Inhibitory Effects of Ginsenoside Rb₁, Rg₃, and *Panax ginseng* Head Butanol Fraction on Inflammatory Mediators from LPS-Stimulated RAW 264.7 Cells

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Abstract – *Panax ginseng* C.A. Meyer (Araliaceae, *P. ginseng*) has been used for the enhancement of vascular and immune functions in Korea and Japan for a long time. Ginsenoside Rb₁ and Rg₃ isolated from *P. ginseng* head-part butanolic extract (PGHB) were investigated for anti-inflammatory activity. Ginsenosides and PGHB did not affect the cell viability within 0 – 100 µg/ml concentration to RAW 264.7 murine macrophage cells. Ginsenosides and PGHB inhibited partly lipopolysaccharide (LPS)-induced nitrite production in a dose-dependent manner. The ginsenosides and PGHB showed partially chemical nitric oxide (NO) quenching (maximum 40%) in the cell-free system. Also, ginsenoside Rb₁ and Rg₃ inhibited markedly approximately 74 and 54% of inducible nitric oxide synthase (iNOS) mRNA transcription from LPS-induced RAW 264.7 cells. Taken together, the inhibitory effect of ginsenosides and PGHB on NO production did not occur as a result of cell viability, but was caused by both the chemical NO quenching and the regulation of iNOS. Additionally, the ginsenoside Rb₁ and PGHB inhibited prostaglandin E₂ (PGE₂) synthesis in a concentration-dependent manner, showed approximately 70-98% inhibition at 100 µg/ml concentration. And the treatment with ginsenosides and PGHB attenuated partially LPS-upregulated cyclooxygenase-2 (COX-2) gene transcription. Ginsenoside Rg₃ suppressed LPS-stimulated interleukin-6 (IL-6) level to the basal in RAW 264.7 cells. From these results, ginsenoside Rb₁, Rg₃, and PGHB may be useful for the relief and retardation of immunological inflammatory responses and its action may occur through the reduction of inflammatory mediators, including NO, PGE₂, and IL-6 production.

Key words: Ginsenoside Rb₁, Ginsenoside Rg₃, Prostaglandin E₂, Nitric oxide, iNOS, COX-2, IL-6

INTRODUCTION

Panax ginseng C.A. Meyer (Araliaceae, *P. ginseng*) has been used as a precious Asian herb medicine for weak physical strength and fatigue in Korea for a long time. Ginsenosides are the main active constituents in *P. ginseng*. The main ginsenosides are glucosides that contain an aglycone with a dammarane skeleton, and include protopanaxadiol-type saponins such as ginsenoside Rb₁, Rb₂, Rc, and Rd, and protopanaxtriol-type saponins such as ginsenoside Re and Rg₁ (Lee *et al.*, 2006). It was reported that ginsenoside Rb and Rc exhibited a seda-

tive effect, whereas ginsenoside Rg showed stimulatory actions on the central nervous system (CNS) (Saito *et al.*, 1974). Rb₁ is the most abundant ginsenoside in *P. ginseng*, and reportedly, it has a neuroprotective effect against ischemia (Lim *et al.*, 1997), glutamate neurotoxicity (Radad *et al.*, 2004), and seizures (Lian *et al.*, 2005). Rg₃ is well known as a potent ginsenoside which has a neuroprotective effect and an anti-stress effect in fermented red-ginseng (Bae *et al.*, 2004B). Ginsenoside Rb₁ is transformed into Rg₃ in the stomach to be absorbed in the form of Rh₂, based on their observation that human intestinal bacteria such as *Bacteroides* sp., *Fusobacterium* sp., and *Bifidobacterium* sp. were able to transform Rg₃ into Rh₂ (Bae *et al.*, 2004A).

Nitric oxide (NO) is involved in various physiological and pathophysiological responses. In inflammation, the

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production of NO from L-arginine results in the formation of L-citrulline and is catalyzed by the inducible nitric oxide synthase (iNOS or type II) in the host or isolated cells stimulated by bacteria or bacterial products such as lipopolysaccharide (LPS) and/or inflammatory cytokines (e.g., TNF- α , IL-1 β , IFN- γ). NO regulates the inflammatory transcription factors in both prokaryotic and eukaryotic cells, and also is involved in NF- κ B pathway, AP-1 pathway, and Jak-STAT pathway (Korhonen *et al.*, 2005). Therefore NO has been a target of intensive research and drug development.

Prostaglandin E₂ (PGE₂), one of the inflammatory mediators, is mainly synthesized from arachidonic acid (AA) by cyclooxygenase-2 (COX-2), and contributes to vasodilation, pain, and fever (Lu and Wahl, 2005). In inflammatory responses, COX-2, normally expressed at low levels, is strongly induced by pro-inflammatory agents, including LPS, tumor promoters, and growth factors.

In addition, the interleukin-6 (IL-6) is a multifunctional cytokine that play a central role in both innate and acquired immune responses, which is triggered by infection and inflammation. Briefly, IL-6 is involved during the subsequent development of acquired immunity against incoming pathogens, including regulation of the expressions of cytokine and chemokine, stimulation of antibody production by B-cells, regulation of macrophage and dendritic cell differentiation, and the response of regulatory T-cells to microbial infection (Grimble, 1998). In addition to these roles in pathogen-specific inflammation and immunity, IL-6 levels are elevated in chronic inflammatory conditions, such as rheumatoid arthritis (Yokota *et al.*, 2006). Due to the significantly involvement of IL-6 in several pathogenesis processes, IL-6 is considered as a drug discovery target.

Previously we reported that *P. ginseng* head butanol fraction (PGHB) had an anti-ulcer activity in rats, based on the premise that the emetics and stomach function are related (Jeong *et al.*, 2003). To elucidate the effects of ginsenoside Rb₁ and Rg₃ isolated from PGHB for an anti-inflammatory activity, we investigated the suppression of NO, PGE₂, and IL-6 production from LPS-stimulated RAW 264.7 cells in this study.

MATERIALS AND METHODS

Materials

Ginsenoside Rb₁, Rg₃, and *P. ginseng* head butanol fraction (PGHB) were supplied from a previous study (Fig. 1, Jeong *et al.*, 2003). Dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Fig. 1. Chemical structure of ginsenoside Rb₁ (A) and ginsenoside Rg₃ (B).

(MTT), 2,2-diphenyl-1-picrylhydrazyl (DPPH), lipopolysaccharide (LPS), and N^G-monomethyl-L-arginine monoacetate (L-NMMA) were purchased from Sigma (Sigma-Aldrich Inc., MO, USA). Cell culture medium and reagents, such as DMEM, fetal bovine serum (FBS), penicillin/streptomycin, and trypsin-EDTA were obtained from GIBCO (Invitrogen Inc., NY, USA). Ethanol and other reagents were used as first grade.

Cell culture

RAW 264.7 murine macrophage cells were obtained from American Type Culture Collection (ATCC, Rockville, MD, USA). These cells were cultured in DMEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a 5% CO₂ humidified incubator at 37°C. For subculture, RAW 264.7 cells were rinsed twice with phosphate buffered saline (PBS, pH 7.4) to remove all traces of serum, which contains trypsin inhibitor, and was subdivided using 0.05% trypsin with 0.53 mM EDTA. They were used at passage numbers 10-20 for this study.

Cell viability assay

Cell viability was examined using MTT assay. RAW 264.7 cells were plated at a density of 1×10^5 cells/well in 96-well tissue culture plate (Corning Inc., USA), and incubated at 37°C for 3-4 h. Plated cells were treated with indicated concentrations of ginsenoside Rb₁, Rg₃, and PGHB. After 24 h incubation, MTT was added to all well at 0.5 mg/ml of concentration, and incubated for 4 h at 37°C. After discarding all medium from the plates, 100 μ L of dimethyl sulfoxide (DMSO) was added to the all well. The plates were placed for 5 min at room temperature on the shaker, so that complete dissolution of formazan was achieved. The absorbance of the MTT formazan was determined at 540 nm by UV-spectrophotometric plate reader (Emax, Molecular Devices Inc., USA). Viability was defined as the ratio (expressed as a percentage) of absorbance of treated cells to untreated cells.

Nitrite assay

RAW 264.7 cells were plated at a density of 2×10^5 cells/well in a 96-well culture plate and incubated for 3–4 h in a 5% CO₂ humidified incubator at 37°C. Plated cells were treated with LPS (1 µg/ml) for stimulation of nitrite-production and the indicated concentrations of ginsenoside Rb₁, Rg₃, and PGHB, followed by 24 h incubation. LPS-stimulated nitrite-production from RAW 264.7 cells was measured by the Griess reaction. Briefly, 100 µl of each supernatant was mixed with 100 µl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride in distilled water), and the absorbance of the mixture was determined with a microplate reader (Emax, Molecular Devices Inc., USA) at 540 nm. In this experiment, 10 µM of L-NMMA, an iNOS inhibitor, was used as a positive control (Sutherland *et al.*, 2001).

Extracellular NO scavenging assay

Extracellular NO radical scavenging activity was measured by the modified protocol (Babu *et al.*, 2001). Briefly, sodium nitroprusside, a NO donor, generates NO when interacts with oxygen in aqueous solution at physiological pH, and NO is measured by Griess reaction. The reaction mixture (3 ml) containing sodium nitroprusside (10 mM in PBS) and ginsenoside Rb₁, Rg₃, or PGHB (50 µM) were incubated at 25°C for 150 min. After incubation, 0.5 ml of the reaction mixture and 0.5 ml of Griess reagent were mixed. The absorbance of the chromophore formed was evaluated at 540 nm. Hemoglobin (Hb, 5 µM) was used as a NO chemical scavenger (Wei *et al.*, 1999).

Measurement of prostaglandin E₂ (PGE₂)

RAW 264.7 macrophage cells were cultured in 24-well culture plates. After reaching confluence, LPS-induced cells were treated with ginsenoside Rb₁, Rg₃, or PGHB, and then incubated in a humidified incubator at 37°C for 48 h. After a 48 h incubation, the supernatant were collected, subdivided into portions and frozen at -74°C. The production of PGE₂, a inflammatory mediator, was measured using commercially available assay (PGE₂: R&D Systems, Wiesbaden, Germany). As a blank we measured cell culture medium from wells without cells that had been treated the same way as the samples according to the procedure described by the manufacturer. Briefly, the supernatant (100 µl) of cell culture that treated with ginsenoside Rb₁, Rg₃, or PGHB were added to the plate, and mixed with 50 µl of a primary mouse monoclonal PGE₂-antibody and PGE₂-conjugated with horse-

radish peroxidase (HRP), and were incubated for 2 h on the shaker at room temperature. And then the supernatant was discarded and washed 4 times. The mixture with hydrogen peroxide and chromogen (tetramethylbenzidine), a substrate, was added to plate and incubated for 30 min at room temperature, protected from light. Sulfuric acid (200 µl, 2 N) was added to all wells for the termination of the reaction, and absorbance was read at 450 nm within 30 min.

Measurement of interleukin-6 (IL-6)

IL-6 production from LPS-induced RAW264.7 macrophage cells was measured by ELISA according to the manufacturer's instruction on a monoclonal antibody based mouse interleukin microplate strip (Quantikine®M, R&D system, Meneapolis, MN, USA). IL-6 coating antibody (100 µl, 4 µg/ml) was added to the 96 well-plate, and incubated at room temperature overnight. After discarding the coating solution, 200 µl of assay solution (4% BSA in PBS, pH 7.4) was added, incubated at room temperature for 1 h, and washed three times with 50 mM Tris buffer (0.2% Tween-20, pH 8.0).

For the blot, 50 µl of IL-6 standards and the supernatant of cell culture that was treated with ginsenoside Rb₁, Rg₃, or PGHB were added to the plate, and incubated for 1 h. Then 50 µl of biotin-labeled detecting antibody (0.6 µg/ml) was added and incubated for another 1 h, and the plate was washed 3 times. And the diluted HRP-conjugated Streptavidine (1:8000) and TMB-substrate solution were added to plate. After a 30 min incubation, 100 µl of H₂SO₄ (0.18 M) was added to stop the reaction, and the absorbance was measured at 450 nm by ELISA plate reader (Emax, Molecular Devices Inc., Sunnyvale, CA, USA).

RT-PCR analysis

For the transcription assay of iNOS and COX-2 gene, the LPS-stimulated RAW 264.7 cells were incubated with ginsenoside Rb₁, Rg₃, and PGHB for 24 h in a 5% CO₂ humidified incubator at 37°C. NMMA (10 µM) and celecoxib (20 µM) were used as a positive control for a selective inhibitor iNOS and COX-2, respectively. Total RNA was isolated from RAW 264.7 cells using RNeasy kit (Qiagen Inc. Valencia, CA) after treated with ginsenoside Rb₁, Rg₃, and PGHB. RT-PCR was performed using One-Step RT-PCR kit (Qiagen Corp.) and primers at a final concentration of 1 µM. For PCR of iNOS and COX-2 in RAW 264.7 cells, the primers were used as followed: iNOS forward primer : 5'-CCCTTCCGAAGTTTCTG-GCAGC-3', iNOS reverse primer : 5'-GGCTGTCAGAGC-CTCGTGGCTT-3', COX-2 forward primer : 5'-GGAGA

GACTATCAAGATAGTGATC-3', COX-2 reverse primer : 5'-ATGGTCAGTAGACTTTTACAGCTC-3'. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers, the house-keeping gene, for RAW 264.7 cells were used to determine PCR efficiency and the quantitation; GAPDH forward primer: 5'-TGAAGGTCGGTGTGACGGATTGGC-3', GAPDH reverse primer: 5'-CATGTAGGCATGAGGTCCACCAC-3'. Thermal cycling was performed as followed. For cDNA synthesis and predenaturation, 1 cycle of 50°C for 30 min and 95°C for 15 min was performed on total RNA. For PCR reactions of iNOS and COX-2 gene were cycled 25 times between 94°C (for denaturation) for 45 s; 60°C (for annealing) for iNOS, 55°C (for annealing) for COX-2 and GAPDH for 1 minute; and 72°C (for extension) for 2 min. And the last 1 cycle was performed for the final extension at 72°C for 10 min using Bio-Rad thermal cycler (MJ Mini, Bio-Rad Inc., USA). And RT-PCR product was stored at 4°C until agarose gel separation. Transcriptional change were calculated using an electrophoresis image quantify program (Bio-Rad Inc., USA)

Statistical analysis

Data are shown as mean $\pm$ SEM (standard error of mean) of three separate experiments, and were analyzed for significance using analysis of variance (ANOVA), followed by Scheffe's test for multiple comparison. Values of $p < 0.05$ were considered significant.

RESULTS AND DISCUSSION

Effect of ginsenoside Rb₁, Rg₃, and PGHB on cell viability for RAW 264.7 macrophage cells

Cytotoxic effect of ginsenosides and PGHB for RAW 264.7 macrophage cells were shown in Table I. Ginsenoside Rb₁ and PGHB did not affect the severe cell viability within tested concentration (IC₅₀>1,000 μ g/ml), whereas ginsenoside Rb₃ showed a significant cytotoxicity for RAW 264.7 cells at more than 100 μ g/ml of concentration (IC₅₀=302.4 μ g/ml). From this result, the ginsenosides and PGHB (6.25 - 100 μ g/ml) was used for further

inhibition of NO, PGE₂, and IL-6 production-study from LPS-stimulated RAW 264.7 cells.

Effect of ginsenoside Rb₁, Rg₃, and PGHB on NO production from LPS-stimulated RAW 264.7 macrophage cells

To investigate the anti-inflammatory effect of ginsenosides and PGHB, we examined whether ginsenoside Rb₁, Rg₃, and PGHB could modulate NO synthesis in LPS-stimulated cultures of the RAW 264.7 murine macrophages cells. Ginsenosides and PGHB had inhibited partly LPS-induced nitrite production in a dose-dependent manner in tested concentration range (6.25 – 100 μ g/ml), as shown in Fig. 2. We observed LPS-stimulated nitrite production from RAW 264.7 cells, as compared to the basal. Also, N^G-monomethyl-L-arginine monoacetate (L-NMMA, 10 μ M), which is one of selective NOS blockers (Sutherland *et al.*, 2001), inhibited nitrite production significantly (approximately 92.4 %) as a positive control. PGHB (100 μ g/ml) inhibited approximately 19% NO production from LPS-induced RAW 264.7 cells (IC₅₀>100 μ g/ml). However ginsenoside Rg₃ (100 μ g/ml), isolated from PGHB, had remarkable inhibitory effect (98.1% %control, IC₅₀=31.0 μ g/ml) on nitrite production. This was superior to inhibitory effect of NMMA. Also, the other ginsenoside Rb₁ (100 μ g/ml), isolated from PGHB, showed partly NO inhibition (22.4% %control, IC₅₀>100 μ g/ml), which was quiet consistent with the report by Park *et al.* (2005).

NO, one of the important inflammatory mediators, is also related to the pathophysiology of inflammation joint disease and plays a key role in cartilage catabolism mediated by inflammation. Also, the inflammatory transcription factors, NF- κ B pathway, AP-1, Jak-STAT pathway, and nitric oxide synthase (NOS) and cellular adhesion molecule (CAM) protein transcription were regulated by NO (Korhonen *et al.*, 2005). NO may represent key regulatory molecules in the inflammatory process of rheumatoid arthritis. Also, NO is either up-regulate or down-regulate cyclooxygenase (COX) activity (Perez-Sala and Lamas, 2001), while PGs can affect iNOS activity (Salvemini, 1997). This finding shows that ginsenos-

Table I. Cell viabilities of ginsenoside Rb₁, Rg₃, and PGHB for RAW 264.7 macrophage cells

μ g/ml	% Control						
	6.25	12.5	25	50	100	500	1,000
Rb ₁	91.91 $\pm$ 7.06	96.79 $\pm$ 4.99	95.03 $\pm$ 1.79	96.85 $\pm$ 10.82	98.65 $\pm$ 4.70	94.30 $\pm$ 6.34	65.46 $\pm$ 3.88
Rg ₃	96.90 $\pm$ 4.89	93.59 $\pm$ 7.31	97.15 $\pm$ 3.72	97.43 $\pm$ 2.31	95.94 $\pm$ 5.52	9.19 $\pm$ 0.27 [#]	8.47 $\pm$ 0.48 [#]
PGHB	104.12 $\pm$ 3.84	102.84 $\pm$ 4.99	101.81 $\pm$ 10.02	100.68 $\pm$ 1.75	107.52 $\pm$ 3.83	138.19 $\pm$ 1.41	139.80 $\pm$ 5.27

Control value was 100 $\pm$ 7.61. [#] $p < 0.05$, as compared to the control.

Fig. 2. Effects of ginsenoside Rb₁, Rg₃, and PGHB on NO production from LPS-stimulated RAW 264.7 macrophage cells. #*P*<0.05, as compared to control, ##*P*<0.05, as compared to only LPS-treated group.

ide Rb₁, Rg₃, and PGHB partly inhibit LPS-induced nitrite production from RAW 264.7 macrophage cells, and, in turn, might affect the NO-mediated inflammatory responses.

Effects of ginsenoside Rb₁, Rg₃, and PGHB on the chemical NO scavenge and the suppression of iNOS mRNA transcription in LPS-stimulated RAW 264.7 cells

Partial inhibition of nitrite production by ginsenosides and PGHB, as shown in Fig. 2, can be explained by two possibilities. One explanation is the NO decrease by the chemical quenching of ginsenosides and PGHB. To demonstrate this possibility, the chemical quenching of NO by ginsenosides and PGHB was investigated in the cell-free system. Briefly, sodium nitroprusside, which is a NO donor, produces nitrite ions in aqueous solution at physiological pH. The chemical quenching of NO by ginsenosides and PGHB was measured by Griess reaction. As shown in Fig. 3A, the ginsenosides and PGHB showed partially chemical NO quenching (maximum 40%) in a concentration-dependent manner in the cell-free system, whereas Hb, a NO scavenger, scavenged most of the NO (approximately 88.6%). However NMMA did not show the chemical NO scavenging activity. From this result, the inhibition of NO production by ginsenosides and PGHB in LPS-induced RAW 264.7 cells was expected to be partially due to chemical scavenge of NO, which was produced by iNOS reaction.

The other explanation for the inhibition of nitrite production from LPS-stimulated RAW 264.7 cells is the suppression of iNOS mRNA transcription by ginsenosides and PGHB. We observed a significant up-regulation of iNOS mRNA transcription in LPS-stimulated RAW 264.7 cells (Fig. 3B). Additionally NMMA, a selective iNOS inhibitor, suppressed approximately 71% of up-regulated iNOS

Fig. 3. Effects of ginsenoside Rb₁, Rg₃, and PGHB on the chemical NO quenching and the suppression of iNOS mRNA transcription. (A) Chemical NO scavenging of ginsenoside Rb₁, Rg₃, and PGHB in the cell-free system. #*P*<0.05, as compared to control. (B) Suppression of iNOS mRNA transcription by ginsenoside Rb₁, Rg₃, and PGHB in LPS-stimulated RAW 264.7 cells. #*P*<0.05, as compared to control. ##*P*<0.05, as compared to the LPS-treated group.

gene transcription. Ginsenoside Rb₁ and Rg₃ (100 µg/ml) inhibited markedly approximately 74 and 54% of iNOS mRNA transcription from LPS-induced RAW 264.7 cells. Additionally PGHB (100 µg/ml) inhibited approximately 59% of iNOS mRNA transcription.

This result suggests that the decrease of NO production by PGHB is due to mainly chemical NO quenching activity by an antioxidant property of PGHB. Ginsenoside Rb₁ and Rg₃ had a similar suppressive effect on iNOS mRNA transcription, but it is supposed that their chemical NO scavenging activity caused the difference of nitrite production inhibitions (Fig. 2). Also, the inhibitory effect of ginsenosides and PGHB on NO production did not occur as a result of cell viability, but was caused by both the chemical NO quenching and the regulation of iNOS. Most studies evaluating the inhibition of NO production have shown only that iNOS protein suppression occurs, but have not included a chemical NO scavenging study by plant extracts. However, chemical NO quenching should be evaluated to determine the inhibition of NO production from LPS-induced RAW 264.7 cells.

Effects of ginsenoside Rb₁, Rg₃, and PGHB on the production of PGE₂ and the suppression of COX-2 mRNA transcription in LPS-stimulated RAW 264.7 cells

The biosynthesis of PGE₂, a typical inflammatory and rheumatoid arthritis mediator, was monitored from LPS-induced RAW 264.7 cells for the indication of inflammatory response (Fig. 4A). LPS stimulated significantly PGE₂ production from RAW 264.7 cells, and celecoxib (10 µM), which is a selective COX-2 inhibitor, inhibited approximately 66.2% PGE₂ synthesis from LPS-induced RAW 264.7 cells. Ginsenoside Rb₁ inhibited PGE₂ synthesis in a concentration-dependent manners (IC₅₀=22.73 µg/ml), showed approximately 98.4% inhibition at 100 µg/ml of ginsenoside Rb₁, which was showed superior effect to celecoxib. Additionally, PGHB suppressed approximately 70-90% PGE₂ synthesis from LPS-induced RAW 264.7 cells. However, ginsenoside Rg₃ did not affect the decline of PGE₂ synthesis with significant difference.

The synthesis of PGE₂ is regulated by COX-2 in acute inflammatory responses. Therefore the effect of ginsenosides and PGHB on COX-2 mRNA transcriptional level was investigated in LPS-induced RAW 264.7 cells. The transcription of COX-2, one of the important enzymes in inflammatory pathogenesis, was not observed in basal RAW 264.7 cells. However LPS increased COX-2 gene transcriptional levels significantly in RAW 264.7 cells, and LPS-stimulated COX-2 expression was suppressed by

Fig. 4. Inhibitory effects of ginsenoside Rb₁, Rg₃, and PGHB on PGE₂ synthesis and COX-2 mRNA transcription in LPS-stimulated RAW 264.7 macrophage cells. (A) Effects of ginsenoside Rb₁, Rg₃, and PGHB on the production of PGE₂ from LPS-stimulated RAW 264.7 macrophage cells, (B) Suppressive effects of ginsenoside Rb₁, Rg₃, and PGHB on COX-2 mRNA transcription in LPS-stimulated RAW 264.7 macrophage cells. #*P*<0.05, as compared to control. ##*P*<0.05, as compared to the LPS-treated group. CLC: Celecoxib (20 µM)

celecoxib (20 µM, 89%), a selective inhibitor of COX-2, depicted in Fig. 4B. The treatment with ginsenosides and PGHB attenuated partially LPS-upregulated COX-2 gene transcription. Ginsenoside Rb₁ and PGHB inhibited approximately 48% and 52% COX-2 mRNA transcription, respectively, and in a concentration-dependent manner in LPS-induced RAW 264.7 cells. Ginsenoside Rg₃ had approximately 25% COX-2 mRNA transcription and relatively less suppressive effect on COX-2 transcription. This result was consistent with PGE₂ synthesis-inhibition by ginsenosides and PGHB.

COX, the rate-limiting enzyme in the conversion of

arachidonic acid (AA) to prostaglandin (PGs), exists as two isoforms. COX-1 is constitutively produced and detectable in most human tissues. In contrast, COX-2, normally expressed at low levels, is strongly induced by inflammatory stimuli, including LPS, tumor promoters, and growth factors. At inflammatory sites, such as human atherosclerotic lesions, high levels of COX-2 have been found compared with low levels in unaffected arteries (Gorgoni *et al.*, 2001). The expression of COX-2 is a key element in the pathophysiology of several inflammatory disorders, and its regulation differs between cell types. COX-2 is expressed during inflammatory disease in many cells, including fibroblasts and macrophages, and mediates the release of large quantities of pro-inflammatory PGs at the site of inflammation.

COX-2 and NO represent key regulatory molecules in the inflammatory process in rheumatoid arthritis. Many studies have demonstrated that some inducible enzyme (COX and iNOS)/cytokines and their reaction products are involved in chronic inflammatory disease. The suppression of COX-2 transcription by ginsenosides and PGHB could be caused by inhibition of Akt activation, or by inhibition of AA release causing suppression of PGs synthesis (Van Dross *et al.*, 2005). Additionally the decrease of exogenously-generated NO by ginsenosides and PGHB exacerbates COX-2 expression in synovial cells (Honda *et al.*, 2000). These results are consistent with a report that decrease of NO production by ginsenosides and PGHB may be caused the down-regulation of COX-2 expression in RAW 264.7 macrophage cells (Chun *et al.*, 2004).

Prostaglandin biosynthesis plays a role in the nociceptive mechanism (Franzotti *et al.*, 2000). The reduction of writhing responses in mice is probably related to the reduced synthesis of the inflammatory mediators by inhibition of cyclooxygenases and/or lipoxygenases (Ojewole, 2006). Additionally, in rheumatoid arthritis, it is known that the third phase of the edema-induced by carrageenan, in which the edema reaches its highest volume, is characterized by the presence of prostaglandins (Spector and Willoughb, 1963). Besides, in the carrageenan-induced rat paw edema model, the production of prostanooids has been through the serum expression of COX-2 by a positive feedback mechanism (Nantel *et al.*, 1999).

This report elucidated that the analgesic and anti-rheumatoid arthritis activities of PGHB is due to the inhibition of PGE₂ synthesis through the suppression of COX-2 mRNA transcription by ginsenoside Rb₁, which is isolated from PGHB, and PGHB. Additionally our results raise the possibility that ginsenosides and PGHB may

retard inflammatory process in rheumatoid synovium by suppression of PGE₂ synthesis COX-2 mRNA transcription.

Effect of ginsenoside Rb₁, Rg₃, and PGHB on the production of IL-6

Ginsenosides and PGHB exacerbated the production of IL-6, an inflammatory cytokine, in LPS-induced RAW 264.7 cells (Fig. 5). The level of IL-6 is remarkably elevated by adding LPS in RAW 264.7 cells. Ginsenoside Rg₃ suppressed the IL-6 level to the basal (more than 99% inhibition) at 100 µg/ml concentration in concentration-dependant manners (IC₅₀ = 13.2 µg/ml). Otherwise ginsenoside Rb₁ did not affect the LPS-induced IL-6 level in RAW 264.7 cells at all. And PGHB suppressed approximately 23-30% IL-6 production in LPS-stimulated RAW 264.7 cells.

In vivo, IL-6 is a multifunctional cytokine that plays a central role in both innate and acquired immune responses. IL-6 is the predominant mediator of the acute phase response, an innate immune mechanism which is triggered by infection and inflammation. IL-6 also plays multiple roles during the subsequent development of acquired immunity against incoming pathogens, including regulation of the expressions of cytokine and chemokine, stimulation of antibody production by B cells, regulation of macrophage and dendritic cell differentiation, and the response of regulatory T cells to microbial infection. In addition to these roles in pathogen-specific inflammation and immunity, IL-6 levels are elevated in

Fig. 5. Effects of ginsenoside Rb₁, Rg₃, and PGHB on the production of IL-6 in LPS-stimulated RAW 264.7 macrophage cells. #*P*<0.05, as compared to control. ##*P*<0.05, as compared to the LPS-treated group.

chronic inflammatory conditions, such as rheumatoid arthritis.

This result suggests that PGHB, containing ginsenoside Rg₃, might have cartilage protection effects in the CIA model by decreasing inflammatory cytokines such as IL-6, and may be helpful to relief of RA.

It is concluded that ginsenosides and PGHB has suppressed NO production and the transcription of iNOS and COX-2 mRNA by LPS-stimulated RAW 264.7 macrophage cells. The potential analgesic and anti-inflammatory activity of PGHB was confirmed by investigating the anti-inflammatory activities of ginsenosides Rb₁ and Rg₃, isolated from PGHB. Taken together, ginsenosides and PGHB may be useful as an alternative medicine for the relief and retardation of immunological inflammatory responses and its action may occur through the reduction of inflammatory mediators, including NO, PGE₂, and IL-6 production.

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