Soybean glyceollins mitigate inducible nitric oxide synthase and cyclooxygenase-2 expression levels via suppression of the NF-κB signaling pathway in RAW 264.7 cells

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Abstract. Glyceollins, produced to induce disease resistance responses against specific species, such as an incompatible pathogen *Phytophthora sojae* in soybeans, have the potential to exhibit anti-inflammatory activity in RAW 264.7 cells. To investigate the anti-inflammatory effects of elicited glyceollins via a signaling pathway, we studied the glyceollin signaling pathway using several assays including RNA and protein expression levels. We found that soybean glyceollins significantly reduced LPS-induced nitric oxide (NO) and prostaglandin E2 (PGE_2) production, as well as the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) via the suppression of NF-κB activation. Glyceollins also inhibited the phosphorylation of IκBα kinase (IKK), the degradation of IκBα, and the formation of NF-κB-DNA binding complex in a dose-dependent manner. Furthermore, they inhibited pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-18, but increased the generation of the anti-inflammatory cytokine IL-10. Collectively, the present data show that glyceollins elicit potential anti-inflammatory effects by suppressing the NF-κB signaling pathway in RAW 264.7 cells.

Introduction

It is now recognized that soybeans (*Glycine max*) and soybean foods, such as tofu and soymilk, can contribute to the better health of the elderly, young people, and pregnant woman (1). The soybean, a legume with a high protein content (~35-40%)

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is excellent at meeting dietary protein needs. Moreover, the importance of soybeans is proven by clinical and preclinical studies of hypocholesterolemia, diabetes, obesity, renal dysfunction and cardiovascular disorders (2,3). Soybeans contain many known components, such as phytoalexins, that are incredibly useful. Genistein, a soybean isoflavone, has been highlighted over the last few years as a potentially beneficial agent due to its wide-ranging effects on breast cancer, osteoporosis, coronary heart disease, diabetes and menopausal discomfort (4-6). Glyceollin, a phytoalexin found in soybeans and grapes, is produced as an immune response to pathogens (7). Glyceollins are isolated as a mixture of glyceollin I, II, and III (Fig. 1A), from germinated soybean from *Rhyzopus* sp. by an elicitor (8). These compounds have been investigated in relation to their anti-estrogenic activity through the inhibition of estrogen receptor α and β compared with well-known phytoestrogenic chemicals such as enterolactones and genistein (9-11). It also has been recognized that glyceollins can suppress human breast and ovarian carcinoma tumorigenesis (12) and may modulate potential estrogenic properties in the breast through an anti-estrogenic effect (13); however, researchers have yet to elucidate the molecular mechanisms of their biological activity.

On the other hand, it has been revealed that NF-κB plays a pivotal role in molecular inflammation by controlling the expression of various genes that encode pro-inflammatory cytokines, chemokines and inducible enzymes, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), in mammalian immune cells (14). NF-κB is ubiquitously located in the cytoplasm of non-stimulated cells because of interactions with inhibitory proteins such as IκBs (15); but, it responds to pro-inflammatory stimuli, when IκBs are rapidly phosphorylated and degraded by the 26S proteasome (16,17), which results in the dissociation of free NF-κB dimers of p65 and p50, which translocate to the nucleus, and then activate the transcription of target genes. The IκBα kinase (IKK) complex contains two catalytic subunits: i) IKKα/β and ii) a regulatory subunit, IKKγ (18-20). Activation of IKK is mediated by phosphorylation through various upstream kinases, such as the NF-κB-inducing kinase, NF-κB-activating kinase, Akt, and protein kinase Cζ, that are involved in cellular signaling in response to pro-inflammatory stimuli (21). Following activation, the NF-κB heterodimer activates the transcription of

target genes, including the genes encoding the pro-inflammatory cytokines, such as interleukin (IL)-1, -6, -8, -18 and tumor necrosis factor-α (TNF-α) as well as iNOS, COX-2 and cell adhesion molecules (22-24). In turn, the products regulated by NF-κB, such as TNF-α and IL-1β, also lead to the activation of NF-κB. This fact means that there is a complex regulatory loop that amplifies and perpetuates inflammatory responses; therefore, it has become a biological target for new types of anti-inflammatory treatment.

Based on this reason, we first hypothesized that glyceollins, overproduced by an elicitor *Rhyzopus* sp., can ameliorate immune cells by an NF-κB signaling pathway. To prove this hypothesis, we compared whether glyceollins have the potential to possess potent anti-inflammatory effects by inhibiting LPS-induced iNOS and COX-2 expression, as well as various inflammatory cytokines like TNF-α, IL-1β, IL-18 in RAW 264.7 cells. We further examined whether glyceollins inhibit the LPS-induced inflammation process by blocking an NF-κB signaling pathway.

Materials and methods

Materials and cell culture. LPS (purified from *Escherichia coli* 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO) and fetal bovine serum (FBS) was purchased from Invitrogen (Carlsbad, CA). Antibodies against iNOS, COX-2, IKKα/β, NF-κB p65, IκBα and phospho-IκBα (Ser-32/36) were obtained from Cell Signaling Technology (Beverly, MA). RAW 264.7 cells were cultured in a RPMI-1640 medium supplemented with 10% of FBS, 2 mM of L-glutamine, 100 U/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 humidified incubator. We measured cell viability by counting, with a stain of 0.2% trypan blue solution.

Preparation of glyceollin mixture by elicitation. Glyceollins I, II and III (Fig. 1A, B and C) were semi-purified from elicited soybeans, with a slight modification, as previously described (8,11). In brief, we used soybeans (AGA, variant no. 3, a gift from KNU Soyventure, Daegu, Korea) that overproduce isoflavones to the rate of 10 mg/g of soybeans. The soybeans were elicited by a *Rhyzopus* sp. for *de novo* synthesis of glyceollins, which produced three kinds of isomers (data not shown). The cultures of *Rhyzopus* sp. were grown at 25°C in a culture room on potato dextrose agar media and the inoculums were harvested after 5 days. We first washed the soybeans by dipping in a 65% ethanol solution for 1 min; thereafter, we washed them with deionized water before the soaking process. Soybean seeds were soaked in sterile, deionized water for 6 h, and then placed into a Sanyo MLR-351H growth chamber (Carlsbad, CA). After saturation with distilled water, the seeds were cut into 4 pieces by a knife. A spore suspension (100 μl) of *Rhyzopus* sp. was dispersed on the cut surfaces of the seeds. The seeds (20 g) were incubated at 26°C for 4 days, extracted with 50 ml of 80% (v/v) ethanol for 1 h, then cooled and centrifuged at 20,000 x g for 10 min. The extracts were filtered by a membrane filter (0.45 μm) from Sartorius (Aubagne Cedex, France). Each crude extract was freeze-dried and dissolved in dimethyl sulfoxide. The extract was 100 μg/ml, and was used for further purification. For purification, we used a high-performance liquid chromatography system, the HPLC, PerkinElmer Series 200 (Waltham, MA). A Brownlee Choice C18 (150x4.6 mm) reverse-phase column and guard column were used. The injection volume and column temperature were 10 and 30 μl, respectively. Detection was monitored at 260 and 285 nm, and the flow rate was 1.0 ml/min at the following solvent condition (A, acetonitrile, B, 1% acetate in water; 0 to 45% A for 10.2 min; 45 to 90% A for 6 min, holding at 90% A for 3.6 min). The glyceollins were separated by thin-layer chromatography (TLC) for use in further experiments because the concentration was low and the amounts that we needed were small. The glyceollin phase was separated by open-column chromatography (OCC). A methanolic extract was fractionated on a Silica gel-60 (<0.063 mm: 0.063 mm, 8:2) developed in methylene chloride:methanol (97-99:1-3, v/v) solution. A sample of OCC extract was fractionated on aluminum-backed Silica gel-60 TLC plates developed in hexane:acetone (4:1.5, v/v). Glyceollins were visualized with 20% aqueous sulfuric acid spray reagent, and UV light at 254 nm. A single glyceollin band (Rf=0.2) was confirmed by a standard glyceollin, and further purified by preparative TLC. The concentration of glyceollin isomers in the fraction was up to ~90% as analyzed by preparative HPLC. The relative ratio of glyceollin I, II and III in the fraction was 17:2:1. Thereafter, we designated the fraction as glyceollins.

Cell viability assay. Cell viability was measured by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (25). Cells were seeded on 96-well plates at a concentration of 5x10^4 cells/well. After seeding, cells were incubated with MTT solution (1 mg/ml) for 2 h. The medium was discarded and the formazan product was solubilized with dimethyl sulfoxide. Viability was assessed by measuring absorbance at 570 nm with a Bio-Rad microplate reader (Hercules, CA).

Nitrite quantification. RAW 264.7 cells were treated with 1 μg/ml LPS plus sample for 16 h. Nitric oxide synthesis can be determined by assaying nitrite in the culture supernatant because nitrite is a stable reaction product of NO (26). Briefly, cell-free culture media were reacted with a 1:1 mixture of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) at room temperature for 10 min. The OD of the assay sample was measured spectrophotometrically at a wavelength of 570 nm. Nitrite concentration was calculated from a standard curve prepared using $NaNO_2$ under the same assay conditions.

Western immunoblot analysis. Proteins were separated by SDS-PAGE and immunoblotted onto a nitrocellulose membrane in a buffer containing 20% methanol, 25 mM of Tris, and 192 mM of glycine, as described elsewhere (27). The membranes were then blocked with 5% non-fat dry milk and incubated with the primary antibody overnight. Subsequently, membranes were washed in Tween-Tris buffer saline (TTBS), incubated for 4 h with horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit (1:4,000) antibodies, and finally developed using an enhanced ECL system (KPL Inc., Gaithersburg, MD). The membranes were then reprobed with a β-actin antibody as a control.
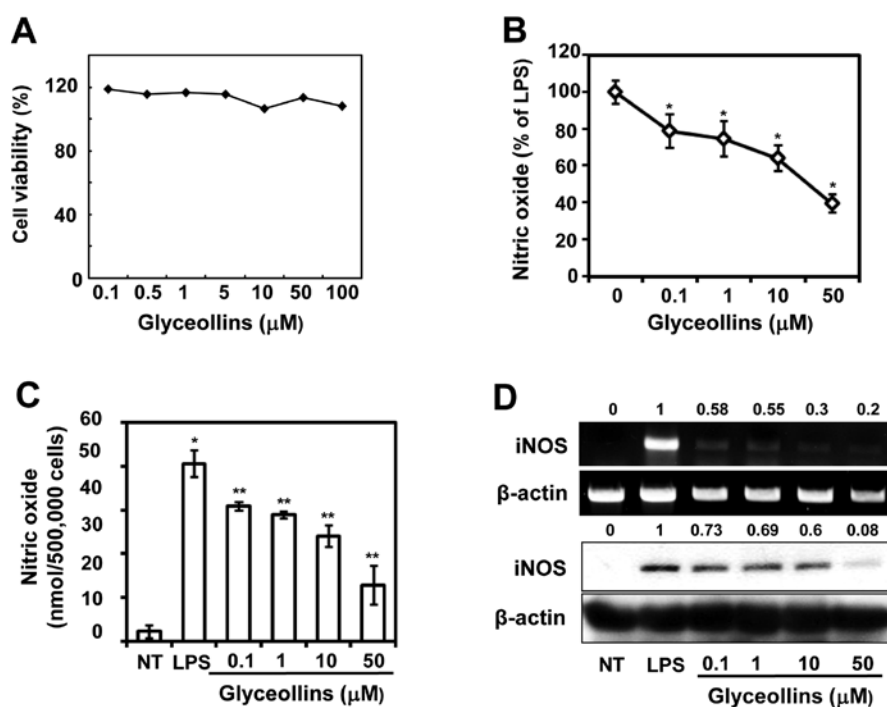
Figure 1. Effect of glyceollins on nitric oxide production and iNOS in LPS-treated RAW 264.7 cells. (A) RAW 264.7 cells were treated with the indicated dose of glyceollins for 48 h. Cell viability was determined using an MTT assay. (B and C) RAW 264.7 cells were pretreated for 2 h with indicated concentrations of glyceollins before LPS (1 μg/ml) stimulation for 16 h, and the amount of nitrite in the supernatant from each treatment group was measured using Griess reagent. Values are the means ± SD of three independent experiments. (B) *P<0.05, compared to the untreated as control. (C) *P<0.05, compared to the control; **P<0.05, compared to the LPS treatment. (D) Nitric oxide synthase mRNA and protein expression were examined by measuring RT-PCR analysis with iNOS primers or western blotting using an anti-iNOS antibody.

Reverse transcription-polymerase chain reaction. Total-RNA was extracted from RAW 264.7 cells using an easy-BLUE total-RNA extraction kit from Intron Biotechnology (Sungnam, Korea) according to the manufacturer's protocols and was quantified by measuring absorbance at 260 nm. RNA was reverse-transcribed using 2.5 μM oligo(dt) primers, 1 mM dNTPs, and Moloney murine leukemia virus (MMLV) reverse transcriptase (Promega, Madison, WI), and the resulting cDNAs were amplified with SuperTherm DNA polymerase SR Product (Kent, UK). β-actin primers were used to standardize the amount of RNA in each sample. PCR products were resolved on 1% agarose gels and visualized by ethidium bromide staining (28).

Measurement of prostaglandin E2 production. RAW 264.7 cells were subcultured in 6-well plates treated with the indicated dose of glyceollins for 2 h in the presence or absence of LPS (1 μg/ml) for 16 h (29). The culture media was collected for determination of prostaglandin E2 (PGE$_2$) concentration by an enzyme immunoassay kit from Cayman (Ann Arbor, MI).

Nuclear extracts. RAW 264.7 cells were incubated with glyceollins and LPS as indicated (30). The cells were harvested in PBS containing 2% serum, washed twice with ice-cold PBS, and resuspended in 500 μl of buffer A (10 mM of HEPES, pH 7.9, 5 mM of MgCl$_2$, 10 mM of KCl, 1 mM of ZnCl$_2$, 0.2 mM of EGTA, 1 mM of Na$_3$VO$_4$, 10 mM of NaF, 0.5 mM of dithiothreitol, 0.5 mM of PMSF and protease inhibitors). After the cells had been incubated on ice for 10 min and lysed by adding 50 μl of 20% Nonidet P-40, to a final concentra-

tion of 2%, their nuclei were harvested by centrifugation. The nuclear pellets were then resuspended in 60 μl of extraction buffer (10 mM HEPES, pH 7.9, 5 mM MgCl$_2$, 300 mM NaCl, 0.2 mM EGTA, 25% glycerol, 1 mM Na$_3$VO$_4$, 10 mM NaF, 0.5 mM dithiothreitol, 0.5 mM PMSF and protease inhibitors), and incubated on ice for 15 min. Nuclear debris was then removed by centrifugation (13,000 rpm, 10 min), and the nuclear extracts were subjected to gel shift analysis. Protein concentrations were determined using a Bradford method (31).

Electrophoretic mobility shift assay. RAW 264.7 cells, in 10-cm diameter dishes (10[7] cells/dish), were pretreated with or without glyceollins for 2 h and then incubated with LPS (1 μg/ml) for 16 h. For the gel shift assay, a consensus sequence for the NF-κB DNA binding site, sc-2505 from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), was used. The mutant binding sequence for NF-κB was identical to sc-2505 except for a G→C substitution in the NF-κB-DNA binding motif (sc-2511, Santa Cruz Biotechnology, Inc.). Beforehand, the NF-κB probe was biotin-end labeled by the biotin 3' end DNA labeling kit from Thermo Scientific Pierce (Rockford, IL). Briefly, EMSA binding reactions were performed by incubating 20 μg of nuclear extract with the annealed oligos with the LightShift EMSA kit from Thermo Scientific Pierce, according to the manufacturer's instructions. The reaction mixture was subjected to electrophoresis on a 4% native gel in a 0.5xTBE buffer. After transfer, the membrane was immediately cross-linked for 15 min on a UV transilluminator equipped with 312 nm bulbs. A chemiluminescence detection method utilizing a luminol/enhancer solution and a stable peroxide solution from Thermo
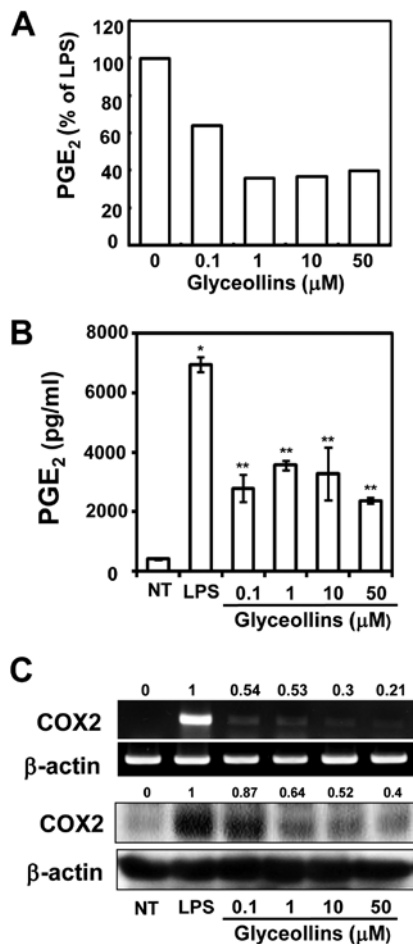
Figure 2. Effect of glyceollins on LPS-induced PGE$_2$ production and COX-2 expression in RAW 264.7 cells. (A and B) RAW 264.7 cells were cultured in 6-well plates and were pre-incubated for 2 h with indicated concentrations of glyceollins before LPS (1 μg/ml) stimulation for 16 h. PGE$_2$ amounts in cell supernatants were analyzed using an ELISA kit. Values are the means ± SD of three independent experiments. *P<0.05, compared to the control; **P<0.05, compared to the LPS treatment. (C) COX-2 mRNA and protein expression were examined by RT-PCR analysis with COX-2 primers or western blotting using an anti-COX-2 antibody.

Scientific Pierce was used as described by the manufacturer's manual, and the membranes were exposed to X-ray films for 2-5 min before developing (32).

Statistical analysis. Statistical differences between mean values ± SD were determined by the Dunnett's multiple range test. The significance was set at P<0.05 (33).

Results

Effect of glyceollins on cell viability. To examine whether glyceollins exhibit cytotoxicity in cells, we first measured whether glyceollins affect cell proliferation in RAW 264.7 cells. The result showed that, at concentrations up to 100 μM, glyceollins had no toxic effect on cell viability (Fig. 1A). Major and/or minor fractions did not have any morphological changes in microscopic observation (data not shown). Therefore, we decided to investigate whether glyceollins have potential in reducing iNOS and COX-2 expressions, which could be a landmark for the assessment of molecular inflammation.
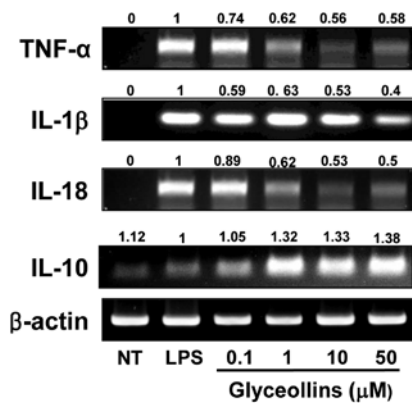

Figure 3. Inhibition of TNF-α, IL-1β and IL-18, and enhancement of IL-10 by glyceollins in RAW 264.7 cells. RAW 264.7 cells were pre-incubated with indicated concentrations of glyceollins for 2 h before LPS (1 μg/ml) stimulation for 16 h. Cells were harvested for RNA preparation. Transcriptional levels of cytokines were detected using RT-PCR. The figure shows a representative of three independent experiments.

Effects of glyceollins on NO production and expression of iNOS in LPS-stimulated RAW 264.7 cells. To assess the inhibitory effect of glyceollins on LPS-induced NO production in RAW 264.7 cells, the cells were treated with LPS (1 μg/ml) for 16 h after treatment in the presence or absence of glyceollins (0.1, 1, 10 or 50 μM) for 2 h. The amount of nitrite, a stable metabolite of NO, was used as the indicator of NO production in the medium. During the 16 h of incubation, RAW 264.7 cells produced up to 6.4±0.02 μM of nitrite in the resting state. When LPS (1 μg/ml) was added, NO production was dramatically increased up to 56.2±0.01 μM (Fig. 1B). In this condition, adding glyceollins inhibited LPS-induced NO production in a concentration-dependent manner corresponding to 10.6 and 58.7% inhibition at 0.1 and 50 μM, respectively (Fig. 1C). To further investigate whether the inhibitory effect of glyceollins on NO production was associated with the inhibition of corresponding gene expression, the protein and mRNA expressions of iNOS were determined by semi-quantitative RT-PCR and western blot analysis, respectively. In unstimulated RAW 264.7 cells, the iNOS mRNA and protein expressions were almost undetectable (Fig. 1D, first bands of each set); however, LPS treatment augmented the protein and mRNA expressions of iNOS remarkably; pretreatment of the cells with different concentrations of glyceollins also dramatically reduced LPS-induced iNOS mRNA and protein expressions in a concentration-dependent fashion (Fig. 1D, compare the second and sixth bands of each set at 0 to 50 μM). The intensity was a 5- and 12.5-fold decreased compared with that of LPS-treated cells. The data suggest that glyceollins can downregulate LPS-induced iNOS expression at the transcription level.

Effects of glyceollins on PGE$_2$ production and COX-2 expression in LPS-stimulated RAW 264.7 cells. To examine whether glyceollins inhibit PGE$_2$ production, the cells were pre-incubated with glyceollins for 2 h and then activated with 1 μg/ml of LPS for 16 h. As shown in Fig. 2A, unstimulated RAW 264.7 cells mildly decreased PGE$_2$ production when compared with treatment with glyceollins alone. In Fig. 2B, LPS induced a 5.2-fold increase in the biosynthesis of PGE$_2$
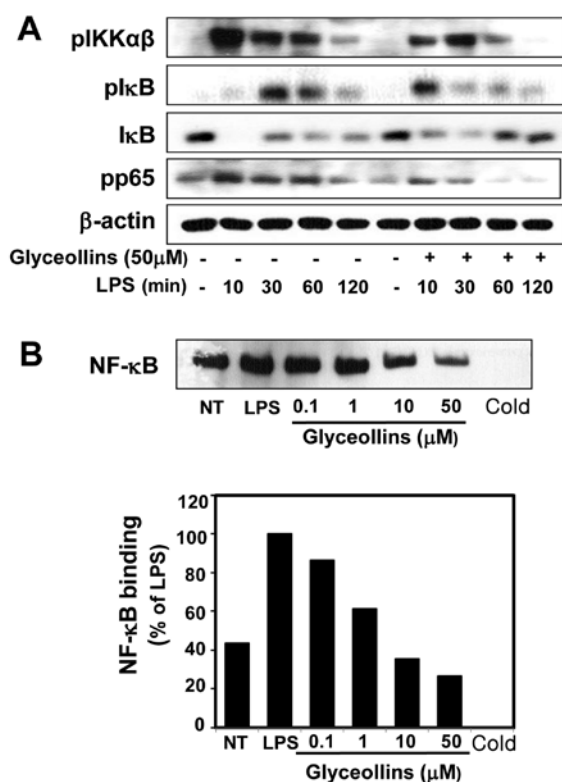
Figure 4. Inhibition of LPS-induced NF-κB activation by glyceollins. (A) Glyceollins blocking LPS-induced IκBα kinase activation and IκBα degradation. RAW 264.7 cells were pretreated with glyceollins at the indicated dose for 2 h and then stimulated with LPS (1 μg/ml) for 10, 30, 60 and 120 min. The protein extract was separated by SDS-PAGE followed by western blot analysis that was performed with specific antibodies. (B) Effect of glyceollins on LPS-induced NF-κB-DNA binding activity. RAW 264.7 cells were pretreated with glyceollins at the indicated doses for 2 h, and then stimulated with LPS (1 μg/ml) for 16 h. Nuclear extracts were prepared and EMSA was performed using a biotin-labeled NF-κB consensus binding sequence. Competitive EMSA using an unlabeled NF-κB consensus sequence at 100-fold excess confirmed the specificity of NF-κB protein binding. Three independent gels showed similar patterns.

as compared with untreated cells, but glyceollins strongly inhibited the production of PGE_2 in a dose-dependent manner. Because the biosynthesis of PGE_2 is catalyzed by COX-1 and COX-2 enzymes, we next measured the effect of glyceollins on LPS-induced COX-2 activities. We also detected that glyceollins inhibited the COX-2 mRNA and protein expressions in a dose-dependent manner (Fig. 2C, 5- and 2.5-fold, respectively). The data suggest that glyceollins can downregulate LPS-induced COX-2 expression at the transcription level. Inhibition of COX-2 expression by glyceollins was responsible for the decrease of PGE_2 production.

Effects of glyceollins on LPS-induced TNF-α, IL-1β, IL-18 and IL-10 mRNA expression. Glyceollins were found to inhibit the pro-inflammatory mediators, such as NO and PGE_2, most potently. Therefore, to test whether glyceollins could effectively for regulate inflammatory and anti-inflammatory cytokines in RAW 264.7 cells, we examined the mRNA levels of TNF-α, IL-1β, IL-18 and IL-10 by RT-PCR. Though IL-1β mRNA expression was only slightly inhibited in a dose-dependent manner (Fig. 3), TNF-α and IL-18 mRNA expression was significantly reduced by pretreatment of RAW 264.7 cells with

glyceollins (0.1, 1, 10 or 50 μM) (Fig. 3). In contrast, in terms of the anti-inflammatory cytokines, the ability of glyceollins to induce IL-10 increased up to 38±0.5% in the LPS-activated RAW 264.7 cells (Fig. 3), while that of β-actin was not altered.

Inhibitory effects of glyceollins on IκBα kinase activation and IκBα phosphorylation. Because the phosphorylation of IKK, and its subsequent phosphorylation of IκBα, are key signals for the activation of NF-κB, we examined the effect of glyceollins on LPS-induced phosphorylation of IKK and the degradation of IκBα protein by western blot analysis. A time-course experiment showed that IκBα in the cytoplasm was almost completely degraded within 10 min, and that it recovered at 30 min, after LPS (1 μg/ml) stimulation (Fig. 4A). Pretreatment with glyceollins prevented the induced degradation of IκBα protein at 5 and 15 min; the recovery of IκBα, which is under the control of NF-κB, was also suppressed (data not shown). IκBα phosphorylation was also examined by western blot analysis. As shown in Fig. 4A, the phosphorylated form of IκBα was hardly detectable in the resting RAW 264.7 cells; however, upon exposure to LPS (1 μg/ml) alone for 10 min, IκBα phosphorylation was initiated. At 30 min after LPS stimulation, pretreatment of glyceollins moderately inhibited LPS-mediated IκBα phosphorylation (Fig. 4A). Because IKK-α and β are the upstream kinases of IκB in the NF-κB signaling pathway and are activated via phosphorylation, we examined the effect of glyceollins on LPS-induced IKKα/β activations by western blot analysis using an a phospho-specific IKKα/β antibody. RAW 264.7 cells were pretreated with glyceollins (50 μM) for 2 h and then stimulated with LPS (1 μg/ml) for various courses of time. As shown in Fig. 4A, LPS was found to strongly induce IKKα/β phosphorylation, whereas glyceollin pretreatment significantly inhibited this phosphorylation. These findings indicate that glyceollins suppressed IKK and IκBα phosphorylation in LPS-induced RAW 264.7 cells.

Effects of glyceollins on LPS-induced NF-κB activation. To investigate whether glyceollins affect the DNA binding ability of the NF-κB complex in the RAW 264.7 cells, we performed an electrophoretic mobility shift assay (EMSA). RAW 264.7 cells were pretreated with 0.1-50 μM of glyceollins for 2 h, and then the cells were stimulated with LPS for 16 h. RAW 264.7 cells with LPS strongly induced the DNA binding activity of NF-κB. In contrast, pretreatment with glyceollins significantly suppressed the induced the DNA-binding activity of NF-κB by LPS in a dose-dependent manner (Fig. 4B). Taken together, the above findings indicate that glyceollins suppress NO production as well as expressions of iNOS, COX-2, TNF-α, IL-1β and IL-18, at least in part via an NF-κB-dependent mechanism.

Discussion

Phytoalexin is now a well-documented self-defense biomaterial that is produced by plants, and plays a critical role in exhibiting various biological events in animal and plant tissues (7,8). Recently, it has been revealed that these compounds have potential in ameliorating antioxidant, antifungal and antidiabetic activities *in vitro* and *in vivo* (34). In soybeans,

glyceollin, a family of lipophilic phytoalexins, as a secondary metabolite, is often accumulated at sites infected by pathogens like *Phytophthora sojae* to inhibit their growth (35). The compound is also known to be induced by countless stress factors or physical stimuli, such as freezing, UV light exposure, and/or microbes (34,36). Because bean, grape, and sunflower seeds have been known to possess various biological activities, many researchers have been focused on the isolation and purification of active compounds; but this process was not convenient to obtain enough material for experimentation. The present approach of obtaining glyceollins deserves further study, especially since the connection between the signaling pathways and glyceollins has not been discovered so far. Although glyceollin and/or their derivatives have been investigated in relation to their biological activities, including anti-estrogenic activity (9-13), their precise mechanisms of signaling are scarcely understood at the present time.

Therefore, in the present study we first hypothesized that glyceollins effectively protect against the generation of pro-inflammatory mediators, specifically, NO and PGE_2, through the inhibition of iNOS and COX-2 expression levels, respectively. We subsequently confirmed that the inhibition of NO production is concurrent with the suppression of iNOS expression at the mRNA and protein levels as shown by RT-PCR and western blot analysis (Fig. 1D). In addition, we investigated another mediator of inflammation, COX-2, which acts as a rate-limiting enzyme in the synthesis of prostaglandin-like PGE_2. In line with our prediction, glyceollins strongly inhibited PGE_2 production and COX-2 mRNA and protein levels (Fig. 2). TNF-α plays a key role in the induction of various genes, such as COX-2, through the activation of NF-κB by T cells and macrophages (37). To alleviate the inflammatory response in LPS-stimulated macrophages, it is also necessary to hamper IL-1β and IL-18 production, which both enhance the body's inflammatory response. These cytokines are known to be increased through the NF-κB signaling pathway (38-40). On the other side, IL-10 is a representative anti-inflammatory cytokine and has pleiotropic effects during the immunoregulation and inflammation processes in various immune cells (41). As shown in our data, glyceollins are effective for inflammation-related cytokines such as TNF-α, IL-1β, IL-18 or IL-10 expression using RT-PCR (Fig. 3). We clearly found that glyceollins are potent inhibitors of TNF-α production by RAW 264.7 macrophages with ~10 μM of an IC_{50} value; this is similar to luteolin and quercetin, which have been the most potent natural products so far at inhibiting TNF-α release, with IC_{50} values of <1 μM (42) and <5 μM, respectively (data not published).

It is well-recognized that NF-κB, a universal transcription factor, plays a pivotal role in the various soluble pro-inflammatory gene expressions and leukocyte adhesion molecules (38,39). As a key step to activate NF-κB functions, studies have been focused on the activation of the IKK complex over the last several years. Many natural phytochemicals, such as luteolin, quercetin, and resveratrol, suppress LPS-induced iNOS and COX-2 expressions as well as inflammatory cytokine expressions in macrophages by inhibiting the NF-κB signaling pathway, such as phosphorylation of IKK, degradation of IκBα, and nuclear translocation of NF-κB (43,44). Because the glyceollin-mediated signaling pathway can provide us with useful information on the function of the agent,

we next studied the NF-κB-mediated signaling pathway in RAW 264.7 cells. In our study, western blot analysis revealed that glyceollins inhibited the LPS-induced phosphorylation of IKK, a series of phosphorylations of IκBα and degradation of p-IκB (Fig. 4A). We also demonstrated that glyceollins inhibit LPS-induced NF-κB in RAW 264.7 cells by performing EMSA. Pretreatment with glyceollins significantly suppressed the induced DNA-binding activity of NF-κB by LPS in a dose-dependent manner (Fig. 4B). Therefore, the above results suggest that glyceollins suppress the inflammation reaction through an NF-κB-dependent signal transduction pathway. Up to now, no data regarding a glyceollin-mediated signaling pathway has been investigated. As a result, our data have the potential to help develop prophylactic and therapeutic anti-inflammatory agents that are both safe and original. Further study of the overall signal transduction pathway promises to be rewarding because the inhibitory mechanism by glyceollins can provide new data for anti-inflammatory therapies.

This study describes a signaling pathway of the anti-inflammatory effects elicited glyceollins. Our results suggest that the glyceollins obtained from soybeans may exhibit increased anti-inflammatory effects. Glyceollins have an appreciable inhibitory activity against the overproduction of inflammatory mediators such as iNOS, COX-2 and various cytokines. Not only can they be used to investigate their effects in various biological areas, but if we uncover the precise mechanisms by glyceollins in immune cells, they can also be used to prevent immune diseases. Use of traditionally fermented soybean products may prove rewarding because we can naturally obtain their high levels of phytoalexins, such as glyceollins.

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