British Journal of Pharmacology (2006) 148, 216–225
www.nature.com/bjp

Anti-inflammatory effect of buddlejasaponin IV through the inhibition of iNOS and COX-2 expression in RAW 264.7 macrophages *via* the NF-κB inactivation

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1 Buddlejasaponin IV isolated from *Pleurospermum kamtschatidum* is an anti-inflammatory compound that inhibits NO, PGE_2 and TNF-α production. Here, we studied the mode of action of this compound.

2 Buddlejasaponin IV (2.5–10 μM) reduced lipopolysaccaride (LPS (1 μg ml^{-1}))-induced levels of iNOS and COX-2 at the protein levels, and iNOS, COX-2, TNF-α, interleukin (IL)-1β and IL-6 mRNA expression in RAW 264.7 macrophages in a concentration-dependent manner, as determined by Western blotting and RT–PCR, respectively.

3 Buddlejasaponin IV inhibited the LPS-induced activation of nuclear factor-κB (NF-κB), a transcription factor necessary for proinflammatory mediators, iNOS, COX-2, TNF-α, IL-1β and IL-6 expression. This effect was accompanied by a parallel reduction in IκB-α degradation and phosphorylation, and by the nuclear translocation of the NF-κB p65 subunit.

4 The effects of buddlejasaponin IV on acute phase inflammation were studied on serotonin- and carrageenan–induced paw edema. The antiedematous effect of buddlejasaponin IV was compared with 10 mg kg^{-1} of indomethacin p.o. Maximum inhibitions of 26 and 41% were noted at a dose of 20 mg kg^{-1} for serotonin- and carrageenan-induced paw edema, respectively.

5 The analgesic effect of buddlejasaponin IV was evaluated using acetic acid-induced writhing and hot-plate tests. Buddlejasaponin IV (10 and 20 mg kg^{-1}, p.o.) was found to have a marked analgesic effect in both models.

6 These results suggest that the inhibitions of the expressions of iNOS, COX-2, TNF-α, IL-1β and IL-6 by blocking NF-κB activation, are responsible for the anti-inflammatory effects of buddlejasaponin IV isolated from *P. kamtschatidum*.
British Journal of Pharmacology (2006) **148,** 216–225. doi:10.1038/sj.bjp.0706718;
published online 6 March 2006

Keywords: Buddlejasaponin IV; *Pleurospermum kamtschatidum*; inducible nitric oxide synthase; cyclooxygenase-2; IL-1β; IL-6; NF-κB

Abbreviations: EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; IL, interleukin; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; MTT, 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; PGE_2, prostaglandin E_2; TNF, tumor necrosis factor

Introduction

Nuclear transcription factor kappa-B (NF-κB) is one of the most ubiquitous transcription factors and regulates the genes involved in cellular proliferation, inflammatory responses, and cell adhesion. Functionally active NF-κB exists mainly as a heterodimer comprised of subunits of the Rel family p50 and p65, which is normally sequestered in the cytosol as an inactive complex due to its binding with inhibitors of κB (IκBs) in unstimulated cells (Baeuerle, 1998). The activation of NF-κB involves the phosphorylation of IκBs at two critical serine residues (Ser32, Ser36) *via* the IκB kinase (IKK) signalosome complex (Brown *et al*., 1995; O'Conell *et al*., 1996). Once IκBs have been phosphorylated, they are ubiquitinated and degraded by 26S proteosome (DiDonato *et al*., 1996; Sanchez-Perez *et al*., 2002). The resulting free NF-κB is then translocated to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes, and induces the transcription of proinflammatory mediators, for example, iNOS, cyclooxygenase-2 (COX-2), TNF-α and interleukin (IL)-1β, -6 and -8 (Baeuerle & Baltimore, 1996; Surh *et al*., 2001; Lappas *et al*., 2002).

During the inflammatory processes, large amounts of proinflammatory mediators, nitric oxide (NO), and prostaglandin E_2 (PGE_2) are generated by the inducible isoforms of NO synthase (iNOS) and COX-2 (Vane *et al*., 1994). In mammalian cells, NO is synthesized by three different isoforms of NO synthase (NOS), namely, neuronal

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NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). Although nNOS and eNOS are constitutively expressed, iNOS is expressed in response to interferon-γ (IFN-γ), lipopolysccharide (LPS) and a variety of proinflammatory cytokines (Moncada *et al*., 1991; Yun *et al*., 1996). COX converts arachidonic acid to PGs, and like NOS, COX exists in two isoforms, that is, COX-1 and COX-2 (Funk *et al*., 1991). COX-1 is expressed constitutively in most tissues and appears to be responsible for maintaining normal physiological functions. In contrast, COX-2 is only detectable in certain types of tissues and is induced transiently by growth factors, proinflammatory cytokines, tumor promoters and bacterial toxins (Prescott & Fitzpatrick, 2000; Hinz & Brune, 2002). Moreover, elevated levels of COX have been detected in different tumor types, and this may account for the excessive production of inflammatory PGs (Subbaranaish *et al*., 2003).

The biological functions of NF-κB are involved in many proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8 and IFN–β. TNF-α plays a key role in the induction and perpetuation of inflammation due to autoimmune reactions by activating T cells and macrophages and by upregulating other proinflammatory cytokines and endothelial adhesion molecules such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, which enhance the recruitment of leukocytes to the site of inflammation (Beutler & Cerami, 1989). Likewise, IL-1β is one of the most important inflammatory cytokines secreted by macrophages, and is induced by LPS in macrophages. During inflammation, increases in the release of IL-1β lead to cell or tissue damage (Molloy *et al*., 1993; West *et al*., 1994; 1995), and thus, reduction in IL-1β release from macrophages may retard inflammatory responses to LPS stimulation. Additionally, the production of IL-6 is induced by several factors, TNF-α, IL-1β as well as the bacterial endotoxin, LPS. IL-6, a proinflammatory cytokine, acts as an endogenous pyrogen in addition to its multiple effects on the immune system and in particular on hematopoiesis (Van Snick, 1990). These cytokines are regulated by the signal transduction pathway of IκB/NF-κB activation (Nolan *et al*., 1991; Dendorfer, 1996; Kellum *et al*., 2004).

During our on-going screening program designed to identify the anti-inflammatory potentials of natural compounds, we previously isolated buddlejasaponin IV from the aerial portion of *Pleurospermum kamtschaticum* by activity-directed fractionation and characterized its structural identity spectroscopically (^{1}H and ^{13}NMR, IR, MS), as described previously (Jung *et al*., 2005). *P. kamtschaticum* Hoffmann (Umbelliferae) is a perennial herb and is found in Kangwon province Korea (Koh & Jeon, 2003). The aerial portion of *P. kamtschaticum* has been used to treat colds, arthritis, atherosclerosis and impotence (Kim, 1996), however, no report has been issued on its anti-inflammatory activity or mode of action, although we previously reported that buddlejasaponin IV significantly inhibits NO, PGE_2 and TNF-α production in LPS-activated RAW 264.7 macrophage cells (Jung *et al*., 2005). Therefore, as a prelude to reveal the underlying mechanisms for the anti-inflammatory effects of buddlejasaponin IV, we evaluated variations in inflammatory proteins, mRNAs, and cytokine expressions *in vitro*, and then evaluated its *in vivo* anti-inflammatory and antinociceptive effects.

Methods

Materials

Dulbecco's-modified Eagle's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin and streptomycin were obtained from Life Technologies Inc. (Grand Island, NY, U.S.A.). iNOS, COX-1, COX-2, IκB-α, p-IκB-α, p65, PARP, β-actin monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Enzyme immunoassay (EIA) kit for IL-1β and IL-6 was obtained from R&D Systems (Minneapolis, MN, U.S.A.), and luciferase assay kit was purchased from Promega (Madison, CA. U.S.A.). pNF-κB-Luc reporter plasmid was purchased from BD Biosciences (San Jose, CA, U.S.A.). Superfect transfection reagent from Qiagen (Qiagen GmbH, Germany); and RNA extraction kits from Intron Biotechnology (Seoul, Korea). iNOS, COX-1, COX-2, TNF-α, IL-1β, IL-6 and β-actin oligonucleotide primers were from Bioneer (Seoul, Korea). 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), aprotinin, leupeptin, phenylmethylsulfonyl-l-fluoride (PMSF), dithiothreitol, *Escherichia coli* LPS, acetylsalicylic acid (aspirin), carrageenan, serotonin, indomethacin and all other chemicals were from Sigma (St Louis, MO, U.S.A.).

Cell culture and sample treatment

The RAW 264.7 murine macrophage cell line was obtained from Korean Cell Line Bank (Seoul, Korea). Cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 U ml^{-1}), and streptomycin sulfate (100 μg ml^{-1}) in a humidified 5% CO_2 atmosphere. Cells were incubated with the test compounds at various concentrations and stimulated with LPS 1 μg ml^{-1} for various times.

Western blot analysis

Cellular proteins were extracted from control and buddlejasaponin IV-treated RAW 264.7 cells. Washed cell pellets were resuspended in extraction lysis buffer (50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 5 mM Na fluoride (NaF), 0.5 mM Na orthovanadate) containing 5 μg ml^{-1} of each of leupeptin and aprotinin, and then incubated for 30 min at 4°C. Cell debris was removed by microcentrifugation, and supernatants were quick frozen. The protein concentration was determined using the Bio-Rad protein assay reagent according to the manufacture's instruction. Of cellular protein, 40 μg from treated and untreated cell extracts were electroblotted onto a nitrocellulose membrane following separation on a 10% SDS–polyacrylamide gel electrophoresis. The immunoblot was incubated overnight with blocking solution (5% skim milk) at 4°C, and then incubated for 4 h with a 1 : 1000 dilution of monoclonal anti-iNOS, a 1 : 1000 dilution of anti-COX-1, COX-2 antibody, a 1 : 1000 dilution of anti-IκB-α, p-IκB-α, β-actin antibody, and a 1 : 1000 dilution of anti-p65, PARP antibody (Santa Cruz Biotechnology Inc.). Blots were washed twice with

Tween 20/Tris-buffered saline (TTBS) and then incubated with a 1 : 1000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc.) for 1 h at room temperature. Blots were again washed three times with TTBS and then developed by enhanced chemiluminescence (Amersham Life Science, Arlington Heights, IL, U.S.A.).

RNA preparation and RT–PCR

Total cellular RNA was isolated using Easy Blue® kits (Intron Biotechnology, Seoul, Korea), according to the manufacturer's instructions. RNA (1 μg) was reverse-transcribed (RT) from each sample using MuLV reverse transcriptase, 1 mM dNTP, and oligo (dT_{12-18}) 0.5 $\mu g\,\mu l^{-1}$. PCR analyses were performed on aliquots of the cDNA preparations to detect iNOS, COX-2, TNF-α, IL-1β and IL-6 (using β-actin as an internal standard) gene expression using a thermal cycler (Perkin Elmer Cetus, Foster City, CA, U.S.A.). Reactions were carried out in a volume of 25 μl containing (final concentrations) 1 U of Taq DNA polymerase, 0.2 mM dNTP, $\times 10$ reaction buffer, and 100 pmol of 5′ and 3′ primers. After an initial denaturation for 2 min at 95°C, 30 amplification cycles were performed for iNOS (1 min of 95°C denaturation, 1 min of 60°C annealing and 1.5 min 72°C extension), COX-1 (1 min of 94°C denaturation, 1 min of 55°C annealing, and 1 min 72°C extension), COX-2 (1 min of 94°C denaturation, 1 min of 60°C annealing, and 1 min 72°C extension), TNF-α (1 min of 95°C denaturation, 1 min of 55°C annealing and 1 min 72°C extension), IL-1β (1 min of 94°C denaturation, 1 min of 60°C annealing and 1 min 72°C extension) and IL-6 (1 min of 94°C denaturation, 1 min of 57°C annealing and 1 min 72°C extension). PCR primers used in this study are listed below and were purchased from Bioneer (Seoul, Korea):

sense strand iNOS, 5′-AATGGCAACATCAGGTCGGCCA TCACT-3′;
antisense strand iNOS, 5′-GCTGTGTGTCACAGAAGTCTC GAACTC-3′;
sense strand COX-1, 5′-AGTGCGGTCCAACCTTATCC-3′;
antisense strand COX-1, 5′-CCGCAGGTGATACTGTCGTT-3′;
sense strand COX-2, 5′-GGAGAGACTATCAAGATAGT-3′;
antisense strand COX-2, 5′-ATGGTCAGTAGACTTTTACA-3′;
sense strand TNF-α, 5′-ATGAGCACAGAAAGCATGATC-3′;
antisense strand TNF-α, 5′-TACAGGCTTGTCACTCGAATT-3′;
sense strand IL-1β, 5′-TGCAGAGTTCCCCAACTGGTACA TC-3′;
antisense strand IL-1β, 5′-GTGCTGCCTAATGTCCCCTTG AATC-3′;
sense strand IL-6, 5′-GAGGATACCACTCCCAACAGACC-3′;
antisense strand IL-6, 5′-AAGTGCATCATCGTTGTTCAT ACA-3′;
sense strand β-actin, 5′-TCATGAAGTGTGACGTTGACAT CCGT-3′;
antisense strand β-actin, 5′-CCTAGAAGCATTTGCGGTGC ACGATG-3′.

After amplification, portions of the PCR reaction products were electrophoresed on 2% agarose gel and visualized by ethidium bromide staining and UV irradiation (Seo *et al*., 2004).

Determination of IL-1β and IL-6 production

IL-1β and IL-6 levels in macrophage culture media were quantified using EIA kits, according to the manufacture's instructions.

Transient transfection and luciferase assay (reporter gene assay)

RAW 264.7 cells were transfected using Superfect reagent (Qiagen GmbH, Germany) and pNF-κB-Luc reporter plasmid (BD Biosciences, San Jose, CA, U.S.A.), as instructed by the manufacturers. Cells were incubated for 2 h before the addition of 5 ml of DMEM/10% FBS. At 48 h after the start of transfection, cells were pretreated with buddlejasaponin IV for 1 h and stimulated with LPS (1 $\mu g\,ml^{-1}$). Following 3 h of stimulation, cells were lysed and the luciferase activity was determined using the Promega luciferase assay system (Promega, Madison, CA. U.S.A.) and luminometer (Perkin Elmer Cetus, Foster City, CA, U.S.A.). Luciferase activity was normalized *versus* sample protein concentrations.

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

RAW 264.7 macrophages in 100-mm dishes (1×10^6 cells ml^{-1}) were preincubated with various concentrations of buddlejasaponin IV (5, 10, 15 μM) and then stimulated with LPS (1 $\mu g\,ml^{-1}$) for 1 h. The cells were washed once with PBS, scraped into 1 ml of cold PBS, and pelleted by centrifugation. Nuclear extracts were prepared as described previously with slight modification (Shin *et al*., 2004). Cell pellets were resuspended in hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF, 0.5 mM DTT, 10 $\mu g\,ml^{-1}$ aprotinin) and incubated on ice for 15 min. They were then lysed by adding 0.1% Nonidet P-40 and vortexing vigorously for 10 s. Nuclei were pelleted by centrifugation at $12{,}000 \times g$ for 10 min at 4°C and resuspended in high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 1 mM NaF, 1 mM sodium orthovanadate). Nuclear extract (10 μg) was mixed with double-stranded NF-κB oligonucleotide. 5′-AGTTGA GGGGACTTTCCCAGGC-3′ end labeled by [γ-^{32}P] dATP (underlying indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric or heterodimeric complex). Binding reactions were performed at 37°C for 30 min in 30 μl of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 μg of poly (dI-dC), and 1 mM DTT. DNA–protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in $0.5 \times$ TBE buffer. Gels were vacuum dried for 1 h at 80°C and exposed to X-ray film at −70°C for 24 h.

Animals

ICR male mice weighing 20–25 g and Sprague–Dawley male rats weighing 100–120 g were purchased from the Korean Experimental Animal Co. and maintained under constant conditions (temperature: 20 ± 2°C, humidity: 40–60%, light/dark cycle: 12 h) for 2 weeks or more. At 24 h before the experiment, only water was provided. All animal experiments were approved by the University of Kyungsung Animal Care

and Use Committee, and all procedures were conducted in accordance with the 'Guide for the Care and Use of Laboratory Animals' published by the National Institute of Health.

Serotonin-induced paw edema in mice

The initial hind paw volumes of ICR mice were determined volumetrically. A 1% solution of serotonin in saline (0.5 μg per rat) was injected subcutaneously into right hind paws 1 h after a test solution (10 or 20 mg kg^{-1}) had been administered orally. Test samples were, first, dissolved in 10% Tween 80 and then diluted with saline. The same volume of vehicle was administered to the control group. Paw volumes were measured for up to 30 min after oral administration at 6 min intervals, and volumes of edema were measured using a plethysmometer. Indomethacin, an anti-inflammatory drug, was used as a positive control (Recio *et al*., 2004).

Carrageenan-induced edema in rats

The initial hind paw volume of the Sprague–Dawley strain rats was determined volumetrically. A 1% solution of carrageenan in saline (0.1 ml per rat) was injected subcutaneously into right hind paws 1 h after administering test solutions (10 or 20 mg kg^{-1}) orally. Test samples were first dissolved in 10% Tween 80 and then diluted with saline. The same volume of vehicle was administered to the control group. Paw volumes were measured for up to 5 h after injections at 1 h intervals, and edema volumes were measured using a plethysmometer. Indomethacin (10 mg kg^{-1}), an anti-inflammatory drug was used as a positive control (Recio *et al*., 2004).

Acetic acid-induced abdominal constriction and hot-plate test

The acetic acid abdominal constriction test was performed using the method described by Atta-ur-Rahman (Eddy & Leimback, 1953) with minor modifications. Vehicle, test solution (10 or 20 mg kg^{-1}) or acetylsalicylic acid (100 mg kg^{-1}), a reference peripheral analgesic, were orally administered 1 h before the experiment, and 10 ml kg^{-1} of 0.7% acetic acid saline was then injected i.p. The frequencies of abdominal constrictions per animals were then counted over the following 20 min starting 10 min after the injection. Aspirin was used as a positive control.

The hot-plate test was used to measure response latencies, as described by Eddy & Leimback (1953), with minor modifications. In these experiments, the hot plate (Ugo Basile, model-DS 37) was maintained at 56$\pm$1°C. The reaction time was noted by observing either the licking of the hind paws or the jumping movements before and after drug administration. The cutoff time was 10 s and 10 mg kg^{-1} of morphine sulfate (Kuju Pharmaceutical Co., Seoul, Korea), administered i.p., was used as a positive control (Yang *et al*., 1996).

Statistical analysis

In vitro experiments were performed three times and analyzed using a nonparametric multiple comparisons test (Kruskal–Wallis test) followed by Dunn's test. All data are expressed as mean$\pm$s.d. of 10 animals *in vivo* experiments. Statistical analysis was performed by analysis of variance for multiple comparisons followed by Dunnett's test. Statistical significance was set at $P<0.05$ (Figure 1).

Results

Effects of buddlejasaponin IV on LPS-induced iNOS, COX-2 and TNF-α expressions

Since buddlejasaponin IV is a major inhibitor of NO, PGE_2 and TNF-α production in *P. kamtschatidum* (Jung *et al*., 2005), we investigated whether the inhibitory effects of buddlejasaponin IV on these proinflammatory mediators (NO and PGE_2) are related to iNOS and COX-2 modulation using Western blot and RT–PCR. In unstimulated RAW 264.7 cells, iNOS and COX-2 protein and mRNA levels were undetectable. However, iNOS and COX-2 were markedly expressed by LPS, and buddlejasaponin IV significantly inhibited these expressions in a concentration-dependent manner (Figure 2a). RT–PCR analysis showed that iNOS and COX-2 mRNA expressions were correlated with their protein levels. In addition, we found that TNF-α mRNA levels were also significantly decreased by buddlejasaponin IV in a similar way (Figure 2b). However, buddlejasaponin IV did not affect on the expression of COX-1, for maintaining normal physiological functions, and β-actin, the housekeeping gene. In general, these results are consistent with the inhibitory effect of buddlejasaponin IV on NO, PGE_2 and TNF-α release (Jung *et al*., 2005).

Effects of buddlejasaponin IV on LPS-induced IL-1β, IL-6 production and mRNA expression

To investigate the effect of buddlejasaponin IV on LPS-induced IL-1β and IL-6 release, we examined its effect on IL-1β and IL-6 by EIA and RT–PCR. We found that pretreating cells with buddlejasaponin IV reduced IL-1β,

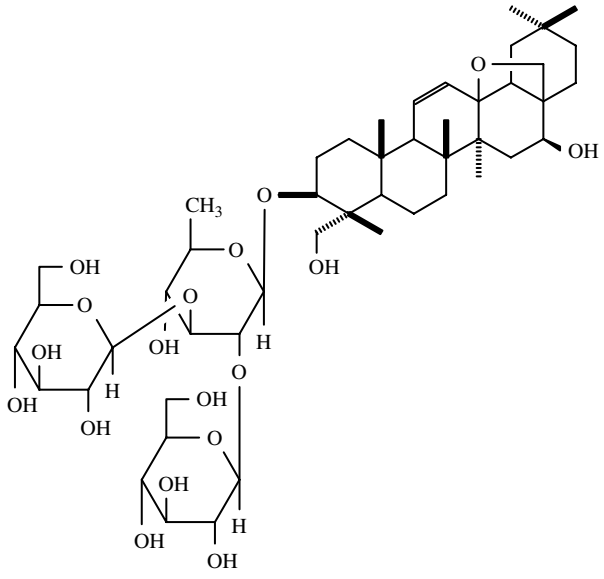

Figure 1 Chemical structure of buddlejasaponin IV.

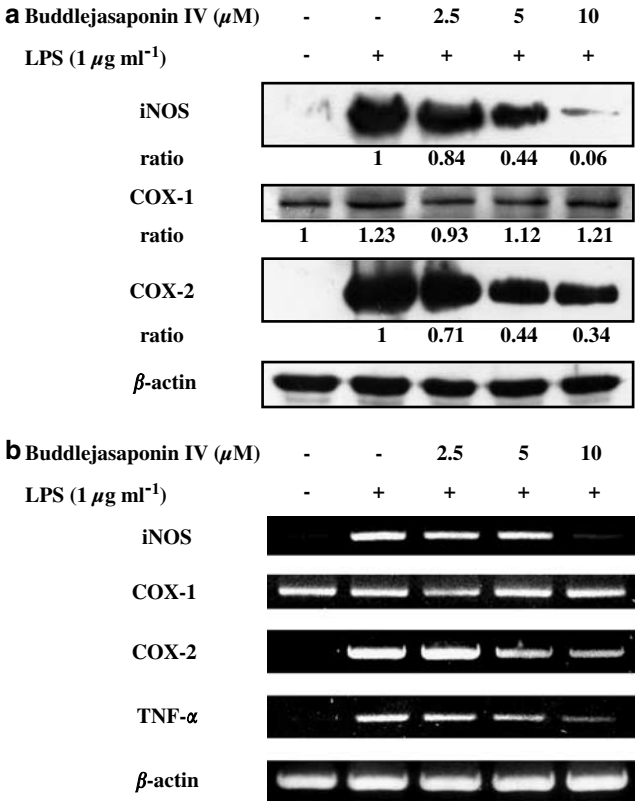

Figure 2 The effects of buddlejasaponin IV on LPS-induced iNOS, COX-1, COX-2 and TNF-α expressions in RAW 264.7 cells. (a) Cells were treated with different concentrations (2.5, 5, 10 μM) of buddlejasaponin IV for 1 h and then LPS (1 μg ml^{-1}) was added and the cells were incubated for 24 h. Total cellular proteins (40 μg) were resolved by SDS–PAGE, transferred to nitrocellulose membranes, and detected with specific antibodies, as described in Methods. A representative immunoblot of three separate experiments is shown. Density ratio of buddlejasaponin IV-treated group over LPS only treated group or control group were measured by densitometer. (b) Total RNA was prepared for the RT–PCR analyses of iNOS, COX-1, COX-2 and TNF-α *gene* expressions in RAW 264.7 macrophages pretreated with different concentrations (2.5, 5, 10 μM) of buddlejasaponin IV for 1 h followed by LPS (1 μg ml^{-1}) treatment for 4 h. iNOS-specific sequences (807 bp), COX-1-specific sequences (382 bp), COX-2-specific sequences (721 bp) and TNF-α-specific sequences (351 bp) were detected by agarose gel electrophoresis, as described in Methods. PCR of β-actin was performed to verify that the initial cDNA contents of samples were similar. Experiments were repeated three times and similar results were obtained.

IL-6 production (Figure 3a and b) and mRNA expression (Figure 3c) in a concentration-dependent manner.

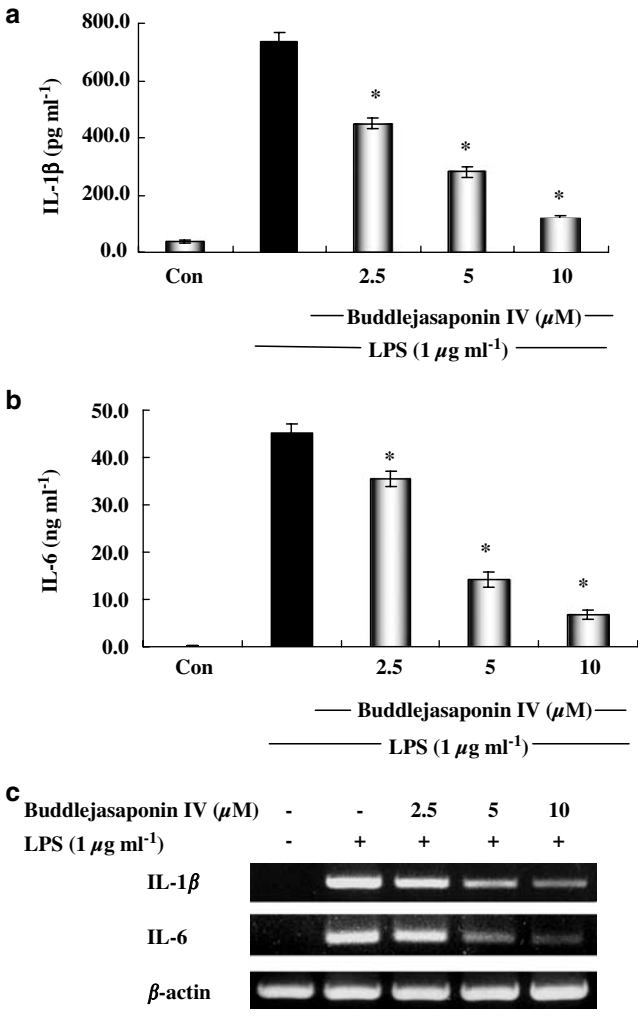

Figure 3 The effects of buddlejasaponin IV on LPS-induced IL-1β and IL-6 in RAW 264.7 cells. (a, b) Cells were pretreated with different concentrations (2.5, 5, 10 μM) of buddlejasaponin IV for 1 h and then LPS (1 μg ml^{-1}) was added and the cells were incubated for 24 h. Control (Con) values were obtained in the absence of LPS or buddlejasaponin IV. The values shown represent the means $\pm$ s.d. of three independent experiments. $^*P<0.05$ compared with the LPS only treated group. (c) Total RNA was prepared for the RT–PCR analysis of IL-1β and IL-6 gene expression from RAW 264.7 macrophages pretreated with different concentrations (2.5, 5, 10 μM) of buddlejasaponin IV for 1 h and then with LPS (1 μg ml^{-1}) for 4 h. IL-1β-specific sequences (387 bp) and IL-6-specific sequences (142 bp) were detected by agarose gel electrophoresis. PCR of β-actin was performed to verify that the initial cDNA contents of the samples were similar. The experiment was repeated three times and similar results were obtained.

Effects of buddlejasaponin IV on LPS-induced NF-κB activation and p65-DNA binding

Since the activation of NF-κB is critically required for the activations of iNOS, COX-2, TNF-α, IL-1β and IL-6 by LPS (Surh *et al*., 2001; Lappas *et al*., 2002), we examined the effect of buddlejasaponin IV on LPS-stimulated NF-κB-dependent reporter gene expression. We used a pNF-κB-luc plasmid, which was generated by inserting four spaced NF-κB-binding sites into pLuc-promoter vector. RAW 264.7 cells were transiently transfected with pNF-κB-luc plasmid and then stimulated with 1 μg ml^{-1} LPS either in the presence or absence of buddlejasaponin IV. Buddlejasaponin IV treatment significantly reduced the LPS-induced increase in NF-κB-dependent luciferase enzyme expression (Figure 4a). In terms of the effect of buddlejasaponin IV on LPS-induced NF-κB-DNA binding and transcriptional activity in RAW 264.7 macrophage cells, we found that the DNA-binding activity of NF-κB induced by LPS was significantly inhibited by buddlejasaponin IV in a concentration-dependent manner (Figure 4b). Taken together, the above findings show that buddlejasaponin IV suppressed NO, iNOS, COX-2, TNF-α, IL-1β and IL-6 expression at least in part *via* an NF-κB-dependent mechanism.

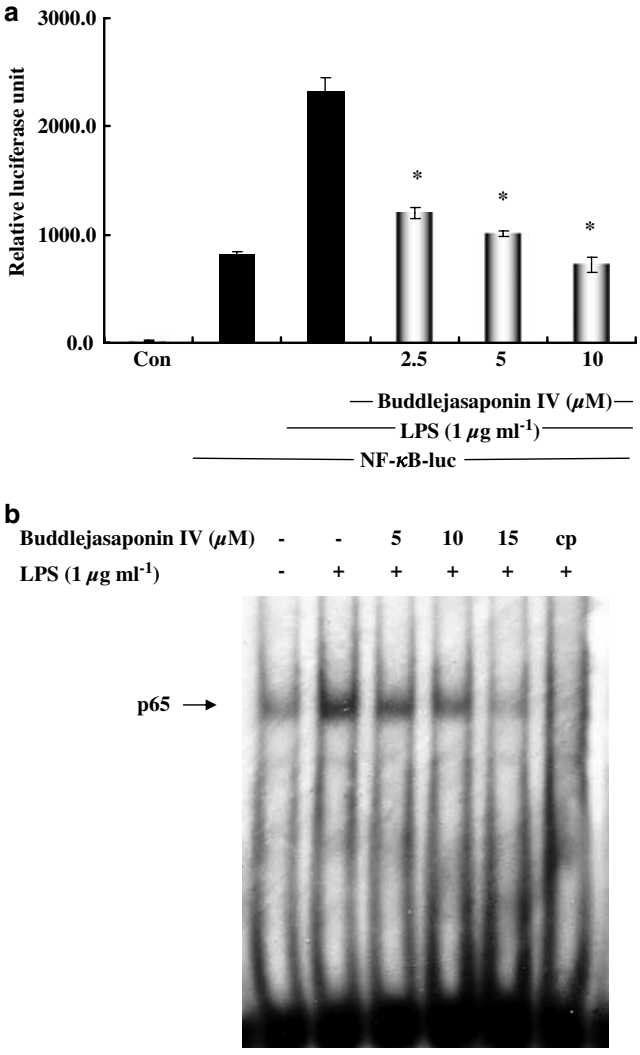

Figure 4 The inhibition of NF-κB activation and p65-DNA binding by buddlejasaponin IV. (a) Cells were transiently cotransfected with pNF-κB-Luc reporter and then left untreated (Con) or were pretreated with different concentrations (2.5, 5, 10 μM) of buddlejasaponin IV. LPS (1 μg ml^{-1}) was then added and the cells were further incubated for 3 h. The cells were then harvested and luciferase activities were determined using a Promega luciferase assay system and a luminometer. The values shown represent the means $\pm$ s.d. of three independent experiments. *$P<0.05$ compared with the LPS only treated group. (b) Nuclear extracts were prepared from controls or pretreated with different concentrations (5, 10, 15 μM) of buddlejasaponin IV for 1 h and then with LPS (1 μg ml^{-1}) for 1 h and analyzed for NF-κB binding by EMSA. The arrow indicates the position of the NF-κB band. The specificity of binding was examined by competition with the 80-fold unlabeled NF-κB oligonucleotide (cp). The data shown are representative of three independent experiments.

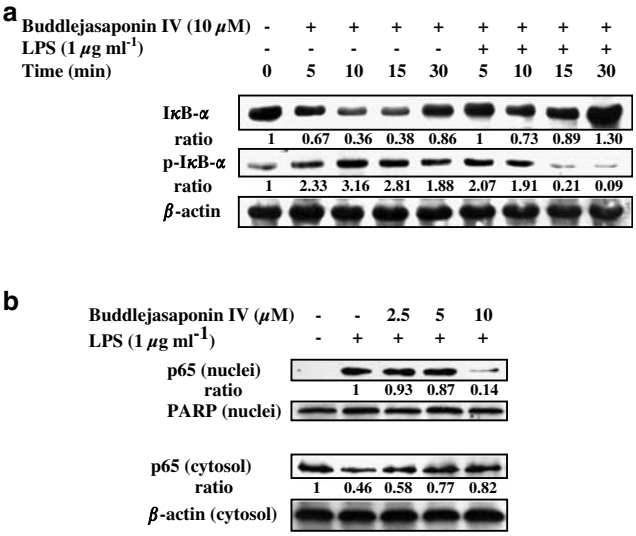

Figure 5 Inhibition of IκB-α degradation, phosphorylation and p65 nuclear translocation by buddlejasaponin IV. (a) RAW 264.7 macrophage cells were treated with LPS (1 μg ml^{-1}) in the absence or presence of 10 μm buddlejasaponin IV for different times (5, 10, 15, 30 min). Total cellular proteins were prepared and Western blotted for IκB-α and p-IκB-α using specific IκB-α and p-IκB-α antibodies. β-actin was used as an internal control. Experiments were repeated three times and similar results were obtained. Density ratio of buddlejasaponin IV-treated group over LPS only treated group or control group were measured by densitometer. (b) Cells were pretreated with different concentrations (2.5, 5, 10 μm) of buddlejasaponin IV for 1 h and then with LPS (1 μg ml^{-1}) for 1 h. Cytosolic and nuclear extracts (40 μg ml^{-1}) were prepared for Western blot analysis of p65 of NF-κB protein using a specific anti-p65 NF-κB monoclonal antibody. Density ratio versus LPS-treated group or control group was measured by densitometer.

Inhibitory effects of buddlejasaponin IV on p65 nuclear translocation and IκB-α degradation

In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor, IκB, which is phosphorylated, upon LPS stimulation, by its inhibitor IκB kinases, ubiquitinated, and rapidly degraded *via* 26S proteosome, to release NF-κB (Janssen-Heininger *et al*., 2000). We further investigated whether buddlejasaponin IV (10 μM) could inhibit the LPS-stimulated degradation of IκB-α in RAW 264.7 macrophage cells by Western blotting with anti-IκB-α antibody. Figure 5a shows that LPS induced IκB-α degradation in 10–15 min and that this degradation was significantly blocked by pretreatment with buddlejasaponin IV (10 μM). Moreover, to determine whether this IκB-α degradation is related with IκB-α phosphorylation, we examined the effect of buddlejasaponin IV on LPS-induced p-IκB-α by Western blotting, and found that buddlejasaponin IV also reduced LPS-induced IκB-α phosphorylation in a time-dependent manner. We also investigated whether buddlejasaponin IV prevented the translocation of the subunit of NF-κB, p65 from the cytosol to the nucleus after its release from IκBs. It was found that treatment with buddlejasaponin IV attenuated p65 levels in the nuclear fraction by Western blotting in a concentration-dependent manner (Figure 5b). β-actin and PARP were used as an internal control, respectively.

Inhibitory effect of buddlejasaponin IV on serotonin- and carrageenan-induced edema in mice

The anti-inflammatory effect of buddlejasaponin IV was examined using the serotonin-induced edema model. Maximal edema inhibition was observed at 24 min after edema induction. In particular, treatment with buddlejasaponin IV (20 mg kg^{-1}, p.o.) reduced edema by 25.7% at 24 min, whereas the positive control indomethacin (at 10 mg kg^{-1}, p.o.) decreased the edema rate by 42.6% at 24 min (Figure 6a). The significances of these reductions were maintained for more

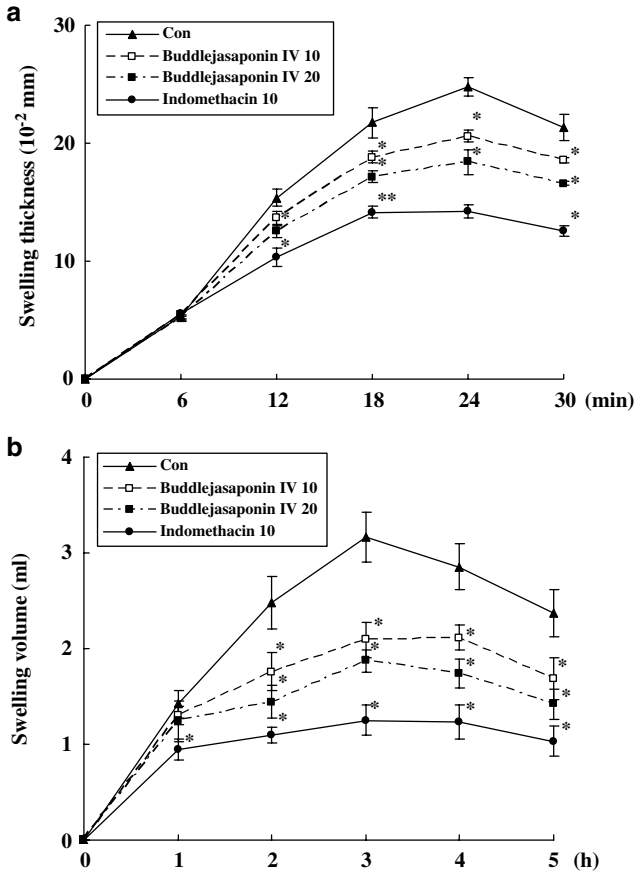

Figure 6 The inhibitory effect of buddlejasaponin IV pretreatment on (a) serotonin- and (b) carrageenan-induced paw edema. Buddlejasaponin IV was administered orally at 10 or 20 mg kg^{-1}. Indomethacin (10 mg kg^{-1}, p.o.) was used as a positive control. Control groups were administered vehicle and edema volumes were measured using a plethysmometer. Values represent mean ± s.d. ($n = 10$). *$P < 0.05$ compared with control group.

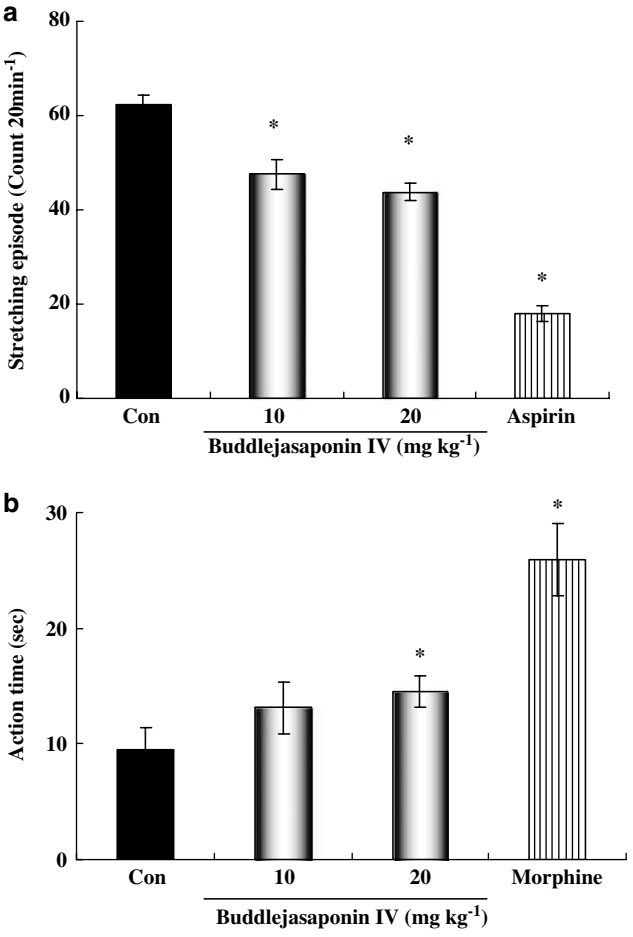

Figure 7 Antinociceptive effects of buddlejasaponin IV in mice according to the acetic acid-induced abdominal constriction test (a) and the hot-plate test in mice (b). Buddlejasaponin IV was administered orally (10 or 20 mg kg^{-1}). Aspirin (100 mg kg^{-1}) or morphine (10 mg kg^{-1}) were used as positive control in this assay. Values represent mean ± s.d. ($n = 10$). *$P < 0.05$ compared with control group.

than 30 min after edema induction. In addition, as shown in Figure 6b, the anti-inflammatory activities of buddlejasaponin IV (10 or 20 mg kg^{-1}, p.o.) were observed 2 h after carrageenan injection. Maximal edema inhibition was observed at 3 h after edema induction. Notably, treatment with buddlejasaponin IV (20 mg kg^{-1}, p.o.) reduced edema by 41.0% at 3 h, whereas the positive control, indomethacin (10 mg kg^{-1}, p.o.) decreased the edema rate by 60.6% at 3 h.

Effects of buddlejasaponin IV on acetic acid-induced abdominal constriction and hot-plate test in mice

The antinociceptive effects of test samples were assayed in two different models; by acetic acid-induced abdominal constriction and by hot-plate test in mice. Buddlejasaponin IV significantly reduced the number of writhings induced by 0.7% acetic acid. Percentage of protection by the oral administration of buddlejasaponin IV (10 or 20 mg kg^{-1}) ranged from 23.7 to 29.8%. Aspirin (100 mg kg^{-1}) also exerted a significant protective effect of 71.2% (Figure 7a). The results of hot-plate testing in mice (Figure 7b) show that buddlejasaponin IV increased jumping response latency at 20 mg kg^{-1} without affecting the animal's ability to detect heat-induced pain, as determined by a licking response, suggesting that buddlejasaponin IV has a central analgesic property. Morphine (10 mg kg^{-1}) also exerted a significant effect in hot-plate latency response. The antinociceptive activity shown by buddlejasaponin IV in these models indicates that this compound possesses peripherally and centrally mediated antinociceptive properties.

Discussion

Diverse saponin-rich crude drugs have been used extensively for their anti-inflammatory properties. The most important of these drugs is probably liquorice root, which contains glycyrrhizin and its aglycone, glycyrrhetinic acid, that have been shown to have corticosteroid-like effects (Edwards *et al.*, 1996). Among the most recent studies in this field is that on the horse chestnut, which decreases the formation of edemas induced by different phlogistic agents and its component, aescine, a mixture of oleanane saponins that prevents neutrophil recruitment, adherence and activation (Bougelet

et al., 1998). Another saponin, esculentoside A from *Phytolacca esculenta*, inhibits antibody production by B lymphocytes, phagocytosis, production of anti-inflammatory mediators and cytokines by macrophages (Ju *et al*., 1994; 1998). As part of our screening program designated to discover anti-inflammatory agents from plant sources, we isolated buddlejasaponin IV from the aerial portion of *P. kamtschaticum* that possess the inhibitory effect on LPS-induced NO, PGE_2 and TNF-α production (Jung *et al*., 2005).

In murine macrophage RAW 264.7 cells, LPS induces iNOS transcription and transduction, and then the NO production. Furthermore, LPS stimulation is well known to induce IκB proteolysis and NF-κB nuclear translocation (Freeman & Natanson, 2000). Therefore, RAW 264.7 cells provide an excellent model for drug screening and for subsequent evaluations of potential inhibitors on the pathway leading the induction of iNOS and NO production. The reactive free radical NO synthesized by iNOS is a major macrophage-derived inflammatory mediator and also has been reported to be involved in the development of inflammatory diseases (Xie *et al*., 1994). The production of TNF-α is crucial for the synergistic induction of NO synthesis in IFN-γ and/or LPS-stimulated macrophages (Jun *et al*., 1995). TNF-α elicits a number of physiological effects including septic shock, inflammation, cachexia and cytotoxicity (Aggarwal & Natarajan, 1996). Moreover, a large body of evidence suggests that PGs are involved in various pathophysiological processes, including inflammation and carcinogenesis, and inducible isoform of COX-2 is mainly responsible for the production of large amounts of these mediators (Simon, 1999). Based on this information, efforts have been made to reveal the anti-inflammatory activities of buddlejasaponin IV on LPS-induced NO, PGE_2 and TNF-α production in murine macrophage RAW 264.7 cells.

In the present study, buddlejasaponin IV downregulated the expression levels of iNOS and COX-2 proteins and of iNOS, COX-2 and TNF-α mRNA, indicating the action of buddlejasaponin IV occurs at the transcriptional level. The inhibition of the LPS-stimulated expressions of these molecules in RAW 264.7 cells by buddlejasaponin IV was not due to buddlejasaponin IV cytotoxicity, as assessed by MTT assay (data not shown) and the expression of the housekeeping gene β-actin. Furthermore, it did not change the expression level of COX-1, constitutively expressed in most tissues and seems to be responsible for housekeeping roles for normal physiological functions including maintenance of the integrity of the gastric mucosa and regulation of renal blood flow (Smith *et al*., 1996).

It has been reported that cytokines, such as TNF-α, IL-1β and IL-6 are proinflammatory *in vitro* and *in vivo* (Feldmann *et al*., 1998; Dinarello, 1999; Mannel & Echtenacher, 2000). In particular, IL-1 is a major proinflammatory cytokine, which is mainly released by macrophages and is believed to play a considerable role in the pathophysiology of endometriosis (Lebovic *et al*., 2000; Bergqvist *et al*., 2001). Moreover, IL-1β is an important component in the initiation and enhancement of inflammatory response to *Helicobacter pylori* infection (Zhang *et al*., 2005). IL-6 is also pivotal proinflammatory cytokine, regarded as an endogenous mediator of LPS-induced fever. In the present study, we found that buddlejasaponin IV also significantly inhibits the production of the proinflammatory cytokines and their mRNA expression.

NF-κB is known to play a critical role in the regulation of cell survival genes and to coordinate the expressions of proinflammatory enzymes and cytokines, such as iNOS, COX-2, TNF-α and IL-1β, -6 (Xie *et al*., 1993; 1994; Chen *et al*., 1995; Roshak *et al*., 1996; Schmedtje *et al*., 1997). Since the expressions of these proinflammatory mediators are known to be modulated by NF-κB, our findings suggest that the transcriptional inhibition of the proinflammatory mediator production by buddlejasaponin IV are due to blocking of the NF-κB signaling pathway. we examined the possibility that buddlejasaponin IV inhibits NF-κB activity. NF-κB is associated and tightly controlled by an inhibitory subunit, IκB, which is present in the cytoplasm in an inactive form. However, IκB is phosphorylated, which targets its proteolysis and allows NF-κB translocation to the nucleus, where it activates the transcription of NF-κB-responsible genes (Henkel *et al*., 1993). We also found that the translocation of NF-κB was inhibited in a concentration-dependent manner and that the degradation of IκB-α is inhibited in a time-dependent manner by buddlejasaponin IV.

While investigating the anti-inflammatory and antinociceptive effects of buddlejasaponin IV *in vivo*, we found that it significantly reduced the edema induced by serotonin or carrageenan (Figure 6), where peak edema is characterized by the presence of PGs (Yang *et al*., 1996). Increased vascular permeability and swelling are common during the early stages of many types of inflammations, and the paw swelling induced by serotonin is principally dependent on increased vascular permeability.

Carrageenan-induced inflammation in the rat paw is a classical model of edema formation and hyperalgesia that has been extensively used in the development of nonsteroidal anti-inflammatory drugs and selective COX-2 inhibitors. Several lines of evidence indicate that COX-2-mediated increases in PGE_2 production in the central nervous system contribute to the severity of the inflammatory and pain responses in this model. COX-2 is rapidly induced in the spinal cord and in other regions of the CNS following carrageenan injection into a paw (Oyanagui, 1981; Ichitani *et al*., 1997). The antinociceptive effects of test sample were assayed using two different models, that is, by the acetic acid-induced abdominal constriction test and by the hot-plate test in mice. The acetic acid-induced abdominal constriction test showed that buddlejasaponin IV had a dose-dependant effect at doses of 10 or 20 mg kg^{-1}. Moreover, a mild reduction in the number of writhings observed in this abdominal constriction model also suggested that analgesic effect of buddlejasaponin IV is related to the sensitization of nociceptive receptors to PGs. Therefore, it is possible that buddlejasaponin IV exerts a peripheral analgesic effect probably by inhibiting the synthesis or action of PGs. The results of the hot-plate test in mice show that buddlejasaponin IV slightly increase jumping response latency when treated at 10 or 20 mg kg^{-1} without affecting the abilities to detect the thermal pain threshold (licking response), which suggests that buddlejasaponin IV had central analgesic properties. The antinociceptive activities shown by buddlejasaponin IV in these models indicate that buddlejasaponin IV may possess peripherally and centrally mediated antinociceptive properties.

In conclusion, we found that buddlejasaponin IV is a potent inhibitor of LPS-induced NO, PGE_2, TNF-α, IL-1β and IL-6

production and that it acts at the transcription level. This inhibition was found to be caused by the prevention of NF-κB activation by the inhibition IκB-α degradation in RAW 264.7 macrophages. In addition, our results demonstrate that buddlejasaponin IV has anti-inflammatory and antinociceptive effects in animals. Therefore, we conclude that buddlejasaponin IV appears to have the potential to prevent inflammatory and pain diseases.

This research was supported by a Grant (PF002104-07) from the Plant Diversity Research Center of the 21st Frontier Research Program funded by the Korean Ministry of Science and Technology and by the Korean Science & Engineering Foundation (No. R13-2002-020-01002-0).

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(Received October 20, 2005
Revised January 16, 2006
Accepted February 2, 2006
Published online 6 March 2006)