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Enhanced Anti-Inflammatory Activities of *Monascus pilosus* Fermented Products by Addition of Ginger to the Medium

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Hypercholesterolemia initiates the atherogenic process; however, chronic inflammation promotes atherogenesis. *Monascus* spp. fermented products are recognized for their anti-hypercholesterolemic effect, but their anti-inflammatory activity is not as significant as that of many plant-derived foods. To enhance the anti-inflammatory function of *Monascus pilosus* fermented products, ginger was added to the PDB medium at a ratio of 20% (v/v). The mycelia and broth were collected, freeze-dried, and extracted by ethanol for assays. Macrophage RAW264.7 was challenged with lipopolysaccharide (LPS) and coincubated with the extracts of fermented product cultured in ginger-supplemented medium (MPG) or extracts of fermented product cultured in regular PDB medium (MP) for 18 h. Human umbilical vein endothelial cell HUVEC was challenged with tumor necrosis factor (TNF)-α and coincubated with the extracts of either MPG or MP for 6 h. The results showed that MPG significantly ($p < 0.05$) lowered the production of macrophage pro-inflammatory cytokines TNF-α, nitric oxide (NO), interleukin (IL)-1, IL-6, and prostaglandin E_2 (PGE_2) by 68.53%, 84.29%, 32.55%, 84.49%, and 69.49%, respectively; however, MP had no inhibitory effect. MPG significantly downregulated the expression of p-IκB, cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) in macrophage by 42.16%, 50.87%, and 51.35%, respectively, while MP had no inhibition on COX-2 expression and only 16.64% and 19.22% downregulatory effect on iNOS and phosphorylated-IκB (p-IκB), respectively. Moreover, MPG significantly suppressed the expression of vessel cell adhesion molecule-1 (VCAM-1) and p-IκB in endothelial cell by 63.48% and 63.41%, respectively. LC/MS/MS analysis indicated that 6-gingerdiol was formed in the ginger-modified medium during fermentation. The results of this study will facilitate the development of *Monascus* spp. fermented products as antiatherosclerotic nutraceuticals.

KEYWORDS: ***Monascus pilosus*; ginger; anti-inflammation**

INTRODUCTION

Atherosclerosis is the root cause of the majority of cardiovascular diseases (CVDs) such as myocardial infarction and stroke. Atherosclerosis develops as a result of various risk factors, including increased plasma low-density lipoproteins (LDL) concentration. LDLs, the major lipoproteins in the body, are small particles and easily trapped in arteries when the concentration increases. The trapped LDL undergoes progressive oxidation and is internalized by macrophages. The internalization leads to the accumulation of lipid peroxides, facilitating the formation of foam cells which can be further converted into complex and thrombus. In addition to the ability to injure endothelial cells, oxidized LDL is chemotactic for monocytes and upregulates the expression of cytokines such as tumor necrosis factor α (TNF-α) and interleukin-1 (IL-1) that expand the inflammatory responses (*1*). After atherogenic process is initiated, a vicious circle of inflammation, LDL oxidation, monocyte recruitment, and further inflammation is maintained in artery. Atherosclerosis has been considered a chronic inflammatory disease (*2*); therefore, efforts to attenuate inflammatory responses are important for the prevention of atherosclerosis.

Because one of the secondary metabolites, monacolin K, is a specific inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase in cholesterol *de novo* synthesis (*3*), *Monascus* spp. fermented product is known for its anti-hypercholesterolemia function; however, its antioxidant and anti-inflammatory activities are not as significant as those of many plant-derived foods. Ginger (*Zingiber officinale*) is one of the world's best known spices, and it was reported for its antioxidant, anti-inflammatory, and anticancer activities (*4*−*7*). In a previous study (*8*), we showed that addition of ginger into culture medium at the ratio of 20% significantly increased the antioxidant activity of *Monascus pilosus* fermented product.

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The network of inflammatory pathways include a variety of transcription factors, cytokines, and enzymes. Modulation of these factors is found to be a viable anti-inflammatory strategy (*9*). In this study, we were interested in evaluating the anti-inflammatory properties of the ginger-modified *M. pilosus* fermented product that possesses antioxidant properties superior to those of the nonmodified product. The proinflammatory parameters chosen in this study are those that play crucial roles in the development of atherosclerosis. The results of this study will facilitate the development of nutraceuticals with antiatherosclerotic function.

MATERIALS AND METHODS

Chemicals. Potato dextrose agar (PDA) and potato dextrose broth (PDB) were purchased from BD (Sparks, MD). Medium 199, lipopolysaccharide (LPS), and tumor necrosis factor-α (TNF-α) were obtained from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium-high glucose (DMEM), penicillin, streptomycin, and fetal bovine serum were purchased from GIBCO (Grand Island, NY). Endothelial cell growth supplement (ECGS) was obtained from Upstate (Temecula, CA). Anti-cyclooxygenase (COX)-2 and the anti-inducible NO synthase (iNOS) polyclonal antibodies were obtained from Cayman (Ann Arbor, MI). Antiphosphor-IκB (p-IκB) polyclonal antibody, antivascular cell adhesion molecule-1 (VCAM-1) polyclonal antibody were purchased from Epitomics (Burlingame, CA) and Abnova (Walnut, CA), respectively. Anti-β-actin monoclonal antibody was obtained from Novus (Littleton, CO). Anti-mouse CD284/MD-2 complex, FITC Rat anti-mouse CD11b, and rat anti-mouse CD16/CD32 were purchased from BD Pharmingen (San Diego, CA).

Extract Preparation. Ginger purchased at a farmer's market (Nanto County, Taiwan) was washed and ground. The juice collected during grinding was filtered twice to make "ginger juice", which was then used for the experiments. *Monascus pilosus* (BCRC 31527) obtained from the Biosource Collection and Research Center of Food Industry Research and Development Institute (Shinchu City, Taiwan) was inoculated onto potato dextrose agar (PDA) slants and incubated at 28 °C. After a pure culture was obtained, the mycelia were reinoculated into potato dextrose broth (PDB) at 28 °C for 7 days. The mycelia and broth from the submerged culture were blended together, and the mixture was added at a ratio of 1:25 to the 2.4% fresh PDB containing 20% ginger juice for further submerged cultivation at 28 °C. At day 7, the mycelia and broth were collected, blended, and then freeze-dried. The dried powder was dissolved in ethanol (1/20, w/v), sonicated for two hours, and centrifuged at 100*g* for 10 min. After centrifugation, the supernatant was collected and referred to as "MPG". Uninoculated ginger-containing medium, uninoculated regular PDB medium, and inoculated regular PDB medium were cultured, collected, freeze-dried, and extracted using the same conditions as used for MPG to obtain "PDBG", "PDB", and "MP", respectively.

Cell Culture. The mouse BALB/c macrophage cell line RAW 264.7 and human umbilical vein endothelial cell line HUVEC were obtained from Biosource Collection and Research Center of Food Industry Research and Development Institute (Shinchu City, Taiwan). RAW 264.7 macrophages were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 0.22% sodium bicarbonate,100 units/mL penicillin, and 100 units/mL streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. HUVEC endothelial cells were cultured in Medium 199 supplemented with endothelial cell growth supplement (30 μg/mL), 10% fetal bovine serum, 0.15% sodium bicarbonate,100 units/mL penicillin, and 100 units/mL streptomycin. Both cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. When 50% confluence was attained, Raw 264.7 and HUVEC were treated with 7-day PDB, MP, PDBG, or MPG extracts (final concentration of 500 ppm) for 18 and 6 h, respectively. The medium was collected for cytokine assays. The cells were washed twice with cold PBS, harvested, and then resuspended in lysis buffer containing 1% Nonidet P 40, 150 mM sodium chloride, and 50 mM Tris-HCl, pH 7.5. The cell suspension was centrifuged at 10000*g* for 30 min, and the supernatant was collected and subjected to further centrifugation at 105000*g* for 60 min. The supernatant was collected for immunoblot analysis. The protein content in samples was determined by Dc Protein Assay Kit (Bio-Rad, Hercules, CA).

Immunoblot Analysis. Equal amount of proteins were denatured and separated by gel electrophoresis before being transferred to polyvinylidene fluoride (PVDF) membranes (GE Healthcare, Pittsburgh, PA). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing Tween (TBST), 20 mM Tris-HCl, pH 8.3, 137 mM NaCl, and 0.1% Tween-20 for 1 h and then incubated sequentially with primary antibody for 2 h and horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Immunoreactive proteins were detected using an enhanced chemiluminescence kit (ECL, PerkinElmer, Waltham, MA) and the film was analyzed using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Measurement of Cytokines, NO, and PGE_2 Production. After the RAW 264.7 macrophages were treated with LPS and various extracts for 18 h, the culture medium was collected for the analysis of TNF-α, IL-1β, IL-6, NO, and PGE_2. The concentration of TNF-α and IL-1β was assayed by ELISA kits purchased from eBioscience (San Diego, CA). Levels of IL-6 and PGE_2 were measured by ELISA kits obtained from Assay Designs (Ann Arbor, MI). The production and release of NO were determined by commercial kit (Cayman, Ann Arbor, MI).

Flow Cytometry. Approximately 10^6 RAW 264.7 cells were first preincubated with 1 μg of anti-CD16/CD32 (clone 2.4G2) at 4 °C for 30 min and then incubated with monoclonal antibodies in a combination of 1 μg of fluorescein isothiocyanate (FITC)-labeled CD11b (FITC-CD11b) (clone M1/70) and 1 μg of phycoerythrin (PE)-labeled CD284/MD-2 (clone MTS510) for 30 min in the dark. Three milliliters of PBS with sodium azide was added to the mixture before centrifugation was carried out at 200*g* for 5 min. The supernatant was removed and gently mixed with 0.5 mL of PBS with 1.0% paraformaldehyde. A total of 20000 events were collected in a FACScaliber (BD Biosciences, Sunnyvale, CA), and the data were analyzed using WinMDI 2.8 software (Scripps Institute, La Jolla, CA).

LC/MS/MS Analysis. All culture filtrates were sampled and analyzed by liquid chromatography−tandem mass spectrometry (LC/MS/MS). Separation of components in fermented filtrates (10 μL, diluted with acetonitrile, 1:1, v/v) was directly conducted on a Luna C18(*2*) column (2.00 mm i.d. × 150 mm, 5 μm) and a guard column (10 × 3 mm i.d., Phenomenex Inc., Torrance, CA) using a HPLC system consists of Finnigan Surveyor module separation system and a photodiode-array (PDA) detector (Thermo Electron Co., MA). A linear gradient from 98% A to 30% B in 45 min was used for the HPLC analysis of ginger extracts. Solvent A was water containing 1% formic acid, and solvent B was acetonitrile containing 0.1% formic acid. The flow rate was 0.2 mL/min. The flow rate was 0.2 mL/min. A solvent gradient for HPLC analysis was programmed as follows: 30% B to 95% B in 30 min, then isocratic of 95% B for 10 min and finally returning to the initial gradient program in 20 min. Absorption spectra of ginger extracts were recorded from 190 to 600 nm with the in-line PDA detector. The system was coupled to a Finnigan LCQ Advantage MAX ion trap mass spectrometer and was operated in electrospray ionization (ESI) with positive ionization mode. Samples of 20 μL of extracts were directly injected into the column using a Rheodyne (model 7725i) injection valve. The ion trap instrument was operated at the following setting: capillary voltage, 41 V; tube lens offset, 55 V; source voltage, 3.5 kV; ion transfer capillary temperature, 320 °C; nitrogen sheath gas, 30; and auxiliary gas, 10 (arbitrary units). Mass spectra were acquired in a m/z range of 150−1000, with five microscans and a maximum ion injection time of 200 ms. The SIM analysis was a narrow scan event that monitored the m/z value of the selected ion, in a range of 1.0 Th centered on the peak for the molecular ion; this function was used in the analysis of molecular ions of the gingerol-related compounds. For MS/MS analysis, helium collision gas was introduced in accordance with the manufacturer's recommendations. The MS/MS fragment spectra were produced using normalized collision energies with an increment of 30% and also with wideband activation "off".

Statistical Analysis. Statistical analyses were performed using the Statistical Analysis System (SAS Institute, Cary, NC). Analysis of variance (ANOVA) and Student−Newmann−Keuls multiple range test were used to determine significant difference among means ($\alpha = 0.05$).

RESULTS

Growth Curve. According to **Figure 1A**, *M. pilosus* inoculated in ginger-containing medium (MPG) grew faster than that inoculated

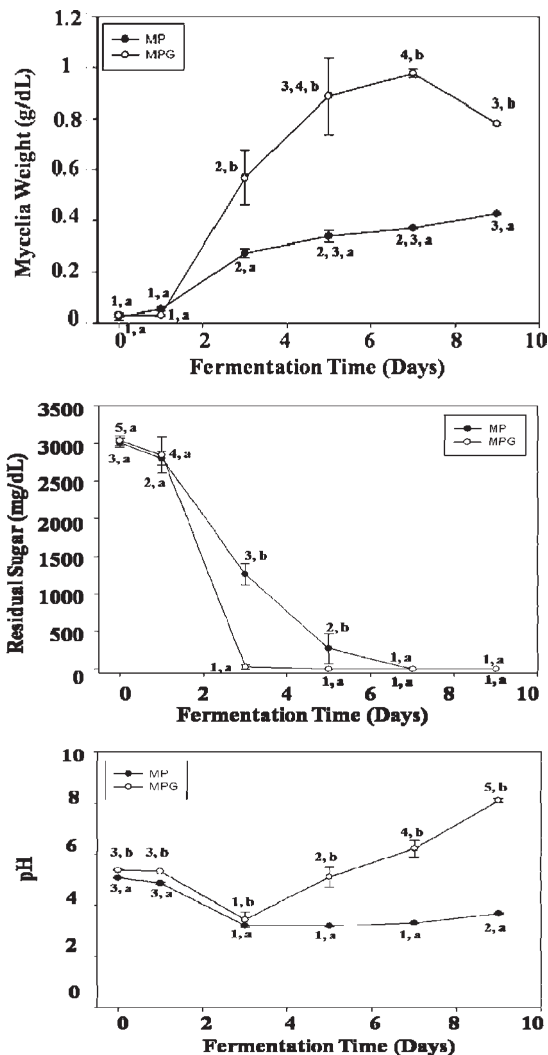

Figure 1. Growth curves of MP and MPG: (top, **A**) mycelia weight, (middle, **B**) residual sugar, and (bottom, **C**) pH of medium. MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; and MPG, fermented products collected from ginger-containing PDB medium inoculated with *M. pilosus*. Values indicate means ± SD. In a group, means having the same number are not significantly different at various time points. At a time point, means having the same letter are not significantly different among groups (α = 0.05).

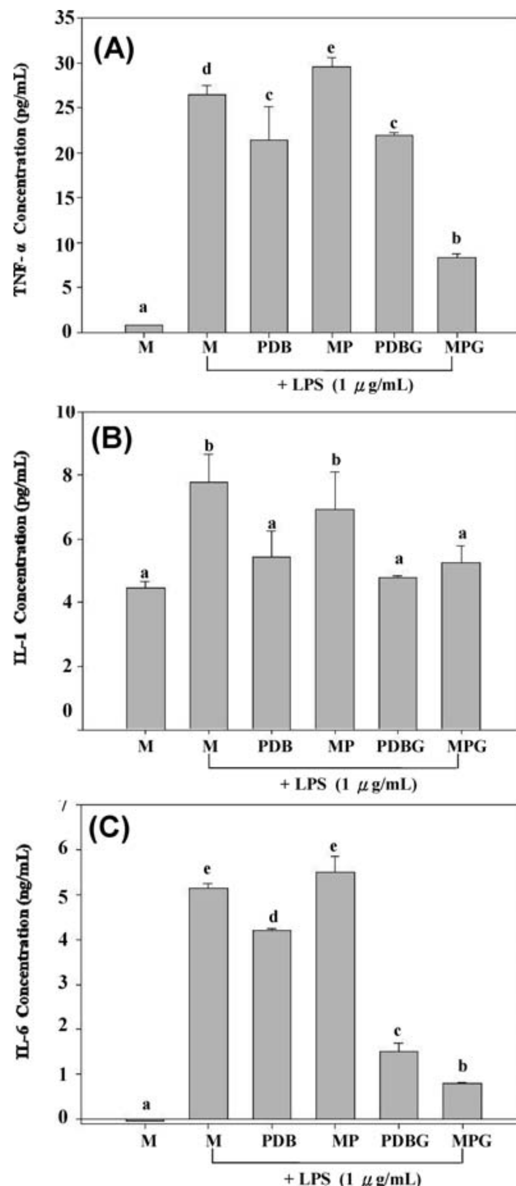

Figure 2. Effects of extracts on the production of (**A**) TNF-α, (**B**) IL-1, and (**C**) IL-6 of RAW 264.7 macrophages treated with PDB, MP, PDBG, or MPG for 18 h. TNF-α, tumor necrosis factor-α; IL-1, interleukin-1; IL-6, interleukin-6. +LPS indicates the stimulation by 1 μg/mL of lipopolysaccharide. M, medium without the addition of extracts; PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBG, fermented products collected from uninoculated ginger-containing medium; MPG, fermented products collected from ginger-containing PDB medium inoculated with *M. pilosus*. Values indicate means ± SD. Means having the same letter are not significantly different (α = 0.05).

in regular medium (MP). Therefore, the depletion of sugar was faster in the ginger-containing medium (**Figure 1B**). The pH of medium started to rise after the sugar was depleted (**Figure 1C**).

Cytokine Production. Inflammation is a key process in atherosclerosis. Biomarkers of inflammation have been adopted for risk prediction of atherosclerosis. Exposure to 1 μg/mL of LPS significantly increased the release of TNF-α, IL-1β, and IL-6 into medium by 30.52-, 1.75-, and 513.50-fold, respectively (**Figure 2A,B,C**). Cotreatment with MPG inhibited the secretion of theses three cytokines by 68.53, 32.55, and 84.62%, respectively, whereas MP did not show inhibitory effect. The effects of PDB and PDBG on the production of TNF-α and IL-1β were not different; however, PDBG had a more significantly inhibitory effect on the release of IL-6. Two-way ANOVA analysis indicated that the interaction between ginger extract and *M. pilosus* fermentation had significant effect on the release of TNF-α and IL-6 by macrophages.

NO and PGE_2 Production. Exposure to 1 μg/mL of LPS significantly increased the release of NO and PGE_2 into medium by 11.75- and 4.08-fold, respectively (**Figure 3A,B**). However, cotreatment with MPG inhibited the secretion of NO and PGE_2 by 84.29% and 69.58%, respectively. MP suppressed the release of NO by 24.53%, but did not have inhibitory effect on the secretion of PGE_2. Comparing PDBG with PDB, cotreatment with PDBG inhibited the production of NO and PGE_2 by 42.89% and 30.51%, respectively, whereas PDB only showed inhibitory effect on the release of NO. The interaction effect of ginger extract and *M. pilosus* fermentation was observed on the production of both NO and PGE_2 by macrophages.

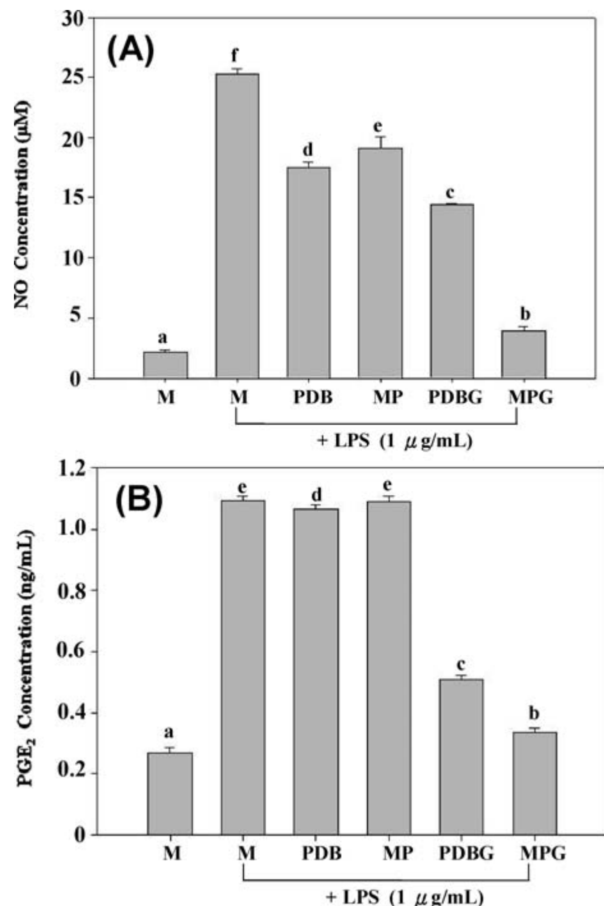

Figure 3. Effects of extracts on the production of (**A**) NO and (**B**) PGE_2 of RAW 264.7 macrophages treated with PDB, MP, PDBG, or MPG for 18 h. PGE_2, prostaglandin E_2. +LPS indicates the stimulation by 1 μg/mL of lipopolysaccharide. M, medium without the addition of extracts; PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBG, fermented products collected from uninoculated ginger-containing medium; MPG, fermented products collected from ginger-containing PDB medium inoculated with *M. pilosus*. Values indicate means $\pm$ SD. Means having the same letter are not significantly different (α = 0.05).

Expression of Inflammatory Proteins. As shown in **Figure 4**, exposure to 1 μg/mL of LPS for 18 h significantly enhanced the expression of P-IκB, iNOS, and COX-2. Cotreatment with MPG downregulated the expression of P-IκB, iNOS, and COX-2 by 51.35%, 50.87%, and 65.02%, respectively, more significant than the downregulation exerted by MP. PDBG showed greater inhibition on the expression of iNOS and COX-2 than MP and PDB. Consistent with the results of NO and PGE_2 producton, the interaction between ginger extract and *M. pilosus* fermentation showed a significant effect on the expression of iNOS and COX-2 in macrophages. The detection of β-actin was also performed as the loading control and noted as a consistent band.

Expression of Adhesion Protein. Exposure to 10 μg/mL of TNF-α for 6 h significantly increased the expression of P-IκB and vascular cell adhesion molecule-1 (VCAM-1) (**Figure 5**). Cotreatment with MPG suppressed the expression of P-IκB and VCAM-1 by 62.46% and 63.48%, respectively. PDBG showed similar inhibitory effect on the expression of VCAM-1 as MPG. Both PDB and MP did not suppress the expression of VCAM-1, but showed 8.33% and 28.3% inhibition on P-IκB expression, respectively.

Expression of Toll-like Receptor-4. FSC/SSC analysis indicated that LPS stimulated the morphological change of macrophages (**Figure 6A**). Cotreatment with MPG decreased the changes induced by LPS while MP showed no effect. TLR4 has been renamed as CD284 and identified to be the transmembrane signal-transducting portion of the receptor for LPS. To study the effect of MPG and MP extracts on the expression of TLR4, murine macrophages RAW264.7 were stained with antibodies against CD284 and CD11b, a receptor for complement protein C3bi and is mainly expressed in monocyte/macrophage. As shown in **Figure 6B**, stimulation of LPS increased the expression of TLR4. The LPS-induced upregulation was suppressed by cotreatment with MPG, but not MP.

LC/MS/MS Analysis. The 7-day MPG and MP extracts were analyzed to detect gingerol-related and pigment-related compounds. Gingerdiol, a metabolite of gingerol, was detected in MPG extract (**Figure 7A**,**B**; Table 1), but not MP extract. The relative percentage of monacolin K production in MPG was higher than that in MP. On the other hand, the relative percentage of monascin (a yellow pigment) production was significantly lower in MPG than in MP (**Figure 7C**,**D**). Because the formation of monacolin k and pigments is competitive during fermentation, the relatively higher production of monacolin K did lead to a difference in color appearance between MPG and MP.

DISCUSSION

In our previous study (*8*), PDBG and MPG already demonstrated greater antioxidant activities than their counterparts, PDB and MP, before fermentation (day 0). This observation may result from the presence of antioxidant ingredients in ginger. During fermentation, the antioxidant activities of both MP and MPG increased gradually; however, the change in MP was not as significant as that observed in MPG. The antioxidant activity of PDBG was not changed during fermentation and significantly lower than that of MPG during fermentation. This implies that the fermentation process promoted the formation of metabolites that exert superior antioxidant activities.

Because antioxidants also possess anti-inflammatory activity, the significant inhibition on the production of cytokines and the expression of pro-inflammatory proteins and adhesion protein was observed in the cells treated with MPG. Although MP suppressed the production of IL-1β and NO and downregulated the expression of iNOS and p-IκB, the inhibitory effect of MP was not as significant as that of MPG. Consistent with the results observed in antioxidant study, PDBG possessed superior anti-inflammatory capacities to PDB.

Cytokines are regulatory polypeptides produced by virtually all immune cells. In the presence of stimuli, such as lipopolysaccharide (LPS), increased production of proinflammatory mediators occurs, leading to initiation of an inflammatory response (*10*). TNF-α, IL-1β, and IL-6 are three of the major cytokines involved in the initiation of inflammation (*11*−*13*), and PGE_2 is a potent immunomodulator that upregulates proinflammatory cytokines (*14*). In the present study, the release of TNF-α, IL-1β, IL-6, and PGE_2 significantly increased when RAW 264.7 macrophages were challenged with LPS; however, cotreatment with the extracts of ginger-modified *M. pilosus* fermented product (MPG) inhibited cytokines and PGE_2 production while regular *M. pilosus* fermented product (MP) had no inhibitory effect. This implies that the modified *M. pilosus* fermented product was more efficient in suppressing inflammation than the regular counterpart.

Nitric oxide is synthesized from L-arginine by nitric oxide synthase in various tissues (*15*). Based on the location and catalytic mechanism, three NOS have been identified: neuronal

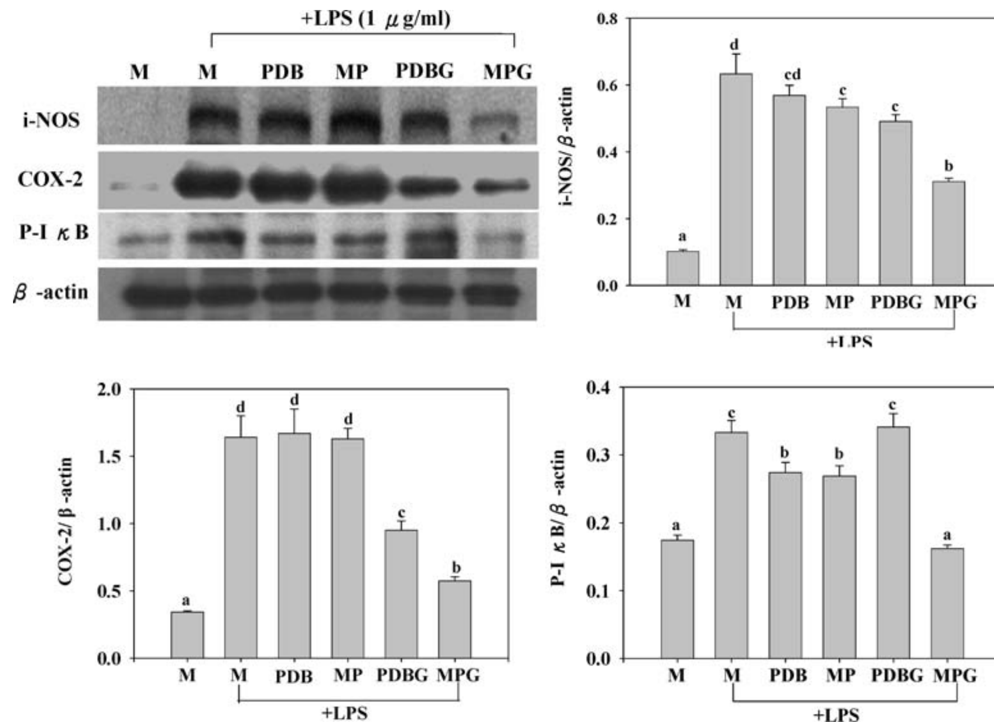

Figure 4. Expression of iNOS, COX-2, and P-IκB in RAW 264.7 cells treated with PDB, MP, PDBG, or MPG for 18 h. +LPS indicates the stimulation by 1 μg/mL of lipopolysaccharide. M, medium without the addition of extracts; PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBG, fermented products collected from uninoculated ginger-containing medium; MPG, fermented products collected from ginger-containing PDB medium inoculated with *M. pilosus*. iNOS, inducible NO synthase; COX-2, cycloxygenase-2; P-IκB, phosphorylated- IκB. Values indicate means ± SD. Numbers having the same letter are not significantly different (α = 0.05).

NOS (nNOS), inducible NOS (iNOS), and endothelial NOS (eNOS). Both nNOS and eNOS are constitutively expressed, producing low levels of NO in the neurons and endothelium for the maintenance of physiological functions. iNOS is expressed in macrophages following exposure to cytokines and/or microbial products, such as LPS. Excessive NO production catalyzed by iNOS causes oxidative damage to endothelial tissues. In the present study, both MPG and MP showed an inhibitory effect on the production of NO; however, the suppression was more significant in the cells treated with MPG. Consistent with the results of NO production, expression of iNOS in macrophages was downregulated more significantly by MPG than by MP. TNF-α has been shown to stimulate the upregulation of iNOS and NO production in human coronary endothelial cells (*16*); therefore, suppression of TNF-α production by MPG contributed to the observed downregulation of iNOS and NO.

In addition to iNOS, the expression of COX-2 was also significantly suppressed by MPG. Cyclooxygenase catalyzes the conversion of arachidonic acids to eicosanoids, including prostaglandins (PG). COX-1 is constitutively expressed in many cell types, whereas the expression of COX-2 is restrictively observed after stimulation of cells with proinflammatory cytokines (*17*). COX-2 enhances the adhesion of monocytes to activated endothelial cells in the presence of oxidized-LDL. The proatherogenic role of monocytic COX-2 in the early stage of atherogenesis is suggested by a study in LDL-deficient mice, in which the formation of vascular lesion was reduced by the use of highly selective COX-2 inhibitor (*18*). Our study indicated that cotreatment with MPG downregulated the LPS-induced COX-2 expression; however, MP did not show an inhibitory effect. This observation is consistent with the results of PGE_2 production

Nuclear factor-κB (NF-κB) is a heterodimeric protein sequestered in the cytosol of unstimulated cells via nonconvalent binding with a class of inhibitor proteins called I-κBs. Signals that induce NF-κB activity cause the phosphorylation of I-κB, leading to the dissociation of NF-κB and I-κB, thereby allowing the translocation of NF-κB into nucleus and binding to the upstream of genes being regulated (*19*). NF-κB is considered to be essential

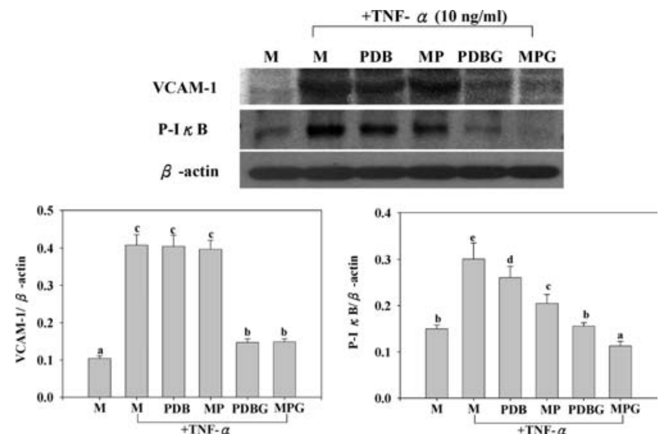

Figure 5. Expression P-IκB and VCAM-1 in HUVEC cells treated with PDB, MP, PDBG, or MPG for 6 h. +TNF-α indicates the stimulation by 10 ng/mL of tumor necrosis factor. M, medium without the addition of extracts; PDB, fermented products collected from uninoculated PDB medium; MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; PDBG, fermented products collected from uninoculated ginger-containing medium; MPG, fermented products collected from ginger-containing PDB medium inoculated with *M. pilosus*. P-IκB, phosphorylated- IκB; VCAM-1, vascular cell adhesion molecule-1. Values indicate means ± SD. Numbers having the same letter are not significantly different (α = 0.05).

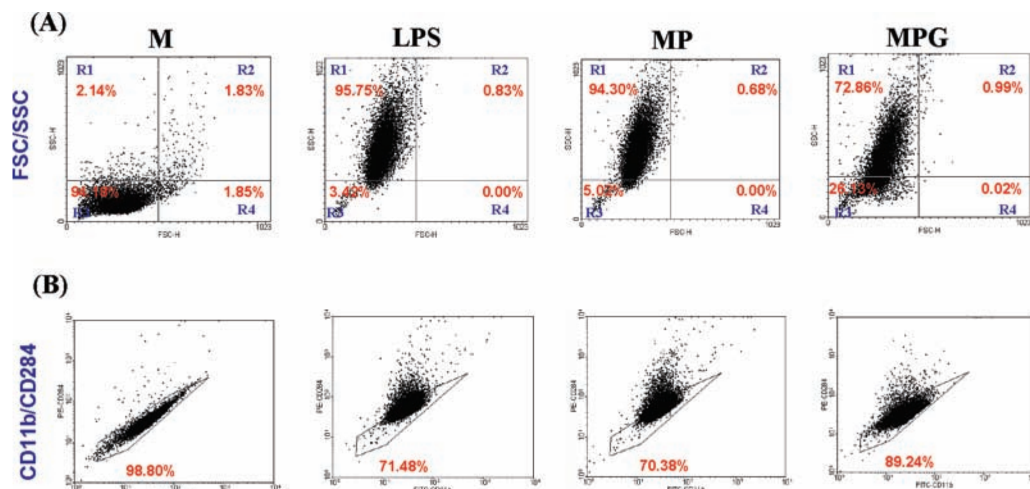

Figure 6. FACS analysis of RAW 264.7 treated with MP or MPG for 18 h. (**A**) Dot plots of FSC-H vs SSC-H. The plots show total events. The number shown in each quadrant indicates the percentage of total cells. (**B**) Cells were stained with PE-conjugated rat anti-mouse CD284 antibody and FITC-conjugated anti-mouse CD11b antibody. The plots show total events. The number indicates the percentage of cells in gating area. M, cells were maintained in medium without the addition of lipopolysaccharide or extracts; LPS, cells were maintained in medium supplemented with lipopolysaccharide (1 μg/mL); MP, cells were maintained in medium supplemented with LPS and MP extract; MPG, cells were maintained in medium supplemented with LPS and MPG extract. The numbers are the results of three independent studies.

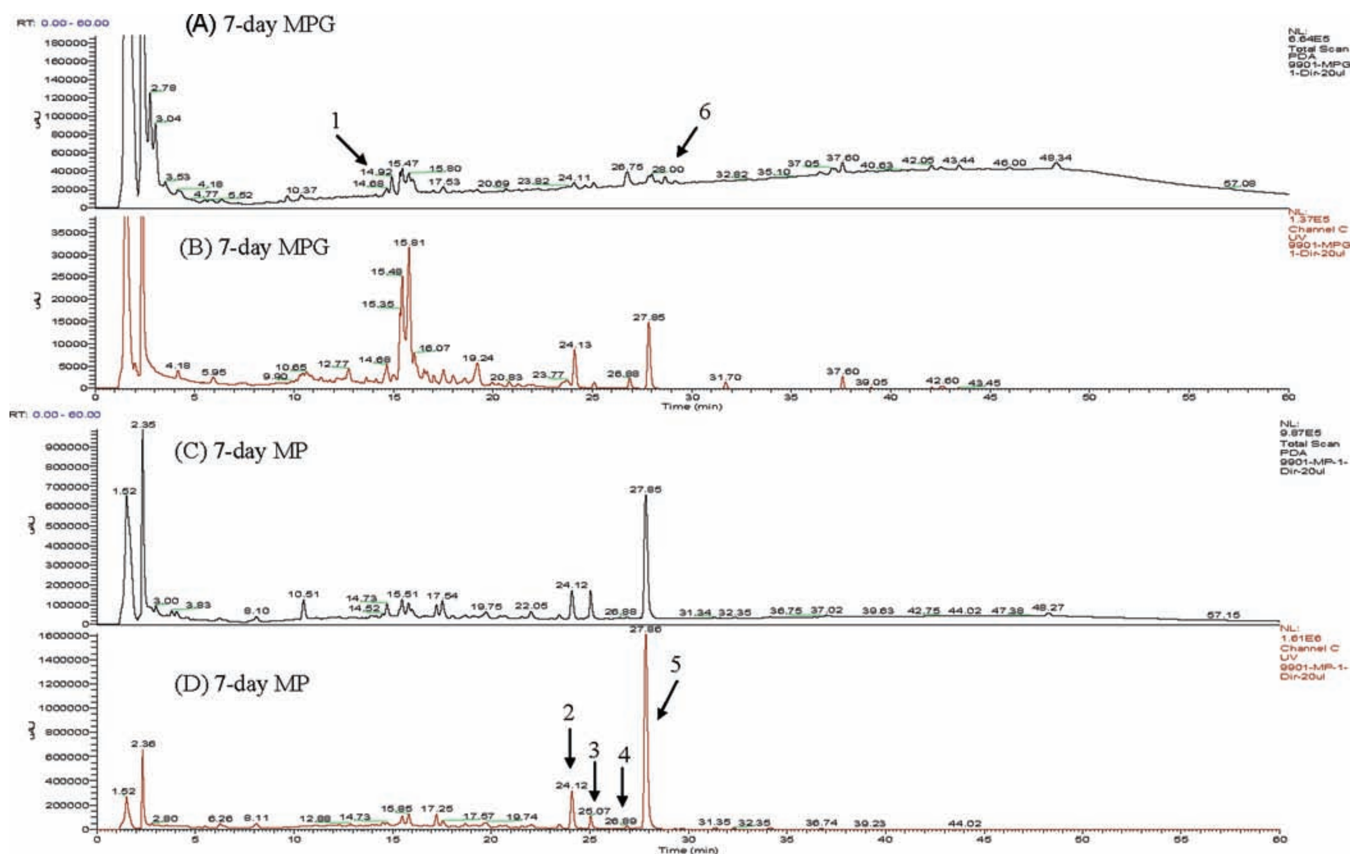

Figure 7. HPLC/UV chromatogram (**A** and **C**, photodiode array detection at 190−600 nm; **B** and **D**, UV detection at 425 nm) of gingerol and pigment related compounds. The peak numbers refer to the identification of compounds in **Table 1**. MP, fermented products collected from regular PDB medium inoculated with *M. pilosus*; MPG, fermented products collected from ginger-containing PDB medium inoculated with *M. pilosus*.

for the enhanced expression of iNOS, COX-2, and proinflammatory cytokines in the macrophages exposed to LPS. Activated has been identified in human atherosclerotic plaques but is present only a very small amount in vessels devoid of atherosclerosis (*20*). In the present study, cotreatment with either MPG or MP inhibited the phosphorylation of I-κB; however, MPG showed a more significant effect. More inhibition on pI-κB formation indicates less activation of NF-κB, resulting in the downregulation of iNOS and COX-2 as observed.

One of the earliest events in the development of atherosclerosis is the expression of adhesion molecules by endothelial cells. This leads to the recruitment of circulating monocytes into the

Table 1. Retention Time, UV−Vis, and Mass Spectral Characteristics for the Composition of *M. pilosus* Culture Extracts

peak	compound	t_R[a] (min)	λ	(+)-ESI-MS
1	6-gingerdiol	14.92	283, 238	297, 279.5
2	unknown	24.12	398, 241	361
3	unknown	25.07	390, 375 sh, 291, 236	357
4	monacolin K	26.88	241, 249 sh, 224 sh	405
5	monascin	27.86	398, 242	359
6	6-gingerol	29.66	279, 241	277, 295

[a] Retention time.

subendothelial space of the artery wall. Recruited monocytes transform into macrophages and absorb excessive lipids to become "foam cells". Smooth muscle cells in normal arterial media do not express adhesion molecules. However, immunohistochemical studies showed the expression of vessel cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1 on smooth muscle cells in atherosclerotic plaques (*21*, *22*). Deletion of any adhesion molecule leads to a significant reduction in atherosclerosis (*23*). In the present study, the increased expression of VCAM-1 stimulated by exposure to TNF-α was significantly suppressed when macrophages were cotreated with MPG. It is known that proinflammatory cytokines such as TNF-α induce the expression of adhesion molecules on endothelial cells (*24*); the inhibited production of TNF-α by MPG may contribute significantly to the downregulation of VCAM-1 in MPG-treated endothelial cells. Since inhibition of VCAM-1 was shown to prevent the development of atherosclerosis in apolipoprotein E-deficient mice (*25*), MPG may reduce the extent of atherosclerotic damage.

Mammalian Toll-like receptors (TLRs) are a family of type-1 transmembrane receptors that play an important role in host recognition of microbial infection (*26*). Each receptor serves to initiate inflammatory signaling in response to the detection of pathogen-associated molecules. TLR-dependent signaling has been implicated in the activation of vascular cells during atherogenesis (*27*). TLR4 initiates the innate immune response against Gram-negative bacterial infection by recognizing LPS (*28*). Stimulation of TLR4 results in activation of NF-κB and upregulation of adhesion molecules and proinflammatory cytokines. Genetic studies in ApoE−/− have shown that specific deletion of TLR4 leads to a significant reduction in atherosclerosis burden (*29*, *30*). In this study, ginger-modified *M. pilosus* fermented products downregulated the LPS-induced expression of TLR4 while regular *M. pilosus* fermented products had no effect. Inhibition on TLR4 led to the downregulation of NF-κB, COX-2, iNOS, and proinflammatory cytokines as observed in the MPG-treated macrophages.

Gingerol-related compounds have been reported as bioactive components from the rhizome of ginger (*31*). To monitor the changes of gingerol-related compounds which might contribute to the enhanced antioxidant and anti-inflammatory capacities of MPG, 7-day MPG and MP extracts were analyzed. 6-Gingerdiol, which carries one more OH group on the side chain than 6-gingerol, was found in 7-day MPG extract. To determine if the 6-gingerdiol present in the extract was a metabolite of 6-gingerol, 6-gingerol was added to the *M. pilosus* inoculated PDB medium at the concentration of 100 ppm at day 0 of fermentation. 6-Gingerdiol was detected in the extract of mycelia and broth starting at day 3, and the concentration increased gradually while the level of 6-gingerol decreased gradually (data not shown). 6-Gingerol was also added to the noninoculated PDB medium at the same concentration; however, production of 6-gingerdiol was not detected during fermentation.

Pfeiffer et al. (*32*) reported that incubation of 6-gingerol with NADPH-fortified rat hepatic microsomes led to the production of eight metabolites, including diastereomers of 6-gingerdiol. Because we were not able to reduce the 6-gingerol to 6-gingerdiol chemically, it is reasonable to infer that the presence of 6-gingerdiol in the MPG extract resulted from the action of enzymatic system in the culture broth. Studies showed that 6-gingerdiol had higher antioxidant activities than 6-gingerol in terms of delaying linoleic acid oxidation and scavenging DPPH radicals; moreover, both 6-gingerdiol and 6-gingerol exhibited higher antioxidant activities than α-tocopherol (*33*). Because 6-gingerdiol is not commercially available, we purified 6-gingerdiol from ginger according to Sekiwa's method (*34*) and treated the LPS-stimulated RAW 264.7 macrophages with either 6-gingerol or 6-gingerdiol (final concentration 100 ppm). After 18 h treatment, the release of NO was inhibited by 85.50% and 86.49%, respectively (data not shown). Since the antioxidant activity of compounds significantly contribute to their anti-inflammatory activity, production of 6-gingerdiol and the synergistic effect of 6-gingerol and 6-gingerdiol may result in the enhanced anti-inflammatory capacities of MPG.

Taking together the results of this study, the ginger-modified fermented product suppressed the expression of transmembrane Toll-like receptor 4, resulting in the downregulation of NF-κB activation and the expression of proinflammatory proteins that are modulated by NF-κB. Moreover, the decreased production of proinflammatory cytokines led to the suppressed expression of adhesion molecule that is crucial to the recruitment of monocytes into subendothelial space of artery wall. These results provide a support for future application of ginger-modified *M. pilosus* fermented product as an antiatherosclerotic nutraceutical.

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