Inhibitory effects of lupane-type triterpenoid saponins from the leaves of *Acanthopanax gracilistylus* on lipopolysaccharide-induced TNF-α, IL-1β and high-mobility group box 1 release in macrophages

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Abstract. *Acanthopanax gracilistylus* (AGS) has long been used in traditional Chinese medicine for the treatment of various inflammatory diseases. 3-O-*β*-*D*-glucopyranosyl 3*α*, 11*α*-dihydroxylup-20(29)-en-28-oic acid, acantrifoside A, acankoreoside D, acankoreoside B and acankoreoside A are major lupane-type triterpenoid saponins derived from AGS. In the present study, these five saponins were isolated from AGS by chromatography and their anti-inflammatory activities were investigated in lipopolysaccharide (LPS)-treated RAW264.7 macrophages. Cell viability was evaluated by MTT assay. Tumor necrosis factor (TNF)-α, interleukin (IL)-1β and NF-κB p65 were measured by ELISA. The gene expression levels of TNF-α and IL-1β was detected by reverse-transcription polymerase chain reaction. And high-mobility group box 1 (HMGB1) were analyzed by western blotting. The results demonstrated that these five saponins significantly suppressed LPS-induced expression of TNF-α and IL-1β at the mRNA and protein level in RAW264.7 cells. Further analysis revealed that acankoreoside A and acankoreoside B were able to reduce the secretion of HMGB1 and NF-κB activity induced by LPS in RAW264.7 macrophages. Taken together, these results suggested that the anti-inflammatory activity of AGS-derived saponins may be associated with the downregulation of TNF-α and IL-1β, and the 'late-phase' proinflammatory cytokine HMGB1, via negative regulation of the NF-κB pathway in RAW264.7 cells.

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Introduction

Inflammation, which is the natural response of living tissue to infection or injury, is able to inactivate toxins, destroy microorganisms and ultimately to restore damaged tissue (1). However, the majority of inflammatory mediators exert adverse effects that can cause further tissue and organ damage, chronic inflammation and hypersensitivity. During onset of the inflammatory response, macrophages serve an important role by releasing proinflammatory mediators and cytokines.

Lipopolysaccharide (LPS) is a major constituent of the outer membrane of Gram-negative bacteria, which induces inflammation through the release of inflammatory mediators, including tumor necrosis factor (TNF)-α and interleukin (IL)-1β (2). When LPS acts on Toll-like receptors, which are expressed on macrophages, various pathways are activated. These activated macrophages often produce downstream proinflammatory mediators and cytokines, which may ultimately result in the development of anti-inflammatory agents.

IL-1β and TNF-α are multifunctional cytokines that are involved in the regulation of the immune response, hematopoiesis and inflammation (3). While numerous cytokines have demonstrated beneficial effects on immune regulation, some have also been implicated in the pathogenesis of acute and chronic inflammatory disease (4).

High-mobility group box 1 (HMGB1) is a protein, which is released by activated monocytes or macrophages, as well as damaged and necrotic cells (5). Within the nucleus, HMGB1 serves an important role in the regulation of gene transcription (6). Upon release by phagocytes and damaged/necrotic cells (7-9), extracellular HMGB1 has a critical role in the initiation of inflammation; HMGB1 can activate macrophages

and upregulate the expression of cytokines such as TNF-α and IL-1β (10), and contribute to the pathogenesis of various inflammatory diseases (11,12). As a late mediator of inflammation, HMGB1 has been reported to be released days after endotoxin exposure (13). HMGB1 amplifies the inflammatory response by stimulating the release of various proinflammatory cytokines in numerous types of cell, including macrophages and monocytes (14). Due to the critical role of HMGB1 in the process of inflammation, HMGB1 represents a promising drug target for the clinical treatment of inflammatory diseases (15). Furthermore, it has previously been demonstrated that the mechanism underlying inhibition of HMGB1 release is associated with the nuclear factor (NF)-κB signaling pathway (16). Therefore, in order to develop a therapeutic strategy to treat inflammatory conditions by inhibiting HMGB1, it is important to determine whether HMGB1 secretion may be attenuated via activation of the NF-κB pathway.

Acanthopanax gracilistylus (AGS) belongs to the Araliaceae family. Its dried roots and stem bark are officially listed in the Chinese Pharmacopoeia as Acanthopanacis Cortex, and have been commonly used to treat paralysis, arthritis, rheumatism, myasthenia gravis, bone pains, lameness and liver disease for several centuries in China (17,18). Furthermore, various *Acanthopanax* Miq. plants have been widely used as a treatment for inflammatory diseases, due to their anti-inflammatory activity. For example, extracts from *A. giraldii* Harms, *A. senticosus*, *A. henryi* and *A. koreanum* have previously been reported to inhibit the production of inflammatory factors, including TNF-α, nitric oxide, prostaglandin E_2, IL-1β, and IL-6, in activated inflammatory cells (19-23). However, the mechanism by which the biologically active components of AGS elicit these anti-inflammatory effects remains to be elucidated.

Previous studies have isolated numerous chemical constituents from AGS (24-27). In our initial screen of medicinal plants for anti-inflammatory compounds, five saponins (compounds 1-5) were isolated from AGS. In order to investigate the anti-inflammatory properties of these saponins, the present study used the murine macrophage-like cell line, RAW264.7. RAW264.7 cells were exposed to LPS to initiate an inflammatory cascade, and the effects of AGS-isolated compounds 1-5 were assessed on the production of TNF-α, IL-1β and HMGB1 in LPS-stimulated cells. In addition, the present study aimed to determine whether the anti-inflammatory effects of these compounds were mediated via the NF-κB signaling pathway.

Materials and methods

Plant material. The leaves of AGS were collected from Changsha, China, in September 2012, and were botanically identified by Professor Chang-Soo Yook (Kyung Hee University, Seoul, South Korea).

Chemicals and regents. The RAW264.7 cell line was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). LPS, dimethyl sulfoxide (DMSO), and dexamethasone (DEX) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). The EZ4 U cell proliferation and cytotoxicity assay kit (cat. no. BI-5000) was obtained from Biomedica Medizinprodukte GmbH & Co KG (Vienna, Austria). Anti-HMGB1 antibody (cat no. ab18256) was purchased from Abcam (Cambridge, UK). The secondary antibody conjugated with horseradish peroxidase, conjugated goat anti-rabbit Immunoglobulin G (cat. no. A0208) and Taq DNA Polymerase (cat. no. D7209) were purchased from Beyotime Institute of Biotechnology (Jiangsu, China). TNF a Mouse ELISA kit (cat. no. KMC3012) and IL-1β Mouse ELISA kit (cat. no. KMC0012) were obtained from Invitrogen; Thermo Fisher Scientific, Inc. (Waltham, MA, USA). The anti-β-actin primary antibody (cat. no. sc-130656) was obtained from Santa Cruz Bioechnology, Inc. (Dallas, TX, USA). Total NF-κB p65 Sandwich ELISA kit was purchased from (cat. no. 7174; Cell Signalling Technology, Inc. (Danvers, MA, USA), RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), OPTI-MEM I medium and fetal bovine serum (FBS) were obtained from Gibco; Thermo Fisher Scientific, Inc. Other reagents used in the present study were endotoxin-free.

Extraction and isolation. The isolation of compounds 1-5 (compound 1, 3-O-*β-D*-glucopyranosyl 3*α*, 11*α*-dihydroxy lup-20(29)-en-28-oic acid; compound 2, acantrifoside A; compound 3, acankoreoside D; compound 4, acankoreoside B; and compound 5, acankoreoside A) was performed as described previously (27). Briefly, the dried leaves of AGS (1,000 g) were extracted three times using hot methanol (3x10 l). The combined methanol extract was evaporated under reduced pressure to obtain a residue (140 g), which was dissolved in water and successively partitioned with petroleum ether, ethyl acetate and n-butyl alcohol, resulting in petroleum ether (6 g), ethyl acetate (42 g) and n-butyl alcohol (58 g) layers. The ethyl acetate fraction (4.0 g) underwent chromatography on a silica gel column (Φ 25x100 mm) using chloroform-methanol (10:1, v/v) to obtain three fractions (E_1-E_3). E_2 was recrystallized to generate compound 1 (65 mg). In addition, 2.0 g n-butyl alcohol fraction underwent chromatography on an minimum dead space (a reversed-phase chromatography material manufactured by Beijing Medicine Technology Center, Beijing, China) column (Φ 30x150 mm) using V (MeOH):V (H_2O)=5:5 to obtain five fractions (B_1-B_5). B_1 and B_4 were recrystallized to obtain compounds 2 (315 mg) and 5 (440 mg), respectively. B_2 and B_3 underwent chromatography on a silica gel, eluted with chloroform-methanol-water (7:3:0.3, v/v), and were recrystallized to obtain compounds 3 (35 mg) and 4 (30 mg), respectively. The structures of compounds 1-5 were identified by analyzing the signals of spectral data [mass spectrometry, ^{1}H- and ^{13}C-nuclear magnetic resonance, and their values were reported in the authors' previous study (27)] and are presented in Fig. 1. The compounds used in this study were checked by HPLC and were >98% pure.

Cell culture and stimulation. RAW264.7 macrophages were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS, 100 U/ml penicillin and 100 U/ml streptomycin in tissue culture dishes at 37˚C, in a humidified atmosphere containing 5% CO_2. For most experiments, cells were plated at a density of $1x10^6$ cells/well in 12-well plates in 200 μl DMEM. Adherent

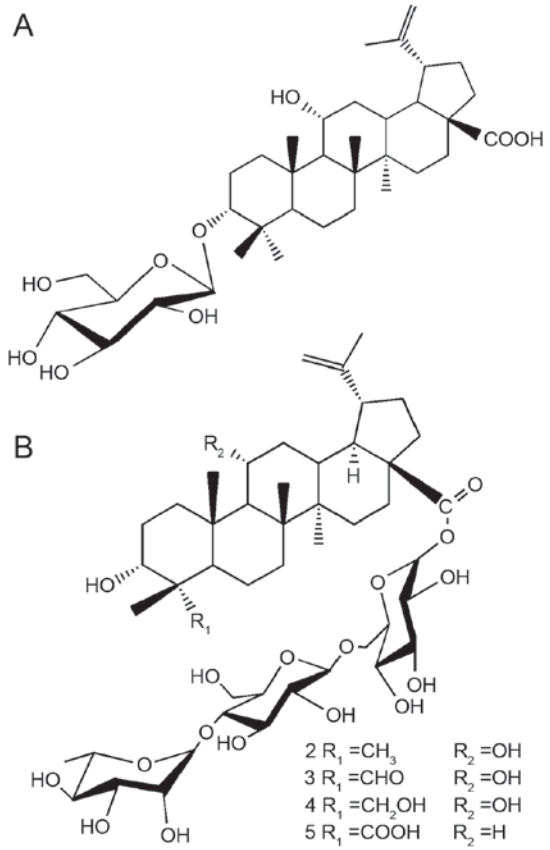

Figure 1. Chemical structures of compounds 1-5. (A) Compound 1 3-O-β-*D*-glucopyranosyl 3α, 11α-dihydroxylup-20(29)-en-28-oic acid; (B) compound 2, acantrifoside A; compound 3, acankoreoside D; compound 4, acankoreoside B; compound 5, acankoreoside A.

RAW264.7 cells in 12-well culture plates were gently washed, and cultured in serum-reduced OPTI-MEM I medium (Gibco; Thermo Fisher Scientific, Inc.) for 8 h prior to treatment with vehicle (0.1% DMSO) or various concentrations of compounds 1-5 (5-150 μM, dissolved in 0.1% DMSO). After 1 h of treatment with various concentrations of compounds 1-5 or vehicle, LPS was added at a final concentration of 100 ng/ml at varying time intervals. Cells were incubated with LPS at 37˚C until further analysis.

Cytotoxicity assay. The cytotoxicity of compounds 1-5 was assessed in RAW264.7 cells using an MTT-based test (EZ4 U cell proliferation and cytotoxicity assay kit; Biomedica Medizinprodukte GmbH & Co KG) according to the manufacturer's protocol. Briefly, $1x10^3$ cells/well were seeded in quadruplicate in 96-well microplates and were cultured with various concentrations of compounds 1-5 (5-150 μM) for 48 h. Subsequently, 20 μl EZ4U reagent was added to each well and the cells were incubated for 4 h at 37˚C. Absorbance was recorded using a Spectrafluor fluorometer (Tecan Group Ltd., Männedorf, Switzerland) at 450 nm; the reference wavelength was 620 nm. The spectrophotometer was calibrated to 0 absorbance using cell-free culture medium. Cell viability (%) relative to control was calculated as follows: (A) test/(A) control x100, where A refers to absorbance. Data are presented as the mean ± standard deviation of three individual experiments performed in triplicate.

Detection of TNF-α and IL-1β production. Macrophages were pretreated with various concentrations of compounds 1-5 or DEX (10 μM, as a positive control) for 1 h, and were then exposed to LPS (100 ng/ml) for 6 h. Subsequently, culture media were collected and assessed using commercially available sandwich ELISA kits, according to the manufacturer's protocols (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, 50 μl incubation buffer, 50 μl standard diluent buffer, and 50 μl standards, controls or samples were added in triplicate to anti-TNF-α or anti-IL-1β-coated ELISA microplates, and 50 μl biotin conjugate solution was added to the monoclonal antibody-coated microtiter wells, with the exception of the chromogen blank wells. Plates were covered and incubated at room temperature for 90 min. Subsequently, the wells were aspirated and washed four times with wash buffer, after which 100 μl streptavidin-horseradish peroxidase working solution was added to each well and incubated for 30 min at room temperature. Solutions were aspirated and the wells were washed a further four times, after which 100 μl stabilized chromogen was added to each well, and the plates were incubated for 30 min at room temperature in the dark. Finally, 100 μl stop solution was added to each well, and absorbance was measured at 450 nm using a plate reader (Perkin Elmer Cetus; PerkinElmer, Inc., Waltham, MA, USA). Absorbance values were normalized using a standard curve.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis. Macrophages were pretreated with various concentrations of compounds 1-5 (10, 20, 30, 40 or 50 μM) for 1 h and were then exposed to LPS (100 ng/ml) for 2 h. After washing twice with PBS, total RNA was extracted from the treated RAW264.7 cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. RNA isolation was conducted in an RNase-free environment. Subsequently, 4 μg RNA was reverse transcribed at 42˚C for 1 h using MuLV reverse transcriptase (Promega Corporation, Madison, WI, USA), oligo (dT)16 primer, dNTP (0.5 μM) and 1 unit RNase inhibitor, and terminated by heating at 70˚C for 15 min. Then PCR analyses were performed on the aliquots of cDNA to detect TNF-α, IL-1β and β-actin (as an internal standard) gene expression using a DNA gene cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Reactions were carried out in a volume of 25 μl containing 1 unit of Taq DNA polymerase (cat. no. D7209; Beyotime Institute of Biotechnology), 0.2 mM dNTP, 10X reaction buffer, and 100 pmol of the 5' and 3' primers. The PCR cycle was as follows: 95˚C for 10 min; 40 cycles of 95˚C for 15 sec and 60˚C for 1 min. The PCR primer sequences used in the present study were as follows (Takara Biotechnology Co., Ltd., Dalian, China): TNF-α, sense 5'-GAATGGGTGTTCATCCATTCT-3', anti-sense 5'-GCT TAAGTGACCTCGGAGCTTACA-3'; IL-1β, sense 5'-TTG ACGGACCCCAAAGAGTG-3', anti-sense 5'-ACTCCTGTA CTCGTGGAAGA-3'; and β-actin, sense 5'-ATGGTGGGA ATGGGTCAGAAG-3' and anti-sense 5'-GGAAGATGT TACTCGACGAGC-3'. After amplification, the PCR reaction products were separated by 1.2% agarose gel electrophoresis, and were visualized by ethidium bromide staining and ultraviolet irradiation.

Western blot analysis. Macrophages were pretreated with compounds 1-5 for 1 h and were then exposed to LPS (100 ng/ml) for 24 h. RAW264.7-conditioned medium was harvested, filtered and concentrated through a Centricon YM-10 ultrafilter (pre-wetted with distilled water; EMD Millipore, Billerica, MS, USA) according to the manufacturer's protocol. Concentrated samples were stored in aliquots at -80˚C.

Cells were harvested and washed three times with cold PBS, after which total protein extracts were isolated from the RAW264.7 cells. Briefly, the cell pellet was resuspended in 100 ml cell lysate buffer (50 mmol/l Tris, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 0.1% SDS, 0.02% sodium azide and 1% Nonidet P-40). After mixing at 4˚C for 30 min on a shaking platform and undergoing ultrasonication at 20 KHz for 30 sec, the cellular extracts were centrifuged at 12,000 x g for 30 min in a microfuge at 4˚C, and the supernatants were stored in aliquots at -80˚C. Protein concentration was determined using the Enhanced Bicinchoninic Acid Protein Assay kit (Beyotime Institute of Biotechnology).

Subsequently, 25 μl processed conditioned medium or 15 μg total proteins from cellular extracts were loaded onto a 12% SDS-PAGE gel. After electrophoretic separation, proteins were transferred to a polyvinylidene fluoride membrane. The membrane was blocked with 5% fat-free skim milk in Tris-buffered saline containing 0.05% Tween-20 (TBST) at room temperature for 1 h, and was then incubated with anti-HMGB1 (cat no. ab18256; 1:1,000; Abcam) or anti-β-actin primary antibodies (cat. no. sc-130656; 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at 4˚C overnight. After washing with TBST, the membrane was incubated with a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary antibody (cat. no. A0208; 1:5,000; Beyotime Institute of Biotechnology) at room temperature for 1 h, followed by extensive washing. The blot was visualized using enhanced chemiluminescence detection reagent (Thermo Fisher Scientific, Inc.) and was semi-quantified by densitometry using Quantity One software (version 4.6.2; Bio-Rad Laboratories, Inc.). The protein expression levels of in cellular extracts were normalized to β-actin levels.

Determination of NF-κB activation. Macrophages were pretreated with compounds 1-5 (10, 20, 30, 40 or 50 μM) for 1 h and were then exposed to LPS (100 ng/ml) for 2 h. Following stimulation, nuclear extracts were prepared using a Cayman Nuclear Extraction kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's protocol. The suspension was centrifuged at 16,000 x g, for 5 min at 4˚C and the supernatants containing cytosolic fractions were stored at -80˚C for subsequent analysis of cytoplasmic NF-κB. Levels of NF-κB were measured using an NF-κB p65 ELISA kit (cat. no. 7174; Cell Signalling Technology, Inc., Danvers, MA, USA), according to the manufacturer's protocol.

Statistical analysis. All the experiments were repeated at least three times. The results are expressed as the mean ± standard deviation. One-way analysis of variance with Duncan's multiple range tests was used to examine the difference between groups, and Student's t-test was also used to examine the difference between vehicle and LPS groups.

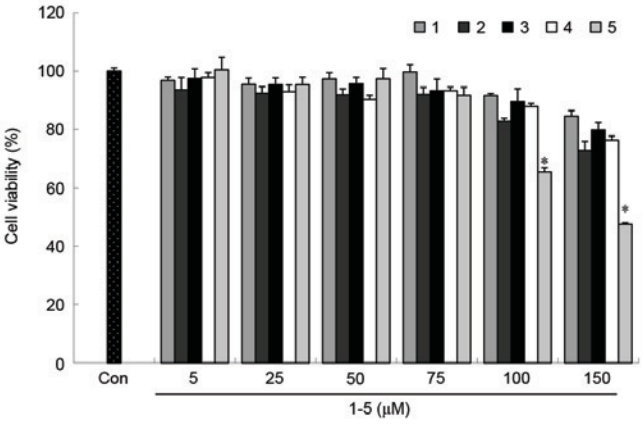

Figure 2. Cell viability of RAW264.7 macrophages treated with compounds 1-5. *P<0.05 vs. the Con group. Con, vehicle control.

In all comparisons, P<0.05 was considered to indicate a statistically significant difference. All statistical analyses were performed using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Effects of compounds 1-5 on cell viability. The viability of RAW264.7 cells was determined following treatment with compounds 1-5 using the MTT-based EZ4 U assay. Cytotoxicity was only observed in RAW264.7 cells treated with concentrations of compound 5 >75 μM (Fig. 2). Therefore, subsequent experiments were performed using concentrations ≤50 μM for all five compounds.

AGS-derived compounds inhibit TNF-α and IL-1β production in LPS-stimulated RAW264.7 macrophages. TNF-α and IL-1β are multifunctional proinflammatory cytokines involved in the regulation of the immune response, hematopoiesis and inflammation. To determine the effects of AGS-derived compounds 1-5 on the release of TNF-α and IL-1β, RAW264.7 cells were incubated with compounds 1-5 for 1 h and were then exposed to LPS (100 ng/ml) for 6 h to induce inflammation. The levels of TNF-α and IL-1β secreted from RAW264.7 cells were determined using an ELISA assay. Dex (10 μM), which is a widely used anti-inflammatory agent, was used as a positive control.

In LPS-stimulated RAW264.7 cells pretreated with compounds 3-5, the secretion of TNF-α was significantly decreased in a concentration-dependent manner compared with the control-treated cells (P<0.05; Fig. 3A). Compound 5 elicited the greatest suppressive effect, and 50 μM compound 5 inhibited TNF-α production by 77%, which was similar to the effects observed in cells treated with the positive control DEX. However, compounds 1 and 2 exhibited no obvious inhibitory effects on TNF-α release when used between 10 and 50 μM (Fig. 3A).

In LPS-treated RAW264.7 cells treated with compounds 1-5, IL-1β secretion was significantly decreased in a concentration-dependent manner (P<0.05; Fig. 3B). Similar to the results regarding TNF-α secretion, compound 5 demonstrated the strongest inhibitory effect with regards to IL-1β secretion. At

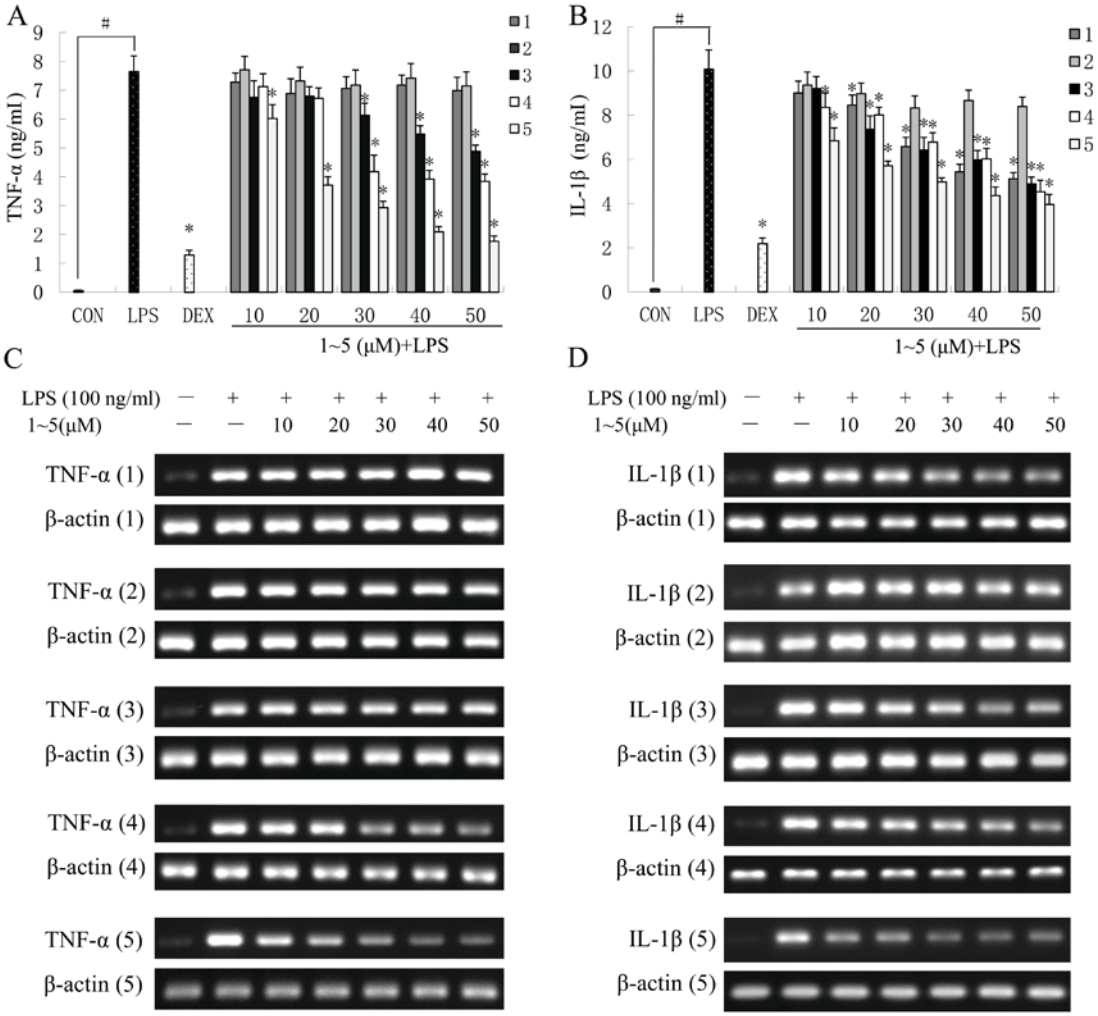

Figure 3. Effects of compounds 1-5 on TNF-α and IL-1β production, and mRNA expression, in LPS-induced RAW264.7 cells. (A and B) Effects of compounds 1-5 on LPS-induced (A) TNF-α and (B) IL-1β production. LPS values refer to cells treated with LPS (100 ng/ml) only. DEX values refer to the positive control group, in which cells were treated with LPS (100 ng/ml) and DEX (10 μM). $^{\#}$P<0.05 vs. the Con group; *P<0.05 vs. the LPS group. (C and D) Effects of compounds 1-5 on LPS-induced (C) TNF-α and (D) IL-1β mRNA expression in RAW264.7 cells. β-actin was used as the internal control. Con, vehicle control; DEX, dexamethasone; IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor.

a concentration of 50 μM, compound 5 significantly inhibited IL-1β production by 60.7% compared with the LPS-stimulated RAW264.7 cells (Fig. 3B).

Compounds 1-5 suppress TNF-α and IL-1β mRNA expression in LPS-stimulated RAW264.7 macrophages. To confirm whether the inhibition of TNF-α and IL-1β production was due to decreased gene expression, the mRNA expression levels of IL-1β and TNF-α were detected in LPS-stimulated RAW264.7 cells. As presented in Fig. 3C and D, pretreatment with the lupane-type triterpenes suppressed the mRNA expression levels of TNF-α and IL-1β, which coincided with the protein levels of TNF-α and IL-1β detected in the cell culture medium. These results indicated that compounds 1-5 were able to inhibit the expression of IL-1β and TNF-α at the transcriptional level, which, in turn, reduced the production of IL-1β and TNF-α in the LPS-stimulated RAW 264.7 cells.

Effects of compounds 1-5 on HMGB1 secretion in LPS-induced RAW264.7 macrophages. To examine the effects of compounds 1-5 on the expression of HMGB1, RAW264.7 cells were incubated with compounds 1-5 and were then exposed to LPS (100 ng/ml). The protein expression levels of HMGB1 in cell supernatants or whole cell lysates were analyzed by western blot analysis.

HMGB1 is an intracellular protein that, when present in the extracellular matrix, acts as a 'necrotic marker' for the immune system. Studies indicate that damaged or necrotic cells can release HMGB1 into the extracellular matrix, where it triggers inflammatory responses (28). The HMGB1 protein was low in the RAW264.7 cell supernatants without LPS-stimulation. However, HMGB1 protein levels in the RAW264.7 cell supernatants were significantly increased following LPS stimulation, and preincubation with compound 5 reversed this effect. The relative increase in the expression of secreted HMGB1 in the supernatant following LPS stimulation was significantly reduced by treatment with compound 5 (Fig. 4A and B).

Following western blot analysis of whole cell lysates, it was demonstrated that in LPS-treated RAW264.7 cells pretreated with compound 5 at 20, 30, 40 or 50 μM, the expression of HMGB1 protein was not obviously altered. These findings

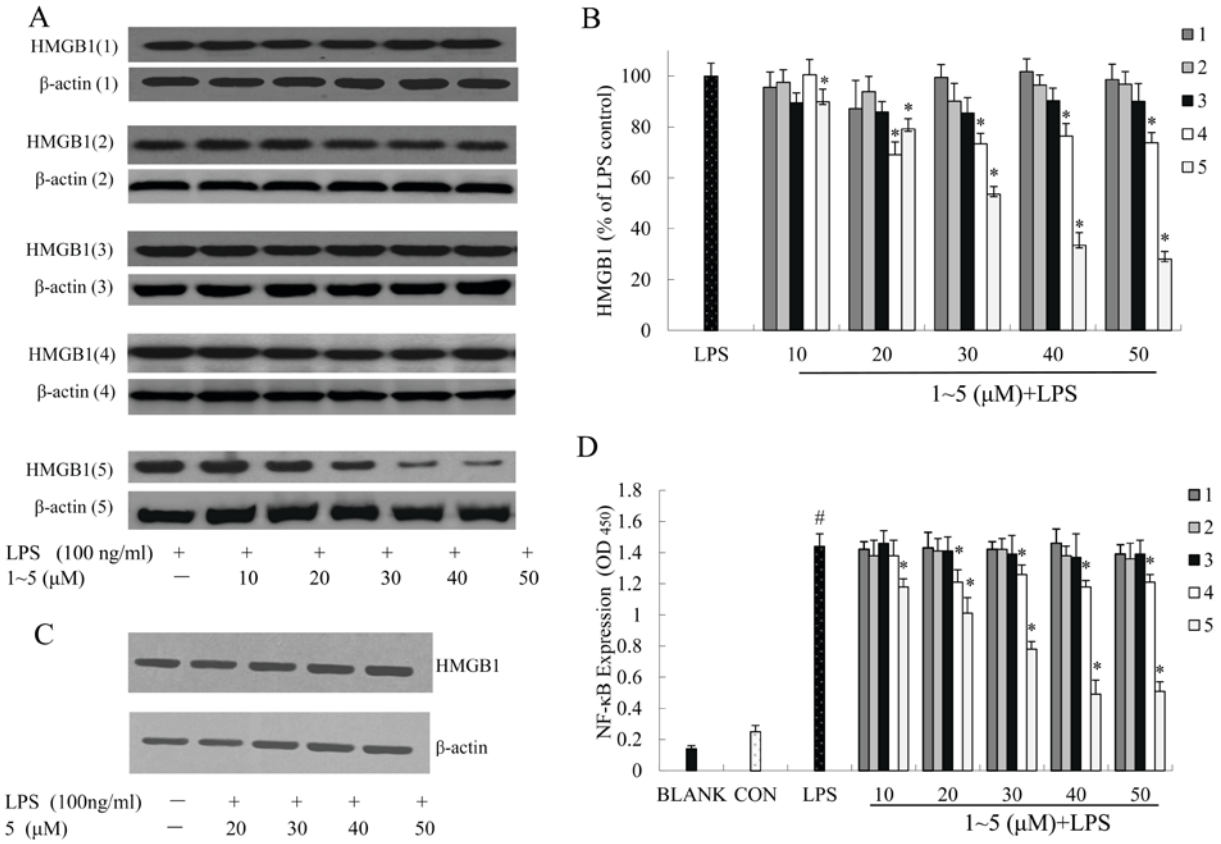

Figure 4. Effects of compounds 1-5 on the protein expression levels of HMGB1 and the transcriptional activity of NF-κB in LPS-treated RAW264.7 cells. (A) Effects of various concentrations of compounds 1-5 on the protein expression levels of HMGB1 in the cell supernatant. (B) Semi-quantitative densitometric analysis of HMGB1 protein expression in LPS-treated RAW264.7 cells. *P<0.05 vs. LPS group. (C) Effects of compound 5 on HMGB1 protein expression in LPS-treated RAW264.7 cells. The protein expression levels of HMGB1 in cellular extracts were normalized to β-actin levels. (D) Effects of compounds 1-5 on NF-κB transcriptional activity in LPS-stimulated RAW264.7 macrophages. #P<0.05 vs. the Con group; *P<0.05 vs. the LPS group. Con, vehicle control; HMGB1 high-mobility group box protein 1; LPS, lipopolysaccharide; NF, nuclear factor; OD, optical density.

suggested that treatment with compound 5 induced a significant decrease in the secretion of HMGB1; however, it did not alter the steady-state levels of HMGB1 protein in RAW264.7 cells (Fig. 4C).

Effects of compounds 1-5 on NF-κB transcriptional activity in LPS-stimulated RAW264.7 macrophages. RAW264.7 cells were stimulated with or without LPS (100 ng/ml) in the absence or presence of compounds 1-5 (10, 20, 30, 40 or 50 μM) for 24 h. Nuclear proteins were extracted, and NF-κB activity was determined using the NF-κB p65 ELISA kit. Control values were obtained in the absence of LPS and compounds 1-5. LPS values were obtained in the presence of LPS (100 ng/ml) and absence of compounds 1-5. Our aforementioned results demonstrated that compound 5 inhibited HMGB1 secretion in LPS-induced RAW264.7 macrophages. Therefore, the present study aimed to determine whether the upstream NF-κB signal transduction pathway was involved. The results indicated that NF-κB activity was increased in RAW264.7 cells stimulated with LPS (100 ng/ml), and that compound 5 significantly reduced NF-κB activity in a dose-dependent manner (Fig. 4D).

Discussion

Macrophages can be stimulated by various agents, including LPS, to produce TNF-α, IL-1β and HMGB1 (29), which are known to serve important roles in the immune response. Previous studies regarding sepsis have largely focused on the suppression of early inflammatory cytokines during the super-acute inflammatory response. The use of antibodies and antagonists specific to these early inflammatory cytokines has had some success in avoiding the development of septic shock in animal models (30-32). However, clinical trials using these antagonists to treat patients with sepsis did not produce satisfactory results (33,34). This may be because the intervention of early inflammatory cytokines is not feasible. HMGB1 is considered a late inflammatory cytokine, which appears relatively late in the inflammatory response, and has a long duration. Serum concentrations of HMGB1 have been revealed to increase late (16-24 h) in patients with sepsis (5,35). In addition, HMGB1 protein inhibitors or antagonists have been reported to significantly reduce the incidence of lethal endotoxemia and resulting acute tissue damage, even when given 24 h following the occurrence of endotoxemia and sepsis in mice (36). Furthermore, HMGB1 may enhance the inflammatory response by stimulating various cells to synthesize other proinflammatory cytokines. Therefore, inhibition of HMGB1 is considered a potential target for reducing mortality and complications in patients with sepsis.

Previous studies regarding herbal medicine have been conducted to identify potential natural anti-inflammatory

properties in various *in vitro* and *in vivo* systems. AGS is an important constituent of traditional Chinese medicine, which has been used since ancient times to treat various diseases. Although numerous pharmacological and biochemical pharmacokinetic studies of AGS-derived compounds have previously been conducted, the potential existence of anti-inflammatory properties of lupane-type triterpenoids has not been explored. The present study demonstrated that saponin compunds 1-5 reduced the production of TNF-α and IL-1β. Acankoreoside A (compound 5) and acankoreoside B (compound 4) were also able to suppress HMGB1 secretion and reduce NF-κB activity induced by LPS in RAW 264.7 macrophages. These results indicated that the anti-inflammatory effects of AGS were due to the saponins present in this plant.

Based on the results of present study, it was hypothesized that the anti-inflammatory effects of these saponins related to their structures. The functional groups at C-3 and C-28 may have no effect on anti-inflammatory activity. However, the presence of functional groups at C-23 or an hydroxyl group at C-11, may affect the anti-inflammatory activity. The authors' of the present study hypothesize that the order of the anti-inflammatory activity of functional groups at C-23 was revealed to be: -COOH>-CH_2OH>-CHO>-CH_3. Acankoreoside A (5), which possesses a carboxyl group at C-23 and no hydroxyl group at C-11, was able to significantly inhibit the expression levels of TNF-α, IL-1β and HMGB1. Further studies are necessary to research the structure-activity relationship of lupane-type triterpenoid with groups at C-3, C-11, C-23, or C-28.

In conclusion, the present results demonstrated that AGS-derived lupane-type triterpenoid acankoreoside A (compound 5), may exert anti-inflammatory effects by inhibiting NF-κB activation in macrophages, and thus, preventing the expression of TNF-α, IL-1β and HMGB1. Accordingly, these results suggested that acankoreoside A is a promising therapeutic agent for the treatment of inflammatory diseases, including rheumatoid arthritis, scapulohumeral periarthritis, cervical spondylosis and slipped disk. Furthermore, the discovery of the anti-inflammatory properties of acankoreoside A (compound 5) indicate that future studies are required to identify other potentially beneficial pharmacological mechanisms underlying AGS-derived compounds.

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