Scutellarein Reduces Inflammatory Responses by Inhibiting Src Kinase Activity

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INTRODUCTION

Inflammation is a natural defense system to protect the body from pathogens. When immune cells such as macrophages encounter pathogens (i.e. bacteria or parasites), inflammatory reactions are spontaneously induced. These reactions include the production of cytokines, chemokines, and inflammatory mediators such as nitric oxide (NO) and tumor necrosis factor (TNF)-α. However, excessively high or sustained inflammation can cause various diseases such as atherosclerosis [2], septic shock [3], and rheumatoid arthritis [4]. Macrophages, which are the major phagocytic cells that defend the body against pathogens, are known to play a critical role in inflammatory responses [5]. Macrophage activation is known to be triggered when pathogen-associated molecules like lipopolysaccharide (LPS) stimulate macrophage receptors, such as Toll-like receptor (TLR) 4. This receptor/ligand interaction activates intracellular signaling cascades mediated by various enzymes, including the non-receptor type protein kinase Src, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K), the serine/threonine-specific protein kinase AKT, and IκB kinase (IKK) [6]. Activated IKK phosphorylates IκBα, which is a suppressor of NF-κB, to stimulate translocation of NF-κB subunits into the nucleus by degradation of IκBα [7]. Eventually, the activation of inflammatory transcription factors such as NF-κB results in the expression of pro-inflammatory genes, including inducible NO synthase (iNOS), cyclooxygenase (COX)-2, and cytokines [8]. Accordingly, various types of new anti-inflammatory drugs targeting NF-κB are currently being developed [9].

KEY WORDS: Anti-inflammatory effect, Flavonoid, Macrophages, NF-κB, Scutellarein, Src
Scutellarein (SCT, Fig. 1A) is the active component of *Erigeron breviscapus*, *Clerodendrum phlomidis* and *Oroxylum indicum* Vent and is used to treat inflammation, diabetes, nervous disorders, asthma, rheumatism, digestive disorders, and urinary disorders; it is also given as a bitter tonic [10,11]. In addition, *Codariocalyx motorius* with structural analogue of SCT (SCT-6-O-glucuronide) was also shown to suppress LPS-induced inflammatory responses in macrophages and a HCl/EtOH-triggered gastritis symptoms [12]. From a pharmacological perspective, SCT has been reported to exert antioxidative, anti-cancer, and neuroprotective activities [13,14]. Although SCT is known to have a flavonoid-derived backbone, the precise anti-inflammatory activity of SCT and its molecular mechanism of action are not yet completely understood. Therefore, in this study, we aimed to determine the immunopharmacological role of SCT and to identify the target of SCT in LPS-activated macrophages.

**METHODS**

**Materials**

SCT (isolated from *Erigeron breviscapus*, Purity >98%), polyethylenimine (PEI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N²-nitro-L-arginine methyl ester (L-NAME), sodium dodecyl sulfate (SDS), and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS), Phosphate buffered saline (PBS), penicillin, streptomycin, TRIzol reagent, and RPMI1640 were obtained from GIBCO (Grand Island, NY, USA). The RAW264.7 murine macrophage cell line and HEK 293 human embryonic kidney cell line were purchased from ATCC (Rockville, MD, USA). Phospho-specific and total protein antibodies raised against p65, p50, inhibitor of κ B α (IκBα), IKK αβ, AKT, p85, Src, lamin A/C, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies against cyan fluorescent protein (CFP) and human influenza hemagglutinin (HA) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

**Expression vectors**

Wild type Src (HA-Src) was used as reported previously [15,16]. The luciferase construct containing binding sites for NF-κB (NF-κB-Luc) and the plasmids encoding adaptor molecules (CFP-TRIF and Flag-hMyD88) for TLR signaling were used as reported previously [17]. All constructs were confirmed by automated DNA sequencing.

**Cell culture and drug preparation**

RAW264.7 cells were maintained in RPMI1640 medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS. HEK 293 cells were maintained in Dulbecco’s Modified Eagle Medium supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, and 10% FBS. Cells were grown at 37°C and 5% CO₂ in humidified air. The SCT stock solution used in the *in vitro* experiments was prepared in DMSO.

**Measurement of NO production**

After preincubation of RAW264.7 cells (1×10⁵ cells/ml)
overnight, the cells were treated with SCT (0 to 200 μM) or L-NAME (0 to 750 μM) for 1 h and then further incubated with LPS (1 μg/ml) for 24 h. The inhibitory effects of these drugs on NO production were determined by analyzing the NO levels using the Griess reagent as previously described [18,19].

mRNA analysis using polymerase chain reaction

To determine cytokine mRNA expression levels, total RNA was isolated from LPS-treated RAW264.7 cells using TRIzol according to the manufacturer’s instructions. All RNA was stored at −70°C until use. Semi-quantitative RT reactions were conducted as previously reported [20,21]. mRNA quantification was performed by real-time RT-PCR with SYBR Premix Ex Taq according to the manufacturer’s instructions (Takara, Shiga, Japan) using a real-time thermal cycler (Bio-Rad, Hercules, CA, USA). Semi-quantitative RT-PCR was conducted as previously reported with minor modifications [22]. All of the primers (Bioneer, Daejeon, Korea) used are listed in Table 1.

Preparation of cell lysates and nuclear fractions for immunoblotting

RAW264.7 or HEK 293 cells (5×10^6 cells/ml) were washed 3 times in cold PBS containing 1 mM sodium orthovanadate and resuspended in lysis buffer (20 mM Tris-HCl (pH 7.4), 2 mM Ethylenediaminetetraacetic acid (EDTA), 2 mM ethyleneglycotetraacetic acid, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μM leupeptin, 10 μM aprotinin, and 100 μM 1,4-dithiothreitol (DTT). Cells were lysed by incubation on ice for 4 min. Cell lysates were then spun by centrifugation at 14,000 rpm for 5 min. The supernatant was collected as the nuclear extract.

Whole cell and nuclear lysates were then analyzed by immunoblotting. Proteins were separated on 10% SDS-polyacrylamide gels and transferred by electroblotting to polyvinylidenefluoride membranes. Nonspecific binding sites on the membranes were blocked for 60 min at room temperature in Tris-buffered saline containing 3% bovine serum albumin (BSA) and 0.1% Tween 20. The membranes were then incubated for 60 min with the appropriate primary antibodies at 4°C, washed 3 times with the same buffer, and incubated for an additional 60 min with HRP-conjugated secondary antibodies. The total and phosphorylated forms of the reaction were then spotted onto a P30 Filtermat, room temperature, the reaction was stopped by the addition of Mg-ATP. After incubation for 40 min at room temperature, the reaction was stopped by the addition of the mix of an 3% phosphoric acid solution. Ten microliters of the reaction was then spotted onto a P30 Filtermat, which was then washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

DNA transfection and luciferase reporter gene activity assay

HEK 293 cells were seeded in 6 cm petri dishes (5×10^5 cells/ml) and transfected with plasmids driving the expression of HA-Src, Flag-hMyD88 or CFP-TRIF (1 μg/ml each). Transfections were performed according to the PEI method as reported previously [25,26]. All experiments were performed 24 h post-transfection. SCT was added to the cells 1 h before termination. For reporter gene assays, HEK 293 cells (5×10^5 cells/ml) were seeded in 24-well plates and transfected with 1 μg each of plasmids expressing NF-κB-Luc and TRIF, as well as β-galactosidase. Transfections were carried out using the PEI method according to the procedures outlined in previous reports [25,26]. Luciferase assays were performed using the Luciferase Assay System (Promega, Madison, WI, USA), as previously reported [27].

In vitro kinase assay with purified enzymes and immunoprecipitated enzymes

To evaluate the abilities of purified enzymes to inhibit Src kinase activity, a kinase profiler service using radioactive ATP from Millipore (Billerica, MA, USA) was used. Purified Src (1~5 μM) was incubated in reaction buffer in a final volume of 25 μl. The reaction was initiated by the addition of Mg-ATP. After incubation for 40 min at room temperature, the reaction was stopped by the addition of the mix of a 3% phosphoric acid solution. Ten microliters of the reaction was then spotted onto a P30 Filtermat, which was then washed three times for 5 min in 75 mM phosphoric acid and once in methanol prior to drying and scintillation counting.

Statistical analyses

All data (Figs. 1B, 1C, 2B, 2C, 3A right panel, 3B right panel, 4A right panel, 4B right panel, 5A, 5B lower panel, 6C right panel) were analyzed using one-way analysis of variance (ANOVA). Statistical analyses were performed using the Statview program (version 5.0; Abacus Concepts, Berkeley, CA, USA). Differences were considered statistically significant at p<0.05.

Table 1. Real-time and semiquantitative RT-PCR primers used in this study

| Name     | Sequence (5’ to 3’) |
|----------|---------------------|
| iNOS     | F GGAGCCTTTAGACCTCAACAGA |
|          | R TGAACGGAGGATCGTGGT |
| TNF-α    | F TGGCTATGTCCTAGGTCTTC |
|          | R GAGGCCATTTGGAACTTCTC |
| GAPDH    | F CAATGAATACGGCTACAGCAAC |
|          | R AGGGGTATCTGCTGTTGG |
| iNOS     | F CCCCCGAAAGTTCGCGACAG |
|          | R GGCCTGGCAGCCCTGCTTG |
| TNF-α    | F TTGACCTACGCCTGGATG |
|          | R CCTTATCCACCGTCTAGC |
| GAPDH    | F CAGTCCACGACATCACTCAGC |
|          | R GACTCCACGACATCACTCAGC |
Effect of SCT on inflammatory gene expression in LPS-treated RAW264.7 cells. (A) RAW264.7 cells (1×10^6 cells/ml) were incubated with LPS (1 μg/ml) for 6 h after treatment with SCT. The mRNA expression levels of iNOS and TNF-α were determined by RT-PCR. (B and C) iNOS and TNF-α mRNA expression levels were measured by real-time PCR. **p < 0.01 compared with the control group.

Fig. 2. Effect of SCT on inflammatory gene expression in LPS-treated RAW264.7 cells. (A) RAW264.7 cells (1×10^6 cells/ml) were incubated with LPS (1 μg/ml) for 6 h after treatment with SCT. The mRNA expression levels of iNOS and TNF-α were determined by RT-PCR. (B and C) iNOS and TNF-α mRNA expression levels were measured by real-time PCR. **p < 0.01 compared with the control group.

RESULTS

Effect of scutellarein on NO production and cell viability

To determine whether SCT has an anti-inflammatory activity in macrophages during inflammatory conditions induced by LPS, we first checked NO production level in LPS-stimulated RAW 264.7 cells in the presence or absence of SCT or L-NAME (as a control drug). As shown in Fig. 1B, SCT strongly suppressed the production of NO in a dose-dependent manner up to 99% at a dose of 200 μM. Dose-dependent NO inhibitory profile was also observed in L-NAME-treated condition (Fig. 1B right panel). Importantly, SCT did not significantly affect the viability of RAW264.7 cells up to 200 μM (Fig. 1C).

Effect of scutellarein on the mRNA expression of inflammatory genes

We next examined the mRNA expression levels of inflammatory genes in LPS-treated RAW264.7 cells. As shown in Fig. 2, LPS alone dramatically triggered the mRNA expression of iNOS and TNF-α, while SCT strongly suppressed the upregulated levels of the inflammatory genes, according to both RT-PCR (Fig. 2A) and Real-time PCR (Fig. 2B to C) analyses.

Effect of scutellarein on the transcriptional activation

In order to investigate how SCT modulated inflammatory gene expression, we employed a reporter gene assay with luciferase constructs containing NF-κB promoter using HEK 293 cells. In fact, NF-κB mediated luciferase activity was revealed to be highly increased under the overexpression of TRIF, a TLR4 adaptor molecule up to 50 folds. Interestingly, however, SCT strongly suppressed this activity by 55% at 200 μM of SCT (Fig. 3A right panel), although the viability of HEK 293 cells was rather enhanced at 200 μM (Fig. 3A left panel). To confirm the possibility that NF-κB pathway can be blocked by SCT treatment, we determined the translocation levels of NF-κB subunits (p65 and p50) in the nucleus of LPS-treated RAW264.7 cells. Expectedly, SCT (200 μM) clearly diminished the translocation levels of p65 and p50 at 15, 30, and 60 min during LPS stimulation (Fig. 3B).

Effect of scutellarein on the activation of NF-κB translocation signaling

Since the phosphorylation of IκBα is a critical step involved in the translocation of NF-κB, we next investigated whether SCT is able to modulate the LPS-induced phosphorylation of IκBα by using immunoblotting analysis. As
Scutellarein as a Src Inhibitor

Fig. 3. Effect of SCT on the transcriptional activation of inflammatory gene expression. (A, left panel) HEK 293 cells (5×10⁵ cells/ml) were incubated with SCT for 24 h. Cell viability was measured by the MTT assay. (A, right panel) HEK 293 cells were treated with SCT for 24 h after cotransfection with NF-κB-Luc, TRIF, or pcDNA for 24 h. Luciferase activity was determined using a luminometer. (B left panel) Nuclear translocation of NF-κB subunits was detected by immunoblot analysis of nuclear fractions. Relative intensity (B right panel) was calculated using total levels by the DNR Bio-Imaging system. *p < 0.05 and **p < 0.01 compared with the control group.

shown in Fig. 4A, the phosphorylation level of IκBα was highly reduced by SCT at 3 to 15 min. Moreover, incubation of SCT (200 μM) also inhibited enhanced phosphorylation of IKK, AKT, p85, and Src, which participate in increasing IκBα phosphorylation, at 3 to 5 min after LPS treatment (Fig. 4A and 4B).

Effect of scutellarein on the activation of Src kinase

Since SCT blocked the phosphorylation of Src (Fig. 4B), we attempted to identify whether there is downregulation of Src autophosphorylation by this compound. To do this, in vitro Src kinase activity was measured. As Fig. 5A shows, 200 μM of SCT significantly inhibited the kinase activity of Src, indicating that Src can directly be a target of SCT. Similarly, the phosphorylation level of Src induced by MyD88 overexpression was also reduced in HEK293 cell (Fig. 5B). In addition, Src phosphorylation mediated by HA-Src was also decreased by 200 μM of SCT (Fig. 5C).

DISCUSSION

Based on the reported findings that SCT tetramethyl ether and SCT 7-O-β-d-glucuronide have anti-inflammatory activity [28-30], we explored whether SCT suppresses inflammatory responses. We first investigated the effect of SCT on NO production in LPS-treated macrophages. As shown in Fig. 1B, SCT inhibited NO secretion by LPS-stimulated RAW264.7 cells in a dose-dependent manner. Importantly, SCT did not affect the viability of RAW264.7 cells at concentrations up to 200 μM (Fig. 1C). Since NO is known to be a key molecule in the inflammatory response [31,32], this finding suggests that SCT could inhibit other inflammatory activities of macrophages. To test this hypothesis, we next determined whether SCT affects inflammatory gene expression in LPS-activated RAW264.7 cells. Testing the profile of inflammatory gene expression patterns is one way to identify the anti-inflammatory spectrum of a compound. As shown in Fig. 2, LPS-induced upregulation of iNOS and TNF-α mRNA was significantly suppressed by SCT (Fig. 2A), as predicted. In addition, quantitative real-time PCR showed that SCT strongly inhibited iNOS (Fig. 2B) and TNF-α (Fig. 2C) expression in a dose-dependent manner. This result implies that SCT inhibits NO and cytokine production with a broad-spectrum mode of action at the transcriptional level.

To further investigate the mechanism by which SCT inhibits the expression of proinflammatory genes, we sought the target transcription factors involved. Interestingly, SCT tetramethyl ether has been reported to suppress NF-κB activation [30]. Moreover, the expression levels of phosphorylated EGFR, ERK, and NF-κB in proliferative lung cancer cells have been found to be reduced during SCT treatment [33]. Therefore, we next examined whether SCT suppresses NF-κB activation. To this end, we tested the effect of SCT
on the activity of an NF-κB-driven luciferase reporter construct in HEK 293 cells. Since HEK 293 cells do not express TLR4, one of its adaptor molecules, TRIF, was also introduced to activate TLR4-induced signaling events leading to NF-κB activation. Indeed, TRIF overexpression strongly induced NF-κB-driven luciferase activity [34]. Under these conditions, TRIF-transfected HEK 293 cells displayed up to 50-fold increased levels of NF-κB-driven luciferase activity (Fig. 3A, right panel). Treatment with SCT clearly decreased this NF-κB-driven luciferase activity (Fig. 3A, right panel). Importantly, SCT did not affect cell viability at this concentration (Fig. 3A, left panel), implying that NF-κB could be targeted by SCT. To test this possibility, we next prepared nuclear fractions to determine the levels of translocated NF-κB by immunoblot analysis. As depicted in Fig. 3B, SCT treatment reduced the nuclear levels of p65 and p50, which are components of the NF-κB transcription factor [35], suggesting that the NF-κB translocation pathway could be targeted by SCT. So far, numerous naturally occurring compounds with anti-inflammatory properties such as quercetin, luteolin, kaempferol, resveratrol, and ginsenoside Rd have been reported to inhibit NF-κB activation [18,36-39]. The active forms of NF-κB have also been identified in many different inflammatory diseases such as colitis, pancreatitis, and gastritis [9,40]. Therefore, our results are consistent with previous results and strongly imply that SCT may exert its anti-inflammatory actions by inhibiting the NF-κB pathway.

Since SCT suppressed the nuclear translocation of NF-κB, we investigated further upstream signaling events, including IκBα, IKK, AKT, p85/PI3K, and Src phosphorylation. These molecules all regulate NF-κB translocation [41,42]. As shown in Fig. 4A, the level of phosphorylated IκBα was greatly reduced by treatment with SCT at 3, 5, and 15 min, although both total- and phospho-IκBα levels were markedly decreased at 15 min, due to increased degradation pathway of IκBα linked to phosphorylation without altering mRNA level (data not shown). In addition, the levels of phosphorylated IKK and AKT, two kinases upstream of IκBα, were also decreased by SCT treatment (Fig. 4B). Similarly, phosphorylation levels of p85, the regulatory subunit of PI3K [43], and Src were also reduced under the
Scutellarein as a Src Inhibitor

Fig. 5. Effect of SCT on the regulation of Src kinase. (A) The inhibitory effect of SCT on Src kinase activity was determined using an established kinase assay with purified Src enzyme. (B and C) HEK 293 cells (5×10^5 cells/ml) were transfected with Flag-hMyD88 or HA-Src plasmids and then incubated for 24 h. The transfected cells were then treated with SCT for 12 h. The level of phosphorylated Src was measured by immunoblotting. Relative intensity (Bottom panels of B and C) was calculated using β-actin levels by the DNR Bio-Imaging system. **p<0.01 compared with the control group.

Fig. 6. Schematic diagram of the inhibitory action of SCT on LPS-induced inflammatory responses in macrophage cells.

In summary, here we showed that SCT blocks NO production and reduces inflammatory gene expression in LPS-stimulated macrophages without decreasing cell viability. These anti-inflammatory activities of SCT appear to result from suppressed NF-κB activation, including inhibition of upstream signaling proteins such as IκBα, IKK, AKT, p85/PI3K, and Src (Fig. 6). Therefore, in agreement with previous reports that SCT is a beneficial pharmacological remedy [49], we propose that SCT can be developed as a novel anti-inflammatory drug.

DISCLAIMER

The authors alone are responsible for the content and writing of the paper.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.
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