Uncaria sinensis(Oli.) Havil Suppressendotoxin-induced Proinflammatory Responses through Blocking ERK Signaling Pathways

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Uncaria sinensis(Oli.) Havil (USH) is used in traditional Korean medicine to treat inflammation such as amebic dysentery. In this study, we investigated the anti-inflammatory effect of USH. The water extract of USH significantly inhibits lipopolysaccharide (LPS)-induced nitrite oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-6and IL-12 productions in murine peritoneal macrophages. Furthermore, USH selectively inhibited activation of the extracellular signal-regulated kinase (ERK) but not of p38 MAPK, c-Jun N-terminal kinase (JNK) and nuclear factor-κB (NF-κB). In murine model, we found that administration of USH reduced serum levels of TNF-α, IL-6 and IL-12 productions in LPS-treated mice. Our results suggest that USH exerts ant-inflammatory effects in macrophages via inhibition of ERK activation and may be a useful therapeutic approach to inflammatory diseases.

Key words : LPS, *Uncaria sinensis(Oli.)* Havil, Inflammation, MAPK

Introduction

Mononuclear cells play a key role in the synthesis of proinflammatory cytokines, particularly TNF-α, IL-12, and IL-6, as well as an array of other inflammatory mediators. These pro inflammatory cytokines can in turn trigger secondary inflammatory cascades, including the production of cytokines, lipid mediators, and reactive oxygen species, as well as the up-regulation of cell adhesion molecules that facilitate the migration of inflammatory cells into tissues[1-4]. However, exuberant production of pro inflammatory cytokines leads to severe immunopathology such as endotoxic shock[5]. Thus, proper regulation of thepro- and anti-inflammatory balance at the appropriate time is assured.

The inflammatory responses to microbial infection are initiated by innate immunity via Toll-like receptors (TLRs) that recognize the pathogen-associated molecular patterns characteristic of microbial products[6]. The inflammatory response to LPS is mediated mainly by a receptor complex composed of LPS-binding protein CD14 and TLR4, which is critically important in the innate immune recognition of Gram-negative pathogens[7]. Upon activation of TLR4, the adaptor protein MyD88 is recruited to the receptor, which in turn triggers a cascade of signaling events leading to the activation of transcription factor NF-κB and mitogen- activated protein (MAP) kinases. NF-κB plays a pivotal role inthe transcription of a variety of proinflammatory cytokines, including TNF-α, IL-12, and IL-6. MAP kinases, including extracellular signal - regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38, also modulate cytokine expression at multiple levels. ERK is required for the transport of TNF-α mRNA from the nucleus to the cytoplasm[8]. p38 is also a crucial mediator for the production of proinflammatory cytokines[9] and has been identified as the target of a class of small molecule inhibitors capable of inhibiting the production of inflammatory cytokines[10].

Uncaria sinensis(Oli.) Havil (USH) are used in traditional Korean medicine to treat inflammation-related diseases. In this study, we investigated the anti-inflammation and immunosuppressive effect of water extract of USH in vitro and in vivo. We demonstrate firstly USH significantly reduce the NO production and suppresses pro inflammatory TNF- α, IL-12 and IL-6 release as well as at mRNA level when response to bacterial LPS in peritoneal macrophage culture medium. Further more, the cytokines from serum of extract fed mouse are reduced, too. In addition we show the USH selectively inhibited activation of the ERK, whereas activation p38 MAPK, JNK and NF-κB were unaffected. Thus USH is able to ameliorate inflammatory disease by exerting anti inflammatory and immunosuppressive activities.

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Materials and Methods

1. chemicals and reagent

RPMI-1640, fetal bovine serum (FBS), penicillin, and streptomycin were obtained from Gibco BRL (Grand Island, NY, U.S.A.). ELISA kits for mouse IL-12, IL-6 and TNF-α, detection were purchased from R&D System. LPS (from Escherichia coli 055:B5), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and Griess reagent were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Abs against total and phosphospecific MAPKs were from Cell Signaling Technology (Beverly, MA). IκB-α monoclonal antibodies and the peroxidase-conjugated secondary antibody were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Prestained SDS-PAGE markers were from Bio-Rad. TRIzol reagent, MML-V reverse transcriptase and PCR reaction kit are purchased from Invitrogen orporation.(Carlsbad, California, U.S.A.). iNOS, IL-12, IL-6, TNF-α and β -actin oligonucleotide primers were purchased from Genotech Co. Ltd. (Daejeon, Republic of Korea). C57BL/6 mice were purchased from Orient bio Co. (Sungnam, KyungKiDo, Republic of Korea).

2. Plant material and extract preparation

The stem of USH were purchased from Ohmi Herb and authenticated by H.J. Song, College of Oriental Medicine Wonkwang University. The 1.0 ㎏ dried prescription of herbs was decocted with boiling distilled water.The duration of decoction was about 3 hours and finally dried by a freeze drier to yield the extract (62 g) The water extracts was suspended in H_2O and filtered with 0.22 um syringe filter.

3. Peritoneal macrophage

Thioglycollate(TG)-elicited macrophages were harvested 4 d after i.p. injection of 2.5 ml TG to the mice and isolated, as reported previously[11]. Using 8 ml of HBSS containing 10 U/ml heparin, peritoneal lavage was performed. The cells were distributed in RPMI which was supplemented with 10% heat-inactivated FBS, in 12-well tissue culture plates .(1 x 10^6cells/well). After incubation for 3 h, non adherent cells were removed and adherent cells were treated LPS (0.5 g/ml) in the absence/presence with USH.

4. Blood Samples and cytokine measurement(*in vivo*)

USH were orally given to mice (n=6 per group) at 500 mg/kg and 50 mg/kg for 1 weeks. The serum samples were taken from the mice at 3 h after challenge with 25 mg/kg LPS and then stored at -70℃ until use. Cytokine levels in the serum were determined according to the manufacturer's instruction.

5. Nitrite Quantification

Nitrite oxide synthesis was determined by assaying culture supernatants for nitrite, the stable reaction product of NO and molecular oxygen. Nitrite concentration was determined by the Griess reaction. Briefly, 100 μl of culture supernatants were incubated with equal volumes of the Griess reagent (1% sulfanilamide/0.1% N-(1-naphthyl)-ethylenediamine dihydrochroride/2.5% H_3PO_4) at room temperature for 10 min and absorbance (540 nm) was determined in a spectrophotometer. Nitrite concentration was determined by using sodium nitrite as a standard

6. Cytokine measurements(ELISA Assay)

Mouse peritoneal macrophages pretreated with various concentrations of PKN were further cultured in medium with LPS for 24 h. Culture supernatants were collected and stored at -70℃ until use for TNF-α, IL-6, IL-12 detection. Cytokine levels in the supernatants were determined according to the manufacturer's instruction.

7. Reverse transcriptase-PCR(RT-PCR)

Total RNA of cultured cells were extracted by using TRIzol reagent, and cDNA was synthesized by MML-V reverse transcriptase followed manufacturer's protocols. For the reverse transcriptase (RT) reaction, total RNA (3 ug) with 0.5 ug of oligo-(dT) was denatured at 70℃ for 10 minutes. Then, 1X single strand buffer, 0.5 mM DTT, 500 mM of each dNTPs and 200 U of MMLV reverse transcriptase were added. The RT reactions were performed at 42℃ for 1 hour. The polymerase chain reactions (PCR) were performed with 1 ml of the cDNA, 1X buffer, 1 mM $MgCl_2$, 200 mM of each dNTPs, and 0.2 mM of each specific primers. Optimal PCR conditions were 30 cycles of 30 seconds at 92℃, 45 seconds at 58℃, and 30 seconds at 72℃. The following primer pairs were used: for inos : TGG GAA TGG AGA CTG TCC CAG (forward) and GGG ATC TGA ATG TGA TGT TTG (reverse); tnf-α : ATG AGC ACAGAA AGC ATG ATC (forward) and TAC AGG CTT GTC ACT CGA ATT (reverse) ; il-6 : CAT CCA GTT GCC TTC TTG GGA (forward) and CAT TGG GAA ATT GGG GTA GGA AG (reverse); il-12p40 : GGA GAC CCT GCC CAT TGA ACT (forward) and CAA CGT TGC ATC CTA GGA TCG (reverse); β-actin: TGT GAT GGT GGG AAT GGG TCA G (forward) and TTT GAT GTC ACG CAC GAT TTC C (reverse). A 10 μl aliquot of the amplified DNA reaction mixture was subjected to 1.5% agarose gel electrophoresis, and the amplified product was visualized by UV fluorescence.

8. Western Blot Analysis

Mouse peritoneal macrophages pretreated with various concentrations of PKN were further cultured in medium with 500 ng/ml LPS for different times to detect phosphorylated-ERK1/2, phosphorylated -JNK and phosphorylated-p38, and IKB-. After washing with PBS, cells were lysed with SDS sample buffer containing 50mM Tris (pH 7.4), 2% SDS (wt/vol), 5% 2-mercaptoethanol and 10% glycerol. Samples were heated at 95℃ for 5 min and centrifuged (13,000 g, 5 min) at 4℃ and the supernatant was analyzed by 10% SDS - PAGE. Proteins were transferred to PVDF membranes, and the membranes were blocked with 5% nonfat dry milk in TBST [20 mM Tris (pH 8.0), 150 mM NaCl and 0.1% Tween-20]. The membranes were blotted with the primary antibody, then with a horseradish peroxidase-conjugated secondary antibody and detected by enhanced chemiluminescence according to the manufacturer's instructions. Molecular weights of proteins were estimated by using prestained SDS - PAGE markers.

9. Statistical analysis

The experiments shown are a summary of the data from at least-three experiments and are presented as the mean S.E.M. Statistical evaluation of the results was performed by independent t-test.

Results

1. USH Have no Cytotoxicity and Reduce the LPS-induced NO production as well as iNOS mRNA in Mouse Peritoneal Macrophage

In order to assess the cytotoxic effect of USH on the mouse peritoneal macrophages, the cells were cultured with PKN for 24 h, and MTT assay was then carried out. The viability of cells incubated with USH extract at concentrations of at final concentrations of 0.01 mg/ml, 0.1 mg/ml and 1 mg/ml was 102%, 99% and 97% of the control value, respectively(Fig. 1A). NO has been associated with many diseases conditions including inflammation and cancer. To examine the effect of USH on LPS-induced NO production in peritoneal macrophages, cells were pretreated with USH (0.01 -1 mg/ml) for 30 min and then stimulated with LPS (500 ng/ml) for 24 h. The cell culture medium was then harvested and NO levels weredetermined using Griess reaction. As shown in Fig. 1B, USHinhibited NO production in dose-dependent manner. Stimulation of macrophages with LPS expresses the iNOS, which is responsible for the production of a significant amount of NO[13,14]. To determine whether the inhibition of LPS-induced NO production by USH was mediated by the regulation of iNOS expression, RT-PCR analyses were performed. The expression of iNOS mRNA was significantly elevated in macrophages treated with LPS (500 ng/ml) compared to unstimulated cells (control). When the cells were pretreated with the USH for 30 minutes before LPS stimulation, the expression of iNOS mRNA (Fig. 1C) were inhibited in a concentration dependent manner in respect to LPS-stimulated macrophages.

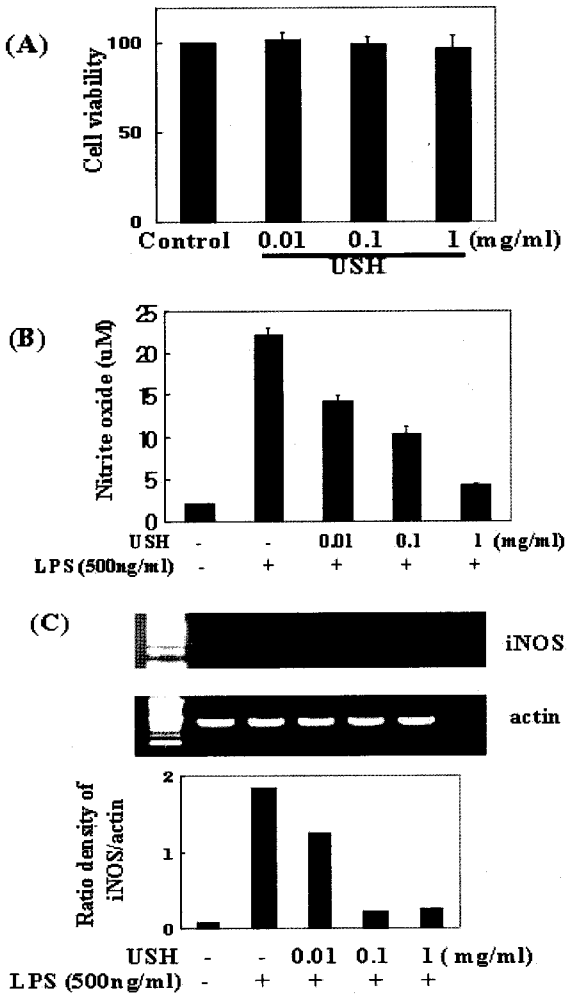

Fig. 1. USH inhibits NO production and iNOS expression in LPS stimulated peritoneal macrophages. (A): Peritoneal macrophages were treated with various USH (as indicated) and the cell viability was detected by MTT assay after 24h exposure. Values are the mean±S.D. of three separate experiments performed in quadruplicate. p(vs. control. (B) Effect of USH on LPS-induced NO production in peritoneal macrophage. Nitrite concentrations in the medium of peritoneal macrophage cultures at 24after treated with LPS (500USH (0.01-1 mg/ml) was applied to the medium 30 min. before LPS treatment. Each bar shows the means±S.D. of triplicate determinations. p(vs. LPS(C): Cells were treated similarly as for (B). RT-PCR was performed as descript in material and methods. The relative density of DNA was analyses with a Quantity One program (Bio-Rad Laboratories, Inc.) The best result of three independence experiments was shown.

2. USH Inhibited TNF-α, IL-6, IL-12 cytokines from LPS-Activated Mouse Peritoneal Macrophages

Stimulation of macrophages by LPS markedly increased the production of both TNF-α, IL-6 and IL-12[15].

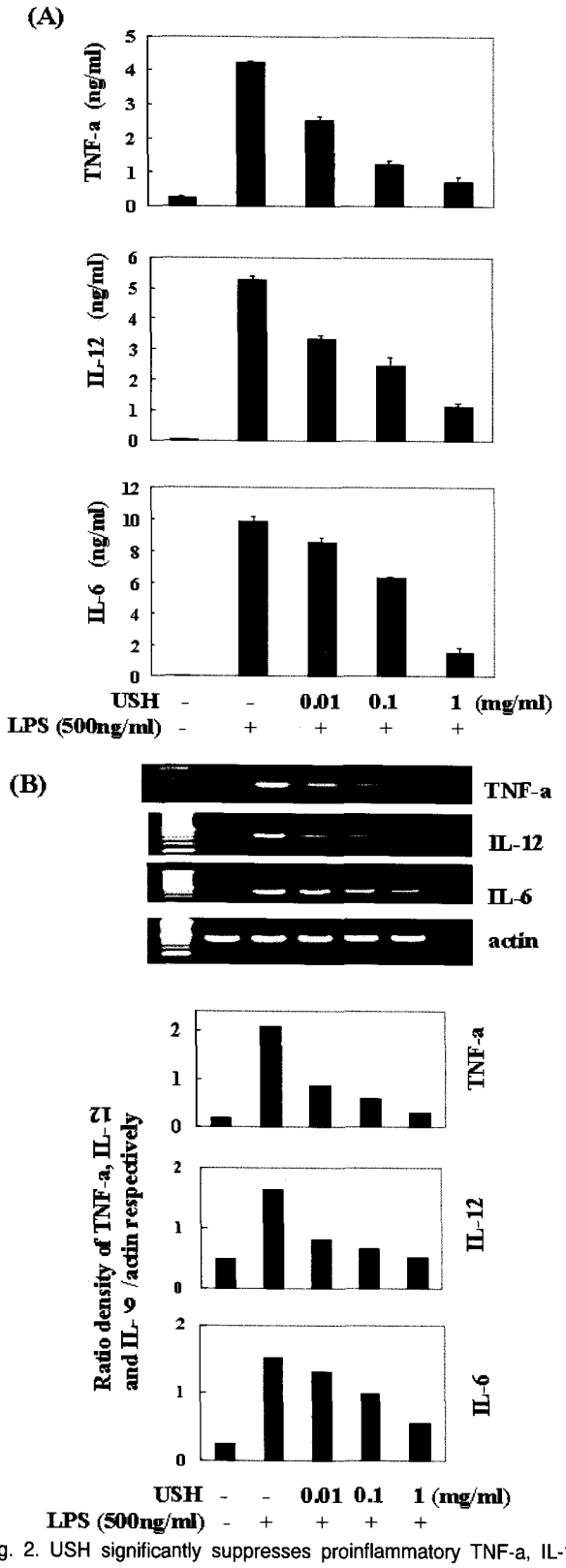

Fig. 2. USH significantly suppresses proinflammatory TNF-a, IL-12 and IL-6 release when response to bacterial LPS in peritoneal macrophage. (A) Mouse peritoneal macrophages pretreated with various concentrations of USH were further cultured in medium with LPS for 24 h. Culture supernatants were collected used for TNF-a, IL-6, IL-12 detection. Each bar shows the means±S.D. of triplicate determinations. p(vs. LPS. (B) Cells were treated similarly as for (A). RT-PCR was performed as descript in material and methods. The relative density of DNA was analyses with a Quantity One program (Bio-Rad Laboratories, Inc.). The best result of three independence experiments was shown.

We examined the effect of water extract of USH used ELISA assay to examine the secretion of TNF-α, IL-6, IL-12 from the culture supernatant of LPS-stimulated mouse peritoneal macrophages. TNF-α, IL-6 and IL-12 in the culture supernatants of LPS-stimulated macrophages were also inhibited by the extract in a concentration-dependent manner(Fig. 2A). At mRNA level, the reduction of TNF-α, IL-6 and IL-12 in the cell lysate was also demonstrated(Fig. 2B).

3. USH Inhibited ERK1/2 Phosphorylation but Failed to Affect Both p38 and JNK Kinases as Well as NF-kB Activation in LPS-Activated Mouse Peritoneal Macrophages

The activation of the MAPKs p38, ERK1/2 and JNK as well as the activation of transcription factor NF-kB are important signaling pathways responsible for the production of pro-inflammatory cytokines when macrophage is activated. When stimulated with LPS, p38, ERK1/2 and JNK were strongly activated in macrophages. P38, ERK1/2 and JNK were all activated in peritoneal macrophages, and the degradation of IkB-α occurred 15 min after LPS treatment(Fig. 3). When 1mg/ml of USH is added, it significantly inhibited ERK1/2 phosphorylation in LPS-stimulated macrophages(Fig. 3). However, as shown in the same figure, USH did not alter the activation of the other three intracellular pathways p38, JNK and NF-kB.

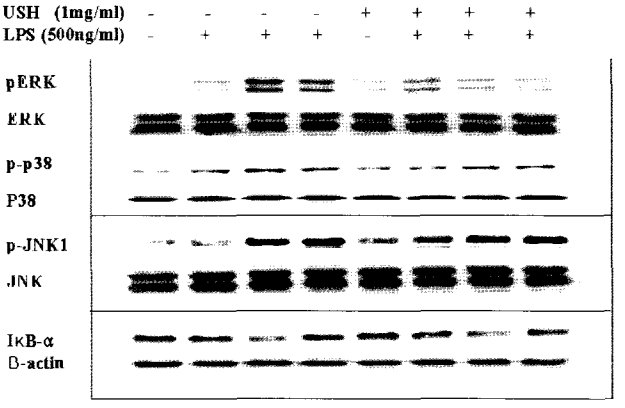

Fig. 3. USH significantly inhibited ERK1/2 phosphorylation in LPS-stimulated macrophages. Cultured peritoneal macrophages were stimulated with 500 ng/ml LPS for the indicated time points. At the compared group 1mg/ml USH was applied to the medium 30 min. before LPS stimulation. The activities of ERK, p38 MAPK and JNK were examined by Western blot analysis using phosphospecific Abs. The total protein levels of ERK, p38 MAPK and JNK also were measured. IκB-α and actin were examined by Western blot analysis, too. The best result of three independence experiments was shown.

4. USH Reduce the LPS-induced cytokines production in vivo

The effect of USH was examined on TNF-α, IL-6 and IL-12 production 3 h after LPS injection. As shown in Fig. 4, the production of TNF-α, IL-6 and IL-12 was inhibited by orally administration of USH.

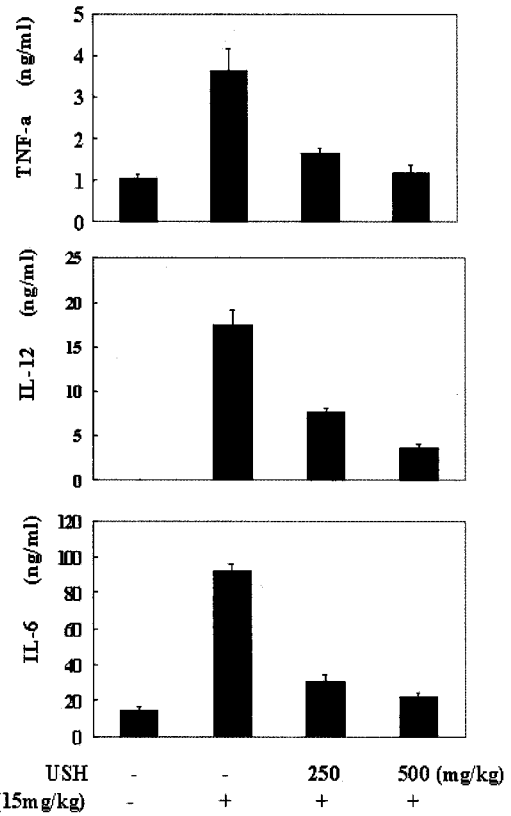

Fig. 4. USH inhibited TNF-α, IL-6 and IL-12 in the serum of bed mice. USH were orally given to mice (n=6 per group) at 250 mg/kg and 50 mg/kg for 1 weeks. The serum samples were taken from the mice at 3 h after challenge with 25 mg/kg LPS. Cytokine levels in the serum were determined by ELISA according to the manufacturer's instruction. Values are the mean±S.D. of 6 serum sample.

Discussion

Activation of macrophages through TLR4, the receptor for the bacterial endotoxin LPS, results in a potent inflammatory response[16]. TLR4 engagement results in activation of the mitogen-activated protein kinases (MAPKs), which, together with the NF-kB pathway, transduce extracellular signals to cellular responses and subsequently results in a potent inflammatory response characterized by the release of proinflammatory molecule and cytokines, such as TNF-a, IL-10, IL-6, IL-12 and nitrite oxide(NO)[17,18]. Activation of the innate immune system is important for subsequent activation of lymphocytes and other cell types and clearance of infectious organisms. However, Excessive production of inflammatory mediators is harmful to host tissue and in extreme cases can result in multiple-organ dysfunction syndromes or lethal septic shock[19]. Thus, study of the proper regulation of the pro- and anti-inflammatory balance response to LPS in macrophage is important and necessary for discovering potential therapeutic targets and drugs.

Uncaria sinensis(Oli.) Havil is used in traditional Korean medicine to treat inflammation-related diseases. But no experimental evidence is provided by now.

An inflammatory mediator NO is particularly important and participates in immunologic reactions, including the ability of macrophages to kill tumor cells and bacteria[20]. Some of the deleterious effects of LPS on organ function have been attributed to NO[21]. However, over expression of the production of NO leads to severe immunopathology such as septic shock, rheumatoid arthritis, and autoimmune disease[22]. Therefore, therapeutic agents that inhibit the NO production may be useful for the relieving these inflammatory conditions. In this study, we demonstrated that USH inhibited NO production in a dose dependent manner(Fig. 1B) in murine activated peritoneal macrophages, suggesting USH as a natural-occurring NO inhibitor. To search for the underlying mechanism for this potent effect of USH, we examined its effects on the transcriptional levels of iNOS enzymes. The inhibition of the expression of iNOS genes was evidenced by reductions in their mRNA levels in a noncytotoxic concentration-dependent manner(Fig. 1C). The inhibition of NO release by USH may be attributed to the inhibition of iNOS mRNA transcription.

MAPKs (ERK, p38 and JNK) as well as transcription factor NF-kB positively control TNF-α ,expression in LPS-activated macrophages, each with a unique signaling pathway. Inhibition of any of the three MAPK pathways or NF-kB pathway is sufficient to block induction of TNF-α by LPS in macrophages[23-25]. In this report, we have demonstrated that USH can negative regulate the innate immune response to LPS. We found that USHselective inhibited activation of the extracellular signal-regulated kinase (ERK), whereas activation of p38 MAPK, c-Jun N-terminal kinase (JNK) and NF-KB were unaffected in peritoneal macrophages response to LPS(Fig. 3). Thus, reduce the production of the proinflammatory cytokines TNF-α and IL-6 in macrophages(Fig. 2). The fact that the addition of USH inhibited LPS-induced mouse serum TNF-α, IL-6 and IL-12 further demonstrated USH can reducethese proinflammatory cytokines in vivo(Fig. 4). These results clearly indicate that USH can decrease the inflammatory responses and thereby protects the host from shock, multiple organ failure, and mortality upon exposure to LPS.

In conclusion, our results first provide experimental evidence, in vitro and in vivo, that Pulsatilla koreana Nakai is able to ameliorate inflammatory disease by exerting an anti-inflammatory activity. These effects of USH caused are through the inhibition of activation of the extracellular signal-regulated kinase (ERK), but not that of p38 MAPK , c-Jun N-terminal kinase (JNK) and NF-kB .

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