have been described in several neurodegenerative diseases, including Alzheimer's disease, Acquired immunodeficiency syndrome dementia and Parkinson's disease (Gao et al., 2002; Nelson et al., 2002). In adults, microglia are distributed throughout the central nervous system (CNS) and represent 5–10% of Glia. Such microglia are said to be resting, but acting as sensors for a range of stimuli that threaten physiological homeostasis, that is, CNS trauma, apoptosis, ischemia, inflammation, and infection (Liu and Hong, 2003). In particular, activated microglia exert cytotoxic effects by releasing various inflammatory mediators, such as nitric oxide (NO), arachidonic acid metabolites, reactive oxygen species, tumor necrosis factor alpha (TNF-α) and interleukin-1 (IL-1) (McGeer et al., 1988; Minghetti and Levi, 1998; Le et al., 2001). Although these immunotoxic factors are necessary for normal function, the microglial response must be tightly regulated to avoid over activation and disastrous neurotoxic consequences (Liu and Hong, 2003). The intracellular signaling mechanisms related to the effects of lipopolysaccharide (LPS) have been well studied in several types of cells including macrophages, microglia and astrocytes (Boulet et al., 1992; Bhat et al., 1998; Chen et al., 1998). LPS is known to activate mitogen-activated protein kinases, nuclear factor-kappa B (NF-κB), protein kinase C and tyrosine kinases, which have been implicated in the release of immune-related cytotoxic factors, such as NO and proinflammatory cytokines (Boulet et al., 1992; Bhat et al., 1998; Chen et al., 1998). BV-2 microglial cells respond to endotoxin, lipopolysaccharide, including the synthesis of inflammatory factors such as nitric oxide (NO), cyclooxygenase-2 (COX-2), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). These microglial products are thought to be responsible for neuroglia-mediated neurotoxicity (Jeohn et al., 2002).

Inflexin, a compound derived from the plant *Isodon excisus* (Max.) Kudo (Labiateae) is a perennial herb that is distributed widely in Korea, China, and Japan. The aerial parts of this plant have been used for detoxification and treatment of gastrointestinal disorders (Jung

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and Shin, 1990), anorexia, indigestion, stomachache, inflammation, and esophageal carcinoma (Lee, 1989 and Yook, 1981). Inflexin has been reported to inhibit LPS-induced NF-κB activation in macrophage RAW264.7 cells (Hong et al., 2007). However the effect of inflexin on LPS-stimulated BV-2 microglial cells and the exact mechanism underlying its action were not studied.

The purpose of the present study is to identify the signal transduction pathway underlying the antiinflammatory effect of inflexin. Inflexin suppressed the LPS-induced release of inflammatory mediators in the microglial cell line, BV-2. It was shown that the inhibitory effect of inflexin on inflammatory mediators was partly mediated through suppression of NF-κB, and Akt but not through mitogen-activated protein kinase (MAPKs) phosphorylation.

2. Materials and methods

2.1. Reagents

Lipopolysaccharide (LPS), Tween-20, bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), p-nitrophenyl phosphate, and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Six-well and 96-well tissue culture plates and 100 mm culture dishes were purchased from Nunc Inc. (North Aurora Road, IL, USA). Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS) and other cell-culture reagents were obtained from Gibco (Carlsbad, CA, USA). The antibodies against iNOS, COX-1, COX-2, and p65 were obtained from Santa Cruz (CA, USA). The IκB-α, phosphor (p)-IκB-α, p38, p-p38, ERK, p-ERK, JNK, p-JNK, Akt, p-Akt and β-actin were supplied by Cell Signaling (Danvers, MA, USA).

Inflexin [($C_{24}H_{32}O_7$); ent-1α-hydroxy-3β, 6a-diacetoxykaur-16-en-11, 15-dione], a white amorphous powder, lipid soluble compound (Takeda et al., 1988), was kindly supplied by Dr. Bang Yeon Hwang, Chung-Buk National University (Cheongju, Korea) (Hong et al., 2007).

2.2. Cell culture and treatments

2.2.1. Primary and immortalized microglia culture

Primary microglia were cultured from the cerebral cortices or substantia nigra of 1- to 3-day-old Sprague–Dawley rats. Briefly, tissues were triturated into single cells in DMEM supplemented with 10% FBS and were plated in 75-cm^2 T-flasks (0.5 hemisphere/flask) for 2 weeks. The microglia were detached from the flasks by mild shaking and applied to a nylon mesh to remove astrocytes and cell clumps. Cells were plated in 6-well plates (5×10^5 cells/well), 60-mm^2 dishes (8×10^5 cells/dish), or 100-mm^2 dishes (2×10^6 cells/dish). One hour later, the cells were washed to remove unattached cells before being used in experiments. The purity of microglia cultures was assessed using CD-11b antibody and more than 90% of cells were stained positively.

The BV-2 cell (a mouse microglial cell line) developed by Dr. V. Bocchini (University of Perugia, Perugia, Italy) was generously provided by Dr. K. Suk (Kyungpook National University, Daegu, Korea). The immortalized murine BV-2 cell line that exhibits both the phenotypic and functional properties of the reactive microglia cells (Bocchini et al., 1992) was grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% heat-inactivated fetal bovine serum (FBS), at 37 °C in a humidified incubator under 5% CO_2.

2.3. Nitrite quantification

Production of NO was assayed by measuring the levels of nitrite, the stable NO metabolite, in culture medium. Accumulation of nitrite in the medium was determined by colorimetric assay with Griess reagent (Green et al., 1982). The BV-2 cells (5×10^4 cells/ml) in 24-well plates in 500 μL culture medium were pretreated with different concentrations of inflexin for 30 min after being stimulated with LPS (100 ng/ml) for 24 h. In brief, 50 μL of culture supernatant reacted with an equal volume of Griess reagent (part 0.1% naphthylethylenediamine and part 1% sulfanilamide in 5% H_3PO_4) in 96-well plates for 10 min at room temperature in the dark. Nitrite concentrations were determined by using standard solutions of sodium nitrite prepared in cell-culture medium. The absorbance at 540 nm was determined using a microplate reader (Molecular device, USA). Each experiment was performed in triplicate.

2.4. MTT cytotoxicity assay

The cell viability of the cultured cells was determined by measuring the reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) to formazan. Briefly, cells were seeded and treated with different concentrations of inflexin (0.1, 1.0, 5 and 10 μM). After incubation with LPS (100 ng/ml) for 24 h, 0.5 mg/ml amount of MTT solution was added to each well. After incubation for 2 h at 37 °C and 5% CO_2, the supernatants were removed and the formed formazan crystals in the viable cells were dissolved in DMSO (dimethyl sulfoxide). The absorbance at 550 nm was determined using a microplate reader (Molecular device, USA). Each experiment was performed in triplicate.

2.5. Immunoblot analysis

To obtain the total cell lysate, 100 μL (or 50 μL) of RIPA buffer [1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, with freshly added protease inhibitor cock-tail (Calbiocam, CA)] was added to the BV-2 cells cultured in 6-well plates. The cells were scraped, incubated for 10 min on ice and centrifuged at 14,000× rpm for 10 min at 4 °C. The protein concentration was determined by the DC protein assay from Bio-Rad (Hercules, CA), and 20 μg of whole cell lysate was separated in 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and transferred to PVDF membranes (Millipore, MA, USA) using an electroblotting apparatus (Biorad, CA). The membranes were blocked for 1 h in TBS containing 0.1% Tween-20 and 5% dry milk, and were then incubated overnight with primary antibodies anti-iNOS, 1:5000 (Upstate, NY); anti-COX-1, 1:5000 (Santa Cruz, CA); anti-COX-2, 1:2000 (Santa Cruz, CA); anti-IκB-α, 1:1000; anti-phospho-IκB-α, 1:1000; anti-phospho-p65, 1:1000; anti-p38, 1:1000; anti-phospho-p38, 1:1000; anti-ERK, 1:1000; anti-phosphor-ERK, 1:1000; anti-JNK, 1:1000; anti-phospho-JNK, 1:1000; anti-Akt, 1:1000; anti-phospho-Akt, 1: 1000; anti-β-actin, 1:5000 (Cell Signaling, MA); anti-p65, 1:1000 (Santa Cruz, CA) followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies (1:10,000) (Cell signaling, MA). The optical densities of the antibody-specific bands were analyzed by a Luminescent Image Analyzer, LAS-3000 (Fuji, Japan).

2.6. Isolation of total RNA and RT-PCR analysis

BV-2 cells (5×10^4 cells/ml) were cultured in 6 well plates, and the total RNA was isolated by extraction with TRIzol kit (Invitrogen, CA). For the reverse transcription-polymerase chain reaction (RT-PCR), 2.5 μg of total RNA from BV-2 cell was used for RT-PCR by using a First Strand cDNA Synthesis kit (Invitrogen, CA). Polymerase chain reaction (PCR) was performed using the above-prepared cDNA as a template. The primer sequences used were presented in Table 1. The PCR conditions were as follows: iNOS and COX-1, 27 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; COX-2, TNF-α, IL-1β, and IL-6, 25 cycles of denaturation at 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 30 s; GAPDH, 19 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. GAPDH was used as an internal control to evaluate the relative expressions of iNOS, COX-1, COX-2, TNF-α, IL-1β, and IL-6.

Table 1
PCR primers used.

Gene target			Size (bp)
iNOS	Forward primer	5′-CTTGCAAGTCCAAGTCTTGC-3′	368
	Reverse primer	5′-GTATGTGTCTGCAGATGTGCTG-3′	
COX-1	Forward primer	5′-CAACTCAGCGCATGACTACATC-3′	348
	Reverse primer	5′-AAGAGCCGCAGGTGATACTGT-3′	
COX-2	Forward primer	5′-ACATCCCTGAGAACCTGCAGT-3′	413
	Reverse primer	5′-ACATCCCTGAGAACCTGCAGT-3′	
IL-1β	Forward primer	5′-AATCTCACAGCACATCAA-3′	671
	Reverse primer	5′-AGCCCATACTTTAGGAAGACA-3′	
IL-6	Forward primer	5′-GGAGGCTTAATTACACATGTT-3′	347
	Reverse primer	5′-TGATTTCAAAGATGAATTGGAT-3′	
TNF-α	Forward primer	5′-TTCGAGTGACAAGCCTGTAGC-3′	369
	Reverse primer	5′-AGATTGACCTCAGCGCTGAGT-3′	
GAPDH	Forward primer	5′-GCAGTGGCAAAGTGGAGATTG-3′	357
	Reverse primer	5′-TGCAGGATGCATTGCTGACA-3′	

Abbreviations: iNOS, inducible nitric oxide synthase; COX-1, cyclooxygenase -1; COX-2, cyclooxygenase-2; IL-1β, interleukin-1β; IL-6, interleukin-6; TNF-α, tissue necrosis factor alpha; GAPDH, glyceraldehydes-3-phosphate dehydrogenase.

2.7. Immunofluorescence assay

For the detection of intracellular location of p65 subunit of NF-κB, BV-2 microglial cells (1×10^5 cells/well in 24-well plate) were cultured on sterile cover slips in 24-well plates and treated with different concentrations of inflexin and LPS. At 60 min after the LPS treatment, the cells were fixed with methanol for 20 min at −20 °C and washed with PBS for 5 min. The fixed cells were then permeabilized with 0.5% Triton X-100 in PBS for 1 h at room temperature, washed with 0.05% Tween-20 in PBS for 10 min and 0.05% Tween-20/1% BSA in PBS for 5 min. The permeabilized cells were then treated with 1 mg/ml of monoclonal mouse anti-human NF-κB (p65) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 60 min at room temperature and washed with 0.05% Tween-20/1% BSA in PBS for 5 min. Cells were then incubated in a 1:2000 dilution of Alexa Fluor 488-labeled goat anti-mouse antibody (Molecular Probes Inc., Eugene, OR) for 60 min at room temperature, and washed with 0.05% Tween-20 in PBS for 5 min and PBS for 5 min. Cells were then stained with 0.5 μg/ml of Hoechst staining solution for 20 min at 37 °C and then washed. Finally, the cover slips with cells were dried at 37 °C in an oven for 45 min and mounted in a 1:1 mixture of xylene and malinol. More than 50 cells per field were counted under a fluorescence microscope.

2.8. Electrophoretic mobility shift assays

Nuclear extracts were prepared from the cells as described previously (Takada and Aggarwal, 2004). Synthetic double-stranded oligonucleotides of a consensus nuclear factor-κB (NF-κB) binding sequence, GAT CCC AAC GGC AGG GGA (Promega), were end-labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase. Labeled nucleic acids were purified using a mini-Quick Spin Column (Roche). The nuclear extract was incubated with the labeled oligonucleotides in the presence of poly (dI–dC) in a binding buffer containing 20 mM N-2-hydroxyethylpiperazine-N0-2-ethanesulfonic acid at room temperature for 30 min. For super shift assays, 0.2 μg of antibodies against the p65 subunit of NF-κB (SC-8008, Santa Cruz Biotechnology) was included in the reaction. DNA–protein complexes were resolved by electrophoresis in a 5% nondenaturing polyacrylamide gel, dried, and visualized by autoradiography.

2.9. Inhibitors of microglia activation

The BV-2 cells were seeded at the density of 5×10^4 cells/ml, pretreated for 30 min with various concentrations of ERK inhibitor, 2′-Amino-3′methoxyflavone (PD98059), p38 inhibitor, 4-(4-Fluorophenyl)-2-(4-methylsulfinyl phenyl)-5-(4-pyridyl) 1H-imidazole (SB203580), JNK inhibitor, Anthra(1,9-cd)pyrazol-6(2H)-one (SP600125) and PI3K/Akt inhibitor (Wortmannin) before incubation in medium containing LPS. And then, the BV-2 cells were tested for viability determined by measuring the reduction of MTT. Production of NO was assayed by measuring the levels of nitrite.

2.10. Statistical analysis

The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. Data were also analyzed using Student's *t*-test (Sigma Plot software). The values given are presented as means ± S.E.M. In all cases, $P<0.05$ was considered significant.

3. Results

3.1. Cell toxicity of the LPS and inflexin in BV-2 microglial cells

To examine the probability that reduced production of proinflammatory mediators in the BV-2 microglia cells was caused by the inflexin, BV-2 microglial cells were treated with various concentrations of inflexin and the cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, which was conducted in triplicate. The viabilities of the control cells were used as the control values at 100%. Inflexin, at concentrations up to 5 μM by itself and in combination with LPS (100 ng/ml), was not significantly cytotoxic (Fig. 1). Although mild reduction of cell viability was observed at 10 μM inflexin in BV-2 microglia cells, cytotoxicity was not evident in human SH-SY5Y cells and murine Neuro-2a (N2A) cells treated with up to 10 μM inflexin, as assessed using the MTT and LDH viability assays (data not shown).

3.2. Inhibition of induced NO and iNOS production by inflexin

The primary microglia cells were pretreated for 30 min with inflexin (0.05 and 0.1 μM) and then stimulated with LPS (25 ng/ml) for another 24 h. The cell-culture medium was then harvested. The content of nitrite in the medium was measured by Griess reaction. Results show that LPS stimulation unregulated the production of NO. Pretreatment with inflexin effectively decreases the LPS-stimulated production of nitrite in primary microglia (Fig. 2A). To investigate the effect of inflexin on LPS-induced NO production and iNOS expression, BV-2 microglial cells were pretreated for 30 min with different concentrations of inflexin, then cells were stimulated with 100 ng/ml LPS for 24 h and the levels of NO in the culture media were determined using the Griess assay. As shown in Fig. 2B, LPS alone markedly induced NO production (68.3 ± 1.4 μM) compared to that in

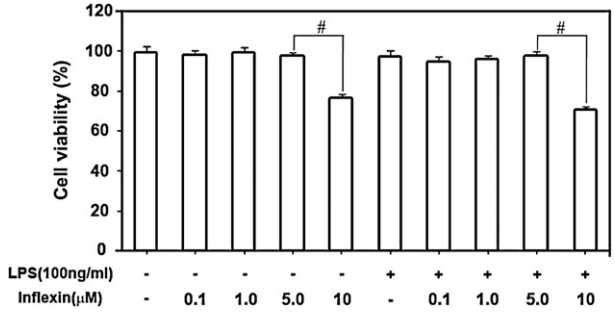

Fig. 1. Effect of inflexin and LPS on the cell viability of BV-2 microglial cells. Viability in inflexin-treated cells was evaluated using the MTT assay. Cells were incubated with 0.1 to 10 μM of inflexin and LPS (100 ng/ml) for 24 h. The results are displayed in percentage of control samples. Data are represented as the mean ± S.E.M. ($n=5$–6) for three independent experiments. Significance was determined using Student's *t*-test ($^{\#}P<0.05$ *vs.* untreated control).

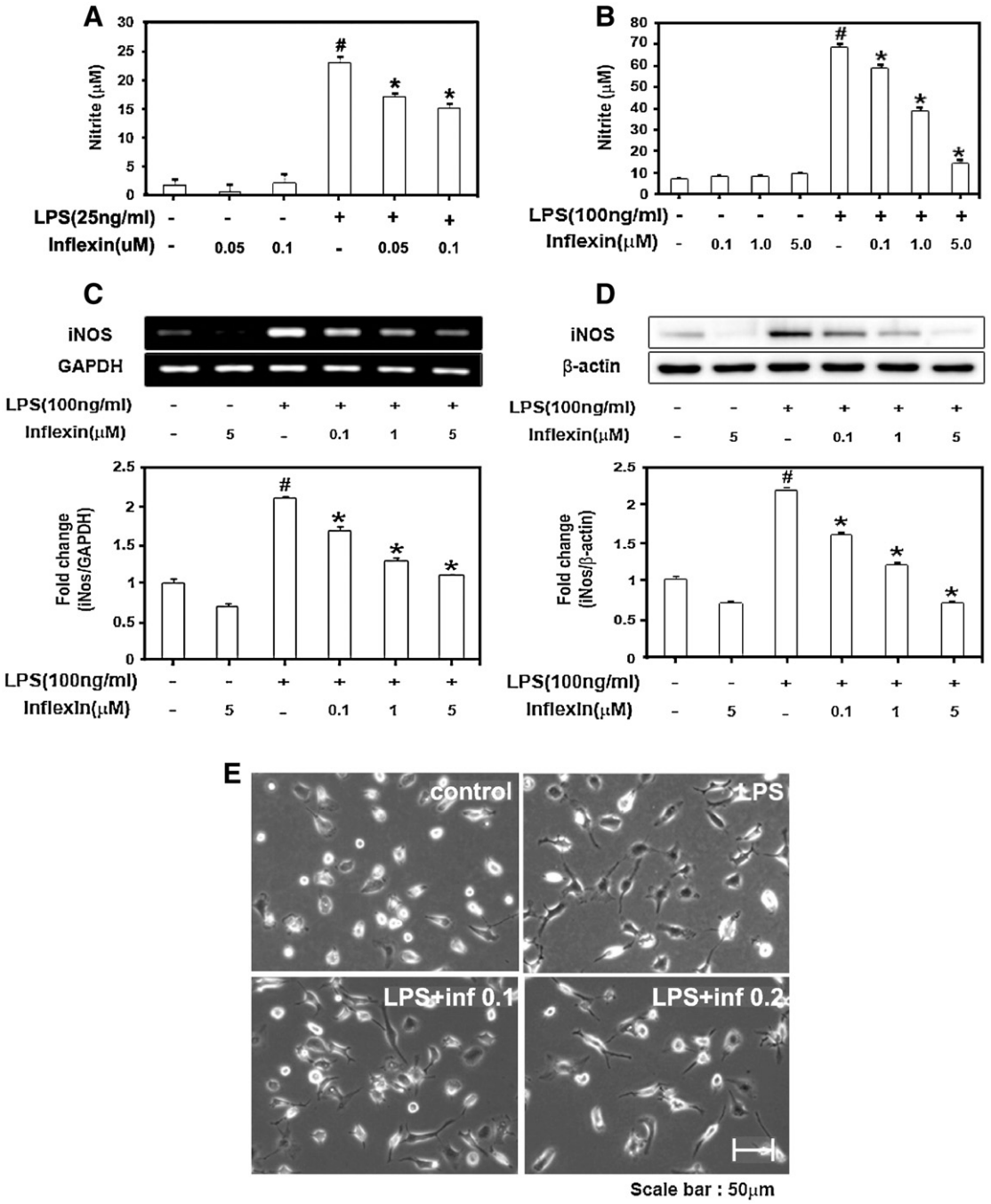

Fig. 2. Inhibition by inflexin of LPS-induced nitrite production in rat primary microglia cultures/BV-2 cell line and iNOS release in the BV-2 cell line. Cells were pretreated with the indicated concentrations of inflexin for 30 min before incubating with LPS (100 ng/ml) for 24 h, the culture medium of primary microglia culture (A) or BV-2 cell line (B) was then collected for the assay of nitrite. BV-2 microglial cells were pretreated with the indicated concentrations of inflexin for 30 min before incubating with LPS (100 ng/ml) for 3 h. Total RNA was prepared and analyzed by RT-PCR(C). BV-2 microglial cells were pretreated with the indicated concentrations of inflexin for 30 min before incubating with LPS (100 ng/ml) for 18 h. The cells were lysed, and the lysates were prepared for mRNA levels (C) and analyzed by immunoblotting with an anti-iNOS antibody (D). The blot was stripped and reprobed with a β-actin antibody to confirm equal loading. The morphological change was represented in primary microglia cells (E). Data are presented as the mean ± S.E.M. ($n = 5–6$) for three independent experiments. *Significantly different from LPS alone group. #Significantly different from control group. Significance was determined using Student's *t*-test, $P<0.05$ was considered statistically significant.

control (8.3 ± 0.3 μM). Inflexin significantly reduced the levels of NO production in LPS-induced BV-2 microglial cells in a dose-dependent manner. The effect of inflexin on iNOS mRNA expression was measured using RT-PCR analysis. The iNOS mRNA was barely detected in unstimulated BV-2 microglial cells, but was expressed at high levels following stimulation with 100 ng/ml LPS for 3 h. Pretreatment with inflexin inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner (Fig. 2C). To further investigate the effects of inflexin on LPS-induced nitrite production, the iNOS protein levels were detected using western blotting. As shown in Fig. 2D, the production of nitrite was in good agreement with the changes in the levels of iNOS protein expression. These results suggest that inflexin inhibited NO production at the transcriptional level. In addition, a small spherical morphology was evident in the collected microglial cells in the absence of LPS. With addition of 25 ng/ml LPS, an amoeboid shape was observed. More than half of the cells changed into process-bearing cell types, often bipolar and sometimes tripolar. This might suggest the involvement of microglial activation induced

by LPS treatment. This morphological change exhibited by cells with LPS treatment was effectively inhibited by 0.05 and 0.1 μM of inflexin in a dose responsible manner (Fig. 2E).

3.3. Inflexin inhibited the expression of COX-2

It is known that increased expression of the cyclooxygenase-2 (COX-2) enzyme and elevated levels of prostaglandin E_2 (PGE_2) play important roles in the inflammatory process (Albazzaz et al., 1994). Several previous reports have also shown that LPS strongly activates microglia and induces COX-2, a rate limiting enzyme in the synthesis of PGE_2 (Bauer et al., 1997; Fiebich et al., 2003). Therefore we investigated the effects of inflexin on the expression of COX-2 in LPS-activated BV-2 microglial cells. We also examined whether inflexin would inhibit the expression of COX-2 mRNA. While the expression of constitutive COX-1, an isoform of COX-2, was not affected by treatment, inflexin significantly decreased the LPS-induced expression of COX-2 mRNA in a dose-dependent manner (Fig. 3A). Moreover, the COX-2 protein level in the LPS-activated BV-2 microglial cells was dramatically reduced dose responsively by inflexin treatment (Fig. 3B). These results imply that inflexin inhibited the expression of COX-2, but not COX-1 expression, in LPS-stimulated BV-2 microglial cells.

3.4. Inflexin repressed the expression of cytokines

To elucidate the potential effects of inflexin on proinflammatory cytokines production, including TNF-α, IL-1β and IL-6, which play central roles in various types of inflammation, the BV-2 microglial cells were incubated with inflexin (0.1, 1.0, 5.0 μM) in the presence or absence of LPS (100 ng/ml). RT-PCR analysis was used to examine whether the suppression of TNF-α, IL-1β and IL-6 by inflexin was due to a decrease in mRNA in the BV-2 microglial cells. While TNF-α, IL-1β, and IL-6 were not expressed at detectable levels under the normal culture conditions, the expression of these cytokines was significantly up-regulated after 3 h of treatment with LPS (100 ng/ml). LPS-stimulated mRNA levels of proinflammatory cytokines (TNF-α, IL-1β and IL-6) were reduced by inflexin (Fig. 4), suggesting that inflexin negatively regulated the production of TNF-α, IL-1β and IL-6 at the transcriptional level.

3.5. Inflexin inhibited NF-κB (p65) activation and IκB-α degradation

It was previously reported that LPS increases transcription factor NF-κB subunits activation via phosphorylation, ubiquitination, degradation and translocation of p65 and the inhibitor protein kappa B alpha (IκB-α), and regulates the expressions of iNOS, COX-2, and proinflammatory cytokines (Nomura, 2001; Fiebich et al., 2002; Carmody and Chen, 2007). NF-κB has been demonstrated to play an essential role in the LPS-induced expression of both iNOS, COX-2, and proinflammatory cytokines genes. Therefore to further elucidate inflexin mechanism with LPS-activated microglia, we examined the effects of inflexin on the transcription factor, NF-κB (p65). NF-κB activity was evaluated by nuclear translocation of p65 subunit of NF-κB (Fig. 5A), formation of DNA binding complex of NF-κB (Fig. 5B), and IκB-α degradation (Fig. 5C). As shown in Fig. 5, LPS enhanced the nuclear translocation of p65 subunit NF-κB, an effect preceded by the cytosolic decrease in IκB-α. Both effects were inhibited by inflexin treatment. These results imply the potential role of inflexin, through the inhibition of NF-κB and IκB-α activation, in suppressing the expressions of iNOS, COX-2, and proinflammatory cytokines (TNF-α, IL-1β and IL-6).

3.6. Inflexin inhibited Akt activation but not MAPKs activation

The Akt signaling molecule is known to regulate NF-κB activation through IκB degradation (Madrid et al., 2000). Here the effect of

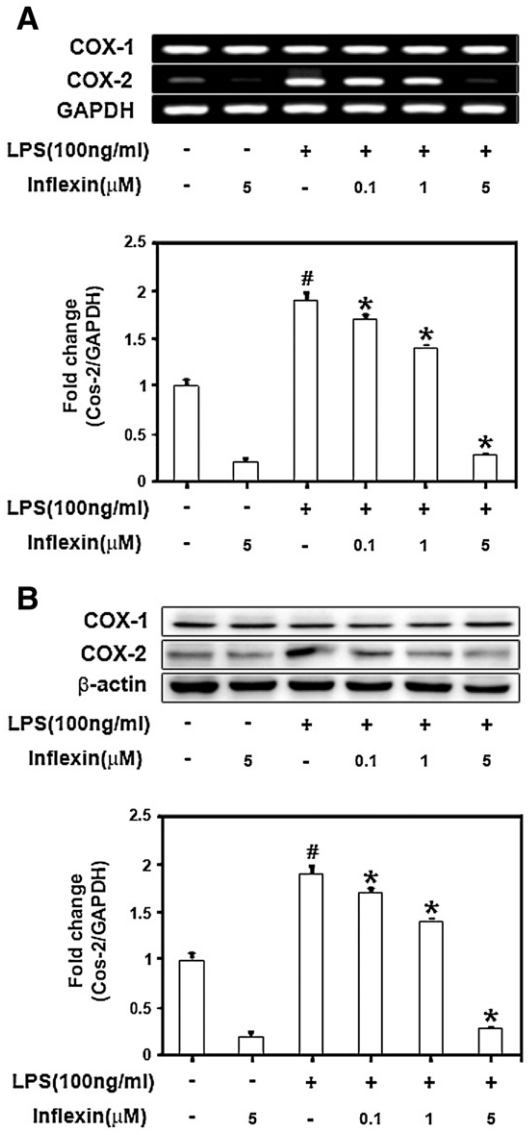

Fig. 3. Effect of inflexin on LPS-induced COX-1 and COX-2 mRNA and protein expressions in BV-2 microglial cells. BV-2 cells were pretreated with vehicle or various concentrations of inflexin for 30 min and then stimulated with LPS (100 ng/ml). Cell lysates were prepared for the determination of mRNA levels and protein expressions for COX-1 and COX-2 (A and B). GAPDH (A) and β-actin (B) were used as internal controls for the RT-PCR assays and western blot analysis, respectively. The band intensity was quantified and is presented as relative to the level of GAPDH and β-actin respectively. Data are presented as the mean ± S.E.M. ($n = 5$) for three independent experiments. *Significantly different from LPS alone group. #Significantly different from control group. Significance was determined using Student's t-test, $P<0.05$ was considered statistically significant.

inflexin on LPS-induced Akt activation was examined. BV-2 microglial cells were pretreated with inflexin (0.1 to 5.0 μM) for 30 min and then stimulated with LPS (100 ng/ml) for 30 min. As shown in Fig. 6A, LPS markedly increased the phosphorylation of Akt at 30 min. Inflexin significantly inhibited LPS-induced Akt phosphorylation. On the other hand, MAPKs are among the most important molecules in the signaling pathways that control the synthesis and release of proinflammatory substances by activated microglia (Koistinaho and Koistinaho, 2002). The possibility was raised that LPS induced proinflammatory mediators by MAPKs. While the MAPKs (ERK, p38 and JNK) signal pathway was not affected by inflexin. As shown in Fig. 6B, BV-2 microglial cells were pretreated with inflexin (0.1 to 5.0 μM) for 30 min and then stimulated with LPS (100 ng/ml) for 30 min. LPS rapidly activated all MAPKs within 30 min after the

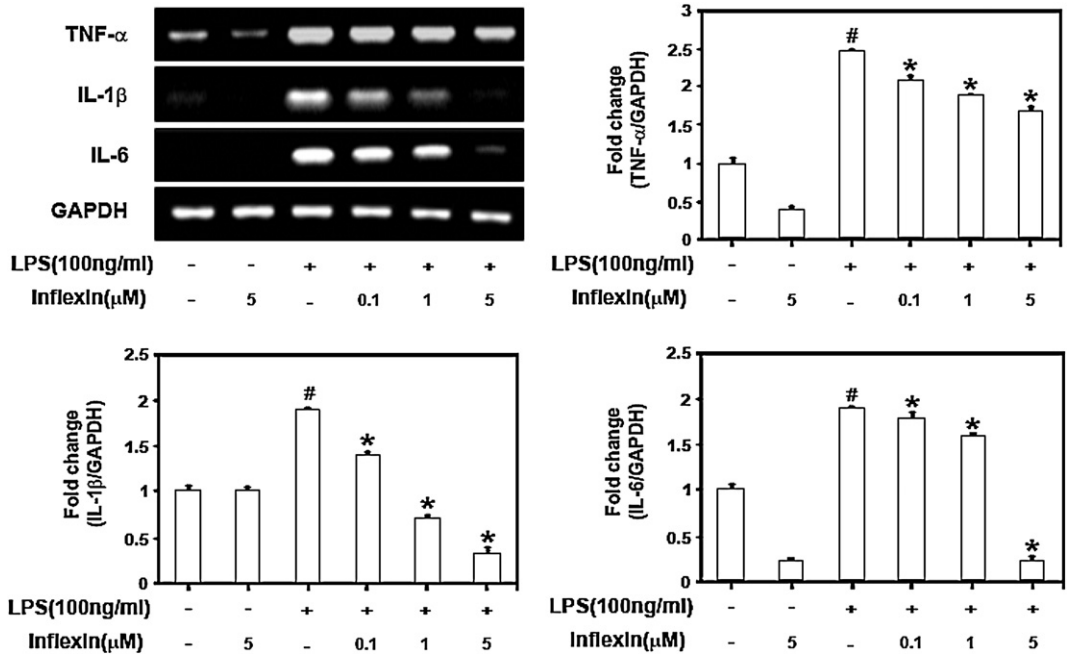

Fig. 4. Effect of inflexin on LPS-induced TNF-α, IL-1β and IL-6 at the transcriptional level in BV-2 microglial cells. BV-2 microglial cells were treated with the indicated doses of inflexin for 30 min before LPS treatment (100 ng/ml) after incubation for 3 h, the mRNA levels of IL-1β, IL-6 and TNF-α were determined by using RT-PCR. GAPDH was used as internal control for RT-PCR assays, respectively. Data are presented as the mean ± S.E.M. ($n = 5$) for three independent experiments. *Significantly different from LPS alone group. #Significantly different from control group. Significance was determined using Student's t-test, $P<0.05$ was considered statistically significant.

stimulation. The results showed that inflexin significantly inhibited the phosphorylation of Akt, but not MAPKs by LPS in BV-2 microglial cells (Fig. 6), which suggests that inflexin is indeed capable of disrupting the key signal transduction pathways elicited by LPS in BV-2 microglial cells, and subsequently preventing the production of pro-inflammatory mediators.

4. Discussion

Microglia activation plays a crucial role in the initiation and progression of brain inflammation, which is thought to aggravate pathologic conditions in the CNS by releasing various proinflammatory cytokines and free radicals. (McGeer et al., 1993; Minghetti and Levi, 1998; Choi et al., 2005). These factors are believed to contribute to microglia-mediated neurodegeneration (McGeer et al., 1988; Minghetti and Levi, 1998; Le et al., 2001). Additionally, proinflammatory cytokines such as TNF-α, IL-1β, IL-6 have an important role in the process of neuroinflammatory diseases.

Inflexin (ent-1α-hydroxy-3β, 6a-diacetoxykaur-16-en-11, 15-dione), a compound derived from the plant *I. excisus* (Max.) Kudo (Labiatae) is a perennial herb that is distributed widely in Asia. Inflexin and other Kaurane diterpenoids have been reported to inhibit LPS-induced NF-κB activation in macrophage RAW264.7 cells (Hong et al. 2007; Chong et al., 2005). However, the effect of inflexin on LPS-stimulated BV-2 microglial cells was not studied.

In the present study, we first examined whether inflexin at various concentrations has any influence on the viability of BV-2 microglial cells. Treatment with inflexin up to 5 μM alone and with LPS (100 ng/ml), showed no signs of toxicity in BV-2 microglial cells which was used in this study. The morphological changes observed in the primary microglia cells with LPS treatment (25 ng/ml) were effectively decreased by inflexin at 0.05 and 0.1 μM respectively, in a concentration-dependent manner. Our results are consistent with the similar observation reported on morphological changes in LPS-induced microglial cells (Nakamura and Kataoka, 1999). It was also showed that inflexin significantly suppressed LPS-stimulated nitrite production and iNOS expression in dose-dependent manners, suggesting that inflexin has potent antiinflammatory activity through inhibition of NO release and iNOS expression. It has been reported that iNOS and COX-2 were induced in various types of central nervous injuries and diseases (Hunot et al., 1996; Teismann et al., 2003). It was also founded that the expression of iNOS and COX-2 has been identified in microglia cells in rodent brain after LPS treatment (Boje and Arora, 1992; Minghetti et al., 1999). Microglia-derived NO and PGE_2 have been presumed to be neurotoxic. Previous studies have demonstrated that iNOS and COX-2 inhibitors (Araki et al., 2001; Arimoto and Bing, 2003; Teismann et al., 2003) provided neuroprotective effects against LPS-induced neurotoxicity. Therefore attenuation of the induction of iNOS, COX-2 and proinflammatory cytokines from microglia could inhibit neuroinflamation after LPS treatment (Lu et al., 2007). It is a well known fact that inflammatory processes associated with increased expression of the COX-2 enzyme and elevated levels of PGE_2 occur in a variety of neurodegenerative disease, including Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (Wyss-Coray and Mucke, 2002; Teismann et al., 2003). It is a well known fact that COX-2 exits in both constitutive and inducible forms. In normal conditions, the constitutive form of COX-2 has low expression or is undetectable in most glia cells of oligodendrocytes and microglia (Ghilardi et al., 2004). The COX-2 expression that dramatically increases in some pathological conditions was proposed to be inducible form of COX-2 (Maria and Gerd, 2005). In the current study we questioned whether inflexin would be associated with PGE_2 production and COX expression in LPS-stimulated BV-2 microglia cells. Though inflexin at 5 μM concentration treated in the absence of LPS was able to suppress constitutive COX-2 expression, it could actually attenuate the increased inducible form of COX-2 expression when stimulated with LPS. The expressions of COX-2 mRNA and protein were gradually suppressed by inflexin treatment (0.1, 1.0, and 5.0 μM) in a dose-dependent manner, but the expression of COX-1 mRNA and protein was not significantly changed by inflexin treatment.

It has been studied that proinflammatory cytokines such as TNF-α, IL-1β and IL-6 cause potent activation of iNOS gene expression in rodent glial cells and muscle cells (Gonzalez-Scarano and Baltuch, 1999; Hunot et al., 2001; Adams et al., 2002). In the present study we

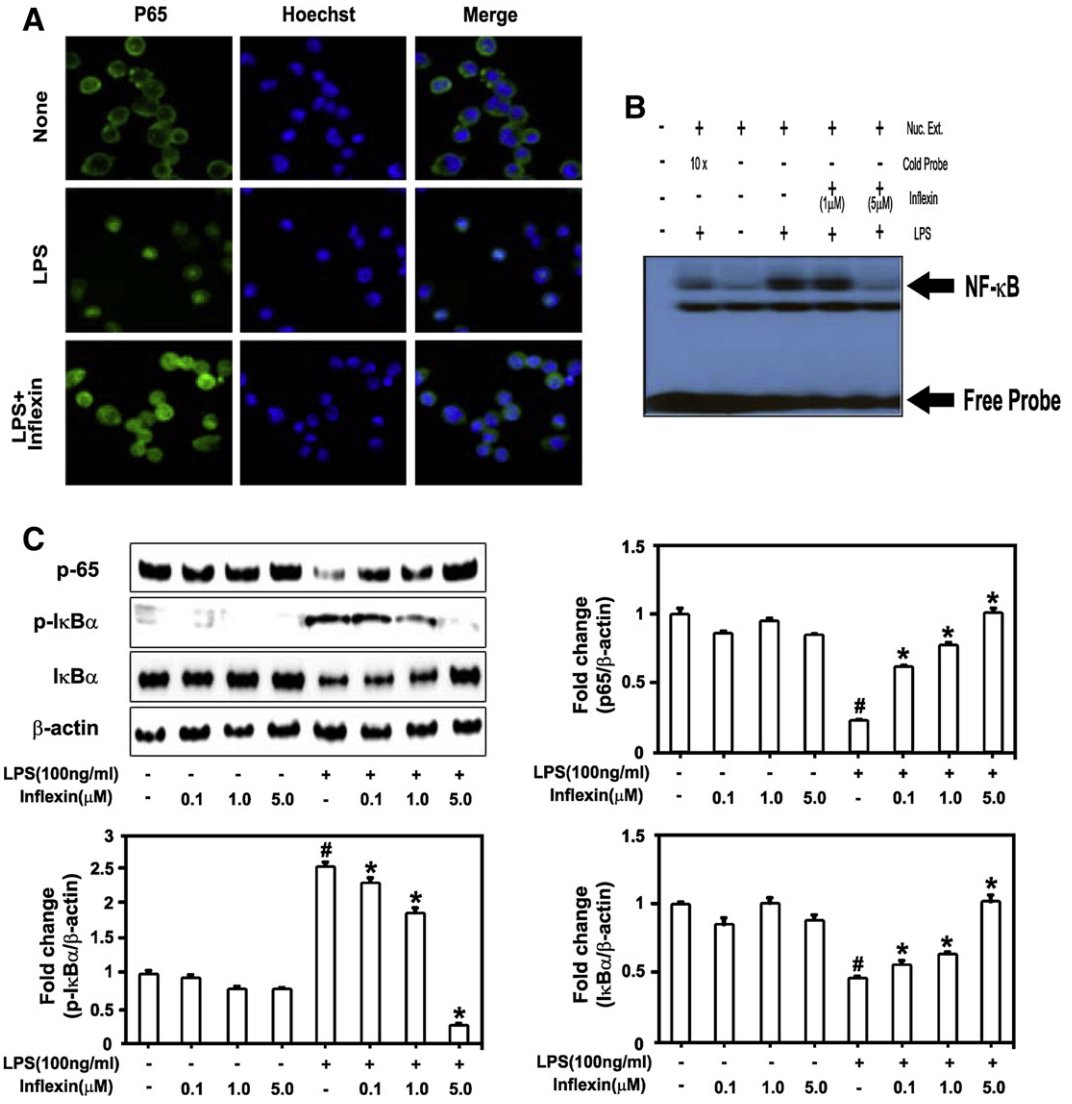

Fig. 5. Inhibition of LPS-induced NF-kB activation by inflexin. BV-2 microglial cells were first treated with 5.0 μM inflexin for 30 min and then exposed to LPS (100 ng/ml) for 30 min. (A) The nuclear translocation of p65 was shown by immunofluorescence, which was inhibited by inflexin. (B) Nuclear extracts from untreated cells or cells treated with inflexin and LPS (100 ng/ml) for 30 min, and an unlabeled NF-κB oligonucleotide probe. They were then assayed for NF-κB activation by electromagnetic shift assay (EMSA). (C) Cell lysates were prepared from untreated control cells and cells treated for 30 min with LPS (100 ng/ml) alone or with different concentrations (0.1 to 5.0 μM) of inflexin. The band intensity was quantified with a densitometric scanner and is presented as relative to the level of β-actin. Data are presented as the mean ± S.E.M. ($n = 5$) for three independent experiments. Significance was determined using Student's *t*-test (* $P<0.05$ *vs.* untreated control).

examined the effects of inflexin on proinflammatory cytokine production, including TNF-α, IL-1β and IL-6. Based on the expression analysis, inflexin suppressed the expression of TNF-α, IL-1β and IL-6 mRNA in the LPS-stimulated BV-2 microglia cells. These results suggest that inflexin suppressed the expressions of TNF-α, IL-1β and IL-6 at the transcriptional level, and thus, perhaps contributes to reduced secretion of TNF-α, IL-1β and IL-6, indicating that the inflexin may be an effective antiinflammatory agent.

NF-κB is an essential and ubiquitous transcription factor for the expression of many inflammation-related genes, including iNOS, COX-2, TNF-α, IL-1β and IL-6. LPS has also been reported to activate NF-κB in microglia (Wang et al., 2002; Lee et al., 2004). It is well established that the nuclear accumulation of NF-κB relies in large part upon IκB kinase-dependent phosphorylation and subsequent degradation of the cytosolic inhibitor, IκB-α. LPS treatment in BV-2 microglial cells led to a rapid degradation of IκB-α and nuclear translocation of p65. Our results show that the signaling pathways of NF-κB activated by LPS were also inhibited by inflexin. Immunofluorescence assay consistently shows that inflexin alone did not affect the translocation of p65 but inhibited the nuclear translocation of NF-κB induced by LPS. Whether the effect of inflexin on NF kappa B translocation was due to a direct inhibition or secondary to inhibition of an upstream event is in question.

Since NF-κB activation and IκB-α degradation are known to be involved in LPS/IFNγ-induced iNOS expression (Nomura, 2001), we examined if inflexin would inhibit iNOS induction by down-regulating NF-κB activity. NF-κB is one of the most widely studied mammalian transcription factors, controlling a number of inflammatory genes, including the iNOS and TNF-α genes (Christman et al., 2000; Carmody and Chen, 2007). NF-κB, a heterotrimer composed of p50, p65 (Rel A), and IκB-α, is shown to be activated by the elimination of the regulatory subunit IκB-α from the heterotrimer. NF-κB activation is achieved through the Phosphorylation of serine 32 of IκB-α by the IκB kinase (IKK) complex, followed by polyubiquitination and 26S proteasomal degradation of IκB-α, resulting in NF-κB translocation into the nucleus (Nomura, 2001). In this study we showed that inflexin inhibited the LPS-induced NF-κB DNA binding activities and also suppresses LPS-stimulated activation of p65 and degradation of IκB-α in a dose-dependent manner, suggesting that inflexin may block NF-κB subunit activation via phosphorylation, ubiquitination, degradation and translocation.

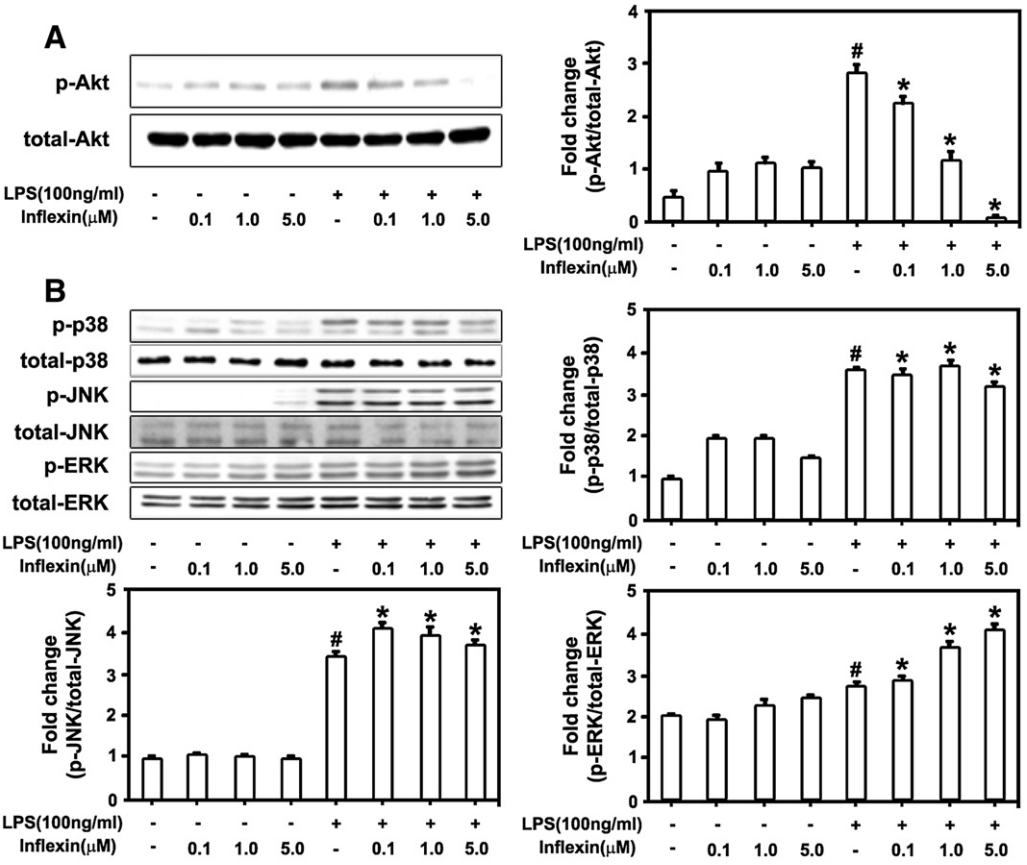

Fig. 6. The effect of inflexin on Akt and MAPKs activation induced by LPS in BV-2 microglial cells. BV-2 microglial cells were treated with the indicated doses of inflexin 30 min before LPS treatment (100 ng/ml) for another 30 min. Total protein was subjected to 10% SDS-PAGE followed by western blotting using anti-Akt and anti-MAPKs (ERK, p38, and JNK). Inflexin inhibited LPS-induced Akt phosphorylation (A), while the MAPKs (p38, JNK and ERK) were not affected by inflexin (B). The quantitative panels were also shown in A and B respectively.

In addition, Akt mediates degradation of IκB through phosphorylation of IκB kinase at Thr23 position (Zimmermann and Moelling, 1999). In our present study inflexin also significantly inhibited Akt activation in LPS-stimulated BV-2 microglia. It is possible that inflexin inhibits LPS-induced NF-kB activation via Akt signal pathway. It has been reported that LPS stimulated NF-κB activation through the phosphorylation and degradation of IκB by increasing IKK or Akt kinase activity (Ardeshna et al., 2000). Recent studies have shown that a signaling pathway culminating in phosphorylation of both IKKα and β by Akt is necessary for activation of NF-κB (Madrid et al., 2001; Ozes et al., 1999). Results show that inflexin significantly decreases the phosphorylation of Akt on Ser 473 and IKKα/β on Ser 32/36 by using their specific antibodies (anti-phospho-Akt which can detect the phosphorylation of Akt on Ser 473 and anti-phospho-IkB-alpha which can recognize on Ser 32/36), and this inhibitory event is accompanied by the suppression of IκB degradation and NF-κB p65 subunit nuclear translocation in BV-2 microglia cells, and showed that Akt and IKK inactivation might be involved in inflexin-mediated antiinflammatory actions. LPS is also known to activate a series of MAPKs such as ERK, p38 and JNK in immune cells (DeFranco et al., 1995; Guha and Mackman, 2001). ERK and p38MAPK, and JNK, have especially been implicated in the signal transduction pathways responsible for increased iNOS, COX-2, TNF-α, IL-1β and IL-6 gene expression in glial cells (Da et al., 1997; Murphy et al., 1998; Xu and Malave, 2000). The present study revealed that inflexin did not affect the activation of MAPKs such as ERK, p38 and JNK induced by LPS stimulation in BV-2 microglial cells.

We tested the effects of inhibitors to MAPKs signaling pathway (ERK, p38 and JNK) and an inhibitor to the phosphoinositol 3-kinase (PI3K)/protein kinase B (Akt) pathway, implicated in regulation of cellular inflammatory responses. Cells were treated with various doses of ERK inhibitor (PD98059), p38 inhibitor (SB203580), JNK inhibitor (SP600125) and PI3K/Akt inhibitor (Wortmannin). These inhibitors did not significantly reduce the levels of NO production in LPS-induced BV-2 microglial cells.

In conclusion, the present study demonstrates that inflexin exhibits antiinflammatory effects suppressing NO release in primary culture of rat microglia cells and BV-2 microglial cells. Inflexin also significantly inhibited the release of proinflammatory mediators and the release of iNOS, COX-2, IL-1β, IL-6 and TNF-α in LPS-stimulated BV-2 microglial cells. The antiinflammatory properties of inflexin are mediated by down-regulation of NF-κB and Akt but not MAPKs activation in BV-2 microglial cells. Since the NO release and the morphological change were found similar in both primary microglia and BV-2 cells, we believe that inflexin might probably behave in the same manner *in vivo*. However, the majority of the data was derived in BV-2 cell line and the same effects may not necessarily reflect the behavior of microglia *in vivo*. Further protective effects of inflexin on LPS-induced animal models and other neurons cannot be excluded. This compound will give rise to a potent therapeutic use and stable derivatives for the treatment of LPS-mediated neuroinflammation and can be developed as therapeutic agent in treating neuroinflammatory diseases.

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References

Adams, V., Nehrhoff, B., Späte, U., Linke, A., Schulze, P.C., Baur, A., Gielen, S., Hambrecht, R., Schuler, G., 2002. Induction of iNOS expression in skeletal muscle by IL-1β and NF-κB activation: an in vitro and in vivo study. Cardiovasc. Res. 54, 95–104.

Albazzaz, M.K., Pal, C., Berman, P., Shale, D.J., 1994. Inflammatory markers of lower respiratory tract infection in elderly people. Age Ageing 23, 299–302.

Araki, E., Forster, C., Dubinsky, J.M., Ross, M.E., Iadecola, C., 2001. Cyclooxygenase-2 inhibitor ns-398 protects neuronal cultures from lipopolysaccharide-induced neurotoxicity. Stroke 32, 2370–2375.

Ardeshna, K.M., Pizzey, A.R., Devereux, S., Khwaja, A., 2000. The PI3 kinase, p38 SAP kinase, and NF-kappaB signal transduction pathways are involved in the survival and maturation of lipopolysaccharide-stimulated human monocyte-derived dendritic cells. Blood 96, 1039–1046.

Arimoto, T., Bing, G., 2003. Up-regulation of inducible nitric oxide synthase in the substantia nigra by lipopolysaccharide causes microglial activation and neurodegeneration. Neurobiol. Dis. 12, 35–45.

Bauer, M.K., Lieb, K., Schulze-Osthoff, K., Berger, M., Gebicke-Haerter, P.J., Bauer, J., Fiebich, B.L., 1997. Expression and regulation of cyclooxygenase-2 in rat microglia. Eur. J. Biochem. 243, 726–731.

Bhat, N.R., Zhang, P., Lee, J.C., Hogan, E.L., 1998. Extracellular signal-regulated kinase and p38 subgroups of mitogen-activated protein kinases regulate inducible nitric oxide synthase and tumor necrosis factor-alpha gene expression in endotoxin-stimulated primary glial cultures. J. Neurosci. 18, 1633–1641.

Bocchini, V., Mazzolla, R., Barluzzi, R., Blasi, E., Sick, P., Kettenmann, H., 1992. An immortalized cell line expresses properties of activated microglial cells. J. Neurosci. Res. 31, 616–621.

Boje, K.M., Arora, P.K., 1992. Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. Brain Res. 587, 250–256.

Boulet, I., Ralph, S., Stanley, E., Lock, P., Dunn, A.R., Green, S.P., 1992. Lipopolysaccharide- and interferon-gamma-induced expression of hck and lyn tyrosine kinases in murine bone marrow-derived macrophages. Oncogene 7, 703–710.

Carmody, R.J., Chen, Y.H., 2007. Nuclear factor-kappa B: activation and regulation during toll-like receptor signaling. Cell. Mol. Immunol. 4, 31–41.

Chen, C.C., Wang, J.K., Chen, W.C., Lin, S.B., 1998. Protein kinase C beta mediates lipopolysaccharide-induced nitric-oxide synthase expression in primary astrocytes. J. Biol. Chem. 273, 19424–19430.

Choi, D.K., Pennathur, S., Perier, C., 2005. Ablation of the inflammatory enzyme myeloperoxidase mitigates features of Parkinson's disease in mice. J. Neurosci. 28, 6594–6600.

Chong, Z.Z., Li, F., Maiese, K., 2005. Stress in the brain: novel cellular mechanisms of injury linked to Alzheimer's disease. Brain Res. Brain Res. Rev. 49, 1–21.

Christman, J.W., Blackwell, T.S., Juurlink, B.H., 2000. Redox regulation of nuclear factor kappa B: therapeutic potential for attenuating inflammatory responses. Brain Pathol. 10, 153–162.

Da, S.J., Pierrat, B., Mary, J.L., Lesslauer, W., 1997. Blockade of p38 mitogen-activated protein kinase pathway inhibits inducible nitric-oxide synthase expression in mouse astrocytes. J. Biol. Chem. 272, 28373–28380.

DeFranco, A.L., Hambleton, J., McMahon, M., Weinstein, S.L., 1995. Examination of the role of MAP kinase in the response of macrophages to lipopolysaccharide. Prog. Clin. Biol. Res. 392, 407–420.

Fiebich, B.L., Lieb, K., Engels, S., Heinrich, M., 2002. Inhibition of LPS-induced p42/44 MAP kinase activation and iNOS/NO synthesis by parthenolide in rat primary microglial cells. J. Neuroimmunol. 132, 18–24.

Fiebich, B.L., Lieb, K., Kammerer, N., Hull, M., 2003. Synergistic inhibitory effect of ascorbic acid and acetylsalicylic acid on prostaglandin E2 release in primary rat microglia. J. Neurochem. 86, 173–178.

Gao, H.M., Jiang, J., Wilson, B., Zhang, W., Hong, J.S., Liu, B., 2002. Microglial activation-mediated delayed and progressive degeneration of rat nigral dopaminergic neurons: relevance to Parkinson's disease. J. Neurochem. 81, 1285–1297.

Ghilardi, J.R., Svensson, C.I., Rogers, S.D., Yaksh, T.L., Mantyh, P.W., 2004. Constitutive spinal cyclooxygenase-2 participates in the initiation of tissue injury-induced hyperalgesia. J. Neurosci. 24, 2727–2732.

Gonzalez-Scarano, F., Baltuch, G., 1999. Microglia as mediators of inflammatory and degenerative diseases. Annu. Rev. Neurosci. 22, 219–240.

Green, L.C., Wagner, D.A., Glogowski, J., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 1982. Analysis of nitrate, nitrite, and [^{15}N] nitrate in biological fluids. Anal. Biochem. 126, 131–138.

Guha, M., Mackman, N., 2001. LPS induction of gene expression in human monocytes. Cell. Signal. 13, 85–94.

Hong, S.S., Lee, S.A., Xiang, H.H., Hui, Z.J., Jeong, H.L., Lee, D.H., Lee, J.J., Hong, J.T., Kim, Y.S., Ro, J.S., Hwang, B.Y., 2007. Kaurane diterpenoids from *Isodon excisus* inhibit LPS-induced NF-kappaB activation and NO production in macrophage RAW264.7 cells. J. Nat. Prod. 70, 632–636.

Hunot, S., Boissiere, F., Faucheux, B., Brugg, B., Mouatt-Prigent, A., Agid, Y., Hirsh, E.C., 1996. Nitric oxide synthase and neuronal vulnerability in Parkinson's disease. Neuroscience 72, 355–363.

Hunot, S., Hartmann, A., Hirsh, E.C., 2001. The inflammatory response in the Parkinson brain. Clin. Neurosci. Res. 1, 434–443.

Jeohn, G.H., Cooper, C.L., Jang, K.J., Liu, B., Lee, D.S., Kim, H.C., Hong, J.S., 2002. Gö6976 Inhibitors LPS-induced microglial TNFα release by suppressing p38 MAP kinase activation. Neuroscience 114, 689–697.

Jung, B.S., Shin, M.K., 1990. Encyclopedia of illustrated Korea Natural Drugs. Yong Lim Sa, Seoul. pp. 845–846.

Koistinaho, M., Koistinaho, J., 2002. Role of p38 and p44/42 mitogen activated protein kinases in microglia. Glia 40, 175–183.

Le, W., Rowe, D., Xie, W., Ortiz, I., He, Y., Appel, S.H., 2001. Microglial activation and dopaminergic cell injury: an in vitro model relevant to Parkinson's disease. J. Neurosci. 21, 8447–8455.

Lee, C.B., 1989. Illustrated Flora of Korea. Hyang Moon Sa, Seoul. pp 661.

Lee, S.M., Yune, T.Y., Kim, S.J., Kim, Y.C., Oh, Y.J., Markelonis, G.J., 2004. Minocycline inhibits apoptotic cell death via attenuation of TNF-alpha expression following iNOS/NO induction by lipopolysaccharide in neuron/glia co-cultures. J. Neurochem. 91, 568–578.

Liu, B., Hong, J.S., 2003. Role of microglia in inflammation-mediated neurodegenerative diseases: mechanisms and strategies for therapeutic intervention. J. Pharmacol. Exp. Ther. 304, 1–7.

Lu, D.Y., Tang, C.H., Liou, H.C., Teng, C.M., Jeng, K.C.G., Kuo, S.C., Lee, F.Y., Fu, W.M., 2007. YC-1 attenuates LPS-induced proinflammatory responses and activation of nuclear factor-kB in microglia. Br. J. Pharmacol. 151, 396–405.

Madrid, L.V., Wang, C.Y., Guttridge, D.C., Schottelius, A.J., Baldwin Jr, A.S., Mayo, M.W., 2000. Akt suppresses apoptosis by stimulating the transactivation potential of the Rel A/p65 subunit of NF-κB. Mol. Cell. Biol. 20, 1626–1638.

Madrid, L.V., Mayo, M.W., Reuther, J.Y., Baldwin Jr, A.S., 2001. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. J. Biol. Chem. 276, 18934–18940.

Maria, B., Gerd, G., 2005. COX-dependent mechanisms involved in the antinociceptive action of NSAIDs at central and peripheral sites. Pharmacol. Ther. 107, 139–154.

McGeer, P.L., Itagaki, S., Boyes, B.E., McGeer, E.G., 1988. Reactive microglia are positive for HLA-DR in the substantia nigra of Parkinson's and Alzheimer's disease brains. Neurology 38, 1285–1291.

McGeer, P.L., Kawamata, T., Walker, D.G., Akiyama, H., Tooyama, I., McGeer, E.G., 1993. Microglia in degenerative neurological disease. Glia 7, 84–92.

Minghetti, L., Levi, G., 1998. Microglia as effector cells in brain damage and repair: focus on prostanoids and nitric oxide. Prog. Neurobiol. 54, 99–125.

Minghetti, L., Walsh, D.T., Levi, G., Perry, V.H., 1999. In vivo expression of cyclooxygenase-2 in rat brain following intraparenchymal injection of bacterial endotoxin and inflammatory cytokines. J. Neuropathol. Exp. Neurol. 58, 1184–1191.

Murphy, G.M., Yang, L., Cordell, B., 1998. Macrophage colony-stimulating factor augments beta-amyloid-induced interleukin-1, interleukin-6, and nitric oxide production by microglial cells. J. Biol. Chem. 273, 20967–20971.

Nakamura, Y., Si, Q.S., Kataoka, K., 1999. Lipopolysaccharide-induced microglial activation in culture: temporal profiles of morphological change and release of cytokines and nitric oxide. Neurosci. Res. 35, 95–100.

Nelson, P.T., Soma, L.A., Lavi, E., 2002. Microglia in diseases of the central nervous system. Ann. Med. 34, 491–500.

Nomura, Y., 2001. NF-kappaB activation and IkappaB alpha dynamism involved in iNOS and chemokine induction in astroglial cells. Life Sci. 68, 1695–1701.

Ozes, O.N., Mayo, L.D., Gustin, J.A., Pfeffer, S.R., Pfeffer, L.M., Donner, D.B., 1999. NF-kappaB activation by tumour necrosis factor requires the Akt serinethreonine kinase. Nature 401, 82–85.

Takada, Y., Aggarwal, B.B., 2004. Flavopiridol inhibits NF-κB activation induced by various carcinogens and inflammatory agents through inhibition of IκBα kinase and p65 phosphorylation: abrogation of cyclin D1, cyclooxygenase-2, and matrix metalloprotease-9. J. Biol. Chem. 279, 4750–4759.

Takeda, Y., Shingu, T., Ichihara, T., Fujita, T., Yokoi, T., Kida, K., Ueno, A., 1988. Revised structures of inflexin and related diterpenoids. Chem. Pharm. Bull. 36, 4576–4580.

Teismann, P., Vila, M., Choi, D.K., Tieu, K., Wu, D.C., Jackson-Lewis, V., Przedborski, S., 2003. COX-2 and neurodegeneration in Parkinson's disease. Ann. N. Y. Acad. Sci. 991, 272–277.

Wang, M.J., Lin, W.W., Chen, H.L., Chang, Y.H., Ou, H.C., Kuo, J.S., 2002. Silymarin protects dopaminergic neurons against lipopolysaccharideinduced neurotoxicity by inhibiting microglia activation. Eur. J. NeuroSci. 16, 2103–2112.

Wyss-Coray, T., L., Mucke, 2002. Inflammation in neurodegenerative disease — a double-edged sword. Neuron 35, 419–432.

Xiong, S., She, H., Takeuchi, H., Han, B., Engelhardt, J.F., Barton, C.H., Zandi, E., Giulivi, C., Tsukamoto, H., 2003. Signaling role of intracellular iron in NF-kappaB activation. J. Biol. Chem. 278, 17646–17654.

Xu, X., Malave, A., 2000. P38 MAPK, but not p42/p44 MAPK mediated inducible nitric oxide synthase expression in C6 glioma cells. Life Sci. 67, 3221–3230.

Yook, C.S., 1981. Flora of Korea Pharmacal Botany. Academy Publishing Co., Seoul. pp 468.

Zimmermann, S., Moelling, K., 1999. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science 286, 1741–1744.