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Betulinic Acid Inhibits LPS-Induced MMP-9 Expression by Suppressing NF-kB Activation in BV2 Microglial Cells

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Abstract

Aberrant activation of microglia has been reported to cause neuronal damages by releasing a variety of pro-inflammatory cytokines. Besides where microglia become active, damages have been also observed in remote places, which is considered due to the migration of activated microglia. Therefore, an agent that could suppress abnormal activation of microglia and their subsequent migration might be valuable in activated microglia-related brain pathologies. The objective of the present study was to evaluate anti-inflammatory effects of betulinic acid on lipopolysaccharide (LPS)-stimulated BV2 microglial cells. Pretreatment of betulinic acid significantly attenuated LPS-induced NO production and protein expression of iNOS. Betulinic acid also significantly suppressed LPS-induced release and expression of cytokines such as TNF-α and IL-1β. Furthermore, betulinic acid significantly suppressed LPS-induced MMP-9 expression, which has been suggested to play an important role in the migration of activated microglia. In order to understand the possible mechanism by which betulinic acid suppresses LPS-induced cytokine production and migration of microglia, the role of NF-kB, a major pro-inflammatory transcription factor, was examined. Betulinic acid significantly suppressed LPS-induced degradation of IKB, which retains NF-kB in the cytoplasm. Therefore, nuclear translocation of NF-kB upon LPS stimulation was significantly suppressed with betulinic acid. Taken together, the present study for the first time demonstrates that betulinic acid possesses anti-inflammatory activity through the suppression of nuclear translocation of NF-kB in BV2 microglial cells.

Key Words: Betulinic acid, MMP-9, BV2 microglial cells, Lipopolysaccharide, Cytokines, NF-kB

INTRODUCTION

Microglia, resident macrophages and immune surveillance cells in the central nervous system, have been reported to play a critical role in host defense and tissue repair in brain (Perry and Gordon, 1988). It has been demonstrated that microglia present in remote areas migrate into the injured sites and clear the cell debris via the process called phagocytosis (Kato and Walz, 2000). However, excessive activation of microglia has also been proposed to play a pathogenic role in neuroinflammatory conditions such as ischemia (Hailer, 2008), multiple sclerosis (Matsumoto, 1992), Parkinson's disease (McGeer and McGeer, 1998), Alzheimer's disease (Itagaki *et al.*, 1989) and HIV-associated dementia (Merrill and Chen, 1991). Under these immunologically mediated CNS diseases, activated microglia have been suggested to contribute to neurodegenerative process by producing excessive amounts of biologically active molecules such as nitric oxide (NO) and several cytokines (Chao, 1992; Merrill and Benveniste, 1996; Tuttolomondo *et al.*, 2008; Graber and Streit, 2010).

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that are involved in the remodeling of the extracellular matrix (ECM) in numerous physiological and pathological processes (Yong *et al.*, 1998; Cuzner and Opdenakker, 1999). MMPs have been reported to play a key role in the microglial migration through the regulation of cellular adhesion, movement, and chemotaxis (Zuo *et al.*, 1998). Furthermore, it has been reported that MMPs could regulate chemotatic properties of chemokines by cleaving or inactivating chemokines (McQuibban *et al.*, 2000) and facilitate the release of chemokines from proteins or other molecules (Li *et al.*, 2002). Especially, increased activity of MMP-9 has been

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reported to significantly contribute to the microglial migration (Choi *et al.*, 2010).

Betulinic acid is naturally occurring pentacyclic triterpene that is widely distributed in the plant kingdom and it has been reported that considerable amount of betulinic acid is available in the outer bark of many tree species such as white-barked birch trees (Yogeeswari and Sriram, 2005). Although betulinic acid has been reported to possess a variety of biological activities including anti-malarial, anti-inflammatory, anthelminthic, anti-tumor, and anti-retrovial properties (Yogeeswari and Sriram, 2005; Fulda, 2008), the exact mechanism for its biological activities has not been clearly examined.

In the present study, in order to bring forth a novel pharmacological agent that could suppress abnormally activated microglia for the treatment of inflammation-related CNS diseases, the anti-inflammatory effects of betulinic acid and its underlying mechanism were examined in LPS-stimulated BV2 microglial cells.

MATERIALS AND METHODS

Reagents and cell culture

Bacterial lipopolysaccharide (LPS, E. coli serotype 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Betulinic acid (Fig. 1) was isolated and identified from *Forsythia koreana*. Briefly, dried *Forsythia koreana* was extracted with methanol and then partitioned with n-hexane, dichlormethane, ethyl acetate, and butanol, successively. Betulinic acid was isolated from n-hexane fraction using silica gel column chromatography. The chemical structure of the compound was determined by means of ^{1}H-NMR (600 MHz) and EI-MS and identified to betulinc acid by comparison with spectral data from the literature (Patra *et al.*, 1988; Do *et al.*, 1991). The compound was dissolved in ethanol and added to the cell culture at the desired concentrations. The BV2 microglia cell line was maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco BRL) and 50 μg/ml gentamicin (Sigma, St. Louis, MO, USA). Cells were maintained in a humidified incubator at 37°C with 5% CO_2. In all experiments, cells were incubated in the presence of the indicated concentration of betulinic acid before the addition of LPS (200 ng/ml).

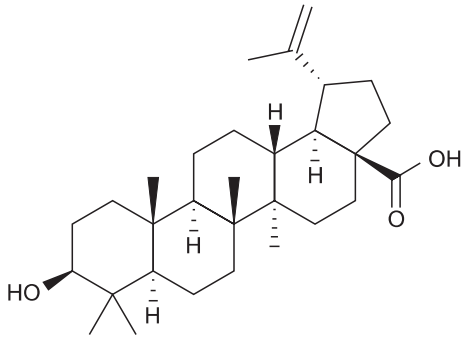

Fig. 1. Chemical structure of betulinic acid.

Cell viability

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The BV2 microglial cells were plated into 96-well plates at a density of 5×10^4 cells per well for 24 hr. BV2 microglial cells incubated with various concentrations of BA for 24 hr. MTT (0.5 mg/ml in PBS) was added to each well, and the cells were incubated for 3 hr at 37°C. The resulting formazan crystals were dissolved in dimethyl sulfoxied (DMSO). The optical density was measured at 540 nm. The results were expressed as a percentage of surviving cells over control cells.

Nitrite quantification assay

The production of NO was estimated by measuring the amount of nitrite, a major stable metabolite of NO, using the Griess reagent (0.1% naphthylethylenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid). BA-pretreated BV2 microglial cells were stimulated with LPS in 12-well plates for 24 hr, and then 100 μl of each culture medium was mixed with an equal volume of Griess reagent. The absorbance at 540 nm was measured on a microplate reader. The results were expressed as percentages of released NO from LPS-stimulated BV2 cells. To prepare a standard curve, sodium nitrite was diluted in culture medium to concentrations.

TNF-α and IL-1β ELISA

The BV2 microglia cells were treated with BA in the absence or presence of LPS. After 24 hr incubation, TNF-α and IL-1β levels in culture media were quantified using monoclonal anti-IL-1β or anti-TNF-α antibody according to the manufacturer's instruction (R &D Systems).

Western blot analysis

The BV2 microglial cells were washed with PBS two times and lysed with lysis buffer [50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% sodium dodecyl sulfate (SDS), 1% NP-40, 0.5% sodium deoxycholate, 1 mM phenyl methylsulfonyl fluoride]. Equal amounts of protein were separated on 10% SDS-polyacrylamide gel. Proteins were transferred to Hypond PVDF membrane (Amersham Biosciences, Piscataway, NJ, USA). The membranes were blocked in 5% skim milk in TBST for 1 hr at room temperature and sequentially incubated with an appropriate antibody; iNOS (BD Bioscience, Franklin Lakes, NJ), IkB-α (Santa Cruz Biotechnology Inc., Eugene, OR, USA), MMP-9 (Affinity BioReagents, Inc., Golden, CO, USA) or β-actin (Sigma, St. Louis, MO, USA). After thoroughly washing with TBST, the membranes were then washed three times with TBST and incubated with HRP-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) or HRP-conjugated goat anti-mouse IgG (Jackson ImmunoResearch) for 2 hr at room temperature. The blots were developed by the enhanced chemiluminescence detection kit (ECL Amersham Biosciences, Piscataway, NJ, USA).

Immunocytochemistry

The effect of betulinic acid on the nuclear translocation of p65 subunit of NF-kB was examined by immunofluorescence assay using confocal microscopy. BV2 microglial cells were cultured in sterile coverslips and pretreated with betulinic acid for 1 hr and stimulated with LPS. At 30 min after the LPS treatment, the cells were fixed in 4% paraformaldehyde for 20 min at room temperature. The fixed cells were then permeabilized

with 0.1% Triton X-100 in PBS and blocked with 3% BSA. Afterwards, the cells were sequentially incubated with rabbit p65 antibody (Santa Cruz Biotechnology Inc., Eugene, OR, USA) at room temperature and FITC-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) at room temperature for 1 hr. After washing with PBS, the sample were mounted and observed by means of confocal microscopy.

RNA isolation and RT-PCR

Total RNA was isolated using the Total RNA Extraction Kit (iNtRON Biotechnology, Inc, USA) according to the manufacturer's instruction. Total RNA (2 μg) was obtained from cells was reverse-transcribed using oligo-(dT) 15 primers (Promega, Madison, WI, USA). The following PCR conditions were applied: β-actin, 24 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extended at 72°C for 30 sec; MMP-9, 25 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extended at 72°C for 30 sec. The β-actin was used as an internal control to evaluate relative expression of MMP-9. PCR primers were as follow: MMP-9, 5'-GAG CTG TGC GTC TTC CCC TTC -3' (forward) and 5'-GGA ATG ATC TAA GCC CAG TGC -3' (reverse) and β-actin, 5'- ATC CTG AAA GAC CTC TAT GC-3' (forward) and 5'- AAC GCA GCT CAG TAA CAG TC-3' (reverse). After amplification, PCR reactions were electrophoresed in an agarose gel.

Statistical analysis

All values shown in the figures are expressed as the mean ± SD obtained from at least three independent experiments. Statistical analysis was carried out by one-way analysis of variance (ANOVA) with Tukey's post-hoc test using SPSS software 12K (SPSS, Chicago, IL, USA). A value of $p<0.05$ was considered as statistically significant.

RESULTS

Betulinic acid inhibits NO production and iNOS expression in LPS-stimulated BV2 cells

The effects of betulinic acid on NO production and iNOS expression were examined in LPS-stimulated BV2 microglial cells. Although higher concentrations of betulinic acid over 25 μM appeared to be cytotoxic, no significant cell death was observed in the concentration range of betulinic acid used in the present study (Fig. 2A). Treatment of LPS resulted in the excessive production of NO and up-regulation of iNOS in protein levels (Fig. 2B, C). Pretreatment of betulinic acid significantly

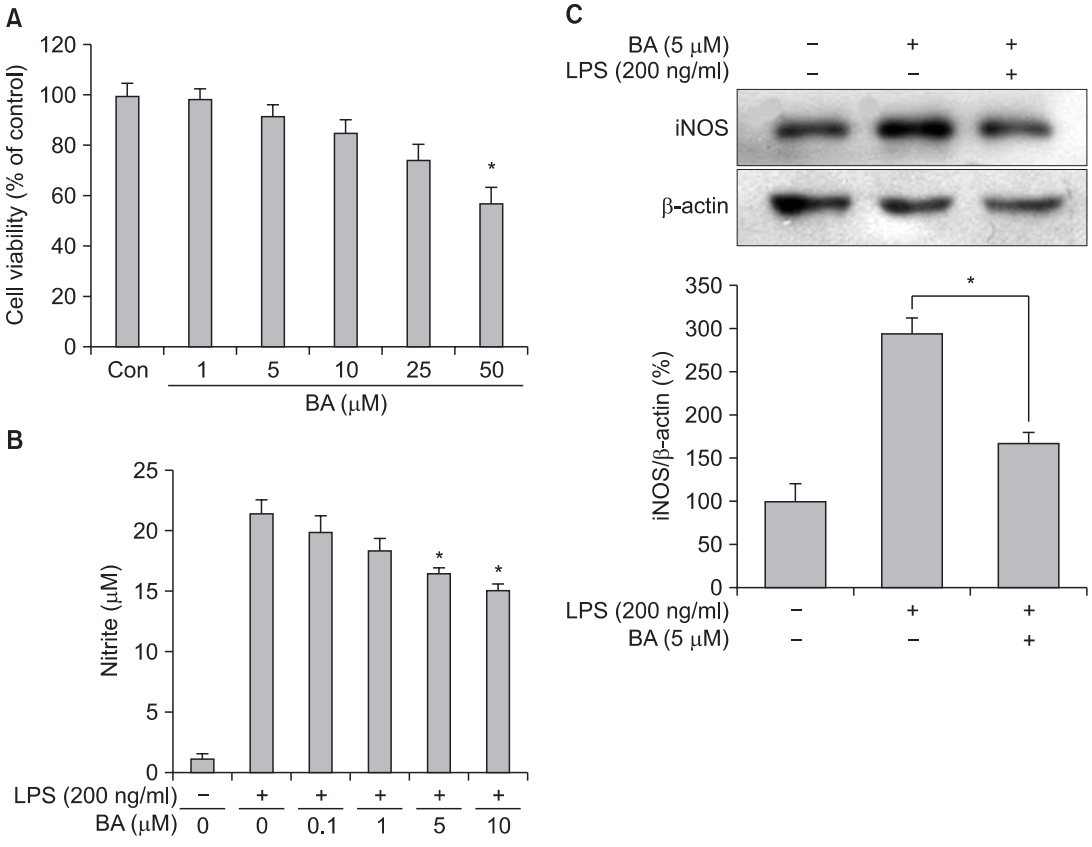

Fig. 2. Effects of betulinic acid on NO production and iNOS expression in LPS-stimulated BV2 microglial cells. (A) Effect of betulinic acid on the viability of BV2 microglial cells. No significant cell death was observed up to 10 μM. (B) Concentration-dependent suppression of LPS-induced NO production by betulinic acid. (C) Suppression of LPS-induced iNOS protein expression by betulinic acid: top, representative immunoblot of iNOS. β-Actin was used as an internal control; bottom, quantitative analysis of immunoblots; Quantitative data represent three independent experiments and are expressed as mean ± SD. *$p<0.05$ indicate statistically significant difference with LPS alone. BA stands for betulinic acid.

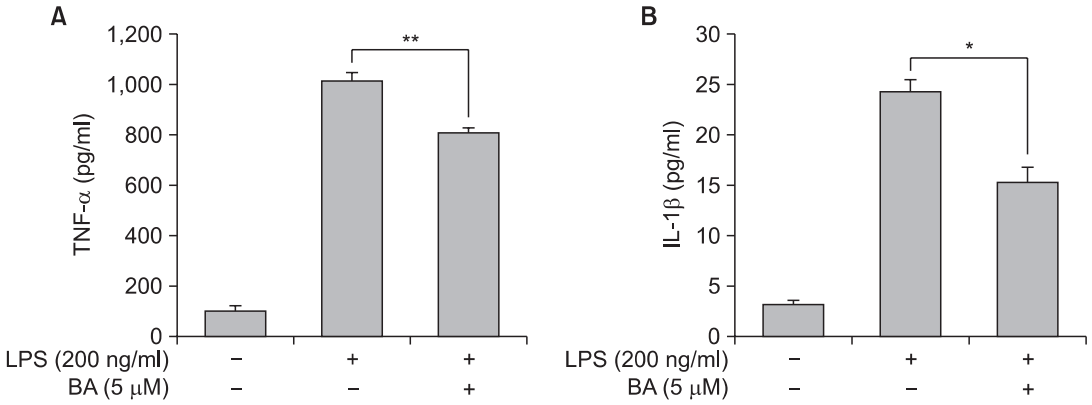

Fig. 3. Inhibitory effects of betulinic acid on the LPS-induced release of TNF-α (A) and IL-1β (B) in BV2 microglial cells. BV2 microglia cells were incubated with 200 ng/ml of LPS in the presence or absence of indicated concentrations of betulinic acid for 24 hr. Cell culture media were collected and subjected to TNF-α and IL-1β sandwich ELISA. Data represent three independent experiments in triplicate and are expressed as mean ± SD. **p*<0.05 and ***p*<0.01 indicate statistically significant difference with LPS alone. BA stands for betulinic acid.

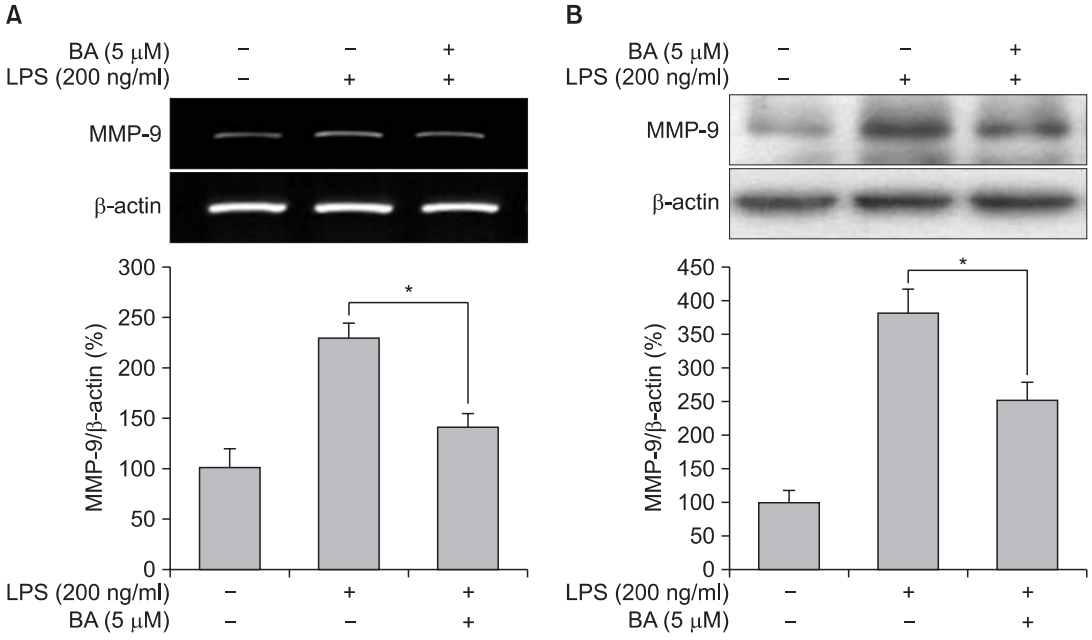

Fig. 4. Effect of betulinic acid on MMP-9 expression in LPS-stimulated BV2 microglia cells. (A) BV2 cells were treated 200 ng/ml LPS in the absence or presence of betulinic acid, and total RNA was isolated at 6 h after LPS treatment. The mRNA expression of MMP-9 was measured by RT-PCR. (B) Western blots was performed using the cell lysates of BV2 cells with LPS in the presence of betulinic acid for 24 hr. Quantitative data represent three independent experiments and are expressed as mean ± SD. **p*<0.05 and ***p*<0.01 indicate statistically significant difference with LPS alone. BA stands for betulinic acid.

suppressed the LPS-induced NO production in a concentration-dependent manner (Fig. 2B). In accordance with the suppression of NO production, betulinic acid also significantly attenuated LPS-induced up-regulation of iNOS expression in protein levels (Fig. 2C). These results demonstrate that betulinic acid exhibits inhibitory effects on LPS-induced iNOS expression and activity in BV2 microglial cells.

Betulinic acid attenuates the expression of pro-inflammatory cytokines in LPS-stimulated BV2 cells

To determine the effect of betulinic acid on the expression of pro-inflammatory cytokines such as TNF-α and IL-1β, extracellular release of these cytokines was examined using ELISA assay. Betulinic acid significantly suppressed LPS-induced extracellular release of TNF-α and IL-1β in BV2 microglial cells (Fig. 3). In accordance with the attenuation of extracellular cytokine release, betulinic acid significantly suppressed LPS-induced up-regulation of these cytokine genes (data not shown).

Betulinic acid suppresses LPS-induced MMP-9 gene expression

Based on the previous report that MMP-9 might play a significant role in the migration of activated microglia to remote

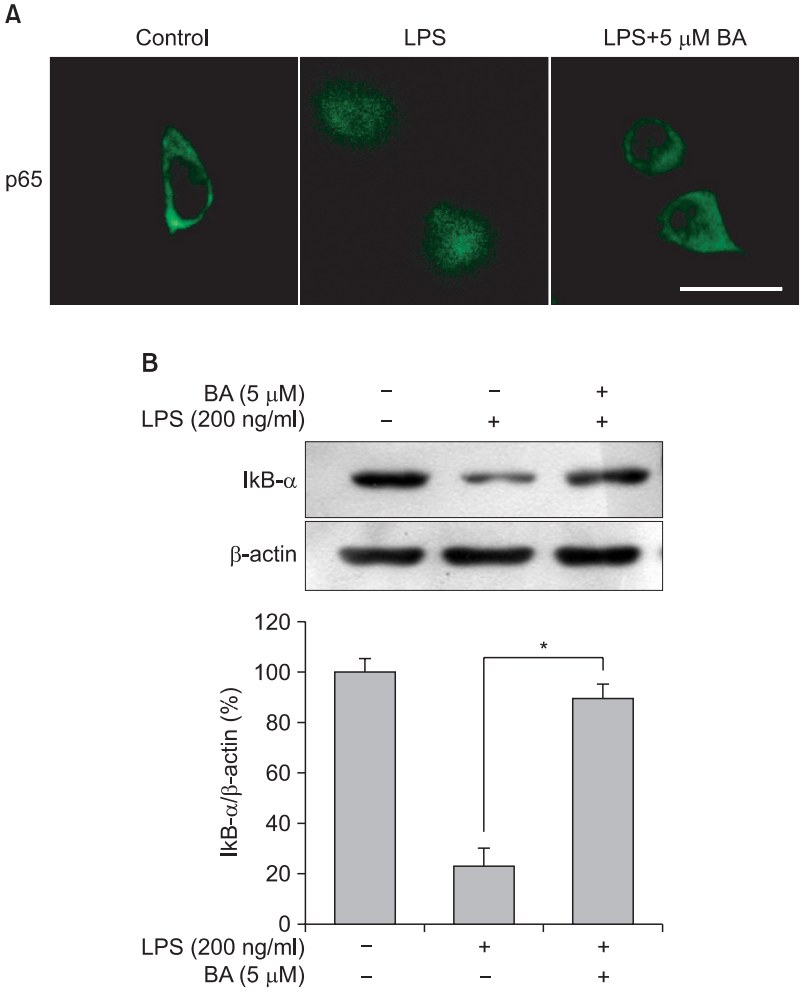

Fig. 5. Blockade of nuclear translocation of p65 subunit of NF-kB in LPS-stimulated BV2 microglial cells by betulinic acid. (A) Localization of NF-kB p65 subunit was determined using a p65 antibody and an FITC-conjugated donkey anti-rabbit IgG antibody. In basal condition, immunostaining of p65 subunit was diffuse throughout the cytoplasm. However, LPS stimulation resulted in the apparent translocation of p65 subunit into the nucleus. Pretreatment of betulinic acid appeared to attenuate LPS-induced nuclear translocation of p65 subunit. Scale bar, 20 μm. (B) Total cell lysates obtained 15 min after the LPS stimulation were subjected to Western blotting to assess the levels of IkB-α proteins (top). Quantification of IkB-α degradation was performed by densitometric analysis (bottom). β-Actin was used as an internal control. Quantitative data represent three independent experiments and are expressed as mean ± SD. *p<0.05 indicate statistically significant difference with LPS alone. BA stands for betulinic acid.

places (Choi *et al.*, 2010), the expression of MMP-9 gene and its extracellular cellular release were examined in the present study. The mRNA expression of MMP-9 was significantly increased with LPS stimulation (Fig. 4A). However, pretreatment of betulinic acid significantly attenuated LPS-stimulated gene expression of MMP-9 in BV2 microglial cells (Fig. 4A). Furthermore, betulinic acid significantly attenuated LPS-stimulated gene expression of MMP-9 as shown in a representative image of western blotting (Fig. 4B). These results strongly suggest that the primary effect of betulinic acid on MMP-9 might be through the suppression of gene expression rather than the inhibition of extracellular release of MMP-9.

Betulinic acid suppresses LPS-induced degradation of IKB and nuclear translocation of NF-kB

The transcription factor NF-kB has been extensively reported to play a predominant role in the expression of numerous inflammatory cytokine genes and IKB has been demonstrated to inhibit the nuclear translocation of NF-kB by retaining it in the cytoplasm (Ghosh *et al.*, 1998; Bonizzi and Karin, 2004). Therefore, in the present study, effects of betulinic acid on LPS-induced nuclear translocation of NF-kB and degradation of cytosolic IKB were examined. To determine the nuclear translocation of NF-kB, the level of nuclear NF-kB was examined using confocal microscopy. Immunostaining of RelA (p65) subunit of NF-kB was present predominantly in the cytoplasm in basal condition (Fig. 5A). LPS stimulation resulted in apparent nuclear translocation of p65 subunit of NF-kB in BV2 microglial cells. However, pretreatment of betulinic acid appeared to attenuate the LPS-induced nuclear translocation of p65 subunit of NF-kB (Fig. 5A). To examine the effect of LPS-induced microglial activation on intracellular level of IKB, intracellular level of IKB was examined upon LPS stimulation using immunobloting assay. LPS challenge resulted in a significantly decreased intracellular level of IKB in BV2 microglial cells (Fig. 5B). However, pretreatment of betulinic acid showed a significant attenuation of LPS-induced degradation of IKB (Fig. 5B).

DISCUSSION

The present study demonstrated that betulinic acid, isolated from *Forsythia koreana*, possesses an anti-inflammatory activity in LPS-stimulated microglia and a suppressive effect on LPS-induced microglial migration, presumably through the suppression of nuclear translocation of transcription factor NF-kB.

Betulinic acid, a pentacyclic terterpene, and its derivatives have been reported to possess a variety of biological properties (Yogeeswari and Sriram, 2005) including inhibition

of human immunodeficiency virus (HIV) (Soler *et al.*, 1996), anti-bacterial, anti-cancer (Chintharlapalli *et al.*, 2007; Fulda, 2008), and anti-inflammatory activities (Recio *et al.*, 1995). Although anti-HIV and anti-cancer activities of betulinic acid and its derivatives have been reported to be through the inhibition of HIV entry, HIV-proteases or reverse transcriptase (Fujioka *et al.*, 1994; Yogeeswari and Sriram, 2005), and through increased degradation of the transcription factors specificity protein 1 (Sp1), Sp3, and Sp4 (Chintharlapalli *et al.*, 2007), respectively, the underlying mechanism of anti-inflammatory property of betulinic acid has not been clearly demonstrated (Recio *et al.*, 1995). The present results evidently demonstrate that betulinic acid significantly suppresses the activity of transcription factor NF-kB, which has been reported to play a key role in the inflammatory responses, through the inhibition of LPS-induced IKB degradation in the cytoplasm (Lee *et al.*, 2011). In accordance with present study, it has been recently reported that betulinic acid exhibits a protective effect in ischemia-induced brain injury by suppressing iNOS expression and oxidative stress (Lu *et al.*, 2011).

Aberrant activation of microglia has been reported to be involved in several pathologic conditions by releasing pro-inflammatory cytokines and oxidants such as TNF-α, interleukin (IL)-6, IL-lβ, IL-6, IL-10, IL-12, interferon-γ, and NO (Kreutzberg, 1996; Graber and Streit, 2010). Accordingly, suppression of microglial activation and subsequent release of cytokines have been suggested to protect neuronal damage in several inflammation-related CNS diseases (Aquilano *et al.*, 2008; Hailer, 2008; Hashioka *et al.*, 2009; Ray and Lahiri, 2009). In the present study, betulinic acid exerts anti-inflammatory property through the significant suppression on LPS-induced expression and activity of iNOS, and the release of TNF-α and IL-1β.

It has been previously reported that MMP-9 plays an important role in the regulation of chemotaxic migration of leukocyte (Sellebjerg and Sorensen, 2003) as well as monocyte (Kappert *et al.*, 2008). Recently, it has been reported that increased expression and activity of MMP-9 are involved in microglial activation and that the inhibition of MMP-9 suppresses LPS-induced expression of pro-inflammatory cytokines in microglia (Woo *et al.*, 2008). In the present study, betulinic acid significantly attenuated the LPS-induced MMP-9 expression.

Aberrant activation of a pro-inflammatory transcription factor NF-kB has been associated with various inflammatory conditions such as autoimmune diseases (Karin *et al.*, 2001; Li and Verma, 2002). NF-kB-dependent microglial activation has been reported to be a crucial contributor to ischemia (Cho *et al.*, 2008). In the present study, betulinic acid significantly attenuated LPS-induced nuclear translocation of NF-kB and subsequent extracellular releases of pro-inflammatory cytokines and MMP-9, through the inhibition of LPS-induced IKB degradation.

In conclusion, the present results strongly demonstrate that betulinic acid exerts the anti-inflammatory activity by suppressing the production of pro-inflammatory mediators and MMP-9 through the inhibition of the nuclear translocation of NF-kB in LPS-stimulated BV2 microglial cells. The present study suggests that betulinic acid might be a valuable therapeutic agent for the treatment of inflammatory diseases in CNS caused by activated microglia.

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