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Research Paper

Suppression of tumour necrosis factor-α by *Schizonepeta tenuifolia* water extract via inhibition of IκBα degradation and Jun N-terminal kinase/stress-activated protein kinase activation

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Abstract

Objectives The anti-inflammatory effects of an aqueous extract of *Schizonepeta tenuifolia* on lipopolysaccharide (LPS)-induced tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) *in vivo* and *in vitro* have been investigated.
Methods C57BL/6 mice were orally administered phosphate-buffered saline (control) or *S. tenuifolia* water extract (50, 200, 500 or 1000 mg/kg) for 10 days before intraperitoneal administration of LPS (1.3 mg/kg). Blood samples were obtained 1 h after LPS challenge, followed by determination of TNF-α and IL-6 levels. Peritoneal macrophages from thioglycollate-injected mice were obtained and stimulated with LPS and *S. tenuifolia* water extract for viability assay, cytokine analysis, real-time RT PCR and Western blotting.
Key findings Oral administration of *S. tenuifolia* water extract to mice significantly reduced LPS-induced serum levels of TNF-α, but not IL-6. When peritoneal macrophages were treated *in vitro* with *S. tenuifolia* water extract, the inhibition of LPS-induced TNF-α was more pronounced than that of IL-6 at the level of secreted protein and mRNA. *S. tenuifolia* water extract reduced the degradation of IκBα and the nuclear relocation of p65 NF-κB, but the phosphorylation of IκBα was not affected. Inhibition of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) by *S. tenuifolia* water extract led secondarily to the inhibition of phospho-c-Jun and phospho-ATF-2.
Conclusions These results indicated that the downregulation of TNF-α by *S. tenuifolia* water extract may have involved the inhibition of both IκBα degradation and activation of c-Jun and ATF-2 involving suppression of JNK/SAPK.
Keywords IκBα; Jun N-terminal kinase/stress-activated protein kinase; macrophages; *Schizonepeta tenuifolia*; tumour necrosis factor-α

Introduction

Inflammation is a normal response to tissue injury, where it plays a critical role in eliminating pathogenic microorganisms, clearing cellular debris, and promoting healing of damaged tissue. However, when inflammation is prolonged or inappropriately controlled, tissue repair is delayed and may be susceptible to the influences of additional harmful stimuli. If the response is not normalized in a timely manner, the condition may progress to a chronic inflammatory state, such as those associated with psoriasis, rheumatoid arthritis, inflammatory bowel diseases, asthma, septic shock and cancer. With a rise in the number of patients experiencing chronic inflammation, there is an increased demand for anti-inflammatory drugs with fewer side effects.

One of the recognized pharmacological effects of anti-inflammatory drugs is the suppression of pro-inflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6.[1] These cytokines stimulate the synthesis of acute-phase proteins by the liver and are produced primarily by macrophages.[2] Expression of these cytokines is controlled at the transcriptional and translational levels. Transcription factors, e.g. nuclear factor (NF)-κB, c-Jun/c-fos and c-Jun/ATF-2, and mitogen-activated protein (MAP) kinases e.g. ERK1/2, p38 and Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), activate or stabilize the mRNA for several pro-inflammatory cytokines including TNF-α.[3–6]

The dried spikes and stem of *Schizonepeta tenuifolia* are used medicinally as a main ingredient for traditional herbal formulae indicated for fever, sore throat, rhinitis, tonsillitis, urticaria and eczema.[7] Despite its extensive use in traditional herbal medicine, limited scientific information exists regarding the pharmacological actions of *S. tenuifolia*. An aqueous extract of *S. tenuifolia* was reported to inhibit the compound 48/80-induced systemic anaphylactic reaction and the anti-DNP-IgE-mediated passive cutaneous anaphylactic reaction *in vivo*.[8] The extract inhibited interferon-γ and IL-4 and increased IL-2 in anti-CD3-stimulated mice.[9] A recent study showed that *S. tenuifolia* water extract decreased the production of nitric oxide (NO)/inducible NO synthase and prostaglandin E_2/cyclooxygenase in lipopolysaccharide (LPS)-stimulated macrophages *in vitro*.[10] According to the same study, *S. tenuifolia* reduced TNF-α and IL-6 secretion through inhibition of p38, ERK1/2, and JNK/SAPK.[10] In this study, we have investigated the suppression of LPS-induced TNF-α and IL-6 by *S. tenuifolia* water extract (STE) *in vivo*. In addition, we have investigated the effects of STE on NF-κB and JNK/SAPK-mediated c-Jun/ATF-2 pathways.

Materials and Methods

Preparation of *S. tenuifolia*

Schizonepeta tenuifolia was purchased from Kyung Hee University Medical Center Medicinal Herbs (Seoul, Korea). Its authentication was confirmed by Professor Choi, of the Department of Herbology, Kyung Hee University. A voucher specimen (ST-2007) was deposited at the Department of Herbology. The plant sample was soaked into 1 vol distilled water overnight, and further dissolved using sonication for 1 h. The material was filtered and evaporated using a freeze dryer at –70°C (EYELA, Japan). The yield of STE was approximately 2.67%. The sample was dissolved in phosphate-buffered saline (PBS) and sterilized by passing through a 0.22-μm syringe filter.

Animals

C57BL/6 mice (male, 8-weeks of age) were obtained from Samtaco (Korea), maintained in a temperature- and humidity-controlled, pathogen-free animal facility at Kyung Hee University. Standard mouse chow was provided and water was freely available. Mice were maintained in accordance with the Guide for the Care and Use of Laboratory Animals issued by the US National Research Council (1996), and the protocol was approved by the Kyung Hee University institutional committee for the care and use of laboratory animals.

In-vivo lipopolysaccharide injection

C57BL/6 mice were divided into seven groups ($n = 7$ in each group). LPS (serotype 055:B5; Sigma, St Louis, MO, USA) (1.3 mg/kg) was injected intraperitoneally 1 h before blood sampling. Blood was obtained by orbital puncture and then mice were killed by CO_2 asphyxiation. The untreated group 1 and control group 2 were administered PBS orally for 10 days before LPS injection. Group 3 (dexamethasone group) was administered dexamethasone (Sigma) 5 mg/kg subcutaneously injected 18 h before LPS injection. Groups 4–7 (STE groups) were administered STE orally at a dose of 50, 200, 500 or 1000 mg/kg, respectively, for 10 days before LPS injection. Blood samples were centrifuged at 800*g* for 20 min. The serum samples obtained were stored at –20°C until used.

Isolation and culture of peritoneal macrophages

C57BL/6 mice were injected intraperitoneally with 0.2 ml sterile thioglycollate medium (BD, France), and three days later macrophages were collected by peritoneal gavage with cold Dulbecco's modified Eagle's medium (DMEM). After the lysis of red blood cells, the remaining cells were resuspended in DMEM with 10% fetal bovine serum and incubated for 90 min in a humidified atmosphere of 5% CO_2 at 37°C. After non-adherent cells were removed, cells were seeded for cytokine production (5×10^5 cells in 1 ml) or for RNA preparation and Western blotting (5×10^6 cells in 3 ml).

Viability assay

Cells (4×10^4 per well) were seeded in 96-well plates and stimulated for 24 h with LPS (1 μg/ml) in the presence of 10, 50, 100, 200 or 400 μg/ml STE. Cell viability was determined using the CellTiter96 One Solution Cell Proliferation assay (Promega, Madison, WI, USA). Optical density was read at 490 nm using a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Cytokine measurement

The levels of TNF-α and IL-6 from serum and cell supernatants were measured by ELISA, according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA).

cDNA preparation and real-time PCR

Real-time PCR was used to quantify TNF-α and IL-6 gene expression 4 h after stimulation. Total RNA from macrophages was isolated using an RNeasy mini kit (Qiagen, Germany) and cDNA was reverse-transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Diluted cDNA was mixed with 2 pmol of primers for TNF-α, IL-6, or GAPDH and Power SYBR Green PCR Master mix (Applied Biosystems, Foster City, CA, USA). The following forward and reverse primer sequences were used: TNF-α, forward :5′- ATG ATC GCG GAC GTG GAA-3′ and reverse: 5′-AGG GCC TGG AGT TCT GGA A-3′; for IL-6, forward: 5′- AGG ATA CCA CTC CCA ACA GAC CT-3′ and reverse: 5′- CAA GTG CAT CAT CGT TGT TCA TAC-3′; for GAPDH, forward: 5′-GGC ATG GAC TGT GGT CAT GA-3′ and reverse: 5′-TTC ACC ACC ATG GAG AAG GC-3′. Amplification of cDNA was performed in triplicate using an ABI PRISM 7300 sequence detector (Applied Biosystems). After initial heat denaturation at 95°C for 10 min, the PCR conditions were set at 95°C for 15 s and 60°C for 1 min for 40 cycles. PCR efficiency was determined using serial dilutions of the template cDNA. For each PCR, a corresponding no-RT mRNA sample was included as a negative control. Quantification of each cDNA copy number was determined according to the manufacturer's protocol. GAPDH gene was used as an endogenous control.

Western blotting

Total cell extracts were prepared by resuspending the cells in lysis buffer (in mM: 50 Tris-HCl, pH 7.5; 150 NaCl; 1 EDTA; 20 NaF; 0.5% NP-40; and 1% Triton X-100) containing a phosphatase inhibitor (Sigma) and a protease-inhibitor cocktail (Sigma). Nuclear protein extracts were prepared using the Nuclear Extract Kit (Active Motif, Carlsbad, CA, USA). Protein concentration was determined using the Bradford assay. Cell extracts were run on a 10% sodium dodecyl sulfate-polyacrylamide gel and were transferred to polyvinylidene fluoride. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST) for 1 h. They were incubated with IκBα, β-tubulin, actin, p65, lamin B (Santa Cruz Biotechnology, CA, USA) or phospho-IκBα, phospho-JNK/SAPK, phospho-c-Jun, phospho-ATF-2 (Cell Signaling Technology, CA, USA) diluted 1/1000 in 5% skim milk in TBST overnight at 4°C. The blots were washed with TBST and incubated for 1 h with anti-rabbit or anti-mouse HRP-conjugated antibody (diluted 1 : 2000 in 5% skim milk in TBST). Immunoreactive bands were developed using the enhanced chemiluminescence system (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

HPLC method

STE (20 mg) was dissolved in 10 ml methanol (HPLC reagent, Duksan Chemical, Korea) and ultrapure distilled water (resistivity > 18 MΩ) and filtered through a 0.45-μm syringe filter (PVDF, Advantec, Japan). The standard materials used for the qualitative analysis of STE were hesperidin, rosmarinic acid and *R*-(+)-pulegone. The standard stock solutions were prepared by dissolving 1-mg samples of hesperidin, rosmarinic acid and *R*-(+)-pulegone, each in 10 ml methanol. The HPLC apparatus was a Gilson System equipped with a 234 Autosampler, a UV/vis-155 detector and a 321 HPLC Pump (Gilson, Korea). A Luna 3.0 × 250 mm C_{18} reversed-phase column with 5-μm particles (Phenomenex, Torrance, CA, USA) was used. Two solvents were used: A, acetonitrile (HPLC grade, Duksan Chemical, Korea); and B, water (with 0.01% formic acid). The flow rate was 0.6 ml/min. The elution profile was 0–70 min, 20–50% B in A (linear gradient), and 5 μl (standard materials) and 20 μl (STE) volumes were analysed. The column eluent was monitored at UV 245 nm and then all solvents were degassed with a micromembrane filter (PTFE, Advantec, Japan).

Statistical analysis

The significance of differences among the means of multiple groups were determined using one-way analysis of variance followed by Dunnet's post hoc test. The difference between means values for two groups was assessed using nonpaired Student's *t*-test. SPSS version 12 was used for all statistical analyses, and values of $P < 0.05$ were considered significant.

Results

S. tenuifolia water extract reduced serum levels of TNF-α, but not IL-6, in lipopolysaccharide-injected mice

STE (50, 200, 500 and 1000 mg/kg) was administered orally to mice for 10 days before intraperitoneal injection of LPS.

Table 1 Effect of *Schizonepeta tenuifolia* water extract on lipopolysaccharide-induced serum cytokines

Treatment	TNF-α (ng/ml)	IL-6 (pg/ml)
Untreated	Not detected	Not detected
LPS+ control	38.22 ± 2.79	5956 ± 337
LPS + STE (50 mg/kg)	39.89 ± 5.23	7815 ± 290
LPS + STE (200 mg/kg)	15.45 ± 1.79*	4580 ± 214
LPS + STE (500 mg/kg)	15.09 ± 0.95*	5812 ± 255
LPS + STE (1000 mg/kg)	24.48 ± 5.85	6912 ± 465
LPS + dexamethasone (5 mg/kg)	3.40 ± 0.02*	2991 ± 300*

S. tenuifolia (STE) water extract was orally given to mice for 10 days before intraperitoneal injection of lipopolysaccharide (LPS; 1.3 mg/kg). Each group consisted of seven animals. Dexamethasone was intraperitoneally injected 18 h before LPS stimulation. Serum tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) were measured 1 h after LPS stimulation. Data are mean ± SEM of three independent experiments. *$P < 0.05$ compared with LPS+ control group.

Dexamethasone was used as a reference anti-inflammatory drug. A previous report demonstrated the in-vitro anti-inflammatory effect of *S. tenuifolia* water extract on the secretion of TNF-α and IL-6 in LPS-stimulated macrophages.[10] TNF-α and IL-6 are produced by many cell types including cells of monocyte/macrophage lineage, fibroblasts, endothelial cells, astrocytes, keratinocytes and epithelial cells.[11,12] Elevation of serum TNF-α and IL-6 must reflect the amount released by all these cells. In our in-vivo experiment, STE significantly reduced the serum levels of TNF-α, but not IL-6, 1 h after challenge with LPS (Table 1). The extract was effective at doses of 200 and 500 mg/kg, but no effect was observed at the higher dose of 1000 mg/kg, suggesting the possibility that one or more additional components of the extract may have acted at this high dose to counteract the efficacy of the active component. Dexamethasone produced a marked, significant reduction in the level of LPS-induced TNF-α and significantly reduced IL-6 levels.

Differential effects of *S. tenuifolia* water extract on lipopolysaccharide-induced TNF-α and IL-6 expression in peritoneal macrophages *in vitro*

Before investigating the in-vitro effect of STE on cytokine production, we confirmed that concentrations of the extract up to 400 μg/ml were not cytotoxic to LPS-stimulated macrophages, as measured by the MTS assay (Figure 1). The LPS-induced secretion of TNF-α was reduced by concurrent exposure of cells to STE (50, 100 and 200 μg/ml) for 6 or 24 h (Table 2). STE reduced the secretion of IL-6 at both time points, but the effect was seen at 200 μg/ml only. To determine if the reductions in TNF-α and IL-6 occurred at the transcriptional level, we quantified the mRNA for each using real-time RT PCR. When assessed 4 h after challenging cells with LPS, STE produced a concentration-dependent (100, 200 μg/ml) inhibition of TNF-α mRNA (Table 3). The expression of IL-6 mRNA was inhibited only at 200 μg/ml.

Table 2 Effect of *Schizonepeta tenuifolia* water extract on lipopolysaccharide-induced tumour necrosis factor-α and interleukin-6 production *in vitro*

Treatment (μg/ml)	TNF-α (ng/ml)		IL-6 (ng/ml)	
	6 h	24 h	6 h	24 h
STE 0	7.78 ± 0.27	10.35 ± 0.61	2.48 ± 0.01	12.10 ± 0.05
STE 50	6.03 ± 0.22*	10.53 ± 0.18	2.34 ± 0.06	9.84 ± 0.18
STE 100	4.90 ± 0.50*	8.50 ± 0.01	2.08 ± 0.11	8.68 ± 0.20
STE 200	4.89 ± 0.44*	7.86 ± 0.38*	1.37 ± 0.01*	7.88 ± 0.05
Dexamethasone	1.72 ± 0.35*	2.33 ± 0.21*	0.47 ± 0.01*	2.12 ± 0.02

Peritoneal macrophages (5×10^5 cells/ml) were stimulated with lipopolysaccharide (LPS; 1 μg/ml) in the presence of *S. tenuifolia* water extract (STE) for 6 or 24 h. Cytokine production in the culture medium was measured by ELISA. TNF-α, tumour necrosis factor-α; IL-6, interleukin-6. Data represent the mean ± SEM of four independent assays. *$P < 0.05$ compared with control cells (0 μg/ml).

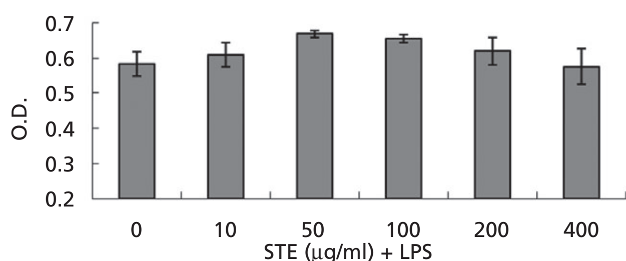

Figure 1 Effect of *Schizonepeta tenuifolia* water extract on lipopolysacccharide-stimulated cell viability. Peritoneal macrophages (4×10^4) were treated with varying concentrations of *S. tenuifolia* water extract (STE) and lipopolysaccharide (LPS; 1 μg/ml) for 24 h. Cell viability was measured by the MTS assay. O.D., optical density. Data are presented as mean ± SEM of three independent assays.

Table 3 Effect of *Schizonepeta tenuifolia* water extract on lipopolysaccharide-induced tumour necrosis factor-α and interleukin-6 mRNA expression *in vitro*

Treatment (μg/ml)	TNF-α/GAPDH	IL-6/GAPDH
STE 0	1.028 ± 0.012	1.097 ± 0.039
STE 50	0.707 ± 0.012	1.246 ± 0.034
STE 100	0.555 ± 0.034*	1.105 ± 0.051
STE 200	0.395 ± 0.007*	0.724 ± 0.023
Dexamethasone	0.408 ± 0.024*	0.159 ± 0.008*

Peritoneal macrophages (5×10^6) were stimulated with lipopolysaccharide (LPS; 1 μg/ml) in the presence of *S. tenuifolia* water extract (STE) for 4 h. Total RNA was obtained and real time RT-PCR was performed. GAPDH was used as an internal control. TNF-α, tumour necrosis factor-α; IL-6, interleukin-6. Data represent the mean ± SEM of four independent assays. *$P < 0.05$ compared with control cells (0 μg/ml).

S. tenuifolia water extract reduced lipopolysaccharide-stimulated IκBα degradation

NF-κB is a major transcription factor required for TNF-α expression. NF-κB is sequestered in the cytoplasm by IκB. Upon stimulation IκB becomes phosphorylated by IκB kinases and is subsequently ubiquitinated and degraded, allowing free NF-κB to move to the nucleus to bind gene promoter regions of TNF-α and other inflammatory cytokines.[13] Once in the nucleus, NF-κB rapidly induces IκBα, which acts as a negative regulator.[14,15] We tried to determine whether STE influenced the breakdown of IκBα in LPS-stimulated macrophages. In control cells, a complete loss of phospho-IκBα and IκBα was detected 15 min after the application of LPS, but phospho-IκBα and IκBα reappeared at 30 and 60 min (Figure 2a). STE treatment reduced IκBα degradation at 15 min, but phospho-IκBα was still detected at the same time point. We also obtained a nuclear protein extract to quantify p65, the major subunit of NF-κB, and found that p65 relocation to the nucleus could be detected as early as 15 min. This translocation was also inhibited by STE (Figure 2a). These data indicated that the inhibitory role of STE on NF-κB signalling may have occurred downstream of IκB kinase activity. Based on our time course data, cells were treated with various concentrations of STE (50, 100 or 200 μg/ml) or dexamethasone (1 μM) 1 h before stimulation with LPS for 15 min. Inhibition of IκBα degradation was observed at all the concentrations tested, and nuclear p65 was reduced by 100 and 200 μg/ml STE (Figure 2b). The mechanism underlying the dexamethasone-induced inhibition of NF-κB signalling was the induction of IκB re-synthesis or its direct interaction to NF-κB in the nucleus.[16,17] Therefore, early IκBα breakdown and translocation of p65 were not affected by dexamethasone.

S. tenuifolia water extract inhibited JNK/SAPK-dependent c-Jun/ATF-2 activity

JNK/SAPK plays an important role in LPS-stimulated TNF-α production, regulating TNF-α mRNA translation and activating the c-Jun/ATF-2 complex, a member of the AP-1 family that binds the promoter regions of various cytokine genes including TNF-α.[6,18] Our kinetic analysis showed that the inhibition of JNK/SAPK activity by STE occurred as early as 15 min after LPS application (Figure 3). Suppression of phospho-c-Jun and phospho-ATF-2 was also detected. These data strongly suggested that the inhibitory role of STE in TNF-α expression may have involved the JNK/SAPK-mediated AP-1 pathway. STE may have interfered with TNF-α mRNA translation through suppression of JNK/SAPK.

HPLC

A liquid chromatogram of STE is shown in Figure 4. The peaks identified in our analysis were hesperidin (retention time 13.36 min), rosmarinic acid (15.38 min) and *R*-(+)-pulegone (54.54 min).

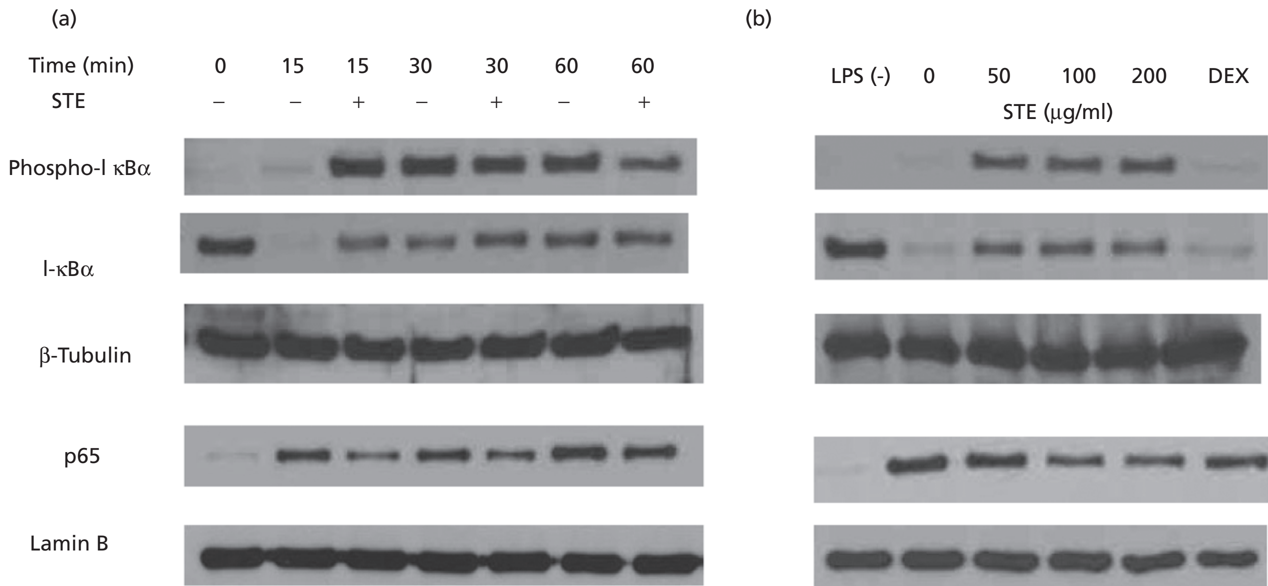

Figure 2 Effect of *Schizonepeta tenuifolia* water extract on the degradation of IκBα and the nuclear relocation of p65 NF-κB in lipopolysaccharide-stimulated macrophages. Phospho-IκBα, IκBα in the whole cell protein and p65 in the nuclear protein were assessed using Western blotting. β-Tubulin or lamin B was used as an internal control. (a) Peritoneal macrophages were pretreated with *Schizonepeta tenuifolia* water extract (STE; 100 μg/ml) for 1 h before stimulation with lipopolysaccharide (LPS; 1 μg/ml) and whole cell protein or nuclear protein was extracted at the indicated time points. (b) Cells were pretreated with STE (50, 100 or 200 μg/ml) or dexamethasone (DEX; 1 μM) for 1 h and later stimulated with LPS for 15 min. One of the five experiments is shown.

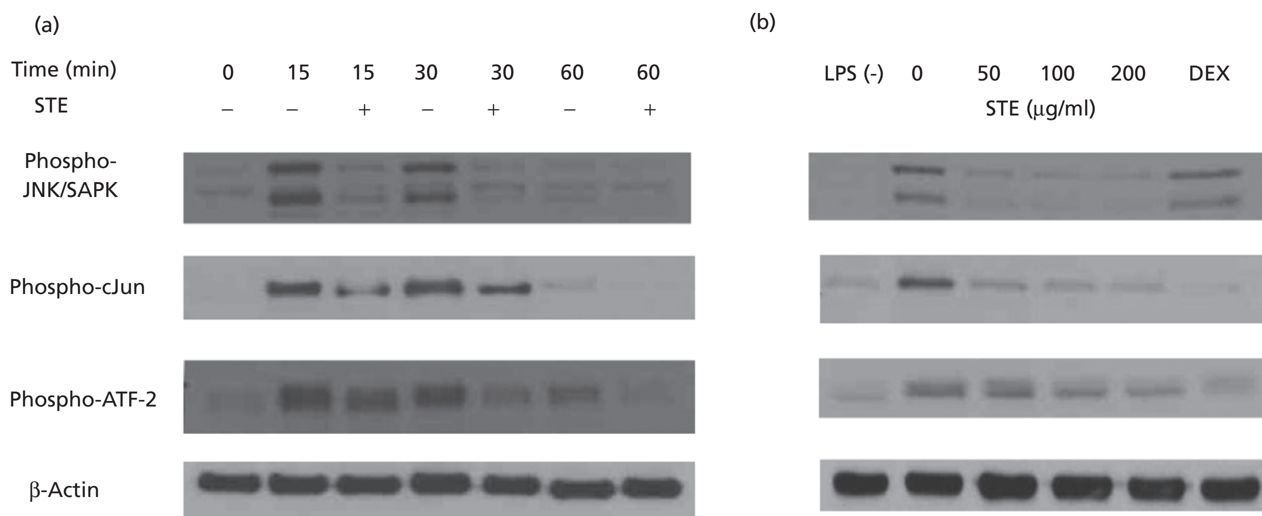

Figure 3 Effect of *Schizonepeta tenuifolia* water extract on the JNK/SAPK-mediated c-Jun and ATF-2 pathways in lipopolysaccharide-stimulated macrophages. Phospho-JNK/SAPK, phospho-cJun and phospho-ATF-2 in the whole cell protein were assessed using Western blotting. β-Actin was used as an internal control. (a) Peritoneal macrophages were pretreated with *Schizonepeta tenuifolia* water extract (STE; 100 μg/ml) for 1 h before stimulation with lipopolysaccharide (LPS; 1 μg/ml) and whole cell protein was extracted at the indicated time points. (b) Peritoneal macrophages were pretreated with STE (50, 100 or 200 μg/ml) or dexamethasone (DEX; 1 μM) for 1 h and stimulated with LPS for 15 min. One of the five experiments is shown.

Discussion

Current anti-inflammatory treatments include fast-acting drugs (nonsteroidal anti-inflammatory drugs, glucocorticoids), slow-acting disease-modifying anti-rheumatic drugs, cytostatic drugs, immunosuppressants (calcineurin inhibitor), and biological response modifiers (anti-TNF-α antibody).[19] Despite their rapid effects, long-term use of anti-inflammatory agents often causes some alterations in the body's response, which can result in reduced efficiency or the emergence of unwanted side effects. There is a need, therefore, to discover safe and effective medicinal herbs as alternatives to be used

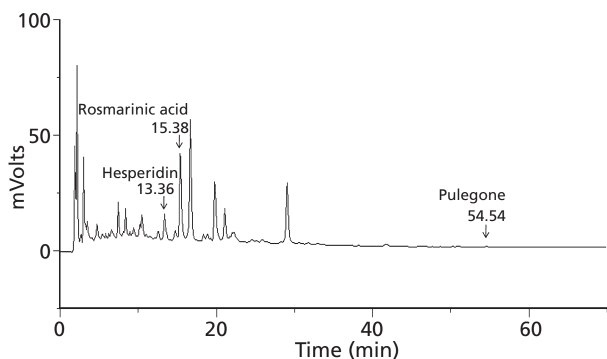

Figure 4 The chromatographic profile of *Schizonepeta tenuifolia* water extract. The numbers indicate retention times. The peaks identified were hesperidin (13.4 min), rosmarinic acid (15.4 min) and *R*-(+)-pulegone (54.5 min).

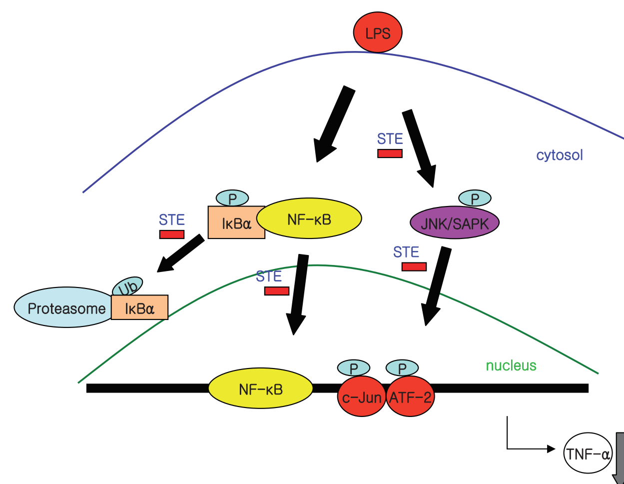

Figure 5 Schematic illustration regarding the signalling pathways involved in the inhibition of TNF-α expression by *Schizonepeta tenuifolia* water extract. *S. tenuifolia* water extract (STE) acted via inhibition of IκBα degradation through suppression of IκBα ubiquitination, and via inhibition of the phosphorylation of c-Jun and ATF-2 secondary to inactivation of JNK/SAPK.

for early or modest inflammatory episodes. The pharmacological principles and mechanism of action associated with herbal medicines used in China, Korea and Japan are described in ancient terminology that is difficult to interpret. Many traditional anti-inflammatory herbal agents are used clinically with no understanding of their mechanism of action. *S. tenuifolia* is a major constituent of herbal formulae used for fever, sore throat, rhinitis, tonsillitis, urticaria and eczema, but the details of its anti-inflammatory action are not fully known. In this article, the downregulation of TNF-α by *S. tenuifolia* has been investigated using the LPS-induced acute inflammation model *in vivo* and *in vitro*.

Recognition of LPS by Toll-like receptors on the macrophages ultimately activates the two main inflammatory pathways: NF-κB and MAP kinases.[20,21] Most anti-inflammatory drugs and drug candidates target the inhibition of inflammatory cytokines or lipid mediators through regulation of NF-κB or MAPK pathways. NF-κB activation occurs through either the classical pathway, mediated by IκBα degradation, or the alternative pathway, which is independent of IκBα.[13] IκBα masks the nuclear localization signal of p65 NF-κB, blocking its nuclear entry. In the classical NF-κB pathway, the β subunit of the IκB kinase complex phosphorylates IκBα, which is then degraded by the ubiquitin–proteasome pathway. A small molecule IκBα ubiquitination inhibitor, Ro106-9920, was reported to suppress the secretion of TNF-α and IL-6 in LPS-stimulated human peripheral blood mononuclear cells *in vitro*, as well as serum TNF-α levels in LPS-injected rats.[22] Our experiments showed that the inhibition of NF-κB signalling by an aqueous extract from *S. tenuifolia* occurred at the level of IκBα degradation, without affecting IκBα phosphorylation. These results indicated that anti-inflammatory constituents of STE may have stabilized the formation of the IκBα/NF-κB complex in the cytoplasm, by interfering with the process of IκBα ubiquitination.

AP-1 is a dimeric transcription factor, consisting mainly of the Jun, Fos and ATF proteins.[23] These members are expressed differentially, depending on cell type and functions. The c-Jun/ATF-2 complex binds the TNF-α promoter region and is activated by JNK/SAPK.[24] Our findings showed that STE inhibited the JNK/SAPK-mediated c-Jun/ATF-2 pathway. In addition to phosphorylating c-Jun and ATF-2, JNK/SAPK participates in the translation of TNF-α mRNA.[6] Based on these results, it appeared that STE may have promoted the downregulation of TNF-α expression by inhibiting the transcriptional activity of AP-1 or by reducing the translation of TNF-α secondary to JNK/SAPK inhibition. Figure 5 summarizes the possible effects of STE on LPS-stimulated JNK/ATF-2 and NF-κB pathways.

In contrast to the observed STE-evoked decreases in macrophage-derived TNF-α and IL-6 production, STE did not affect serum IL-6 levels. Although we observed that serum IL-6 levels were reduced in dexamethasone-treated animals, the magnitude of the reduction was much smaller than observed for serum TNF-α. Other studies in which steroid treatment was performed 30 min or 6 h before LPS application have reported no reduction in serum IL-6.[25,26] The reason for the IL-6 reduction in our dexamethasone-treated animals may have been partly due to the 18-h waiting period before LPS application. Compared with TNF-α, the systemic IL-6 response seemed to be less sensitive, at least, to the short-term effect of anti-inflammatory agents. Generally, blockade of IL-6, like that of TNF-α, is therapeutically beneficial for chronic inflammatory states, such as rheumatoid arthritis and Crohn's disease.[27,28] However, it is noteworthy that IL-6 has anti-inflammatory properties during an early phase of inflammation.[29] IL-6 is reported to block neutrophil infiltration, prevent the development of septic shock, and inhibit LPS-induced TNF-α production *in vitro* and *in vivo*.[30–32] Thus, an elevation of serum IL-6 levels during an early stage of inflammation may be beneficial and could contribute to the downregulation of TNF-α. IL-6 and TNF-α have overlapping biological activities and signalling pathways, but given the differential effects of anti-inflammatory agents, it is clear that they can be produced independently. For example, MEKK3, an upstream molecule of the NF-κB and MAP kinase pathways, is a specific regulator of the production of IL-6, but not that of TNF-α, in macrophages.[33] It is possible that STE and

other anti-inflammatory agents may specifically activate IL-6 signalling at early time points or produce different effects in cells other than macrophages.

According to a recent characterization of phytochemical constituents of *S. tenuifolia* Briq, the major components of the aerial part of this plant are terpenes (pulegone, menthone, ursolic acid), flavonoids (hesperidin, luteolin) and phenolic compounds (rosmarinic acid).[34] Pulegone and menthone are the major essential oils of *S. tenuifolia*, but we found that these compounds did not affect LPS-induced IκB degradation nor JNK activation in peritoneal macrophages (data not shown). Ursolic acid was reported to have dual effects: it was able to inhibit NF-κB in LPS-stimulated macrophages and to activate NF-κB in resting macrophages.[35] Luteolin inhibited LPS-stimulated TNF-α and IL-6 in RAW 264.7 cells through inhibition of NF-κB, ERK and p38, and intraperitoneal injection of luteolin suppressed serum levels of LPS-induced TNF-α *in vivo* without affecting serum IL-6.[36–38] Hesperidin was demonstrated to show anti-inflammatory effects in several animal studies and to inhibit phosphorylation of IκB, JNK and p38 in TNF-α-stimulated lung epithelial cells.[39,40] Rosmarinic acid is known to show anti-inflammatory effects in 12-tetradecanoylphorbol 13-acetate-induced skin response and atopic dermatitis patients and inhibit activation of NF-κB in LPS-stimulated dendritic cells and TNF-α-stimulated dermal fibroblasts.[41–44] All the ingredients mentioned above inhibit NF-κB activity through blockade of IKK activity. Therefore, it appears that STE contains some compounds that can counteract the activity of IKK blocking agents. Further study is required to identify the active constituents of STE for such activity.

Conclusions

Our data demonstrated that oral administration of STE to LPS-injected mice suppressed serum levels of TNF-α, without affecting IL-6. In macrophages, STE treatment inhibited the NF-κB pathway by blocking IκBα degradation and suppressed the activation of c-Jun and ATF-2, secondary to downregulation of JNK/SAPK. Our data provides mechanistic evidence for the anti-inflammatory actions of *S. tenuifolia* observed clinically. Further study is required to identify the active anti-inflammatory constituents.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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