In vitro anti-inflammatory activity of *Pothos scandens* extract in RAW 264.7 cells

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Received: 20 September 2016 / Revised: 29 January 2017 / Accepted: 8 February 2017 / Published online: 29 May 2017
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Abstract *Pothos scandens*, an edible plant, has been traditionally used for reducing swelling and treating wounds, muscle catches, sprains, bone fractures, blisters, and diarrhea. *P. scandens* ethanol extract (PSE) was evaluated for its anti-inflammatory potential in lipopolysaccharide-stimulated murine RAW 264.7 cells. Present studies showed that PSE reduced the mRNA expression of inducible nitric oxide synthase and cyclooxygenase-2, followed by a decrease in production of nitric oxide and prostaglandin E₂. In addition, the secretion of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-1 β was suppressed by PSE treatment. Immunoblotting analyses demonstrated that PSE inhibited the phosphorylation of extracellular signal-regulated kinase and signal transducer and activator of transcription 3 protein, without altering the phosphorylation of inhibitor of κ B α , c-Jun N-terminal kinase, and p38 protein kinase. In conclusion, this study demonstrated that *P. scandens* exhibited anti-inflammatory activity, which might be useful for the development of anti-inflammatory agents.

Keywords Inflammation · *Pothos scandens* · Cytokine · Nitric oxide synthase · Cyclooxygenase

Introduction

Inflammation is a vital host defense mechanism against cell injury, irritants, or pathogens. It involves immune cells such as macrophages and T cells, and inflammatory mediators including pro-inflammatory cytokines, prostaglandins (PG), and nitric oxide (NO) [1]. Although the primary objective of inflammatory response is to remove harmful stimuli and initiate healing process, excessively induced chronic relapsing inflammation may cause inflammatory diseases. For example, NO concentration in serum and synovial fluid of rheumatoid arthritis patients is higher than that in serum and synovial fluid of osteoarthritis patients and healthy volunteers [2]. The production of pro-inflammatory cytokines such as interleukin (IL)-6, IL-1 β , and tumor necrosis factor (TNF)- α by lamina propria macrophages and CD4⁺ T cells significantly increase in inflammatory bowel disease patients [3].

Blood mononuclear cells enter the peripheral tissue and subsequently differentiate into macrophages. Macrophages engulf and digest pathogens or cellular debris for antigen presentation to naïve lymphocytes. The first step in the phagocytosis is the recognition of pathogens through pattern recognition receptors (PRRs) expressed on macrophages [4]. Among several types of PRRs, toll-like receptor 4 (TLR4) recognizes lipopolysaccharide (LPS) on the outer membrane of gram-negative bacteria. Upon LPS stimulation, macrophages secrete pro-inflammatory cytokines resulting in their own activation or other leukocytes and endothelial cells [5]. In addition, activated macrophages up-regulate inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) that lead to an increase in NO and PGE₂ production [5, 6]. Hence, macrophages play pivotal role in triggering immune response through diverse pathways against pathogens. However, over-activation of

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macrophages may cause life-threatening disorders [1]. Therefore, therapeutic strategy targeting excessively activated macrophages and their products could be an applicable approach for controlling inflammatory diseases [5].

Pothos scandens is a climbing shrub that belongs to the family Araceae. *P. scandens* is widely distributed in Asian countries and Madagascar [7]. *P. scandens* is an edible plant, and Dai people drink boiled water decoctions of its leaves as tea [8]. The leaves of the plant are used to reduce swelling in traumatic areas in Sri Lanka [9], and the compressed fruits and leaves are used as blood coagulants in China [7]. Whole plants grounded into paste is used to treat muscle catches and sprains [10], while decoction prepared from the whole plant is used to treat diarrhea [11]. In Thai, people take aerial parts of the plant in the form of tea to treat cancer [12]. Indians use an infusion of the leaves of this plant for curing convulsions and epilepsy, paste of leaves to cure wounds, and whole plant for bone fracture [13–15]. In Malaysia, the plant is also used to treat blister, convulsions, small pox, and asthma [16].

Previous studies have reported that *P. scandens* possess wound healing and anti-oxidative activity [17, 18]. Ethanol and aqueous extract of aerial part of the plant exhibited peritoneal mast cell stabilization in rats, and ethanol extract had inhibitory potential on ovalbumin-induced airway hyperresponsiveness in balb/c mice [14]. In addition, methanol extract of root exhibited antipyretic activity in pyrexia induced rats [18]. However, despite its wide usage as a folk medicine for reducing swelling and improving wound healing, anti-inflammatory activity of *P. scandens* has not been confirmed experimentally. Given that one of the characteristics of inflammation includes swelling, and uncontrolled inflammation inhibits wound healing process and prevents tissue repairing [19], *P. scandens* is highly likely to retain anti-inflammatory activity.

Therefore, in the present study, we delineated the anti-inflammatory effect of *P. scandens* ethanol extract (PSE) on LPS-stimulated RAW 264.7 cells. In order to elucidate the molecular mechanism underlying anti-inflammatory activity, we further assessed the effects of PSE on LPS-induced mitogen-activated protein kinase (MAPK) pathway, inhibitor of κB ($\text{I}\kappa\text{B}$) mediating Nuclear factor- κB (NF- κB) signaling pathway, and signal transducer and activator of transcription 3 protein (STAT3).

Materials and methods

Cell culture

Murine RAW 264.7 cells were cultured in Dulbecco's modified Eagle's medium (Wellgene, Gyeongsan, Gyeongsangbuk-do, Korea) supplemented with 10% fetal

bovine serum (Wellgene), 100 $\mu\text{g}/\text{mL}$ streptomycin, 100 U/mL penicillin (Gibco, Grand Island, NY, USA) at 37 °C and 5% $\text{CO}_2/95\%$ humidified air.

Ethanol extract of *P. scandens*

The ethanol extract of whole plant of *P. scandens* (Code No. FBM047-016) was obtained from the International Biological Material Research Center (<http://www.ibmrc.re.kr>, Daejeon, Korea). Whole plant of *P. scandens* was dried, pulverized, and sieved with 60 mesh screen. Reflux extractions were carried out with 95% ethanol for 2 h, twice. After extraction, the extracts were concentrated with reduced pressure at 40 °C. The extract was dissolved in dimethyl sulfoxide (DMSO; Duchefa, Haarlem, The Netherlands) to prepare 100,000 $\mu\text{g}/\text{mL}$ stock solution. Final concentration of DMSO in cell culture was .5% to avoid cell damage.

Cytotoxicity assay

RAW 264.7 cells seeded in 96-well plate at a density of 5×10^4 cells/100 μL per well were treated with 25, 50, 100, and 200 $\mu\text{g}/\text{mL}$ PSE for 4 h and rinsed with phosphate buffered saline (PBS). Subsequently, cells were incubated with or without LPS (1 $\mu\text{g}/\text{mL}$). After 20 h, 10 μL of 5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma-Aldrich, St Louis, MO, USA) was added and incubated for 5 h at 37 °C in a 5% CO_2 incubator. Formazan crystals were dissolved by adding 100 μL of .04 N HCl in isopropanol. The optical density was measured at 540 nm to quantify viable cells.

Measurement of NO production

RAW 264.7 cells seeded in 96-well plate at a density of 5×10^4 cells/100 μL per well were treated with PSE for 4 h, rinsed with PBS, and then treated with 1 $\mu\text{g}/\text{mL}$ of LPS. After 20 h, 50 μL of supernatant was harvested and mixed with 50 μL of Griess reagent (.1% naphthylethylenediamine and 1% sulfanilamide in 5% phosphoric acid solution). The optical density at 540 nm was measured after 5 min. The production of NO was quantified based on NaNO_2 (Junsei Chemical, Chuo-ku, Tokyo, Japan) standard reference curve.

Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells seeded in 24-well plate at a density of 3×10^5 cells/500 μL per well were treated with PSE, followed by 1 $\mu\text{g}/\text{mL}$ LPS stimulation, and the culture supernatant was collected to measure the production of IL-6, IL-1 β , and TNF- α . Each microplate well was coated

overnight at 4 °C, with purified rat anti-mouse IL-6 antibody (eBioscience, San Diego, CA, USA), purified Armenian hamster anti-mouse/rat IL-1 β antibody (BD Biosciences, San Diego, CA, USA), and purified Armenian hamster anti-mouse/rat TNF- α antibody (eBioscience) in coating buffer. After washing and blocking with PBS containing 3% bovine serum albumin (BSA; Sigma-Aldrich), samples were loaded in each well and incubated overnight at 4 °C. After washing, plate was incubated with biotinylated anti-mouse IL-6 antibody (BD Biosciences), IL-1 β antibody (eBioscience), and TNF- α antibody (eBioscience) for 30 min at room temperature (RT). After incubation, the plate was washed and subsequently incubated with streptavidin-alkaline phosphatase (BD Biosciences) for 20 min at RT. The plate was washed and phosphatase substrate, *p*-nitrophenyl phosphate (Sigma-Aldrich) was added. After 10 min incubation, optical density was measured at 405 nm. Cytokine production level was quantified based on standard reference curve made by recombinant murine IL-6 (Peprotech, Rocky Hill, NJ, USA), IL-1 β (Peprotech), and TNF- α (Peprotech). To measure PGE₂ production, PGE₂ ELISA kit (Cayman Chemical Company, Ann Arbor, MI, USA) was used following the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RAW 264.7 cells seeded in 24-well plate at a density of 3×10^5 cells/500 μ L per well were treated with PSE for 4 h, followed by 1 μ g/mL LPS stimulation. After 20 h, cells were lysed in 250 μ L of RNAiso (TAKARA bio Incorporation, Otsu, Shiga, Japan), and total RNAs were extracted and reverse transcribed into cDNA by using M-MLV RTase, dNTP, RNase inhibitor, and reaction buffer (Promega, Madison, WI, USA). qRT-PCR was conducted by using TOPrealTM qPCR 2X premix (Enzygnomics, Daejeon, Korea) according to the manufacturer's instructions with a CFX ConnectTM Real-Time PCR Detection System (Bio-Rad Laboratories, San Francisco,

CA, USA). Forward and reverse primer sequences for mouse COX-2, iNOS, and β -actin are listed in Table 1.

Immunoblotting analysis

RAW 264.7 cells seeded in 6-well plate at a density of 2×10^6 cells/2 mL per well were treated with PSE for 4 h, followed by 1 μ g/mL LPS stimulation for 15 min. For STAT3 activation, cells were stimulated with 10 ng/mL IL-6 for 30 min. Cells were lysed in RIPA lysis and extraction buffer (Thermo Scientific, Rockford, IL, USA) and incubated on ice for 15 min. Samples were centrifuged at 12,000 rpm for 15 min at 4 °C and supernatants were transferred to clean microtubes. Protein concentration was measured by PierceTM BCA Protein Assay Kit (Thermo Scientific) following the manufacturer's instructions. Cell lysates were loaded to a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to Immune-Blot[®] PVDF Membrane for Protein Blotting (Bio-Rad, Hercules, CA, USA). Membrane was blocked with Tris-buffered saline containing .1% tween 20 and 5% BSA, overnight at 4 °C. After blocking, the membrane was incubated overnight at 4 °C with primary antibody and with horse radish peroxidase conjugated goat anti-rabbit IgG antibody (Bio-Rad) for 1 h at RT. Protein bands were visualized by West-Q Pico ECL solution (GenDEPOT, Barker, TX, USA), and detected by ChemiDoc XRS densitometer (Bio-Rad). Quantification of protein was conducted by using Quantity One software (Bio-Rad).

Statistical analysis

All data were represented as mean $\pm$ standard deviation (S.D.). Differences between experimental conditions are assessed by two-tailed Student's *t* test. Results were considered significantly different when *p* values were less than .05.

Results

Cytotoxicity of PSE

MTT assay was performed to evaluate the cytotoxic effects of PSE in RAW 264.7 cells. The PSE showed no cytotoxic effect at concentrations up to 100 μ g/mL when compared to DMSO control both in the presence or absence of LPS [Fig. 1(A), (B)]. However, 200 μ g/mL of PSE demonstrated significant cytotoxic effects in RAW 264.7 cells, irrespective of LPS. Therefore, 200 μ g/mL of PSE was not included in subsequent experiments.

Table 1 The list of primers used for quantitative real-time PCR

Primer	Direction	Sequence (5'–3')
β -actin	Forward	ACCCACACTGTGCCCATCTAC
	Reverse	GCCATCTCCTGCTCGAAGTC
iNOS	Forward	AGACGGATAGGCAGAGATTGG
	Reverse	ACTGACACTTCGGACAAAGC
COX-2	Forward	AAGAAGAAAGTTCATTCTGATCCC
	Reverse	TGACTGTGGGAGGATACATCTCTC

Fig. 1 Cytotoxicity of PSE. RAW 264.7 cells were treated with 25, 50, 100, and 200 µg/mL PSE for 4 h and then stimulated with (A) or without (B) LPS (1 µg/mL) for 20 h. Cell viability was measured by

MTT assay, and presented compared to DMSO control. The data are representative of three experiments with similar results, and expressed as mean $\pm$ S.D. ** $p < .01$, and *** $p < .001$

Inhibition of iNOS expression and NO production

iNOS expression in macrophages during infectious or inflammatory conditions leads to the production of NO, a major mediator and indicator of inflammation [6]. qRT-PCR and western blot analysis were conducted to measure iNOS

mRNA and protein expression, respectively. The results revealed that PSE markedly reduced the level of iNOS at mRNA and protein level upon LPS-stimulation in RAW 264.7 cells in a dose dependent manner [Fig. 2(A), (B)]. In accordance with the decrease in iNOS expression, LPS-induced NO production was also suppressed by PSE [Fig. 2(C)].

Fig. 2 Inhibition of iNOS expression and NO production. RAW 264.7 cells were treated with PSE for 4 h, and incubated with LPS for 20 h. qRT-PCR was conducted to evaluate iNOS mRNA expression level (A). Expression level of iNOS mRNA was normalized by β -actin. Total cell lysates were subjected to western blot analysis (B). Expression level of iNOS protein was normalized by β -tubulin. NO

production was measured in culture supernatant by Griess reagent (C). Percentage of NO production was normalized to the value of control DMSO-treated cells. The data are presented as the mean $\pm$ S.D. of three representative experiments with similar results. * $p < .05$, ** $p < .01$, and *** $p < .001$

Inhibition of COX-2 and PGE₂ production

COX-2 induced by LPS or cytokine stimulation, synthesizes PG from arachidonic acid. PGE₂ is well known for mediating inflammatory responses, influencing the development of some cancers, and inducing fever [20]. Therefore, selective inhibitor of COX-2 is being used as anti-inflammatory and analgesic agent [21]. As shown in Fig. 3(A), (B), PSE (100 µg/mL) treatment inhibited the expression of COX-2 at mRNA and protein levels simultaneously compared to DMSO control. Consequently, PGE₂ production was also decreased by PSE treatment at a concentration of 100 µg/mL [Fig. 3(C)].

Decrease in pro-inflammatory cytokine production

During inflammation, macrophages release pro-inflammatory cytokines such as IL-6, IL-1β, and TNF-α to stimulate immune response. Similarly, LPS treatment stimulated the production of IL-6, IL-1β, and TNF-α, and the increase in IL-6 and IL-1β was repressed by PSE in a dose dependent manner ranging from 25 to 100 µg/mL [Fig. 4(A), (B)]. However, TNF-α production was not affected by PSE even at a concentration of 100 µg/mL [Fig. 4(C)].

Inhibitory effect of PSE on ERK and STAT3 signaling pathways

To investigate the inhibitory effects of PSE on intracellular signaling pathways, phosphorylation of MAPKs such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), and p38, IκBα mediating NF-κB signaling pathway, and STAT3, an essential mediator of inflammatory signaling pathway, was evaluated in PSE-treated RAW 264.7 cells. As shown in Fig. 5(A), the ratios of phosphorylated ERK (p-ERK) to ERK was decreased by PSE treatment. These results indicate that the anti-inflammatory effect of PSE was due to suppression of ERK phosphorylation in response to LPS stimulation. However, PSE treatment did not alter the phosphorylation of p38, JNK, and IκBα [Fig. 5(A), (B)]. In addition, as shown in Fig. 5(C), PSE treatment reduced the phosphorylation of STAT3 without altering the total STAT3 protein levels after stimulation with IL-6. The data indicate that PSE modulates inflammatory responses by inhibiting the activation of MAPK such as ERK and STAT3.

Fig. 3 Decrease in COX-2 and PGE₂ expression. RAW 264.7 cells were treated with PSE for 4 h, and incubated with LPS. After 20 h incubation, COX-2 mRNA expression (A), COX-2 protein expression (B), and PGE₂ production (C) were determined. Experimental values were presented as the relative value to that of DMSO control.

Expression level of COX-2 mRNA was normalized by β-actin and COX-2 protein was normalized by β-tubulin. Each data is representative of three experiments with similar results. Data are expressed as the mean ± S.D. **p* < .05 and ***p* < .01

Fig. 4 Inhibition of pro-inflammatory cytokine production. RAW 264.7 cells were treated with PSE for 4 h, and incubated with LPS for 20 h. After 20 h incubation, supernatant was collected. The production of IL-6 (A), IL-1 β (B) and TNF- α (C) was measured by ELISA. Data are representative of four experiments with similar results, and expressed as the mean $\pm$ S.D. * p < .05, ** p < .01, and *** p < .001

Discussion

During inflammation, activation of macrophage is induced by a combination of transcription factors that are mediated by NF- κ B and MAPK pathways [22]. In macrophages, TLR activation through external stimuli such as LPS phosphorylates I κ B α via I κ B kinase. The phosphorylated

I κ B α is then ubiquitinated and degraded by the proteasome, and the degradation of I κ B α allows to release NF- κ B and translocate to the nucleus. NF- κ B acts as a transcription factor that induces inflammation via pro-inflammatory cytokines [22].

MAPK pathways consist of the ERK pathway, the JNK pathway, and the p38 pathway. Each pathway is activated through sequential phosphorylation by external stimuli. Activated MAPKs can phosphorylate downstream targets including protein kinases and transcription factors, and consequently facilitate the transcription of MAPK-regulated genes, leading to production of pro-inflammatory mediators such as NO and PGE₂, and pro-inflammatory cytokines including TNF- α , IL-6, and IL-1 β in activated macrophages [23, 24]. Pro-inflammatory cytokines play a major role in cell signaling and promoting systemic inflammation, and often used as indicators of inflammation [25]. However, excessive production of pro-inflammatory cytokines may cause tissue destruction, cancer, and even death, and become novel targets of inflammatory diseases [3].

In this study, PSE suppressed LPS-induced iNOS and COX-2 protein expression leading to a decrease in NO and PGE₂ production in murine macrophage RAW 264.7 cells. Furthermore, the production of IL-6 and IL-1 β was also reduced by PSE treatment, but TNF- α production was not affected by PSE.

Previous studies revealed that iNOS, IL-6, and IL-1 β genes contain NF- κ B and STAT3 binding sites in their promoter regions, whereas TNF- α gene has a binding site for NF- κ B and not for STAT3 [22, 26]. The immunoblotting analysis depicted that PSE suppressed STAT3 phosphorylation without affecting I κ B α phosphorylation. This result, in part, explains the observation that PSE decreased the expression of iNOS, IL-6, and IL-1 β , but did not influence TNF- α production. Another possible explanation for the unchanged or increased (at 100 μ g/mL) level of TNF- α may attribute to a study reporting that simultaneous blockade of ERK and p38 is required for suppression of TNF- α production in RAW 264.7 cells [27]. Since PSE only inhibited the ERK pathway and not the p38 pathway, TNF- α production was not reduced in our study. Additionally, cell type-dependent differential regulation of TNF- α production by ERK signaling may relate to the inability of PSE to reduce TNF- α level. It has been shown that ERK inhibitor blocked the expression of LPS-induced TNF- α gene in alveolar macrophages, but not in non-pulmonary macrophages [28]. As RAW 264.7 cells originate from murine ascites, PSE treatment may not be able to inhibit TNF- α production despite the decrease in ERK phosphorylation. Moreover, PSE is a complex mixture of multiple organic compounds. In this regard, it cannot be overlooked that PSE may contain compounds which

Fig. 5 Inhibitory effect of PSE on the activation of ERK and STAT3. RAW 264.7 cells were treated with 25, 50, and 100 $\mu\text{g/mL}$ PSE for 4 h, and incubated with 1 $\mu\text{g/mL}$ of LPS. After 15 min, total cell lysates were subjected to immunoblotting analysis. For STAT3 analysis, cells were treated with 10 ng/mL of IL-6 for 30 min and cell lysates were prepared. p-ERK, ERK, p-p38, p38, p-JNK, JNK (**A**), p-I κ B α , I κ B α (**B**), and p-STAT3, STAT3 (**C**) levels in total cells were

determined by western blot analysis. p-MAPK and p-STAT3 expression was normalized by total MAPK and total STAT3 expression level, respectively. p-I κ B α and I κ B α expression level was normalized by β -tubulin. Three independent experiments were conducted. The intensity of protein bands from western blots was quantified and expressed as bar graph. Data are expressed as the mean $\pm$ S.D. * $p < .05$

counteract the anti-inflammatory effects of PSE through ERK signaling pathway and specifically stimulate the production of TNF- α . For example, mollugin isolated from *Rubia cordifolia* has been shown to stimulate TNF- α production while inhibiting the production of NO, IL-6, and IL-1 β [29].

STAT3 is an essential mediator of inflammatory signaling pathway induced by LPS [30]. Activated STAT3 translocates to the nucleus and regulates the transcription of inflammation-related genes [31]. Upon LPS stimulation, macrophages are induced to increase IL-6 production followed by augmented activation of STAT3 through IL-6

signaling pathway, and the activated STAT3, in turn, upregulates IL-6 production [32]. Our study demonstrates that PSE downregulates IL-6 production by inhibiting ERK phosphorylation in LPS-stimulated RAW 264.7 cells, and also represses STAT3 phosphorylation upon IL-6 stimulation, indicating that PSE exerts dual effects on IL-6 regulation.

About 19 compounds, including phytol, linoleic acid, and α -linoleic acid, in the ethanol extract from leaves of *P. scandens* have been detected by GC-MS analysis [33]. Linoleic acid and α -linolenic acid were shown to inhibit the production and gene expression of pro-inflammatory cytokines in THP-1 cells [34]. Phytol inhibited carrageenan-induced inflammatory response by reducing IL-1 β and TNF- α production in mouse model [35]. Therefore, the inhibitory effects of PSE on pro-inflammatory cytokine production may be attributed to the presence of these compounds. Further studies are required to identify and isolate single compounds responsible for the anti-inflammatory effects observed.

In summary, the results demonstrate that PSE exerts anti-inflammatory activity in LPS-induced murine RAW 264.7 cells. PSE reduced the production of NO, PGE₂, and pro-inflammatory cytokines such as IL-6 and IL-1 β by downregulating the phosphorylation of ERK and STAT3. Therefore, this study suggests that *P. scandens* can be a promising therapeutic agent for inflammatory diseases. Further studies are required to isolate and assess single compounds that are responsible for anti-inflammatory activities.

Acknowledgement This work was supported by the National Research Foundation (NRF) funded by the Ministry of Science, ICT, and Future Planning (2015R1C1A2A01054457 to H.M.), and by the Chung-Ang University Research Scholarship Grants in 2016 (W.L.).

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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