Pulchellamin G, an amino acid-sesquiterpene lactone, from *Saussurea pulchella* suppresses lipopolysaccharide-induced inflammatory responses via heme oxygenase-1 expression in murine peritoneal macrophages

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ABSTRACT

*Saussurea pulchella* (Asteraceae) is widely distributed in Korea and has been used in Korean folk medicine for the treatment of inflammation, hypertension, hepatitis, and arthritis. Pulchellamin G is an amino acid-sesquiterpene lactone conjugate isolated from *S. pulchella*. In the present study, we focused on the anti-inflammatory effect of pulchellamin G, which acts by inducing the expression of heme oxygenase (HO)-1. HO-1 plays important roles in cytoprotection since it has antioxidant, anti-inflammatory, antiproliferative, and antiapoptotic properties. Pulchellamin G inhibited the mRNA and protein expression of inducible nitric oxide synthase (iNOS), iNOS-derived nitric oxide (NO), and cyclooxygenase (COX)-2 and COX-derived prostaglandin E2 (PGE2) production in lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. The compound also reduced tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) production and suppressed the phosphorylation and degradation of IkBα and nuclear translocation of p65 in murine peritoneal macrophages in response to LPS stimulus. The inhibitory effects of pulchellamin G on nuclear factor kappa B (NF-κB) translocation was impaired by co-treatment of the cells with HO activity inhibitor tin protoporphyrin (SnPP). By using SnPP, we verified that the inhibitory effects of pulchellamin G on the pro-inflammatory mediators NO, PGE2, TNF-α, and IL-1β are associated with induction of HO-1 expression. Our data suggest that pulchellamin G might have potent therapeutic effects and it should be considered in the development of treatments for various inflammatory diseases.

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1. Introduction

Heme oxygenase (HO)-1 and its enzymatic by-products appear to play important roles in cytoprotection through their various properties, including antioxidant (Clark et al., 2000), anti-inflammatory (Kapturczak et al., 2004), antiproliferative (Deng et al., 2004), and antiapoptotic properties (Choi et al., 2004). Thus, considering the cytoprotective role of HO-1, induction of HO-1 expression by pharmacological agents may represent a novel therapeutic mechanism for the treatment of various diseases. When HO-1 catabolizes heme, 3 products that can mediate anti-inflammatory effects are released: carbon monoxide (CO), biliverdin, and free iron (Fe2+). HO-1 expression and CO administration have potent anti-inflammatory effects in monocytes and macrophages (Otterbein et al., 2000). The antioxidant effects of biliverdin/bilirubin and Fe2+ contribute to the overall cytoprotective effect of HO-1 (Singleton and Laster, 1965; O’Carra and Colleran, 1971; Stocker et al., 1987; Otterbein et al., 2002). Induction of HO-1 expression is primarily regulated at the transcriptional level and its induction by various agents is mediated by the nuclear transcription factor E2-related factor 2 (Nrf2), a master regulator of antioxidant responses (Venugopal and Jaiswal, 1996; Itoh et al., 1997; Ishii et al., 2000).

Macrophages, activated by stimulation with bacterial lipopolysaccharide (LPS) or in many inflammatory conditions, produce several pro-inflammatory cytokines and mediators, such as tumor
necrosis factor (TNF-α), interleukin (IL)-1β, nitric oxide (NO), and prostaglandins (PGs) (Beutler et al., 1985; Remick et al., 1990). The production of NO and PGs is increased by increased activity of their inducible enzymes, such as nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 (Nathan and Xie, 1994; Korhonen et al., 2005). The production of these cytokines is also regulated by the transcription factor nuclear factor kappa B (NF-κB) (Pahl, 1999; Blackwell and Christman, 1997). Previous studies suggested that when macrophages are induced to over-express HO-1 prior to stimulation with LPS, the subsequent pro-inflammatory response is markedly inhibited (Minamino et al., 2001). In addition, the upregulation of HO-1 expression reduces the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), thereby attenuating the production of NO and prostaglandin E2 (PGE2), respectively (Suh et al., 2006; Oh et al., 2006).

Saussurea pulchella Fisch (Asteraceae) is widely distributed in Korea and has been used in Korean folk medicine for the treatment of inflammation, hypertension, hepatitis, and arthritis (An, 1998). Although there have been a number of studies on the chemical constituents and biological activities of the genus Saussurea, there have been few phytochemical investigations on S. pulchella, and to date, only a sesquiterpene lactone (Kushnir and Kuzovkov, 1996, 1968) and 4 phenolics (Agafonova et al., 1996; Basargin and Tsiklauri, 1990) have been reported. In our previous study, we reported the isolation of seven new amino acid-sesquiterpene lactone conjugates, pulchellamines A–G, from the methanol extract of the aerial parts of S. pulchella (Yang et al., 2008). We conducted several biological evaluations of these compounds, and in the course of these evaluations, pulchellamines G has identified as an only compound having suppressive effect on LPS-induced NO production. Therefore, we further investigated whether pulchellamine G downregulates the expression of pro-inflammatory mediators in murine macrophages obtained from C57BL/6 mice. To the best of our knowledge, this is the first report on the biological and pharmacological value of pulchellamine G.

2. Materials and methods

2.1. Materials

Pulchellamin G (≥95% pure) (Fig. 1A) was isolated from S. pulchella as described previously (Yang et al., 2008). S. pulchella was collected at Mt. Odae, Korea, in August 2005. A voucher specimen (SKK-05-080) was deposited at the College of Pharmacy at Sungkyunkwan University. Sulfuretin (≥95% pure) isolated from Rhus verniciflua was used as a positive control (Lee et al., 2010). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco BRL Co (Grand Island, NY, USA). Tin protoporphyrin IX (SnPP IX), an inhibitor of HO activity, was obtained from Porphyrin Products (Logan, UT, USA). Thioglycollate (TG) was purchased from BD Pharmingen (San Diego, CA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA) unless stated otherwise. Primary antibodies, including those raised against HO-1, COX-2, iNOS, IkB-α, p-IkB-α, and p65, and the appropriate secondary antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for PGE2, TNF-α, and IL-1β were purchased from R&D Systems (Minneapolis, MN, USA).

2.2. Peritoneal macrophage culture

C57BL/6 mice were purchased from Orient Bio Co. (Sungnam, Kyung-Kido, Republic of Korea). Animal care and experimental protocols approved by the Wonkwang University Ethics Committee for Animal Experiments (WKU11-29). The authors further attest that all efforts were made to minimize the number of animals used and their suffering. TG-elicited peritoneal macrophages were harvested 4 days after an intraperitoneal (i.p.) injection of 3 ml of TG (Narumi et al., 1990). Peritoneal lavage was performed using 8 ml of Hanks’ Balanced Salt Solution containing 10 U/ml heparin. The cells were distributed in Roswell Park Memorial Institute medium (RPMI) supplemented with 10% heat-inactivated FBS, in 6-well tissue culture plates (5 × 10⁶ cells/ml).

2.3. Cell viability assay

The effects of various experimental modulations on cell viability were evaluated by determining mitochondrial reductase function with an assay based on the reduction of tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) into formazan crystals (Berridge and Tan, 1993). The synthesis of formazan is proportional to the number of functional mitochondria in living cells. For the determination of cell viability, 50 mg/ml of MTT was added to 1 ml of cell suspension (1 × 10⁶ cells/ml) in 96-well plates for 4 h. The formazan synthesized was dissolved in acidic 2-propanol and the optical density was measured at 590 nm. The optical density of the formazan formed in control (untreated) cells was considered as 100% viability.

2.4. Determination of nitrite (NO production)

Production of nitrite, a stable end product of NO oxidation, was used as a measure of iNOS activity. The nitrite present in a conditioned...
medium was determined using a method based on Griess reaction (Titheradge, 1998). An aliquot (100 μl) of each supernatant was mixed with the same volume of Griess reagent (0.1% (w/v) N-(1-naphthyl)-ethylenediamine and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid) for 10 min at room temperature. The absorbance of the final product was measured spectrophotometrically at 525 nm by using an ELISA plate reader, and the nitrite concentration in the samples was determined from a standard curve of sodium nitrite made up in phenol red-free DMEM.

2.5. PGE2 assay

The level of PGE2 present in each sample was determined using a commercially available kit from R&D Systems. The assay was performed according to the manufacturer’s instructions. Briefly, murine peritoneal macrophages were cultured in 24-well plates, pre-incubated for 12 h with different concentrations of pulchellamin G, and then stimulated for 18 h with LPS. The cell culture supernatants were collected immediately after treatment and centrifuged at 13,000g for 2 min to remove the particulate matter. The medium was added to a 96-well plate pre-coated with affinity-purified polyclonal antibodies specific for PGE2. An enzyme-linked polyclonal antibody specific for PGE2 was added to the wells and left to react for 2 h, followed by a final wash to remove any unbound antibody–enzyme reagent. A substrate solution was added, and the intensity of color produced, which was measured at 450 nm (the correction wavelength was set at 540 nm or 570 nm), was proportional to the amount of PGE2 present.

2.6. TNF-α and IL-1β assays

The levels of TNF-α and IL-1β present in each sample were determined using a commercially available kit from R&D Systems (Sawle et al., 2005). The assay was performed according to the manufacturer’s instructions. Briefly, murine peritoneal macrophages were cultured in 24-well plates, pre-incubated for 12 h with different concentrations of pulchellamin G, and then stimulated for 18 h with LPS. The cell culture supernatants were collected immediately after treatment and centrifuged at 13,000g for 2 min to remove particulate matter. The medium was added to a 96-well plate pre-coated with affinity-purified polyclonal antibodies specific for mouse TNF-α or IL-1β. An enzyme-linked polyclonal antibody specific to mouse TNF-α or IL-1β was added to the wells and left to react for 2 h, followed by a final wash to remove any unbound antibody–enzyme reagent. A substrate solution was added, and the intensity of color produced, which was measured at 450 nm (the correction wavelength was set at 540 nm or 570 nm), was proportional to the amount of TNF-α or IL-1β present.

2.7. Preparation of cytosolic and nuclear fractions

Murine peritoneal macrophages were homogenized (1:20, w-v) in PER-Mammalian Protein Extraction buffer (Pierce Biotechnology, Rockford, IL, USA) containing freshly added protease inhibitor cocktail 1 (EMD Biosciences, San Diego, CA, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cytosolic fraction of the cell was prepared by centrifugation at 15,000g for 10 min at 4°C. Nuclear and cytoplasmic extracts of murine peritoneal macrophages were prepared using NE-PER nuclear and cytoplasmic extraction reagents (Pierce Biotechnology). After treatment, the murine peritoneal macrophages (3 x 10⁶ cells/3 ml in 60 mm dishes) were collected and washed with phosphate-buffered saline (PBS). After centrifugation, cell lysis was performed at 4°C by vigorous shaking for 15 min in radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl [pH 7.4], 50 mM glycerophosphate, 20 mM NaF, 20 mM ethylene glycol tetraacetic acid [EGTA], 1 mM dithiothreitol [DTT], 1 mM Na₂VO₄, and protease inhibitors). After centrifugation at 15,000g for 15 min, the supernatant was separated and stored at −70°C until further use. The protein content was determined using the bicinchoninic acid (BCA) protein assay kit.

2.8. Western blot analysis

Western blot analysis was performed by lysing the cells in 20 mM Tris–HCl buffer (pH 7.4) containing a protease inhibitor mixture (0.1 mM PMSF, 5 mg/ml aprotinin, 5 mg/ml pepstatin A, and 1 mg/ml chymostatin). The protein concentration was determined using a Lowry protein assay kit (P5626; Sigma Chemical Co.). An equal amount of protein for each sample was resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electrophoretically transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk and sequentially incubated with primary antibody (Santa Cruz Biotechnology) and horseradish peroxidase-conjugated secondary antibody followed by ECL detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

2.9. RNA quantification

Total RNA for reverse transcription (RT)-PCR was extracted from harvested cells by using Trizol (Invitrogen, Carlsbad, CA, USA). The RNA isolation protocol included DNase I treatment. Total RNA was quantified and 1 μg was reverse transcribed into cDNA in a 20 μl reaction volume by using an oligo(dT)₁₂₋₂₀ primer and the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). RT-PCR was conducted in a 25-μl solution containing 67.7 mM Tris–HCl (pH 8.8); 16.6 mM (NH₄)₂SO₄; 0.01% Tween-20; 200 nM each of dATP, dCTP, and dGTP and 400 nM of dUTP; 4.5 mM MgCl₂; 300 nM of each primer; 200 nM probe; 2 U Taq DNA polymerase; and 1/10 (by volume) of the cDNA synthesis reaction. The thermal cycling conditions consisted of 4 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 60°C. PCR was carried out by using following primers: iNOS, 734 bp, forward 5'-GAC CAG ATA ACG CAA GCA C-3', reverse 5'-CTT GTC TTT GAC CCA GTA GC-3', COX-2, 696 bp, forward 5'-ATG CTC CTG CTT GAG TAT GT-3', reverse 5'-CAC TAT ATG ACC CAC TT-3', and actin, 514 bp, forward 5'-TGT GAT GGT GGG TCA GGC-3', reverse 5'-TTT GAT GTC ACG CAC GAT TTC C-3'. The annealing temperatures were 55°C for iNOS, 65°C for COX-2, and 58°C for actin.

2.10. Real-time PCR

Total RNA was isolated from the cells by using Trizol (Invitrogen), in accordance with the manufacturer’s recommendations, and quantified spectrophotometrically at 260 nm. Total RNA (1 μg) was reverse transcribed using the High Capacity RNA-to-cDNA kit (Applied Biosystems, Carlsbad, CA, USA). The cDNA was then amplified using the SYBR Premix Ex Taq kit (TaKaRa Bio Inc., Shiga, Japan) by using a StepOnePlus Real-Time PCR system (Applied Biosystems). Briefly, each 20 μl of reaction volume contained 10 μl of SYBR Green PCR Master Mix, 0.8 μM of each primer, and diethyl pyrocarbonate (DEPC)-treated water. The primer sequences were designed using PrimerQuest (Integrated DNA Technologies, Cambridge, MA, USA). The primer sequences were as follows: HO-1, forward 5'-CTGTTGGTATCGTGGAAGGACT-3', reverse 5'-GTAGAGGCAGGGATGATGTTCT-3'. The optimum conditions for PCR amplification of the cDNA were
established by following the manufacturer’s instructions. The data were analyzed using StepOne software (Applied Biosystems), and the cycle number at the linear amplification threshold (Ct) values for the endogenous control gene (glyceraldehyde 3-phosphate dehydrogenase [GAPDH]) and the target gene were recorded. Relative gene expression (target gene expression normalized to the expression of the endogenous control gene) was calculated using the comparative Ct method \(2^{-\Delta\Delta Ct}\).

2.11. DNA-binding activity of NF-κB

The DNA-binding activity of NF-κB in the nuclear extracts was measured using the TransAM kit (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions.

2.12. Assay for HO activity

HO enzyme activity was measured as previously described (Kutty and Maines, 1982). Briefly, microsomes from harvested cells were added to a reaction mixture containing NADPH, rat liver cytosol as a source of biliverdin reductase and the substrate hemin. The reaction was carried out in the dark for 1 h at 37°C and terminated by the addition of 1 ml chloroform. The extracted bilirubin was calculated from the difference in absorbance between 464 and 530 nm.

2.13. Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.) of at least 3 independent experiments. To compare 3 or more groups, one-way analysis of variance (ANOVA) followed by the Newman–Keuls post hoc test was used. Statistical analysis was performed using GraphPad Prism software, version 3.03 (GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Effects of pulchellamin G on viability of murine peritoneal macrophages

In this experiment, we focused on the possible anti-inflammatory effects of pulchellamin G. First of all, the possible cytotoxic effects of pulchellamin G on murine peritoneal macrophages were determined using MTT assay. TG-elicited peritoneal macrophages were harvested from C57BL/6 mice. Cell viability was not significantly decreased at pulchellamin G concentrations of up to 40 μM (Fig. 1B). Thus, for all our subsequent experiments, we used a concentration range of 5–40 μM.

3.2. Effects of pulchellamin G on expression of protein and mRNA pro-inflammatory enzymes, and production of pro-inflammatory cytokines in murine peritoneal macrophages stimulated with LPS

The expression of pro-inflammatory enzymes, including COX-2 and iNOS, plays an important role in immune-activated macrophages through the production of COX-2-derived PGE_2 and iNOS-derived NO (Suh et al., 2006; Oh et al., 2006). Murine peritoneal macrophages were stimulated with LPS (1 μg/ml) for 18 h, in the presence or absence of non-cytotoxic concentrations of pulchellamin G (5–40 μM), and the iNOS and COX-2 mRNA and protein expression levels were measured (Fig. 2). Pre-treatment of the macrophages with pulchellamin G for 12 h resulted in decreased iNOS expression (Fig. 2A, C) and also attenuated the production of iNOS-derived NO (Fig. 3A). Under the same conditions, pulchellamin G suppressed COX-2 expression (Fig. 2B, D) and reduced COX-derived PGE_2 production (Fig. 3B). This finding that pulchellamin G suppressed LPS-induced pro-inflammatory mediators, such as NO, iNOS, COX-2, and PGE_2, led us to further test the effects of pulchellamin G on LPS-induced TNF-α and IL-1β production. Murine peritoneal macrophages were pre-treated with pulchellamin G for 12 h and then stimulated with LPS. Enzyme immunoassay revealed that pulchellamin G decreased TNF-α (Fig. 3C) and IL-1β (Fig. 3D) production in a concentration-dependent manner. The natural products-derived sulfuretin at 40 μM, as the positive control, decreased LPS-induced pro-inflammatory mediators, such as NO, iNOS, COX-2, and PGE_2, and pro-inflammatory cytokines, such as TNF-α and IL-1β production (Figs. 2 and 3).

3.3. Effects of pulchellamin G on the protein expression level of p-IκB-α and IκB-α, NF-κB activation and NF-κB-DNA binding activity in murine peritoneal macrophages stimulated with LPS

We next set out to determine the mechanisms by which pulchellamin G suppresses the production of LPS-induced pro-inflammatory enzymes and mediators, including iNOS, COX-2, NO, PGE_2, TNF-α, and IL-1β. We focused on the phosphorylation and degradation of IκB-α, an inhibitor of NF-κB nuclear translocation. As shown in Fig. 4A, IκB-α in murine peritoneal macrophages was degraded after treatment with LPS (1 μg/ml for 1 h). However, pulchellamin G pre-treatment for 12 h, at concentrations ranging from 5 to 40 μM, markedly inhibited the LPS-induced phosphorylation and degradation of IκB-α, thereby
preventing NF-κB (p65) translocation into the nucleus. The nuclear p65 protein level increased after treatment with LPS for 1 h. However, this response was gradually inhibited by increasing the concentration of pulchellamin G. We also observed an increase in the DNA-binding activity of NF-κB in nuclear extracts from murine peritoneal macrophages stimulated with LPS for 1 h. Compared with the control treatment, treatment with LPS increased NF-κB binding activity by approximately 3-fold. However, pulchellamin G impaired this activity in a concentration-dependent manner (Fig. 4B).

3.4. Effects of pulchellamin G on HO-1 expression through nuclear translocation of Nrf2 and HO activity in murine peritoneal macrophages

Next, we examined whether pulchellamin G induces HO-1 expression and HO activity in murine peritoneal macrophages. In cells treated with non-cytotoxic concentrations of pulchellamin G (5–40 μM for 12 h), we found a concentration-dependent increase in HO-1 mRNA expression (Fig. 5A), and this increase coincided with an increase in the induction of HO activity (Fig. 5B) and HO-1 protein (Fig. 5C). At pulchellamin G concentrations above 20 μM, HO-1 expression was considerably increased. By using a pulchellamin G concentration of 40 μM, we studied the temporality of the increase in HO-1 expression (Fig. 5D). HO-1 protein expression started to increase 6 h after pulchellamin G treatment, and the maximum level of HO-1 protein expression was reached 18 h after treatment. Thus, we also tested the effects of pulchellamin G on cells pre-treated with 40 μM of pulchellamin G for 12 h in the absence or presence of SnPP, a competitive inhibitor of HO activity (Fig. 5A and B). As shown in Fig. 5A and B, SnPP treatment attenuated the inhibitory activity of pulchellamin G on the HO activity. In addition, Nrf2 is as an indispensable regulator of the coordinated induction of phase II enzyme genes, including HO-1 (Venugopal and Jaiswal, 1996; Itoh et al., 1997; Ishii et al., 2000).
Therefore, we investigated whether the treatment of murine peritoneal macrophages with pulchellamin G induced the nuclear translocation of Nrf2. When macrophages were incubated with pulchellamin G for 0.5, 1.0, and 1.5 h, at a concentration of 40 μM, the nuclear fractions of the macrophages showed a gradual increase in Nrf2 levels with a concomitant decrease in cytoplasmic Nrf2 levels (Fig. 6).

3.5. Effects of SnPP on inhibition of pro-inflammatory mediators production and NF-κB signaling by pulchellamin G pre-treatment of LPS-stimulated murine peritoneal macrophages

To confirm the suppressive effect of HO-1 on pro-inflammatory mediators, such as NO, PGE₂, TNF-α, and IL-1β, we used SnPP, which is a competitive inhibitor of HO activity (Sato et al., 2001). Murine peritoneal macrophages were pre-treated with pulchellamin G (40 μM) for 12 h in the absence or presence of SnPP. The suppressive effects of pulchellamin G on LPS-stimulated NO, PGE₂, TNF-α, and IL-1β production were partially, but not totally, reversed by SnPP (Fig. 7). It is well known that the production of NO, PGE₂, TNF-α, and IL-1β is predominantly regulated by the NF-κB signaling pathway (Pahl, 1999). We proposed that if SnPP impaired the suppression of the pro-inflammatory mediators by pulchellamin G, it would also reduce the inhibition of NF-κB translocation by pulchellamin G. Macrophages were pre-treated for 12 h with pulchellamin G (20 or 40 μM) in the presence or absence of SnPP (50 μM) and stimulated for 1 h with LPS (1 μg/ml). The addition of SnPP significantly impaired the inhibitory effect of pulchellamin G on NF-κB translocation. Moreover, treatment with LPS decreased the cytosolic IκB-α expression, leading to the translocation of NF-κB into the nucleus and subsequent NF-κB activation. Treatment with pulchellamin G inhibited the LPS-induced IκB-α degradation and NF-κB translocation and this was markedly reversed by SnPP (Fig. 8A). We also observed the DNA-binding activity of NF-κB in nuclear extracts from murine peritoneal macrophages under the same conditions. SnPP also significantly reduced the inhibitory effect of pulchellamin G on NF-κB DNA-binding activity (Fig. 8B). Therefore, it is possible that the induction of HO-1 may contribute to the inhibitory effect of pulchellamin G on NF-κB translocation and the subsequent transcriptional activation of NF-κB target genes.

4. Discussion

Natural products can be used for the development of novel drugs and have, therefore, played a significant role in drug discovery and development for the treatment of various diseases, including inflammatory diseases. S. pulchella Fisch is widely distributed in Korea and has been used in Korean traditional
medicine for the treatment of inflammation (An, 1998). Sesquiterpenes (Yin et al., 2005; Sun et al., 2003; Wang et al., 2005; Matsuda et al., 2000, 2003), lignans (Li et al., 2006; Duan et al., 2002; Takasaki et al., 2000), and flavonoids (Fan and Yue, 2003; Xie et al., 2005) have been reported as constituents of the genus Saussurea and been found to possess a wide range of biological activities, including cytotoxic (Sun et al., 2003; Wang et al., 2005; Matsuda et al., 2000), anti-inflammatory (Matsuda et al., 2003), and antioxidant activities (Fan and Yue, 2003; Xie et al., 2005). Although there have been a number of studies on the chemical constituents and biological activities of the genus Saussurea, there have been few phytochemical investigations on S. pulchella, and to date, only a sesquiterpene lactone (Kushnir and Kuzovkov, 1996, 1968) and 4 phenolics (Agafonova et al., 1996; Basargin and
Tsiklauri, 1990) have been reported. Sesquiterpene lactones are chemically distinct from other sesquiterpenes owing to the presence of a γ-lactone moiety. Many sesquiterpene lactones have been shown to have a wide range of biological activities, such as antitumor, mutagenic, and genotoxic activities (Rodriguez et al., 1976; Picman, 1986; Lee et al., 1971). Pulchellamin G, an amino acid-sesquiterpene lactone conjugate isolated from S. pulchella, has an α-methylene-γ-lactone moiety. In our previous study, we reported the isolation of pulchellamines G from the methanol extract of the aerial parts of S. pulchella (Yang et al., 2008). In the present study, we investigated whether pulchellamine G down-regulates the expression of pro-inflammatory mediators in murine macrophages obtained from C57BL/6 mice. Thus, this study aimed to investigate potential anti-inflammatory actions of pulchellamin G and its ability to induce HO-1 expression. To the best of our knowledge, no study has yet been published on the anti-inflammatory effects of pulchellamin G in murine macrophages.

Macrophages, which play significant roles in host defense mechanisms, are widely used as a cellular model to screen compounds that potentially have anti-inflammatory effects. Inflammatory mediators and pro-inflammatory cytokines take charge of host defense, but their overproduction can be involved in the pathogenesis of various diseases, such as rheumatoid arthritis, periodontitis, chronic inflammation, otitis media, auto-immune diseases, hearing loss, and bacterial sepsis (Nathan and Xie, 1994; Korhonen et al., 2005). In many inflammatory conditions, the pro-inflammatory and cytotoxic mediators NO and PGs are released through the activity of their inducible enzymes, iNOS and COX-2 (Pahl, 1999; Blackwell and Christman, 1997). The transcription factor NF-κB (the p50/p65 heterodimer is the most common) is implicated in the regulation of many genes that code for mediators of immune, acute-phase, and inflammatory responses, including iNOS and COX-2 (Surh et al., 2001). Under basal conditions, NF-κB is sequestered in the cytoplasm by inhibitor proteins, usually IκB, but when it is released by a stimulus, NF-κB dimers can translocate to the nucleus to activate target genes by binding with high affinity to κB elements in their promoters (Verma et al., 1995).

HO-1 is cytoprotective because of its anti-inflammatory, anti-apoptotic, and antiproliferative effects that are, in turn, mediated by one or more of its heme products. When HO-1 catalyzes the heme, 3 products that can mediate its effects are released: CO, biliverdin, and Fe2+. CO has been shown to be highly protective against several rodent diseases, mimicking the actions of HO-1 (Sato et al., 2001). CO suppresses pro-inflammatory responses and promotes anti-inflammatory responses in macrophages. Biliverdin is converted to bilirubin by biliverdin reductase, with the bilirubin being recycled back into biliverdin, suggesting a mechanism to amplify the antioxidant effects. Source of Fe2+ from heme upregulates both the iron-transporter pump and the ferritin functioning as decreasing the intracellular iron accumulation, thereby protecting the cell from death; in addition, the expression of iron-transporter as well as ferritin contributes to anti-oxidant activity following the induction of HO-1 (Ferris et al., 1999; Balla et al., 1992).

The transcription factor Nrf2 regulates the basal and inducible expression of numerous detoxifying and antioxidant genes, including HO-1. Under basal conditions, Nrf2 is bound to kelch-like ECH-associated protein 1 (Keap1), an actin-binding cytoplasmic protein that represses its transcriptional activity thereby preventing it from activating target genes. Moreover, Nrf2 is ubiquitinated by E3 ubiquitin ligase, and degraded by the 26S proteasomes, thus Nrf2 levels are usually low. However, exposure to oxidative stress or other stimuli lead to the dissociation of Nrf2 from Keap1 and its stabilization and phosphorylation. Unbound Nrf2 translocates into the nucleus and binds to the antioxidant response element (ARE) in the upstream promoter region of many antioxidative genes, initiating their transcription (Itoh et al., 1999; Villeneuve et al., 2010).

To date, there have been no published studies on the anti-inflammatory effects of pulchellamin G or the underlying mechanisms. We investigated a potential involvement of increased HO-1 expression, mediated by the nuclear translocation of Nrf2, in the anti-inflammatory effect of pulchellamin G. We showed that pulchellamin G pre-treatment of LPS-stimulated murine peritoneal macrophages obtained from C57BL/6 mice inhibited NO production (Figs. 2 and 3). In addition, pulchellamin G also suppressed LPS-induced TNF-α and IL-1β production (Fig. 3). The natural products-derived sulforufin at 40 μM, as the positive control, also decreased LPS-induced pro-inflammatory mediators, such as NO, iNOS, COX-2, and PGE2, and pro-inflammatory cytokines, such as TNF-α and IL-1β production (Figs. 2 and 3). In previous study, sulforufin isolated from Rhus verniciflua inhibited LPS-induced inflammatory effects in murine peritoneal macrophages (Lee et al., 2010). Key steps in the signaling pathway leading to iNOS and COX-2 gene expression, i.e., NF-κB activation and DNA binding, could mediate its anti-inflammatory actions. Therefore, we examined the effects of pulchellamin G on the phosphorylation of IκB-α and the nuclear translocation of the p65 subunit of NF-κB in murine peritoneal macrophages. Our results showed that the LPS-induced phosphorylation and degradation of IκB-α, and the nuclear translocation of p65, were significantly reduced by pre-treatment with pulchellamin G for 12 h (Fig. 4A). We also showed that pulchellamin G inhibited the LPS-induced increase in NF-κB DNA-binding activity. These findings suggest that pulchellamin G, at least in LPS-stimulated macrophages, exerts its anti-inflammatory effects by inhibiting the expression of the pro-inflammatory enzymes iNOS and COX-2 and the secretion of the pro-inflammatory cytokines NO, PGE2, TNF-α, and IL-1β. If so, we curious about the inter-relationships between these anti-inflammatory effects of pulchellamin G and HO-1 expression. We found pulchellamin G to be a powerful HO-1 inducer (Fig. 5). The mechanism underlying HO-1 expression is known to involve nuclear translocation of activated Nrf2, and we found that pulchellamin G increased the levels of Nrf2 in the nucleus of murine peritoneal macrophages (Fig. 6). Our results indicate that inhibition of HO activity by the HO activity inhibitor SnPP partially reversed the inhibitory effects of pulchellamin G on NO, PGE2, TNF-α, and IL-1β production in LPS-stimulated macrophages (Fig. 7). Since SnPP blocks HO enzymatic activity, these data confirm that pulchellamin G inhibits NO, PGE2, TNF-α, and IL-1β production, at least partially, via modulation of HO-1 expression. Moreover, treatment with pulchellamin G inhibited LPS-induced IκB-α degradation and NF-κB translocation, which were markedly reversed by SnPP (Fig. 8). As shown in our Figs. 7 and 8, the treatment of SnPP, which inhibits HO enzyme activity, partially reversed the inhibitory effects of pulchellamin G on NO, PGE2, TNF-α, and IL-1β production and NF-κB translocation. This result suggested that HO-1 expression was involved in the LPS-induced cell responses. When cells are subjected to a variety of oxidative environmental stresses and inflammatory conditions, they typically respond by inducing a coordinated expression of genes encoding the set of phase II detoxifying enzymes, principally involved in activation of the transcription factor such as Nrf2 (Copple et al., 2008). Nrf2 plays a critical part in basal activity and coordinated induction of genes encoding numerous antioxidant and phase II detoxifying enzymes and related proteins, such as HO-1, glutathione (GSH), catalase, superoxide dismutase (SOD), glutathione-S-transferase (GST), γ-glutamyl cysteine ligase (GCL), UDP-glucuronosyltransferase, NAD[P]H:quinone oxidoreductase-1 (NQO1), glutamate cysteine ligase, glutathione peroxidase, glutathione reductase (GR), thioredoxin, and so on (Li et al., 2008).
These phase II detoxifying enzymes and related proteins play important roles in protecting cells from free radical and oxidative stress imposed by reactive oxygen species and NO. Therefore, in our Figs. 7 and 8, SnPP partly attenuated the LPS-induced cell responses, meaning that HO-1 expression as well as the other phase II detoxifying enzymes may be involved in the mechanism of the anti-inflammatory effects by pulchellamin G. However, in this study, we provided evidence to support the view that HO-1, one of the key phase II detoxifying enzymes, expression through Nrf2 signaling pathways plays an important role in mediating the anti-inflammatory effects of pulchellamin G.

In conclusion, we demonstrated that an increase in HO-1 expression through Nrf2 translocation by an amino acid-sesquiterpene lactone conjugate pulchellamin G, isolated from *Saussurea pulchella*, at least in part, suppresses NO, PGE2, TNF-α, and IL-1β production through the inhibition of NF-κB translocation in LPS-induced murine peritoneal macrophages. We suggest that pulchellamin G might have potent therapeutic effects and should be considered for the further development of treatments for various inflammatory diseases.

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If you need further assistance or have any more questions, feel free to ask! 😊


