British Journal of Pharmacology

DOI:10.1111/j.1476-5381.2010.01195.x
www.brjpharmacol.org

RESEARCH PAPER

Kalopanaxsaponin A ameliorates experimental colitis in mice by inhibiting IRAK-1 activation in the NF-κB and MAPK pathways

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Keywords
kalopanaxsaponin A; inflammation; colitis; IRAK-1; TNBS

Received
23 July 2010
Revised
7 November 2010
Accepted
24 November 2010

BACKGROUND AND PURPOSE

Kalopanaxsaponin A, a triterpenoid saponin isolated from *Kalopanax pictus* (family *Araliaceae*), potently inhibited nuclear factor-kappa B (NF-κB) activation in lipopolysaccharide (LPS)-stimulated peritoneal macrophages during a screening programme for anti-colitis agents from natural products. Its anti-inflammatory mechanism remains unknown. Therefore, we investigated its anti-inflammatory effects in lipopolysaccharide (LPS)- or peptidoglycan-stimulated murine peritoneal macrophages and trinitrobenzene sulphonic acid (TNBS)-induced colitic mice.

EXPERIMENTAL APPROACH

Peritoneal macrophages from male ICR mice were stimulated with LPS or peptidoglycan *in vitro* and treated with kalopanaxsaponin A. Colitis was induced *in vivo* by intrarectal administration of TNBS in male ICR mice. Mice were treated daily with kalopanaxsaponin A, sulphasalazine or phosphate-buffered saline. Inflammatory markers, cytokines, enzymes and transcription factors were measured by ELISA, immunoblot, flow cytometry and immunofluorescent confocal microscopy.

KEY RESULTS

Kalopanaxsaponin A potently inhibited the expression of the pro-inflammatory cytokines, interleukin (IL)-1β, tumour necrosis factor (TNF)-α and IL-6, induced by LPS, but not that induced by TNF-α, in peritoneal macrophages. However, it potently increased the expression of the anti-inflammatory cytokine IL-10. Kalopanaxsaponin A inhibited activation of the IL-1 receptor-associated kinase (IRAK)-1, inhibitor of κB kinase-β, NF-κB and mitogen-activated protein kinases (extracellular signal-regulated kinase, c-Jun NH_2-terminal kinase, p-38), but LPS/Toll-like receptor-4 interaction and IRAK-4 activation were not affected. Oral administration of kalopanaxsaponin A (10 and 20 $mg \cdot kg^{-1}$) improved the clinical parameters and histology *in vivo*. Kalopanaxsaponin A inhibited NF-κB and mitogen-activated protein kinase activation induced by TNBS by suppressing IRAK-1 activation.

CONCLUSIONS AND IMPLICATIONS

Kalopanaxsaponin A may improve inflammatory diseases, such as colitis, by inhibiting IRAK-1 activation.

Abbreviations

COX-2, cyclooxygenase-2; ERK, extracellular signal-regulated kinase; IL, interleukin; IRAK, interleukin-1 receptor-associated kinase; JNK, c-Jun NH_2-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; PGE_2, prostaglandin E_2; TLR, Toll-like receptor; TNF, tumour necrosis factor; TNBS, trinitrobenzene sulphonic acid

Introduction

The pathogenic mechanisms of inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, involve the dysregulation of the intestinal immune response to environmental antigens such as intestinal microflora (Gorbach and Nahas, 1968; Benno *et al.*, 1993; Berrebi *et al.*, 2003). IBD occurs most frequently in the terminal ileum and

colon where many intestinal microbes reside (Chandran *et al.*, 2003; Binder, 2004). IBD does not develop or progress significantly in germ-free animals, indicating that intestinal microflora may play an important role in initiating and perpetuating colonic inflammation. Normal intestinal microflora comprise an estimated 400 different bacterial species and reach their highest concentrations in the terminal ileum and colon (Hill and Drasar, 1975; Simon and Gorbach, 1984). Intestinal microflora produces toxic compounds, such as Gram-negative bacterial endotoxins (Andou *et al.*, 2009; Ilg *et al.*, 2009; Pasternak *et al.*, 2010). Endotoxins may interact at the apical intestinal surface and induce responses in intestinal epithelial cells, which produce pro-inflammatory cytokines and other mediators that induce inflammatory activation of the mucosal immune system and suppress the expression of anti-inflammatory mediators such as interleukin (IL)-10 via signalling through Toll-like receptors (TLRs) and/or cytokine receptors (Kühn *et al.*, 1993; Jung *et al.*, 1995). The TLRs are a group of single, membrane-spanning, non-catalytic receptors that recognize structurally conserved pathogen-associated molecular patterns derived from microbes and subsequently activate immune cell responses (Muzio *et al.*, 2000). Among this family of receptors, TLR-4 and TLR-2, which are linked to the activation of the transcription factor nuclear factor-kappa B (NF-κB) via interleukin-1 receptor-associated kinases (IRAKs), TLR4 may serve as the main mediator of lipopolysaccharide (LPS) signalling in IBD (Ingalls *et al.*, 1999; Cario and Podolsky, 2000) while TLR2 recognizes lipoproteins and lipopeptides from Gram-positive bacteria, mycoplasma and mycobacteria (Schwandner *et al.*, 1999). IRAKs are protein kinases involved in signalling innate immune responses from TLRs. After TLR-4 and TLR-2 recognize pathogen-associated molecular patterns, such as LPS and peptidoglycan (O'Neill and Dinarello, 2000), all IRAK members form multimeric receptor complexes. Phosphorylated IRAK-1 is then degraded via an ubiquitin-dependent mechanism and activates a multimeric protein complex composed of TRAF6, TAK1, TAB1 and TAB2. Activated TAK1 phosphorylates both the inhibitor of κB kinases (IKKs), as well as specific mitogen-activated protein kinase kinases (MKKs). IKKs phosphorylate the NF-κB inhibitor IκB-α, leading to its ubiquitination and subsequent degradation by the proteasome. This allows NF-κB to translocate to the nucleus and bind to specific promoter sequences. Activated MKKs phosphorylate and activate members of the c-Jun NH_2-terminal kinase (JNK)/p38 mitogen-activated protein kinase (MAPK) family (Janssens and Beyaert, 2003). The activation of NF-κB in mucosal macrophages is accompanied by an increased capacity of these cells to produce and secrete IL-1β, tumour necrosis factor (TNF)-α and IL-6.

Kalopanaxsaponin A isolated from *Kalopanax pictus*, which has been used for inflammatory diseases in East Asia, has several pharmacological effects, including anti-carcinogenic, anti-inflammatory, anti-rheumatoid and anti-diabetic activities. The anti-inflammatory activity of kalopanaxsaponin A has been studied in RAW 264.7 cells by inhibiting expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) through NF-κB (Kim *et al.*, 2002). However, its complete mechanism of action remains unknown.

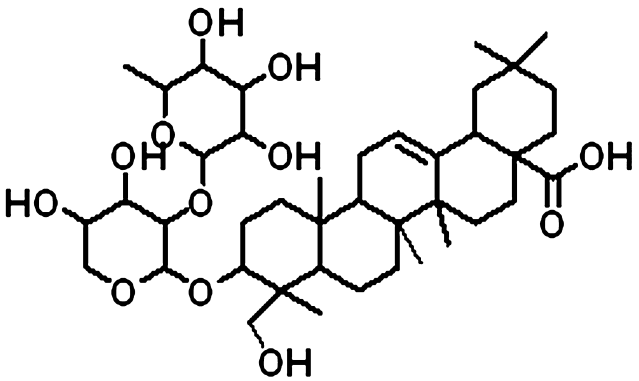

Figure 1
The structure of kalopanaxsaponin A.

Kalopanaxsaponin A potently inhibited NF-κB activation in LPS-stimulated peritoneal macrophages during screening programme for the discovery of anti-colitis agents from natural products. Therefore, we investigated the amelioration of trinitrobenzene sulphonic acid (TNBS)-induced colitis in mice by orally administered kalopanaxsaponin A. We found that the anti-inflammatory effects of this compound involved inhibition of the degradation of phosphorylated IRAK-1 in peritoneal macrophages.

Methods

Isolation of kalopanaxsaponin A

The dried bark of KP (3 kg) was extracted five times with methanol with heating. The methanolic extracts were combined and evaporated to dryness under reduced pressure, which yielded 110 g. This dried powder was extracted with two phase solvent system (H_2O and ethyl acetate). The aqueous phase was extracted with butanol and the BuOH extract (18 g) was fractionated into seven subfractions (FB1–FB7) by silica gel column chromatography using a dichloromethane–methanol solvent gradient. FB5 (2.8 g) was further separated by a medium pressure liquid chromatography (a linear-gradient applied by 10% CH_3CN in H_2O to 70% CH_3CN in H_2O at a flow rate of 4 mL·min^{-1} over 4 h) to afford a kalopanaxsaponin A (69 mg; m/z, 751.4[M]$^-$) (Figure 1).

Animals

All animal care and experimental procedures were in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University. Male ICR mice (20–25 g, 5 weeks old) were supplied from Orient Animal Breeding Center (Sungnam, Korea). All animals were housed in wire cages at 20–22°C and 50 ± 10% humidity, fed standard laboratory chow (Samyang Co., Seoul, Korea), and allowed water *ad libitum*.

Isolation and culture of peritoneal macrophages

Male ICR mice were injected (i.p.) with 2 mL of 4% thioglycolate solution. Mice were killed 4 days after injection and

the peritoneal cavities were washed with 10 mL of RPMI 1640. The peritoneal lavage fluids were centrifuged at 200× *g* for 10 min and the cells were resuspended with RPMI 1640 and plated. After incubation for 1 h at 37°C, the cells were washed three times and non-adherent cells were removed by aspiration. Cells were cultured in 24-well plates (0.5×10^6 cells·well^{-1}) at 37°C in RPMI 1640 plus 10% fetal bovine serum. The attached cells were used as peritoneal macrophages (Park *et al.*, 2009).

Determination of NO and cytokines from peritoneal macrophages induced by LPS

Production of NO was measured (as nitrite) in culture media using the Griess reagent (Bakker *et al.*, 2009). The culture medium (100 µL) was combined with 100 µL of Griess reagent [mixture of equal volume of 1% sulphanilamide in 5% H_3PO_4 and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in H_2O] in a 96-well plate, and then the absorbance was measured spectrophotometrically at 550 nm. Nitrite concentration was determined using sodium nitrite as a standard.

Cytokines, TNF-α, IL-1β, IL-6, IL-10 and prostaglandin (PG)E_2, were assessed using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

Immunoblot analysis for peritoneal macrophages

The cell supernatant extracts prepared from macrophages were separated by 9% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried-milk proteins in phosphate buffered saline Tween-20 (PBST), then probed with COX-2, iNOS, IRAK-4, IRAK-1, phosphorylated IKK-β (p-IKK-β), IκB-α, p65, p-p65, p38, p-p38, JNK, p-JNK, extracellular signal-regulated kinase (ERK), p-ERK or β-actin antibody. After washing with PBST, proteins were detected with horseradish peroxidase-conjugated secondary antibodies for 1 h. Bands were visualized with enhanced chemiluminescence reagent (Shin *et al.*, 2005).

Immunofluorescent confocal microscopy

For the assay of p65, peritoneal macrophages were stimulated with LPS (50 ng·mL^{-1}) in the presence or absence of kalopanaxsaponin A for 60 min. The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. The cells were stained with goat polyclonal anti-p65 antibody for 2 h at 4°C and then incubated with secondary antibodies conjugated with Alexa 488 and propidium iodide (10 µg·mL^{-1}, Calbiochem Co., San Diego, CA, USA) for 1 h. Images were observed by confocal microscopy.

For the assay of IRAK-1, peritoneal macrophages plated on cover slides were incubated at 37°C overnight. Macrophages were stimulated with LPS (5 µg·mL^{-1}) for 30 min in the presence or absence of kalopanaxsaponin A. The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. The cells were stained with goat polyclonal anti-IRAK-1 antibody for 2 h at 4°C and then incubated with secondary antibodies conjugated with Alexa 488 for 1 h. The stained cells were observed by confocal microscopy.

Flow cytometry

Mouse peritoneal macrophages were incubated with Alexa Fluor 488-conjugated LPS (10 µg·mL^{-1}, Invitrogen, Carlsbad, CA, USA) for 10 min in the presence or absence of kalopanaxsaponin A at room temperature. The cells were then fixed in phosphate-buffered saline (PBS) containing 4% paraformaldehyde and 3% sucrose for 20 min. After washing with PBS, the macrophages were incubated with propidium iodide (10 µg·mL^{-1}, Calbiochem Co., CA, USA) for 10 min and then analysed by flow cytometry.

Transient transfection of small interference RNA (siRNA)

Cells were seeded at 3×10^5 cells per well in 24-well plates and allowed to rest for 1 day before transfection. Cells were transfected with 50 nM siRNA for IRAK-1 using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. At 24 h after transfections, cells were treated with or without kalopanaxsaponin A and/or LPS for 30 min.

Preparation of experimental colitis in mice

The curative and preventive effects of kalopanaxsaponin A were investigated in male ICR mice. In each experiment, the mice were divided into five groups: normal and TNBS-induced colitis groups treated with or without kalopanaxsaponin A or sulphasalazine. TNBS-induced colitis was induced by the administration of 2.5% (w·v^{-1}) TNBS solution (100 µL) in 50% ethanol into the colon of lightly anaesthetized mice via a thin round-tip needle equipped with a 1 mL syringe (Fukata *et al.*, 2006). The normal group was treated with vehicle alone. The needle was inserted so that the tip was 3.5–4 cm proximal to the anal verge. To distribute the agents within the entire colon and caecum, mice were held in a vertical position for 30 s after the injection. Using this procedure, >95% of the mice retained the TNBS enema. If an animal quickly excreted the TNBS–ethanol solution, it was excluded from the remainder of the study. Kalopanaxsaponin A (10 or 20 mg·kg^{-1}) or sulphasalazine (50 mg·kg^{-1}) was orally administered once a day from 1 day after TNBS treatment to the day before killing the mice (when evaluating its curative effect) or from 3 days before TNBS treatment to the day before killing the mice (when evaluating prevention). The mice were killed on the third day after TNBS administration. The colon was quickly removed, opened longitudinally, and gently cleared of stool by washing with PBS. Macroscopic assessment of the disease grade was scored according to a previously reported scoring system (0, no ulcer and no inflammation; 1, ulceration and local hyperaemia; 2, ulceration without hyperaemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm (Hollenbach *et al.*, 2005), and the colon tissue was then used for immunoblot and ELISA analysis.

For the histological examination, the colons were fixed in 10% buffered formalin solution, embedded in paraffin

using standard methods, cut into 5 μm sections, stained with haematoxylin-eosin, and then assessed under light microscopy.

Assay of myeloperoxidase (MPO) activity in colon

Colons were homogenized in a solution containing 0.5% hexadecyl trimethyl ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0), and then centrifuged for 30 min at 20 000× *g* and 4°C. An aliquot (50 μL) of the supernatant was added to a reaction mixture of 1.6 mM tetramethylbenzidine and 0.1 mM H_2O_2 and incubated at 37°C; the absorbance was obtained at 650 nm over time. MPO activity was defined as the quantity of enzyme degrading 1 μmol·mL^{-1} of peroxide at 37°C and expressed in units (mg protein^{-1}) (Mullane *et al.*, 1985). The protein content was assayed by the Bradford method (Bradford, 1976).

ELISA and immunoblotting of colonic tissue

To measure IL-1β, TNF-α, IL-10 and IL-6 by ELISA, colons were homogenized in 1 mL of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate was centrifuged (15 000 × *g*, 4°C) for 15 min, and the supernatant was transferred to 96-well ELISA plates. IL-1β, TNF-α, IL-10 and IL-6 concentrations were determined using commercial ELISA kits (Pierce Biotechnology, Rockford, IL, USA). For immunoblotting of IRAK-1, IRAK-4, iNOS, phospho-NF-κB (pp65), NF-κB (p65), COX-2 and β-actin, the colon tissue was carefully homogenized to obtain viable single cells, which were resuspended in 1 mL of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. After centrifugation, the supernatant was used for the immunoblot assay. The protein from collected cells was analysed by electrophoresis on an 8–10% sodium dodecyl sulphate polyacrylamide gel and then transferred to nitrocellulose membrane. Levels of IRAK-1, IRAK-4, pp65, p65, COX-2, iNOS and β-actin were assayed as previously described (Shin *et al.*, 2005). Immunodetection was performed using an enhanced chemiluminescence detection kit.

Statistical analysis

Results are presented as the means ± standard deviation of at least three replicates. ANOVA was used for comparisons between multiple groups The Student's *t*-test was used for the statistical analysis of the difference noted. *P*-values of 0.05 or less were considered statistically significant.

Materials

RPMI 1640, penicillin-streptomycin and LPS purified from *Escherichia coli* O111:B4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for IRAK-4, IRAK-1, COX-2, iNOS and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, LA USA). Antibodies for p-IKK-β, IκB-α, p65, p-p65, p38, p-p38, JNK, p-JNK, ERK and p-ERK were purchased from Cell Signaling Technology (Beverly, MA, USA). Cytokine ELISA kits were purchased from R&D Systems (Minneapolis, MN, USA). Other chemicals were of the highest grade available.

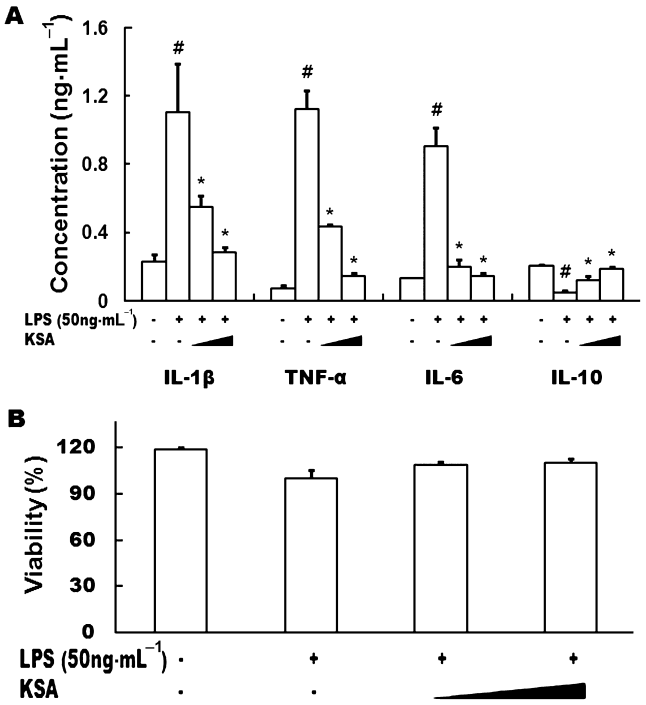

Figure 2

Inhibitory effects of kalopanaxsaponin A on the production of pro-inflammatory cytokines (A) and on cell viability (B), in lipopolysaccharide (LPS)-induced peritoneal macrophages. Peritoneal macrophages (0.5 × 10^6 cells) were treated with 50 ng·mL^{-1} LPS in the absence or presence of kalopanaxsaponin A (KSA, 5 or 10 μM) for 20 h. Levels of interleukin (IL)-1β, tumour necrosis factor-α (TNF-α), IL-6 and IL-10 in culture supernatants were measured by ELISA. All data are expressed as mean ± SD (n = 4 in a single experiment).). #P < 0.05, significantly different versus control group. *P < 0.05 versus LPS control.

Results

Effect of kalopanaxsaponin A on protein expression of pro-inflammatory cytokines in LPS-induced peritoneal macrophages

To investigate the anti-inflammatory effect of kalopanaxsaponin A in peritoneal macrophage cells, we measured the inhibitory effect on protein expression of the pro-inflammatory cytokines, IL-1β, TNF-α, IL-6 and of IL-10. Stimulation of peritoneal macrophages with LPS increased IL-1β, TNF-α and IL-6 expression, but IL-10 expression was reduced. When cells were treated with LPS in the presence of kalopanaxsaponin A, the expression of IL-1β, TNF-α and IL-6 was significantly decreased, but IL-10 expression significantly increased (Figure 2A). No cytotoxic effects of kalopanaxsaponin A were observed in the cell viability test (Crystal Violet method) under the conditions used in these experiments (Figure 2B).

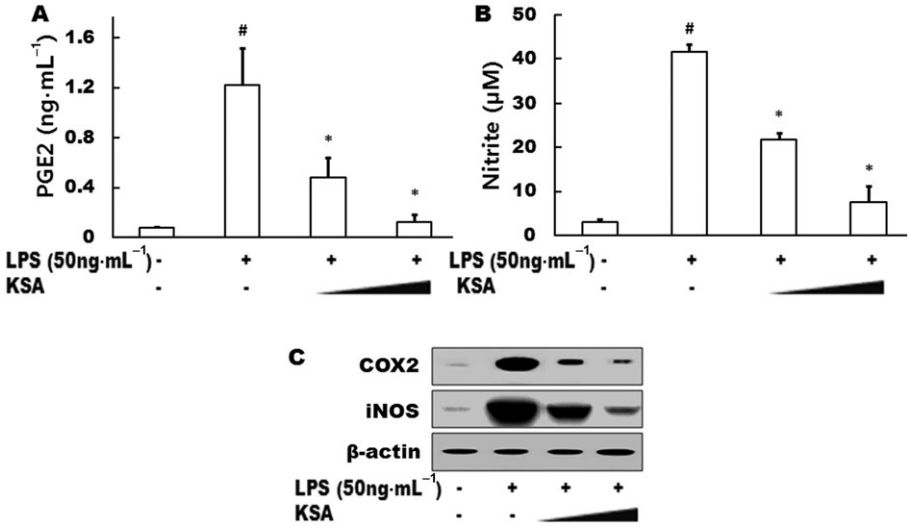

Figure 3

Effects of kalopanaxsaponin A on inflammatory mediators, prostaglandin E2 (PGE_2) (A) and NO (as nitrite; B), and expression of the enzymes, cyclooxygenase-2 (COX-2) and inducible NO synthase (iNOS) (C), in lipopolysaccharide (LPS)-stimulated peritoneal macrophages. Peritoneal macrophages isolated from mice were incubated with LPS in the absence or presence of kalopanaxsaponin A (KSA, 5 or 10 μM) for 20 h, and then the levels of PGE_2 and nitrite in the culture medium were measured using ELISA kit and Griess reagent, respectively. Protein expression of COX-2 and iNOS levels was measured by immunoblot analysis. All data are expressed as mean ± SD ($n = 4$ in a single experiment). #$P < 0.05$, significantly different versus control group. *$P < 0.05$ versus LPS control.

Inhibitory effects of kalopanaxsaponin A on inflammatory enzyme production in LPS-stimulated peritoneal macrophages

Mouse peritoneal macrophages were stimulated with LPS in the presence or absence of kalopanaxsaponin A to assess whether inflammatory enzymes such as COX-2 and iNOS were down-regulated. When these macrophages were stimulated with LPS in the absence of kalopanaxsaponin A, PGE_2 and NO levels were significantly increased. Co-treatment with LPS and kalopanaxsaponin A significantly reduced PGE_2 and NO levels (Figure 3). Kalopanaxsaponin A (10 μM) inhibited the LPS-induced production of PGE_2 and NO by 88% and 82% respectively.

To determine whether kalopanaxsaponin A suppresses protein expression of COX-2 and iNOS, thus regulating the production of these pro-inflammatory mediators, we examined the protein levels of COX-2 and iNOS in peritoneal macrophages stimulated with LPS in the presence or absence of kalopanaxsaponin A (Figure 3C). LPS induced these enzymes; however, kalopanaxsaponin A significantly inhibited the expression of these enzymes in LPS-stimulated cells.

Inhibitory effects of kalopanaxsaponin A on IKK-β phosphorylation, IκB-α degradation and NF-κB activation in LPS-stimulated peritoneal macrophages

Phosphorylation of IKK-β and IκB-α (through ubiquitination and proteolytic degradation) leads to the nuclear translocation of NF-κB, which exists as a complex of NF-κB•IκB-α in the cytoplasm (Wang *et al.*, 2001). To investigate the inhibitory effects of kalopanaxsaponin A on the phosphorylation of IKK-β and subsequent degradation of IκB-α in LPS-induced peritoneal macrophages, the cells were treated with LPS in the presence and absence of kalopanaxsaponin A (Figure 4A). Exposure to LPS increased phosphorylation of IKK-β and degradation of IκB-α in the cells. Co-treatment with LPS and kalopanaxsaponin A reduced the LPS-induced phosphorylation of IKK-β and degradation of IκB-α.

We next examined whether kalopanaxsaponin A could inhibit the phosphorylation and the nuclear translocation of NF-κB in LPS-stimulated peritoneal macrophages through immunoblot analysis (Figure 4B). The p-p65 levels in the macrophages of kalopanaxsaponin A-treated cells were decreased compared with those in cells treated with LPS alone. As further confirmation, p65 translocation into the nucleus was detected by confocal analysis using p65 antibody (Figure 4C). Kalopanaxsaponin A significantly inhibited the translocation of the p65 subunit of NF-κB into the nucleus.

We also investigated the effect of kalopanaxsaponin A on LPS-induced MAPK (ERK, JNK and p38 MAP kinases) activation. While LPS activated MAPKs, kalopanaxsaponin A inhibited this activation (Figure 4D). Of the MAPKs, p-ERK was most potently inhibited.

Inhibitory effects of kalopanaxsaponin A on activation of IRAKs

To determine the anti-inflammatory mechanism of kalopanaxsaponin A, we investigated the LPS–TLR4 interaction and measured the level of IRAK-4. When macrophages were treated with LPS in the presence and absence of kalopanaxsaponin A, LPS was significantly located on the cell membrane. The LPS location on the cell membrane was not affected by the treatment with kalopanaxsaponin A (Figure 5A). Also, the expression level of IRAK-4 was not

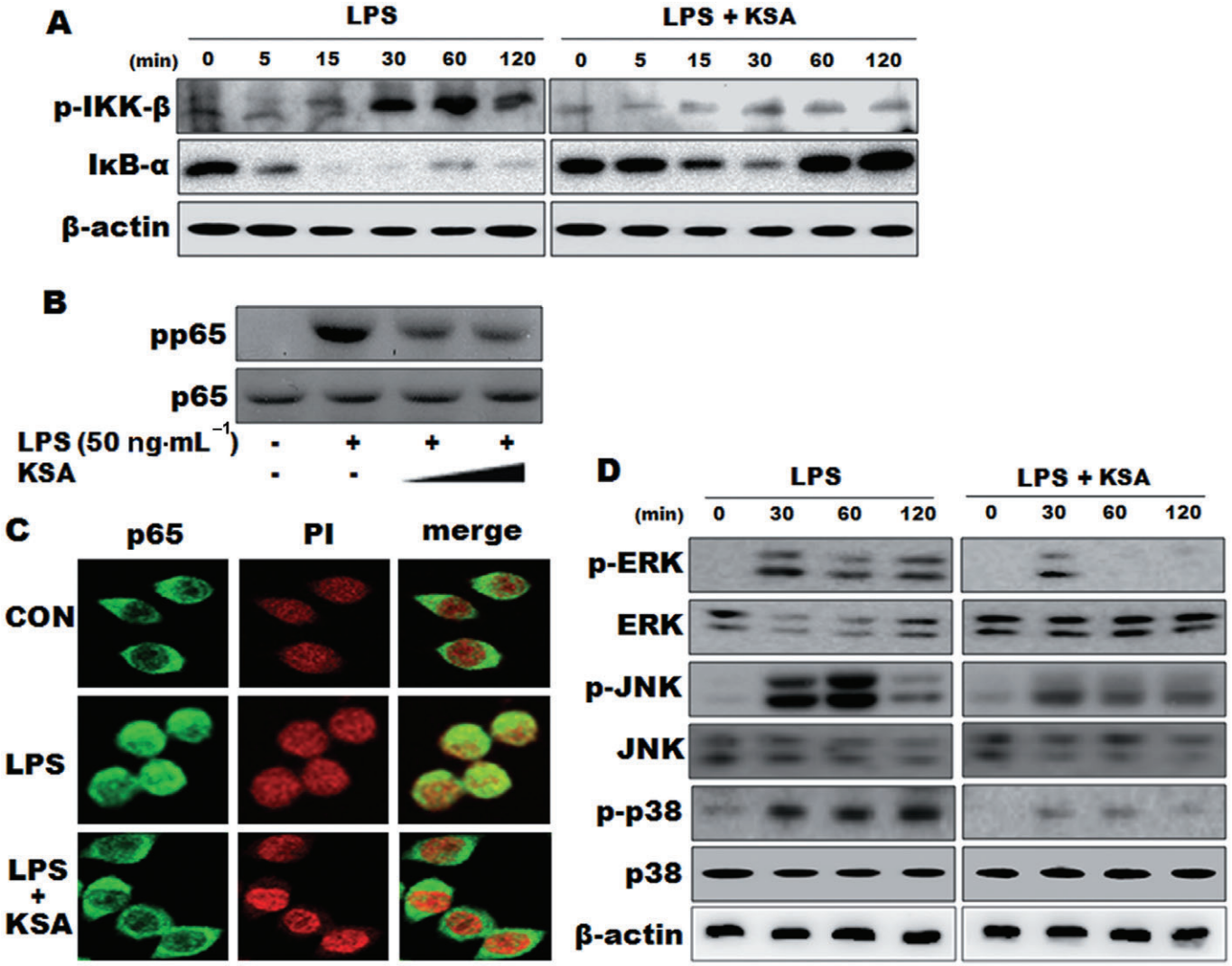

Figure 4

Effects of kalopanaxsaponin A on inhibitor of κB kinase-β (IKK-β) phosphorylation, IκB-α degradation and nuclear factor-kappa B (NF-κB) activation. The peritoneal macrophages isolated from mice were treated with 50 ng·mL^{-1} lipopolysaccharide (LPS) in the absence (LPS) or presence of kalopanaxsaponin A (KSA, 5 or 10 μM). (A) Effect in IKK-β phosphorylation and IκB-α degradation. Cells were treated with kalopanaxsaponin A (10 μM) for 0, 5, 15, 30, 60 and 120 min. IKK-β phosphorylation and IκB-α degradation were assessed by immunoblot analysis. β-Actin was used as a control. (B) Effect on phosphorylation of NF-κB was determined 30 min after treatment with LPS. (C) Effect on NF-κB nuclear translocation was detected by confocal analysis using an antibody for p65 subunit. Peritoneal macrophages were incubated with 50 ng·mL^{-1} LPS in the absence or presence of kalopanaxsaponin A (10 μM) for 30 min. (D) Effects on mitogen-activated protein kinase expression. Peritoneal macrophages were incubated with 50 ng·mL^{-1} LPS in the absence or presence of kalopanaxsaponin A (10 μM) for 120 min. ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase.

affected by the presence of kalopanaxsaponin A in LPS- or peptidoglycan-stimulated macrophages (Figure 5B).

We next examined whether kalopanaxsaponin A inhibited the phosphorylation of IRAK-1, which leads to the phosphorylation of IKK-β, in peritoneal macrophages using immunoblot and confocal microscopy analyses. Exposure to LPS or peptidoglycan increased the phosphorylated degradation of IRAK-1 in the cells. However, treatment with kalopanaxsaponin A inhibited the degradation of IRAK-1 in LPS- or peptidoglycan-treated cells (Figure 5B and C).

To confirm whether kalopanaxsaponin A inhibited IRAK-1 activation, we investigated its effects in IRAK-1 siRNA-treated peritoneal macrophages (Figure 5D). Transfection of IRAK-1 siRNA significantly inhibited IRAK-1 expression by 87%. Treatment of these peritoneal macrophages with LPS did not potently activate NF-κB or IRAK-1, compared with those in normal peritoneal macrophages. Kalopanaxsaponin A did not significantly inhibit LPS-induced p65 phosphorylation in IRAK-1 siRNA-treated peritoneal macrophages.

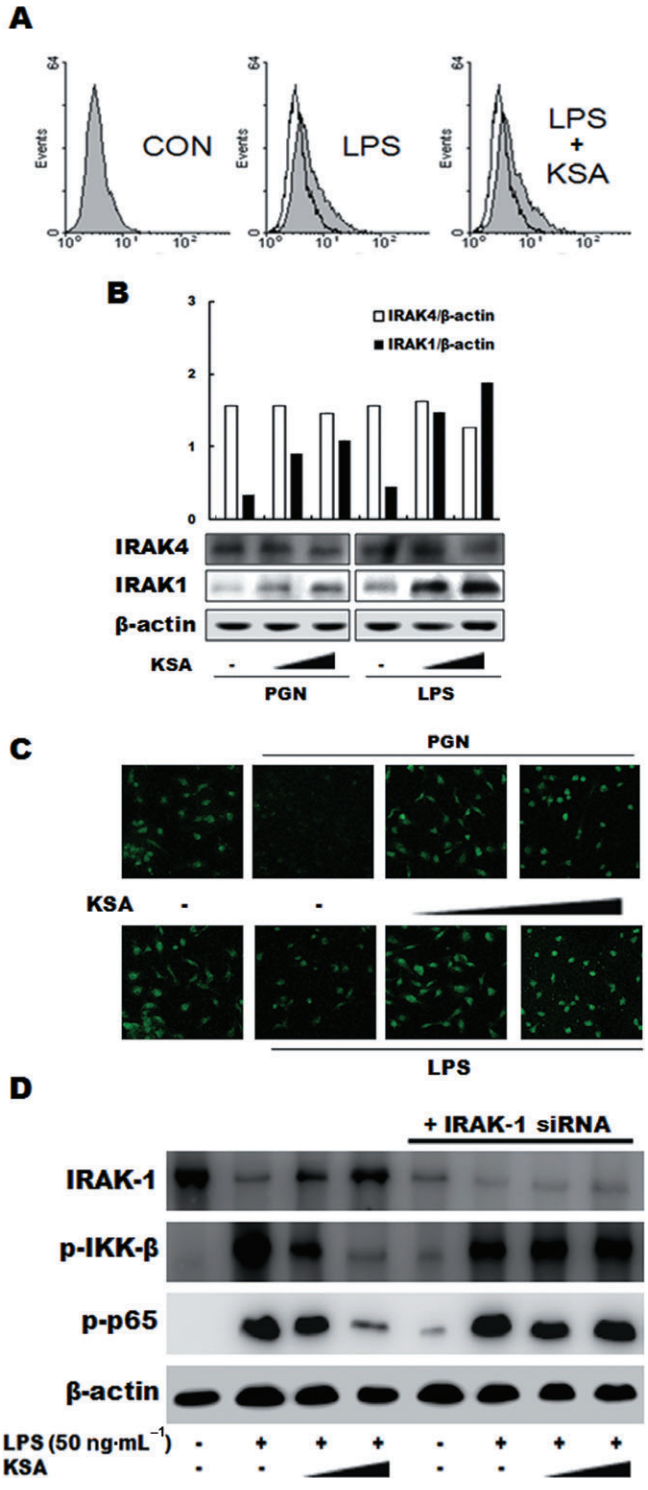

Figure 5

Inhibitory effects of kalopanaxsaponin A on phosphorylated interleukin-1 receptor-associated kinase-1 (IRAK-1) degradation and lipopolysaccharide (LPS)/Toll-like receptor4 (TLR4) interaction in peritoneal macrophages. Peritoneal macrophages isolated from mice were incubated with Alexa Fluor 488-conjugated LPS for 20 min in the absence (LPS) or presence of kalopanaxsaponin A (KSA, 5 or 10 μM), and then LPS binding on the surface of peritoneal macrophages (A) was measured by flow cytometry (10 μM). (B) Effect on degradation of IRAK-1 was determined 30 min after treatment with 50 ng·mL^{-1} of LPS or peptidoglycan (PGN). (C) Effects on degradation of phosphorylated IRAK-1 was detected by confocal analysis using an antibody for IRAK-1 subunit. (D) Effect on IRAK-1 activation in small interference RNA (siRNA)-transfected peritoneal macrophages was detected by immunoblot analysis. IKK-β, inhibitor of κB kinase-β.

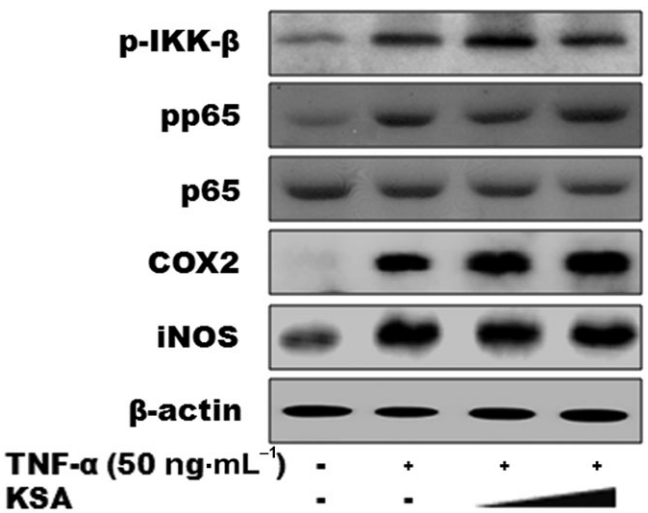

Figure 6

The effect of kalopanaxsaponin A (KSA, 5 or 10 μM) on inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expression and inhibitor of κB kinase-β (IKK-β) and nuclear factor-kappa B activation in TNF-α-induced peritoneal macrophages. Peritoneal macrophages isolated from mice were assessed 30 min or 21 h after treatment with TNF-α. p-IKK-β, p65, p-p65 (30 min), COX-2, iNOS, and β-actin (21 h) expression levels were measured by immunoblot analysis. TNF-α, tumour necrosis factor.

Effects of kalopanaxsaponin A on inflammatory markers in peritoneal macrophages stimulated by TNF-α

To assess whether kalopanaxsaponin A inhibited NF-κB activation via IRAK-1, murine peritoneal macrophages were stimulated with TNF-α in the presence or absence of kalopanaxsaponin A and inflammatory markers were investigated by immunoblot analysis. When these macrophages were stimulated with TNF-α, levels of COX-2, iNOS, p-IKK-β and p-p65 were significantly induced. Responses to co-treatment with TNF-α and kalopanaxsaponin A did not differ from those in the absence of kalopanaxsaponin A (Figure 6).

Kalopanaxsaponin A inhibits pro-inflammatory cytokines and NF-κB activation in mice with TNBS-induced colitis

We tested the curative and preventive effects of kalopanaxsaponin A in the model of colitis induced by TNBS in mice. TNBS caused loss of body weight and severe inflammation

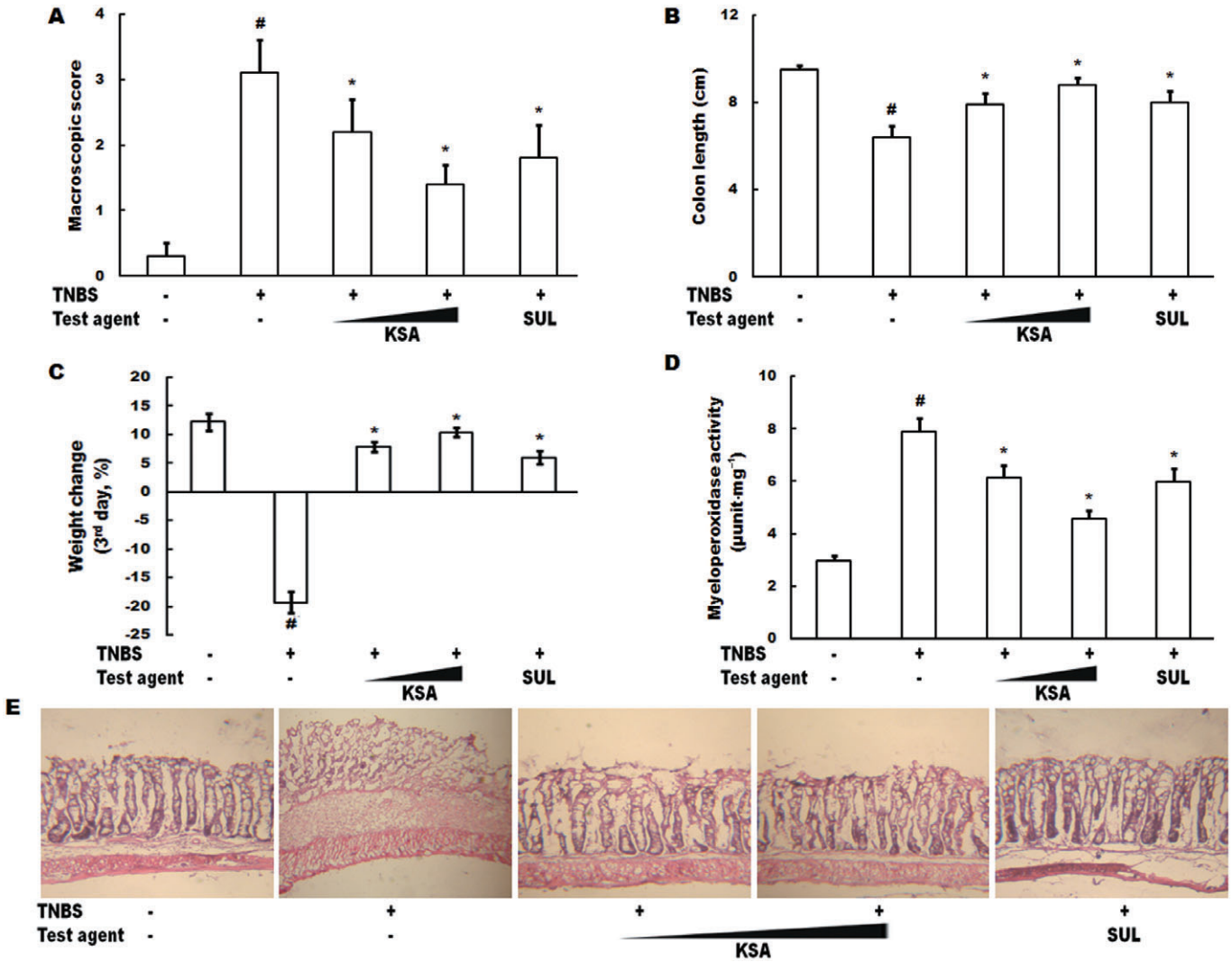

Figure 7

The effects of kalopanaxsaponin A on macroscopic disease (A), colon length (B), body weight (C), colonic myeloperoxidase activity (D) and histology (E) in trinitrobenzene sulphonic acid (TNBS)-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline, kalopanaxsaponin A or sulphasalazine. Kalopanaxsaponin A (KSA, 10 mg·kg^{-1} or 20 mg·kg^{-1}), sulphasalazine (SUL, 50 mg·kg^{-1}) or saline was orally administered for 3 days after TNBS treatment. The mice were anaesthetized with ether and killed 3 days after TNBS treatment. All values are mean ± SD (n = 7). #P < 0.05, significantly different versus control group; *P < 0.05, significantly different versus TNBS group.

manifested by shortened, thickened and erythematous colons. Colon histology showed massive bowel oedema, dense infiltration of the superficial layers of the mucosa and epithelial cell disruption by large ulcerations. To investigate the curative effect of kalopanaxsaponin A, it was orally administered for 3 days beginning 1 day after TNBS treatment. Treatment with kalopanaxsaponin A inhibited body weight reduction, colon shortening, inflammation and thickening (Figure 7). Treatment with kalopanaxsaponin A at a dose of 20 mg·kg^{-1} inhibited the colonic MPO activity by 67%, compared with that in the group treated with TNBS alone (P < 0.05, Figure 7D). Kalopanaxsaponin A showed more potent inhibition than sulphasalazine, a commercially available drug for colitis. We next measured the levels of the pro-inflammatory cytokines, IL-1β, TNF-α IL-6 and IL-10, in the colons of TNBS-induced colitic mice (Figure 8). TNBS increased the expression of IL-1β, TNF-α and IL-6 by 6.7-fold, 4.1-fold and 12.6-fold, respectively, but inhibited IL-10 expression by 76%. Treatment with kalopanaxsaponin A inhibited the TNBS-induced expression of these cytokines, but did not affect β-actin expression. Treatment with kalopanaxsaponin A at a dose of 20 mg·kg^{-1} inhibited expression with these cytokines by 94% (P < 0.05), 95% (P < 0.05) and 91% (P < 0.05), respectively, but increased IL-10 expression by 95% (P < 0.05). TNBS also increased the expression of COX-2, iNOS and NF-κB (pp65). Kalopanaxsaponin A blocked the induction of COX-2, iNOS and pp65 by TNBS. Kalopanaxsaponin A was more potent than sulphasalazine. Moreover, unlike sulphasalazine, kalopanaxsaponin A inhibited degradation of IRAK1 (Figure 9).

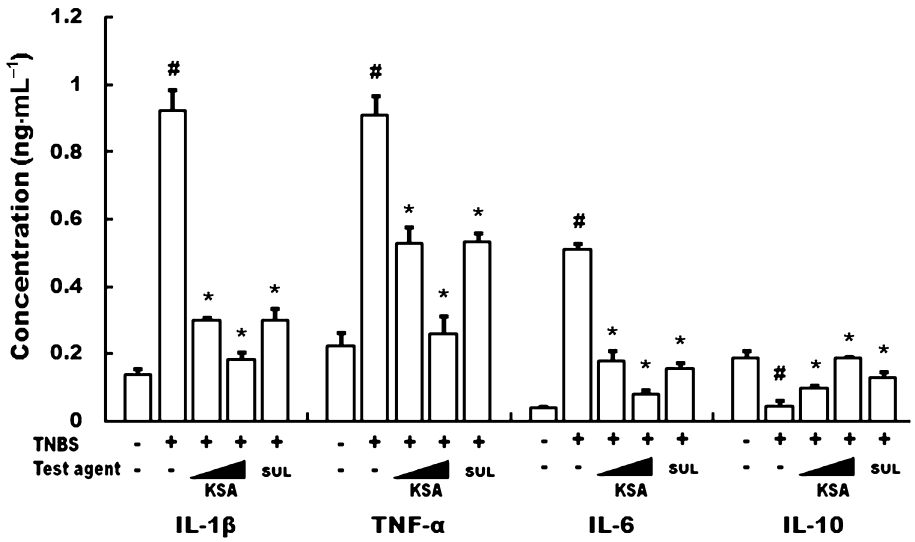

Figure 8

The effect of kalopanaxsaponin A on inflammatory cytokines in trinitrobenzene sulphonic acid (TNBS)-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline, kalopanaxsaponin A (KSA, 10 mg·kg^{-1} or 20 mg·kg^{-1}), or sulphasalazine (SUL, 50 mg·kg^{-1}). Kalopanaxsaponin, sulphasalazine or saline was orally administered for 3 days after TNBS treatment. The mice were anaesthetized and killed 3 days after TNBS treatment. Colons were collected and IL-1β, TNF-α, IL-6 and IL-10 were measured by ELISA. All values are mean ± SD ($n = 7$). #$P < 0.05$, significantly different versus control group; *$P < 0.05$, significantly different versus TNBS group. IL, interleukin; TNF-α, tumour necrosis factor-α.

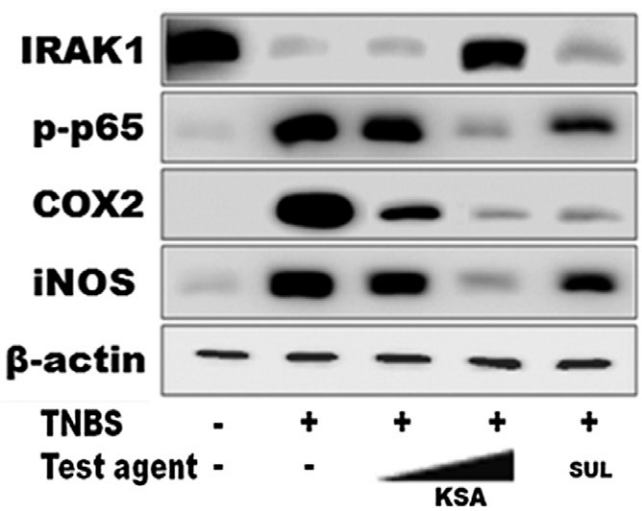

Figure 9

The effect of kalopanaxsaponin A on interleukin-1 receptor-associated kinase-1 (IRAK-1), inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) expression and nuclear factor-kappa B (NF-κB) activation in trinitrobenzene sulphonic acid (TNBS)-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline, kalopanaxsaponin A (KSA, 10 mg·kg^{-1} or 20 mg·kg^{-1}) or sulphasalazine (SUL, 50 mg·kg^{-1}). Kalopanaxsaponin A, sulphasalazine or saline was orally administered from 1st day after TNBS treatment for 3 days. The mice were anaesthetized and killed 3 days after TNBS treatment. Colons were collected and IRAK-1, iNOS and COX-2 expression and NF-κB activation were measured by immunoblot analysis.

When kalopanaxsaponin A was orally administered beginning 3 days before TNBS treatment to the third day after TNBS treatment to evaluate its preventive effect, it potently inhibited the production of inflammatory markers and colon shortening as well as the expression of pro-inflammatory cytokines IL-1β, TNF-α and IL-6, and the activation of NF-κB in TNBS-induced colitic mice (Figure S1).

Discussion

There is an increased interest in finding an alternative treatment for IBD using herbal medicines and their components, because the current medicinal therapies for IBD have toxicities and side effects (Kim *et al.*, 2005; Kwon *et al.*, 2005; Bukovská *et al.*, 2007; Bai *et al.*, 2008). However, studies of anti-inflammatory effects have not clearly provided the underlying mechanism. Therefore, we searched for new therapies for IBD in the constituents of herbal medicines used for inflammation and described a more complete mechanism.

Kalopanaxsaponin A is a major constituent of *Kalopanax pictus*, which is used as an herbal medicine for inflammatory diseases such as arthritis in China, Japan and Korea. It inhibits the expression of the inflammatory markers enzymes, COX-2 and iNOS, in RAW264.7 cells (Kim *et al.*, 2002). In our present study, we found that kalopanaxsaponin A, orally administered before or after treatment with TNBS, improved TNBS-induced colitis symptoms, including diarrhoea and colon shortening, as well as TNBS-stimulated MPO activity, an index of polymorphonuclear leukocyte accumulation, in the intestines. Kalopanaxsaponin A also reversed the expres-

sion of tight junction-associated proteins reduced by treatment with TNBS (Figure S2). Similar to tanshinone IIA, kalopanaxsaponin A reduced the expression of pro-inflammatory cytokines such as IL-1β, IL-6 and TNF-α, as well as NF-κB activation in the colon of mice treated with TNBS (Bai *et al.*, 2008). Based on these results, kalopanaxsaponin A may inhibit TNBS-induced colitis by regulating NF-κB activation. TNBS-induced inflammation was mediated by inflammatory mediators, including TNF-α, IL-1β, IL-6, NO and PGs, as previously reported (Fairweather and Rose, 2005). Among these inflammatory mediators, pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6 may be activated through NF-κB, but they also activate NF-κB (Baldwin, 1996; Fairweather and Rose, 2005).

Blood TNF-α and IL-1β levels are barely detectable in normal mice (Blanqué *et al.*, 1996). However, treatment with LPS increased serum TNF-α and IL-1β levels and caused inflammation, as previously reported (Kotanidou *et al.*, 2002). Co-treatment with LPS and kalopanaxsaponin A suppressed the blood levels of TNF-α and/or IL-1β, compared with those in mice intraperitoneally injected with LPS (Figure S3). Furthermore, kalopanaxsaponin A inhibited TNF-α, IL-1β and IL-6 in LPS-stimulated peritoneal macrophages; however, IL-10 expression was increased. Kalopanaxsaponin A also reduced the expression levels of COX-2 and iNOS, as well as their products, PGE_2 and NO_2, in LPS-treated peritoneal macrophages. Kalopanaxsaponin A inhibited the LPS-induced IKK-β phosphorylation and IκB-α degradation, as well as the translocation of the p65 subunit of NF-κB into the nucleus. This agent also inhibited LPS-induced activation of the MAP kinases, JNK, ERK and p38. Among them, ERK activation was most potently inhibited. These results suggest that kalopanaxsaponin A may regulate inflammatory markers by inhibiting LPS-induced IKK-β phosphorylation or its upstream signal transduction. Therefore, we investigated the effect of kalopanaxsaponin A in LPS/TLR4 interactions and on IRAK activation. IRAK-1 is a key molecule in the signalling cascade of the Toll/IL-1 receptor family. After binding appropriate ligands, such as LPS to TLR-4 or peptidoglycan to TLR-2, IRAK-1 is activated via IRAK-4. The activated IRAK-1 phosphorylates IKKs, which lead to the nuclear translocation of NF-κB. Kalopanaxsaponin A inhibited degradation of phosphorylated IRAK-1 in LPS- or peptidoglycan-induced peritoneal macrophages. However, kalopanaxsaponin A did not affect the interaction between LPS and its receptor, TLR4, and between peptidoglycan and its receptor, TLR-2, or the expression of IRAK-4, which leads to phosphorylation of IRAK-1, on the cell membrane of peritoneal macrophages. Kalopanaxsaponin A did not significantly inhibit LPS-induced p65 phosphorylation in IRAK-1 siRNA-treated peritoneal macrophages, in which IRAK-1 expression was significantly inhibited. Based on these findings, kalopanaxsaponin A may inhibit IRAK-1 activation. Furthermore, kalopanaxsaponin A did not affect the expression of the inflammatory markers, p-IKK-β, p-p65, COX-2 and iNOS, in peritoneal macrophages induced by TNF-α, which does not involve IRAK1. Inflammatory diseases involve over-expression of pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, and inflammatory mediators, such as NO and PGE_2, via NF-κB and MAPK pathways in macrophages (Moynagh, 2005; Tak and Firestein, 2001). Thus, kalopanaxsaponin A inhibited IRAK-1 activation, IKK-β phosphorylation, IκB-α degradation, MAPKs (JNK, ERK and p38) and NF-κB activation, but did not affect TLR-4–LPS complex formation and IRAK-4 activation. Its inhibitory effects against MAPK and NF-κB activation may inhibit the expression of the pro-inflammatory cytokines, IL-1β and TNF-α, and the inflammatory enzymes, COX-2 and iNOS, but increased expression of the anti-inflammatory cytokine IL-10. Kalopanaxsaponin A may inhibit MAPK and NF-κB pathways by inhibiting the degradation of phosphorylated IRAK-1.

Based on these findings, kalopanaxsaponin A may improve inflammatory diseases, such as colitis, by inhibiting IRAK-1 activation in TLR-4-linked NF-κB and MAPK pathways.

Acknowledgements

This study was supported by a grant from World Class University Program through the National Research Foundation of Korea funded by the Ministry of Education, Science and Technology (R33-2008-000-10018-0).

Conflict of interest

The authors state no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The effects of kalopanaxsaponin A on macroscopic disease (A), colon length (B), body weight (C), colonic MPO activity (D) and histology (E) in TNBS-induced colitic mice. TNBS, except in the control group (group treated with vehicle alone), was intrarectally administered to mice treated with saline (TNBS), kalopanaxsaponin A, or sulphasalazine. Kalopanaxsaponin A (KSA, [10 mg·kg^{-1} or 20 mg·kg^{-1}]), sulphasalazine (SUL, 50 mg·kg^{-1}) or saline was orally administered from 3 days prior to TNBS treatment. The mice were anaesthetized with ether and killed 3 days after TNBS treatment. All values are mean ± SD (n = 7). #P < 0.05, significantly different versus control group; *P < 0.05, significantly different versus TNBS group. The effect of kalopanaxsaponin A on inflammatory cytokines in TNBS-induced colitic mice

(F). TNBS, except in the control group, was intrarectally administered to mice treated with saline, kalopanaxsaponin A (KSA, [10 mg·kg^{-1} or 20 mg·kg^{-1}]), or sulphasalazine (SUL, 50 mg·kg^{-1}). Kalopanaxsaponin, sulphasalazine or saline was orally administered from 3 days prior to TNBS treatment. The mice were anaesthetized and killed 3 days after TNBS treatment. Colons were collected and IL-1β, TNF-α and IL-6 were measured by ELISA. All values are mean ± SD (n = 7). #P < 0.05, significantly different versus control group; *P < 0.05, significantly different versus TNBS group. The effect of kalopanaxsaponin A on IRAKs, iNOS and COX-2 expression and NF-κB activation in TNBS-induced colitic mice (G). TNBS, except in the control group, was intrarectally administered to mice treated with saline, kalopanaxsaponin A [KSA, (10 mg·kg^{-1} or 20 mg·kg^{-1})], or sulphasalazine (SUL, 50 mg·kg^{-1}). Kalopanaxsaponin A, sulphasalazine or saline was orally administered from 3 days prior to TNBS treatment. The mice were anaesthetized and killed 3 days after TNBS treatment. Colons were collected and IRAKs, iNOS and COX-2 expression and NF-κB activation were measured by immunoblot analysis.

Figure S2 The effects of kalopanaxsaponin A on tight junction associated proteins expression in TNBS-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline, kalopanaxsaponin A [KSA, (10 mg·kg^{-1} or 20 mg·kg^{-1})], or sulphasalazine (SUL, 50 mg·kg^{-1}). Kalopanaxsaponin A, sulphasalazine or saline was orally administered for 3 days after TNBS treatment. The mice were anaesthetized with ether and killed 3 days after TNBS treatment. Colons were collected and Claudin-1 and ZO-1 expression was measured by immunoblot analysis.

Figure S3 Inhibitory effects of kalopanaxsaponin A on the production of inflammatory cytokines IL-1β (A) and TNF-α (B) in mice. ICR mice were intraperitoneally injected with LPS (4 mg·kg^{-1}) and/or kalopanaxsaponin A (5 mg·kg^{-1}; KSA, kalopanaxsaponin A). Normal control (CON) was treated with vehicle alone instead of LPS and kalopanaxsaponin A. Mice sacrificed 4 h after LPS injection, whole blood was obtained by cardiac puncture and serum was obtained by centrifugation at 12 000× g for 20 min. The serum levels of IL-1β and TNF-α were measured using ELISA kit. All data shown are the mean ± SD (n = 6). #P < 0.05, significantly different versus CON group. *P < 0.05 versus LPS group.