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ORIGINAL ARTICLE

Ginsenoyne C, a Polyacetylene Isolated from *Panax Ginseng* Inhibit Inflammatory Mediators via Regulating Extracellular Regulated Kinases Signaling

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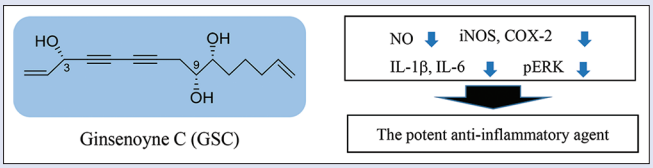
ABSTRACT

Background: *Panax ginseng* has been used as a traditional medicine for various pathological conditions. Ginsenoyne C (GSC) is a polyacetylene, minor constituent. **Objective:** The objective of the present study was to determine the anti-inflammatory activities of GSC for the protection of inflammation. **Materials and Methods:** The effects of GSC on cell viability and nitric oxide (NO) production in lipopolysaccharide (LPS)–induced RAW 264.7 cells were investigated through MTT assay and the Griess reaction, respectively. The levels of inflammatory cytokines such as interleukin (IL-1β) and IL-6 and inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and pERK were measured using enzyme-linked immunosorbent assay and Western blot analysis, respectively. **Results:** GSC suppressed NO production in LPS-induced RAW 264.7 cells. GSC suppressed the production of inflammatory mediators through downregulating phosphorylation of extracellular regulated kinases signaling in LPS-induced RAW 264.7. **Conclusion:** GSC has a potential therapeutic agent for protection of inflammation.

Key words: Ginsenoyne C, inflammation, *Panax ginseng*, polyacetylene, RAW 264.7 cells

SUMMARY

- Ginsenoyne C (GSC) was polyacetylene isolated from powders of fermented ginseng extract (FGE)
- GSC inhibited NO production with the concentration-dependent manner and down-regulated the levels of the proinflammatory cytokines, IL-1β and IL-6, and the iNOS, cyclooxygenase-2 and pERK protein levels in LPS-induced RAW 264.7 cells
- GSC has potential as an anti-inflammatory agent to protect inflammation.



Abbreviation used: CCK: Cell counting kit; COX-2: Cyclooxygenase-2; ELISA: Enzyme linked-immunosorbent assay; ERK: Extracellular regulated kinases; FBS: Fetal bovine serum; FEG: Fermented ginseng extract; GSC: Ginsenoyne C; IL: Interleukin; iNOS: Inducible nitric oxide synthase; LPS: Lipopolysaccharide; MAPK: Mitogen-activated protein kinases; NO: Nitric oxide; PVDF: Polyvinylidene difluoride; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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INTRODUCTION

Panax ginseng, belonging to Araliaceae, has been used as traditional remedies of various pathological conditions for centuries. Many studies were reported on the pharmacological effects of *ginseng* such as antineoplastic, antitumor, antioxidant, neuroprotective, anti-inflammatory, antinociceptive, and anti-stress activities.[1-7] Ginsenosides, triterpenoidal saponins, are known to be major active components of *P. ginseng*.[8-11] On the other hand, polyacetylenes were considered to be minor constituents in *P. ginseng*, and their biological effects have only been evaluated in the recent years.[12] It was reported that Ginsenoyne C (GSC) was a polyacetylene isolated from *P. ginseng*; however, its bioactivities have not yet been reported.

When the immune system malfunctions, leading to the activation of various stimuli such as proinflammatory cytokines and other mediators, the process is known as inflammation.[13,14] This defense response of the body can arise in response to injuries of multiple pathologies and if misdirected, can cause autoimmune diseases and other illnesses. Macrophages are major effector cells in the inflammatory reaction and are activated in response to lipopolysaccharide (LPS), interferon (IFN)-γ, and β-amyloid. Activated macrophages produce proinflammatory enzymes involving cycyooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and proinflammatory cytokines involving tumor necrosis factor (TNF)-α and interleukin (IL)-1β, IL-6, and inflammatory mediators relating NO.[15-17] Production of excessive inflammatory mediators could lead to decreased expression of the anti-inflammatory response and are associated with rheumatoid arthritis, autoimmune disorders, microcirculatory dysfunction, neuropathological diseases, and tissue damage.[18,19] Inhibition of inflammatory mediator release may be an effective therapeutic strategy to protect diseases caused by an inflammatory response.

Mitogen-activated protein kinases (MAPK) plays a pivotal role in inflammation and a family of serine/threonine protein kinases has

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been identified that mediate biological processes and cellular reaction to external stress stimulus.[20-24] Regulation of activated MAPK such as extracellular signal-regulated kinase (ERK), protein 38 (p38), c-jun NH_2-terminal kinase (JNK), and inflammatory mediators at activation of signal transcription make them potential targets for anti-inflammatory therapeutics.

As a part of our ongoing searching program to investigate the anti-inflammatory properties of phytochemicals, we investigated the anti-inflammatory activities of GSC in LPS-induced murine macrophages. In the study, we assessed anti-inflammatory activities of GSC from *P. ginseng* roots in LPS-induced murine macrophages.

MATERIALS AND METHODS

Ginseng extract preparation

The fermented ginseng extract (FGE), prepared by patented process was provided from Greencrosshs (Sungnam, Korea). Concisely, the roots of *P. ginseng* was continually extracted with ethanol and then reacted with an enzyme including ginsenoside-β-glucosidase. The residue was conducted acid hydrolysis reaction and the reactant was eluted from Diaion HP-20 resin using water-95% ethanol gradient and as a mobile phase.[25]

Isolation of ginsenoyne C from the fermented ginseng extract

Powders of the FGE (90 g) were used for silica gel column chromatography (20 cm × 5 cm column) using a gradient elution of solvents ($CHCl_3$: CH_3OH = 10:1, 7:1, 5:1, 3:1 → 100% CH_3OH) to yield 24 fractions (FGE1–24). FGE 3 was chromatographed by RP C_{18} column chromatography with a mixture of CH_3OH and H_2O (30:70 → 100:0) and obtained 17 subfractions (FGE3-1-17). FGE 3–7 was purified on a preparative HPLC (CH_3CN: H_2O = 45:55, 2 mL/min) and yielded GSC (8.7 mg).

GSC – brownish syrup; $C_{17}H_{24}O_3$; ESIMS (negative mode): m/z 276.17 $[M-H]^-$; ^{1}H-NMR ($CDCl_3$, 500 MHz) δ_H 6.21 (1H, ddd, *J* = 17.1, 10.2, 5.3 Hz, H-2), 5.83 (1H, ddd, *J* = 17.0, 10.0, 6.5 Hz, H-16), 5.62 (1H, ddd, *J* = 17.1, 1.9, 1.4 Hz, H-1a), 5.20 (1H, ddd, *J* = 10.1, 1.4, 1.4 Hz, H-1b), 5.31 (1H, br d, *J* = 3.3 Hz, H-3), 5.21 (1H, d, *J* = 17.0 Hz, H-17a), 5.10 (1H, d, *J* = 10.0, H-17b), 4.07 (1H, br q, H-9), 3.99 (1H, br q, H-10), 3.01 (1H, dd, *J* = 16.9, 5.5 Hz, H-8a), 2.89 (1H, dd, *J* = 16.9, 6.9 Hz, H-8b), 2.25 (2H, q, *J* = 5.9 Hz, H-15), 1.18 (2H, m, H-11), 1.70 (2H, m, H-12), 1.51 (4H, m, H-13,14); ^{13}C-NMR ($CDCl_3$, 125 MHz) δ_C 138.9 (C-16), 136.9 (C-2), 117.8 (C-1), 115.4 (C-17), 80.4 (C-7), 76.4 (C-4), 73.4 (C-10), 73.2 (C-9), 70.6 (C-5), 66.4 (C-6), 63.0 (C-3), 34.1 (C-11), 32.0 (C-15), 30.1 (C-13), 29.6 (C-14), 26.6 (C-12), 25.3 (C-8).

Cells and cytotoxicity determination

RAW 264.7 cells were obtained from the KCLB. The cells were maintained in DMEM supplemented 10% heat-inactivated FBS, penicillin, and streptomycin solution, and cultured at 37°C humidified atmosphere with 5% CO_2. Cells were cultured in serum-free DMEM medium with various concentration of GCS for 1 h and stimulated with LPS for indicated times. Cell viability was evaluated using a cell counting kit-8 assay kit (Dojindo. Japan). All experiment was used 1 μg/mL of LPS.

Nitric oxide measurement

The cells were treated with GSC for 1 h and were stimulated with LPS for 24 h. After incubation, the culture media was mixed with an equal amount of Griess reagent, incubated at RT for 15 min, and measured at 550 nm using a microplate reader (BioTek Instruments, Inc, Winooski, USA).

Western blotting analysis

RAW 264.7 cells were seeded and pretreated with or without GSC and stimulated with LPS. The whole cell lysates were suspended in a protein extraction solution. Equal amounts of protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, MA, USA). The membrane was blocked with 5% skim milk in plain buffer (20 mM Tris (pH 7.4), and 136 mM NaCl) and incubated with primary antibodies (SantaCruz Biotech, CA, USA). The membrane was incubated with specific-HRP conjugated antibodies (SantaCruz Biotech, CA, USA). The immunoreactive bands were exposed to enhanced chemiluminescence Western blot analysis detection reagents (ThermoFisher Scientific, Waltham, MA, USA), and were analyzed using a Bio imaging-system (MicroChemi 4.2 Chemilumineszenz-System, Israel).

The measurement of pro-inflammatory cytokines

The concentration of cytokines in culture media of murine macrophage was measured using enzyme-linked-immunosorbent assay (ELISA) kits (Cusabio, Wuhan, China).

Statistical analysis

The statistical differences were determined through a "one-way ANOVA" test using a computerized statistical package. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Isolation of ginsenoyne C from fermented ginseng extract

GSC was obtained as brownish syrup from powders of the FGE through repeated column chromatography. From the negative ESIMS at m/z 276.17 $[M-H]^-$, its molecular formula was determined as $C_{17}H_{24}O_3$. The structure of GSC was confirmed as a compound of the polyacetylene series based on comparison with data from the previous literature [Figure 1]. Polyacetylene-based compounds have been reported to be isolated from ginseng extract, but their pharmacological activity is not yet known.

Cytotoxic effects

The cytotoxicity of GSC was assessed by quantitating cell viabilities in the presence of the GSC, as shown in Figure 2. GSC exhibited no toxic effects on the cells over a concentration range from 1 to 10 μM.

Effects on nitric oxide production

Proinflammatory macrophages produce high levels of a pleiotropic molecule, nitric oxide (NO), that plays an important role a range of physiological and pathological inflammatory reactions.[26,27] This molecule is classically generated from iNOS and L-arginine.[15,16] To assess its inhibitory activities on NO production, cells were treated with GSC for 1 h, stimulated with LPS for 24 h, and NO production in culture media was evaluated through the Griess reaction. NO production was found to have been concentration-dependently reduced by this compound [Figure 3].

Effects on inducible nitric oxide synthase and cyclooxygenase-2 activation

The enzymes, iNOS and COX-2, are involved in the generation of pro-inflammatory mediators in activated cells.[15,16] To further study the inhibitory activities of GSC, expression of COX-2 and iNOS was measured through Western blot. Cells were pretreated with GSC for

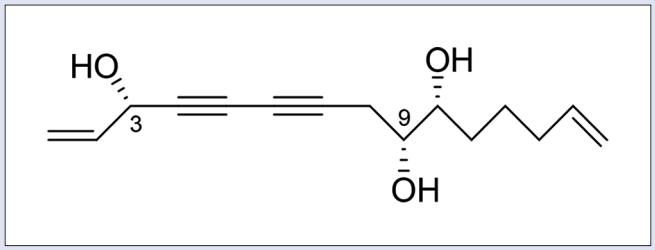

Figure 1: Chemical structure of ginsenoyne C. Ginsenoyne C was polyacetylene isolated from powders of fermented ginseng extract

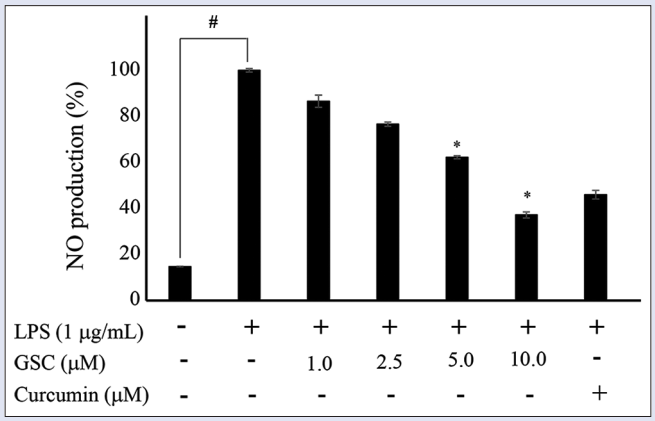

Figure 3: Effects of ginsenoyne C on nitric oxide production in lipopolysaccharide-stimulated RAW 264.7 cells. The cells were treated with ginsenoyne C for 1 h and stimulated with lipopolysaccharide (1 mg/mL) for 24 h. The culture media were measured the nitric oxide production according to Griess reaction. Each determination was made in triplicate and the data are expressed as means ± standard deviation $^{\#}P < 0.05$ indicates significant differences from the control group. $^{*}P < 0.05$ indicates significant differences from the lipopolysaccharide-treated group

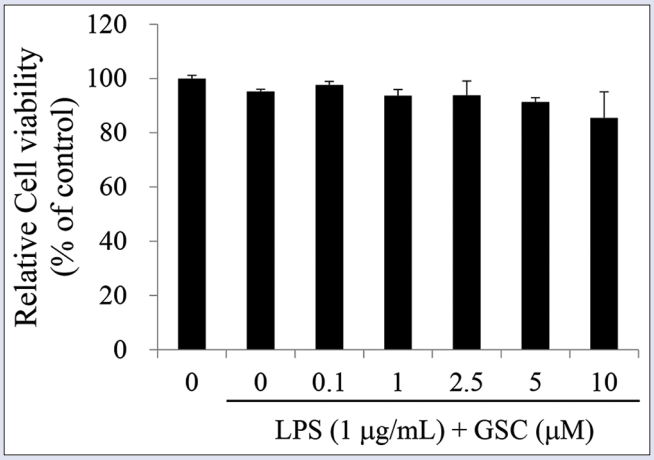

Figure 2: Effects of ginsenoyne C on the cell viability. RAW 264.7 cells were cultured in the presence of ginsenoyne C for 1 h and stimulated lipopolysaccharide (1 μg/mL) for 24 h under serum-free conditions. The cell viability in lipopolysaccharide-induced RAW 264.7 cells treated with various concentration of ginsenoyne C for 24 h. ginsenoyne C cytotoxicity was determined cell counting kit-8 assay according to manufacturer's instruction. Each determination was made in triplicate and the data are expressed as means ± standard deviation

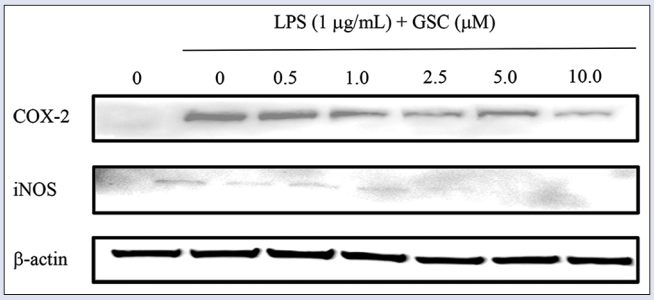

Figure 4: Effects on inducible nitric oxide synthase and cyclooxygenase-2 activation. Cells were pretreated with the indicated concentrations of ginsenoyne C for 1 h and stimulated lipopolysaccharide (1 μg/mL) for 24 h under serum-free conditions. Lysates were prepared, and the expression of inducible nitric oxide synthase, cyclooxygenase-2 and β-actin was detected by Western blot analysis using corresponding antibodies. The results presented are representative of three independent experiments

1 h followed by addition of LPS and incubated for 24 h. Protein levels of COX-2 and iNOS were not detectable in basal murine macrophages, but LPS stimulus caused a dramatic increase in activation of COX-2 and iNOS. However, the corresponding level of GSC-treated cells was shown to be concentration-dependently reduced [Figure 4]. These results showed that GSC-mediated inhibition of NO production was associated with inhibition of COX-2 and iNOS level.

Effects on pro-inflammatory cytokines production

The levels of pro-inflammatory cytokines are increased during inflammatory and immune responses, and play an important role in the development of inflammatory diseases.[20] It is well established that cellular exposure to LPS causes the secretion of inflammatory cytokines that lead to the inflammatory reaction. To assess the anti-inflammatory effects of GSC on cytokine production in LPS induced macrophages, the cells were treated with GSC for 1 h and stimulated LPS for 24 h, and the content of cytokines in culture media was measured using ELISA. Treatment of GSC concentration-dependently suppressed these IL-1β and IL-6 production, compared to LPS alone [Figure 5].

Effects on mitogen-activated protein kinases activation

MAPK activation is considered to be major mechanisms underlying inflammation and are involved in the regulation of pro-inflammatory mediators.[21-24] To examine whether the inhibition of inflammatory mediator secretion by GSC is mediated through a MAPK pathway, we assessed the effect of GSC on LPS-induced activation of ERK 1/2, p38, and JNK by the Western blot analysis. Murine macrophages were pretreated with GSC for 24 h and then followed by LPS stimulation for 15 min. As shown in Figure 6, treatment of GSC resulted in the inhibition of LPS-induced activation of ERK 1/2, whereas it did not affect p38 and JNK phosphorylation. These results suggest that the suppression of one of the MAPK constituents plays a crucial role in decreasing GSC-induced anti-inflammatory actions. Studies regarding the anti-inflammatory activities of GSC also included the modulation of inflammatory proteins and cytokines. Moreover, GSC suppressed ERK 1/2 phosphorylation in LPS-stimulated murine macrophage cells.

The study results were the first results that GSC isolated from the root of *P. ginseng* plays a pivotal role in the anti-inflammatory activities of LPS-induced murine macrophages, and that GSC may reduce inflammatory mediators during exposure to LPS stimulation through

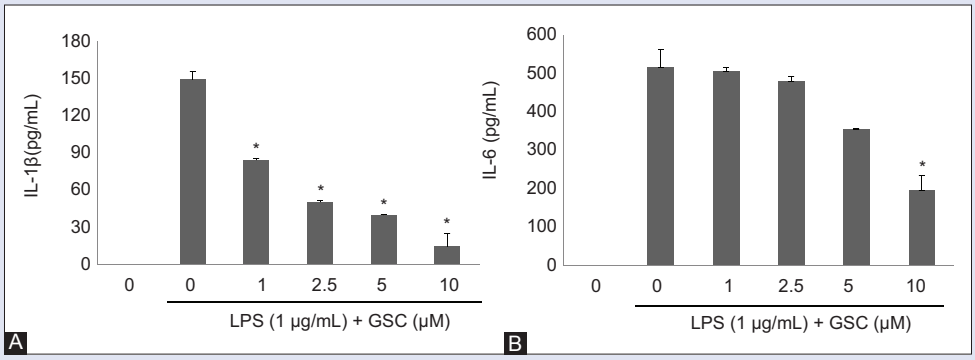

Figure 5: Effects on pro-inflammatory cytokine production. Cells pretreated with various concentrations of ginsenoyne C for 1 h were stimulated with lipopolysaccharide (1 μg/mL) for 24 h. The production of interleukin-1β (A) and interleukin-6 (B) in the cultured media were determined by enzyme linked-immunosorbent assay according to the manufacture's instrument. Data are means ± standard deviation of three independent experiments. *$P < 0.05$ indicates significant differences from the lipopolysaccharide-treated group

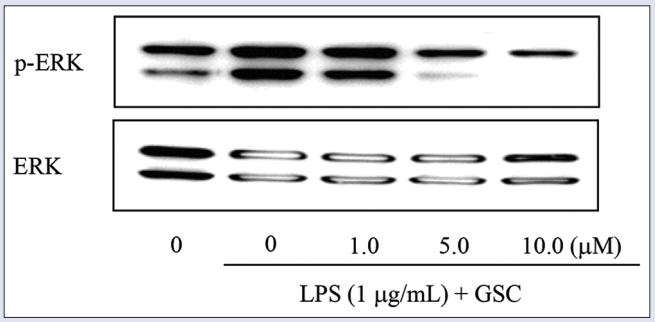

Figure 6: ginsenoyne C inhibits mitogen-activated protein kinases activation. RAW 264.7 cells were cultured in the presence of ginsenoyne C for 24 h and stimulated with lipopolysaccharide (1 mg/mL) for 15 min under serum-free conditions. Whole cell lysates were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the phosphorylation of extracellular regulated kinases 1/2, p38, and c-jun NH_2-terminal kinase was measured by Western blot analysis. The results presented are representative of three independent experiments

down-regulation of MAPK phosphorylation. We suggested that GSC is highly promising for development as therapeutic agents in inflammation.

Acknowledgements

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Nil.

Conflicts of interest

There are no conflicts of interest.

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