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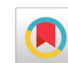

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Anti-inflammatory effect of *Malus domestica* cv. Green ball apple peel extract on Raw 264.7 macrophages

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Abstract We examined the anti-inflammatory effect of the peel extract of the newly bred Korean apple (*Malus domestica* Borkh.) cultivar Green ball. To test its possible use as anti-inflammatory functional material, Raw 264.7 macrophages were treated with pro-inflammatory lipopolysaccharide (LPS) in the presence or absence of Green ball apple peel ethanol extract (GBE). Notably, up to 500 μg/mL of GBE did not result in any signs of inhibition on cellular metabolic activity or cytotoxicity in Raw 264.7 macrophages. Supplementation with GBE to LPS-treated Raw 264.7 macrophage significantly suppressed various pro-inflammatory responses in a dose-dependent manner, including i) nitric oxide (NO) production, ii) accumulation of inducible NO synthase and cyclooxygenase-2, iii) phosphorylation of nuclear factor-kappa B (NF-κB) subunit p65, and iv) expression of pro-inflammatory biomarker genes, including tumor necrosis factor alpha, interleukin 1 beta, interleukin 6, monocyte chemoattractant protein-1, and prostaglandin E synthase 2.

Keywords Anti-inflammation · Apple peel · Biomarker genes · Green ball · Lipopolysaccharide

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Introduction

Apple (*Malus domestica* Borkh.) fruits are nutritious and beneficial for human health, as they contains various physiologically active substances, such as vitamins, minerals, organic acids, sugars, and anti-oxidative polyphenolic compounds [1,2]. Anti-oxidative polyphenolic compounds are reported to effectively reduce the risk of human diseases like skin aging, obesity, inflammation, arteriosclerosis, diabetes, high blood pressure, and certain cancers [3]. The majority of previous studies on apple were conducted on the whole apple or tree, which basic part of the user of most and contains flavonoids, anthocyanin, and carotene as well as phenolic compounds (viz., procyanidin, chlorogenic acid, caffeic acid, quercetin, rutin) [2,3]. In particular, quercetin flavanol is an active ingredient in many plant medicines and is used in a variety of products (‘Condition’, CJ HealthCare, Seoul, Korea; ‘Quercetin with bromelain’, iHerb, Moreno valley, CA, USA) [1,4]. In addition, polyphenols, which are highly contained in apple peels, have preventive effects on diseases like endogenous and/or exogenous aging, skin aging, inflammation, and cancer caused by oxidative stress [4].

Human cells are continuously exposed to various oxidative stresses, and insufficient levels of antioxidants lead to accumulation of reactive oxygen species (ROS), causing cell damage and inflammation [4-6]. The inflammation process is mediated by immune-related cells, such as macrophages and T cells [7]. The inflammatory response is primarily caused by microbial infection, resulting in the local release of pro-inflammatory chemical mediators, such as histamine, bradykinin, arachidonic acid, prostaglandin (PG), leukotriene, cytokine, chemokine, nitric oxide (NO), and serotonin by immune-related cells. These pro-inflammatory chemical mediators increase vascular permeability and vasodilation and also induce fever, redness, pain, and swelling [8,9]. Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are well known inflammation-related enzymes which produce pro-inflammatory NO and PG, respectively [10,11].

Expression of COX-2, in particular, leads to expression of inflammatory biomarker genes for compounds like tumor necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), interleukin 6 (IL-6), monocyte chemoattractant protein-1 (MCP-1), interferon-γ (IFN-γ), and prostaglandin E synthase 2 (PTGES2) [10,11]. Under inflammatory conditions, ROS induces structural and functional changes in DNA and protein molecules via oxidation and enhances the cellular levels of malondialdehyde produced by lipid peroxidation [12]. Ultimately, oxidative stress can cause reversible and/or irreversible damage to cells, lowers cell functioning, and causes chronic diseases like arteriosclerosis and cancer.

Thus, in this study, we aimed to evaluate the anti-inflammatory function of apple peel extract of the newly bred Korean apple (*Malus domestica* Borkh.) cultivar Green ball by measuring alteration of lipopolysaccharide (LPS)-induced inflammatory responses in Raw 264.7 macrophage.

Materials and Methods

Samples and sample preparation

In this study, the apple cultivar 'Green ball' was used. This cultivar was developed by Apple Research Institute (National Institute of Horticultural & Herbal Science, Gunwi 39000, Korea) in 2008 by crossing the 'Golden Delicious' and 'Fuji' varieties. The apple fruit samples were harvested from trees (/M.9, 8 years old) grown at Apple Research Institute, washed with distilled water, and apple peels were collected for further experiments. Collected apple peels were lyophilized in a freeze dryer (FD8518; ilShin BioBase, Yangju, Korea). After lyophilization, the samples were passed through a 40 mesh screen and stored at −80 °C until use.

Preparation of total phenolic extracts from Green ball apple peels

Green ball apple peel ethanol extract (GBE) were prepared as previously reported [1,13]. Briefly, 1 g of fruit powder was mixed with 100 mL of 10-100% ethanol and stored at 4 °C for 24 h with shaking. Ethanol extracts were filtered through Whatman No. 1 filter paper (Whatman Inc., Piscataway, NJ, USA), lyophilized in a freeze dryer (FD8518; ilShin BioBase, Yangju, Korea), and stored at −20 °C until use. GBE were tested at concentrations ranging from 100 to 500 μg/mL.

Raw 264.7 macrophage cell culture

The Raw 264.7 macrophages line was purchased from Korean Cell Line Research Foundation. Cells were cultured in DMEM high glucose medium (HyClone Laboratories, Inc., Logan, UT, USA) supplemented with 10% FBS (HyClone Laboratories, Inc.) and 1% penicillin-streptomycin (HyClone Laboratories, Inc.). All incubations were performed at 37 °C with 95% humidity and an atmosphere of 5% CO_2 (311, Thermo Fisher Scientific, Rockford, IL, USA). Cells were passaged after reaching 80-90% confluence. Experiments were conducted using cells that did not exceed 20 passages.

MTT assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was performed to test the cytotoxicity of GBE on Raw 264.7 macrophages, as previously reported [14]. Raw 264.7 macrophages were seeded in 48-well tissue culture plates at 5×10^3 cells/mL in complete medium (DMEM medium supplemented with 10% heat-inactivated FBS). Plates were incubated for 24 h in a CO_2 incubator at 37 °C. The following day, cells were treated with different concentrations of GBE (100-500 μg/mL) and allowed to grow for 18 h. After incubation, 50 μL of 5 mg/mL MTT (Sigma-Aldrich Co., St. Louis, MO, USA) was added. The plates were incubated for 4 h, incubation media was discarded, and 500 μL of dimethyl sulfoxide was added to solubilize the formazan. After 10 min of shaking at room temperature, the OD_{590nm} was measured using an ELISA reader (SPECTRO star Nano, BMG LABTECH, Ortenberg, Germany). Cell viability (%) was calculated as follows: cell viability (%)=[(OD_{590} of GBE treated cell)/(OD_{590} of GBE untreated cell)]×100.

Measurement of NO production

NO production was measured as nitrite (NO_2^-) and nitrate (NO_3^-) using the Griess reagent system kit (Promega, Madison, WI, USA). Raw 264.7 macrophages were seeded in 96-well tissue culture plates at 5×10^4 cells/mL in complete medium, and plates were incubated for 24 h in a CO_2 incubator at 37 °C. Cells were treated the following day with indicated concentrations of LPS (1 μg/mL) and GBE (100-500 μg/mL) and allowed to grow for 18 h. Griess reagent (1% sulfanilamide+0.1% naphthylendiamine dihydrochloride) was mixed with the cell culture supernatant at a 1:1 ratio, and the NO level was measured at $OD_{540\ nm}$ using an ELISA reader (SPECTRO star Nano, BMG LABTECH). The concentration of NO in the cell culture supernatant was calculated using a sodium nitrite ($NaNO_2$) standard curve. The inhibition rate was calculated as follows: Inhibition rate (%)=[1−(Absorbance of sample/Absorbance of control)]×100.

Immunoblotting

Measurements of iNOS and COX-2 protein expression levels were conducted using Raw 264.7 macrophages seeded in 6-well tissue culture plates at 5×10^5 cells/mL. After incubating for 24 h in a CO_2 incubator at 37 °C, 1 μg/mL LPS and/or indicated concentrations of GBE (100-500 μg/mL) were added, and the plates were incubated for 18 h and washed with PBS. Seventy microliters of a mixture of M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific) with 1× protease inhibitor (Thermo Fisher Scientific) was added to each well and cells were lysed at 4 °C, followed by centrifugation at 16,000×g for 20 min at 4 °C (Gyrozen, Seoul, Korea) to remove insoluble debris. NF-

κB protein phosphorylation levels were measured using Raw 264.7 macrophages seeded in 100×20 mm tissue culture plates at 2×10^6 cells/mL and incubated for 24 h in a CO_2 incubator at 37 °C. After incubation, 1 μg/mL LPS and/or indicated concentrations of GBE (200-500 μg/mL) were added, and the plates were incubated for 0.5 h and washed with ice-cold PBS. Cytoplasmic and nuclear proteins were extracted by using nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific) with 1× protease and 1× phosphatase inhibitors (Thermo Fisher Scientific). The protein concentrations were measured using a BCA Protein Assay Kit (Thermo Fisher Scientific). Forty micrograms of protein per sample was separated using 10% SDS-PAGE and transferred to a PVDF membrane for immunoblotting (IB) using anti-iNOS, anti-COX-2, anti-NF-κB p65, anti-p-NF-κB p65, anti-GAPDH, and anti-β-actin antibodies (Santa Cruz Biotechnology, Dallas, TX, USA). For quantification of relative iNOS, COX-2, NF-κB p65, and p-NF-κB p65, densitometry was performed using an imaging system (C300, Azure Biosystems Inc., Dublin, CA, USA).

Expression analysis of inflammatory biomarker genes by using real-time PCR

Raw 264.7 macrophages were seeded in 100×20 mm tissue culture plates at 5×10^6 cells/mL. After a 24 h incubation in a CO_2 incubator at 37 °C, 1 μg/mL LPS and/or indicated concentrations of GBE (100, 300, and 500 μg/mL) were added, and the plates were incubated for 18 h and washed with ice-cold PBS. Total RNA was extracted by using a GeneAll® Ribospin RNA extraction kit (GeneAll Biotechnology Co., Seoul, Korea) and used for cDNA synthesis with a qPCRBIO cDNA synthesis kit (PCR Biosystems, London, UK). One microliter of 1:1 diluted cDNA was used for real-time PCR with 5 μL Real-time PCR master mix (GeneAll Biotechnology Co.) and the PCRmax Eco 48 real-time PCR system (PCRmax, Staffordshire, UK). PCR conditions and primer sequences are listed (Tables 1, 2).

Statistical analysis

All experiments were repeated at least three times. SPSS 23 for Windows was used for analysis (Statistical Package for Social Science, SPSS Inc., Chicago, IL, USA). One-way analysis of variance (ANOVA) and Duncan's multiple range test were used to determine the significance of differences between the samples at $p<0.05$ or $p<0.01$.

Results

GBE is not cytotoxic to Raw 264.7 macrophages

To confirm the cytotoxic effect of GBE, cell viability of Raw 264.7 macrophages was measured using an MTT assay (Fig. 1). Treatment with different concentrations of GBE did not alter cellular metabolic activities. No significant alterations in the metabolic activity were observed in cells treated with the highest

Table 1 The sequences of primers used for real-time PCR

Gene name	Accession	primer	Sequence (5'-3')	Amplicon (b.p.)
TNF-α	NM_001278601.1	Forward	5'-TCTACTGAACTTCGGGGTGA-3'	87
		Reverse	5'-AGGGTCTGGGCCATAGAACT-3'	
IL-1β	NM_008361.4	Forward	5'-CAACCAACAAGTGATATTCTCCATG-3'	152
		Reverse	5'-GATCCACACTCTCCAGCTGCA-3'	
IL-6	NM_031168.2	Forward	5'-TAGTCCTTCCTACCCCAATTTCC-3'	76
		Reverse	5'-TTGGTCCTTAGCCACTCCTTC-3'	
MCP-1	NM_011333.3	Forward	5'-TTCCTCCACCACCATGCAG-3'	64
		Reverse	5'-CCAGCCGGCAACTGTGA-3'	
PTGES2	NM_133783.2	Forward	5'-CCGTGAGAAGGACTGAGATC-3'	162
		Reverse	5'-AAGTGATGACCTCTTCCAGG-3'	
β-actin	NM_007393.4	Forward	5'-CGTGCGTGACATCAAAGAGAA-3'	137
		Reverse	5'-GCTCGTTGCCAATAGTGATGA-3'	

Table 2 Real-time PCR conditions

Factors	Real-time PCR condition
TNF-α	95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, followed by 95, 55, 95 °C for 15 s, each
IL-1β	95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, followed by 95, 55, 95 °C for 15 s, each
IL-6	95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, 72 °C for 15 s, followed by 95, 55, 95 °C for 15 s, each
MCP-1	95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, followed by 95, 55, 95 °C for 15 s, each
PTGES2	95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s, followed by 95, 55, 95 °C for 15 s, each
β-actin	95 °C for 5 min, followed by 40 cycles of 95 °C for 10 s, 60 °C for 20 s or 95 °C for 10 s, 60 °C for 20 s, 72 °C for 15 s, followed by 95, 55, 95 °C for 15 s, each

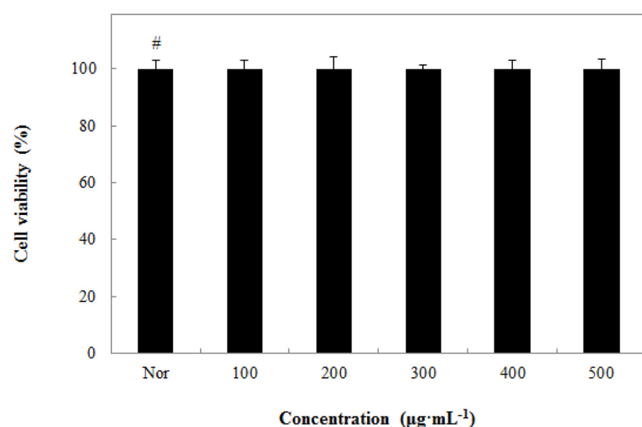

Fig. 1 MTT assay of Raw 264.7 macrophages treated with GBE. Raw 264.7 macrophages were stimulated with LPS (1 μg/mL) in the presence of different concentrations of GBE (100-500 μg/mL) for 18 h. Data presented as means ± SD of three independent experiments (n =3). No significant differences were observed between GBE-untreated and GBE-treated groups (p <0.01). Nor: Normal GBE-untreated

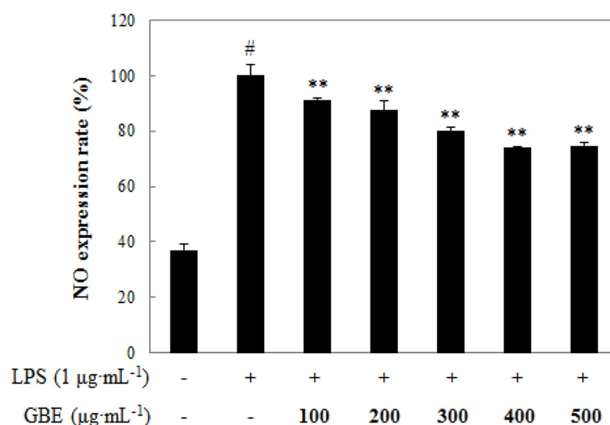

Fig. 2 Effect of GBE on NO production in LPS-stimulated Raw 264.7 macrophages. Raw 264.7 macrophages were treated with LPS (1 μg/mL) in the presence of various concentrations GBE (100-500 μg/mL) for 18 h. LPS- and GBE-untreated cells served as negative control. LPS-treated cells served as a positive control. The values are mean ± SD of three independent experiments. $^{\#}p$ <0.05 compared with negative control group, *p <0.05, **p <0.01 compared with positive control group

concentration of GBE, suggesting GBE is not cytotoxic. Therefore, 500 μg/mL was used in the following experiments.

GBE suppresses NO production in LPS-stimulated Raw 264.7 macrophages

NO generation was measured in Raw 264.7 macrophages stimulated with 1 μg/mL LPS in the presence of different concentrations of GBE (Fig. 2). NO production was significantly reduced to 91.02, 87.84, 80.29, 74.14, and 74.73% by 100, 200, 300, 400, and 500 μg/mL GBE, respectively, as compared to the GBE-untreated control. At the highest concentration of GBE (500 μg/mL), approximately a 25.2% reduction in LPS-induced NO production was observed. This suggests that GBE can significantly attenuate the inflammatory response in Raw 264.7 macrophages. NO is produced during inflammatory responses and functions as an antibacterial, anticancer, and vasodilation agent in the immune system [15]. However, excessive production of NO can damage cells or tissues and induce mutations in DNA. Thus, NO is often used as a biomarker for inflammatory responses [15].

GBE suppresses accumulation of iNOS and COX-2, as well as activation of NF-κB

To confirm the phosphorylation status of nuclear factor-kappa B (NF-κB) involved in iNOS and COX-2 protein expression, different concentrations of GBE were used to treat LPS-stimulated Raw 264. 7 macrophages (Fig. 3). Consistent with GBE's suppressive effect on LPS-induced NO production in Raw 264.7 macrophages, iNOS expression was significantly reduced by 99.97, 72.74, 54.96, 41.51, and 31.73% at 100, 200, 300, 400, and 500 μg/mL, respectively (Fig. 3A). The highest concentration of GBE (500 μg/mL) reduced iNOS expression up to 68.27%. GBE treatment also significantly reduced LPS-induced COX-2 expression in a dose-dependent manner (Fig. 3B). Expression of COX-2 was significantly reduced to 93.69, 79.20, 57.82, 43.26, and 18.44% by 100, 200, 300, 400, and 500 μg/mL GBE, respectively. Although the endogenous level of NF-κB subunit p65 was not significantly altered by LPS treatment, the level of phosphorylation (or activation) was significantly increased (Fig. 4). However, LPS-induced p65 phosphorylation was significantly reduced to 61.73, 63.02, 54.70 and 43.41% by 200, 300, 400 and 500 μg/mL GBE treatment, respectively. In summary, LPS-induced expression of pro-inflammatory iNOS and COX-2 and phosphorylation of NF-κB subunit p65 were significantly suppressed by GBE in a dose-dependent manner. This suggests that GBE-mediated suppression of NF-κB activation might lead to suppression of downstream iNOS and COX-2 production. Taken together, our results suggest that GBE can be used as a potential anti-inflammatory functional material.

GBE downregulates expression of inflammatory biomarker genes

Expression of pro-inflammatory biomarker genes TNF-α, IL-1β, IL-6, MCP-1, and PTGES2 were examine in LPS-stimulated Raw 264.7 macrophages treated with different concentrations of GBE (Figs. 5A-E). Expression of TNF-α was significantly reduced to 88, 42 and 6% by treatment with 100, 300, and 500 μg/mL GBE, respectively. Notably, 500 μg/mL GBE treatment reduced LPS-induced TNF-α expression to levels similar to that of LPS-untreated Raw 264.7 macrophages. Similarly, expression of IL-1β, IL-6, MCP-1, and PTGES2 were also significantly inhibited by GBE treatment in a dose-dependent manner. In particular, the highest concentration of GBE (500 μg/mL) inhibited expression of MCP-1at a level similar to that observed in LPS-untreated cells

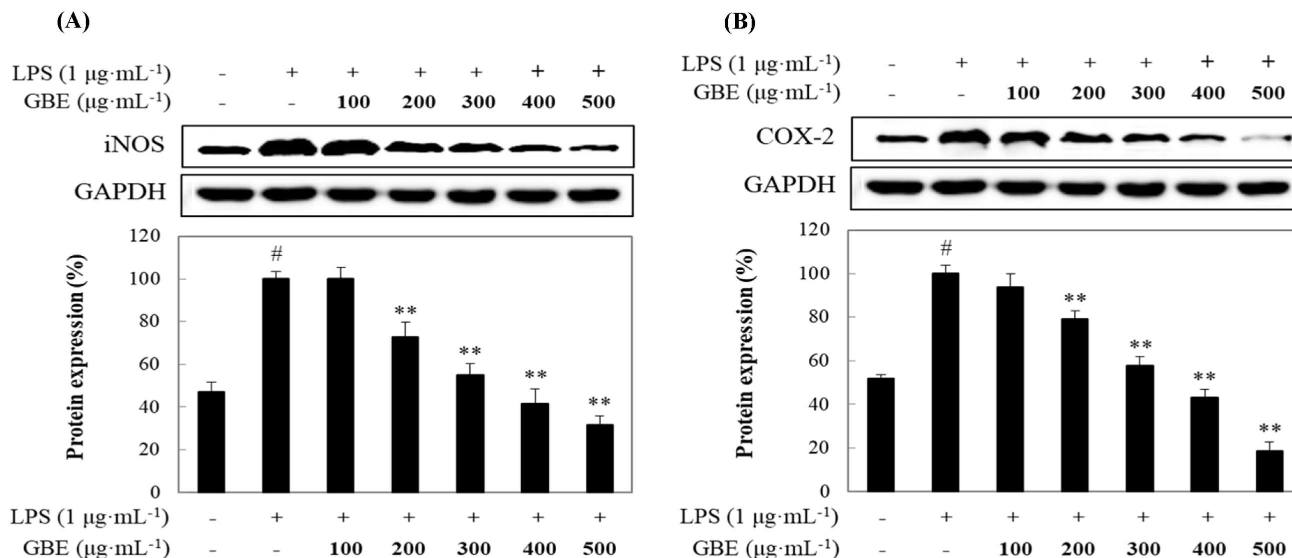

Fig. 3 Effect of GBE on the protein expression of iNOS (A) and COX-2 (B) in Raw 264.7 macrophages stimulated with LPS. Raw 264.7 macrophages were treated with LPS (1 μg/mL) in the presence of various concentrations of GBE (100-500 μg/mL) for 18 h. LPS- and GBE-untreated cells served as a negative control. LPS-treated cells served as a positive control. The values are mean ± SD of three independent experiments. $^{\#}p<0.05$ compared with negative control group, $^{*}p<0.05$, $^{**}p<0.01$ compared with positive control group

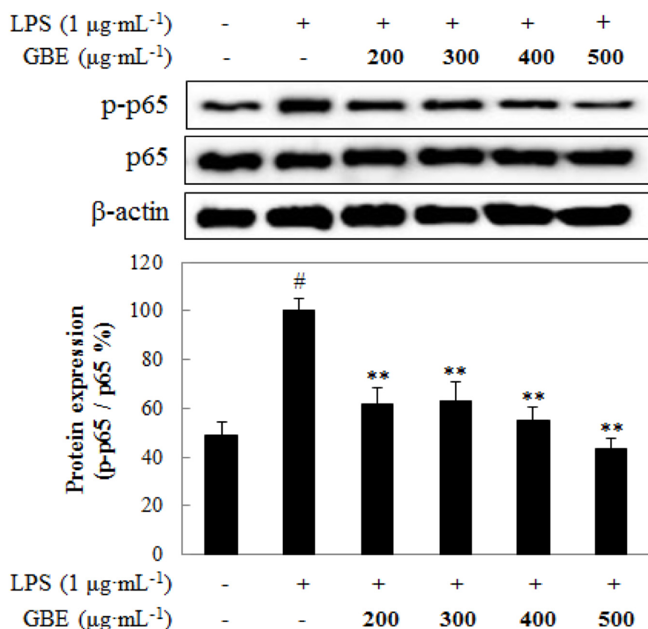

Fig. 4 Effect of GBE on the phosphorylation of NF-κB subunit p65 in Raw 264.7 macrophages stimulated with LPS. Raw 264.7 macrophages were treated with LPS (1 μg/mL) in the presence of different concentrations of GBE (200-500 μg/mL) for 30 min. LPS- and GBE-untreated cells served as a negative control. LPS-treated cells served as a positive control. The values are mean ± SD of three independent experiments. $^{\#}p<0.05$ compared with negative control group, $^{*}p<0.05$, $^{**}p<0.01$ compared with positive control group

or, in the case of IL-1β, IL-6 and PTGES2, levels were even lower than those of the control.

Discussion

As a result of infection and injuries, inflammatory responses are activated, which in turn cause pain, fever, redness and swelling. LPS, also known as lipoglycans and endotoxins, are large molecules consisting of a lipid and a polysaccharide produced by Gram-negative bacteria [16]. Macrophage cells are recruited to the site of infection where they can recognize the presence of LPS via its cognate toll-like receptor (TLR)-4. iNOS produces NO from L-arginine and plays a key role in regulation of inflammatory responses [15]. COX-2 catalyzes prostaglandin H_2 (PGH_2) generation from arachidonic acid (AA) [17]. Then, PGH_2 is further processed to prostacyclin, prostaglandin E_2 (PGE_2), thromboxane A, and prostaglandin D_2, which have different physiological roles in humans, including in immune response [17]. Expression of both iNOS and COX-2 are induced by inflammatory stimuli, such as LPS, and their expression is known to be regulated by the phosphorylation status of NF-κB [18]. NF-κB is found in almost all animal cell types and is activated by various external and internal stimuli, such as biotic and abiotic stresses, cytokines, free radicals, and UV irradiation. It is also a major transcription factor of various pro-inflammatory proteins and/or cytokine genes involved in the inflammatory response [19]. Under the normal conditions, NF-κB is sequestered in the cytoplasm by an inhibitor (IκB); however, LPS-induced phosphorylation of IκB by the IκB kinase (IKK) complex promotes IκB degradation and NF-κB phosphorylation. Phosphorylated NF-κB is translocated into the nucleus where it can transcriptionally activate iNOS, COX-2, and pro-inflammatory cytokine genes [20,21]. This recognition activates downstream pro-inflammatory cytokine gene expression of TNF-α, IL-6 and

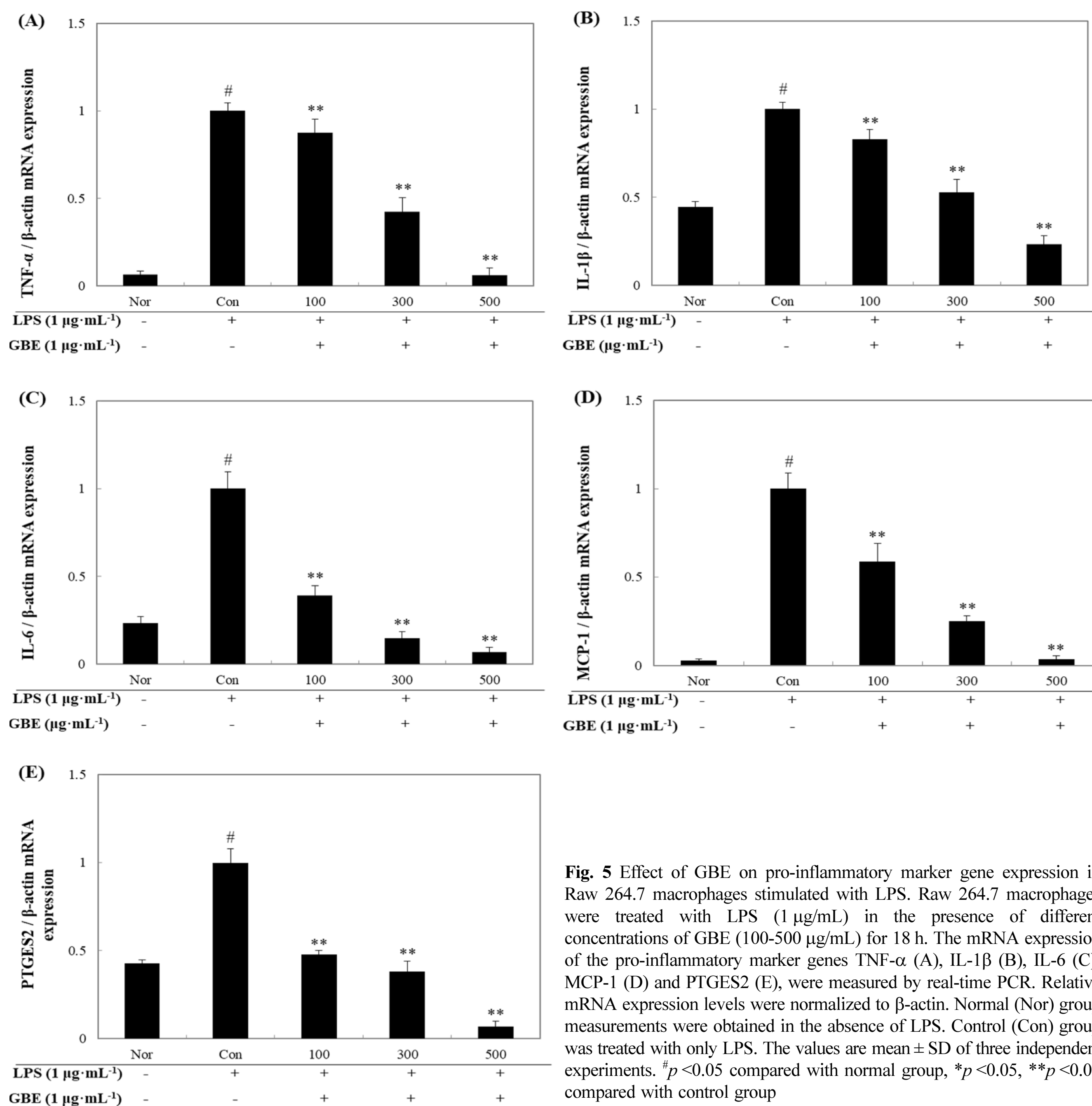

Fig. 5 Effect of GBE on pro-inflammatory marker gene expression in Raw 264.7 macrophages stimulated with LPS. Raw 264.7 macrophages were treated with LPS (1 μg/mL) in the presence of different concentrations of GBE (100-500 μg/mL) for 18 h. The mRNA expression of the pro-inflammatory marker genes TNF-α (A), IL-1β (B), IL-6 (C), MCP-1 (D) and PTGES2 (E), were measured by real-time PCR. Relative mRNA expression levels were normalized to β-actin. Normal (Nor) group measurements were obtained in the absence of LPS. Control (Con) group was treated with only LPS. The values are mean ± SD of three independent experiments. $^{\#}p$ <0.05 compared with normal group, *p <0.05, **p <0.01 compared with control group

IL-1β [22]. During activation of inflammatory responses, cytokines promote recruitment of circulating white blood cells to the infected site. IL-1β and IL-6 are known as endogenous pyrogens, whereas TNF-α enhances the permeability of the peripheral vascular epithelium, thus enhancing the migration of cells and water-soluble molecules from blood vessels to tissues [23]. Prostaglandin H_2 (PGH_2), the first intermediate of all prostaglandins (PGs), is synthesized from AA by COX (or PTGS) enzymes. Then, prostaglandin E synthases (PTGES) are involved in further modification of PGH_2 to pro-inflammatory PGE_2. PGES include cytosolic PTGES (cPTGES), microsomal PTGES1 (mPGES1), and mPTGES2 [24]. Resulting PGE_2 increases blood flow, pain, redness, and edema of tissues [13]. MCP-1 functions as a chemokine that controls the movement of immune-related cells, such as monocytes, to the infection site in the early stage of the inflammatory response [25]. Monocytes recruited by MCP-1 differentiate into macrophages and secrete inflammatory cytokines (TNF-α, IL-6, IFNs) to intensify the inflammatory response. Therefore, MCP-1 plays an important role in the early inflammatory response, which is involved in the pathogenesis of several diseases

characterized by mononuclear cell infiltration, such as psoriasis, rheumatoid arthritis, and atherosclerosis [26].

This evidence suggests that GBE, a physiologically active phenolic substance contained in Green ball apples, inhibits the expression and/or activation of iNOS, COX-2, and NF-κB, thereby suppressing the expression of various pro-inflammatory biomarker genes. Our results suggest that GBE is a valuable health-related functional material that can be used for treating inflammation-related disorders like skin aging.

Inflammation-inhibitory effects were measured utilizing ELISA, western blotting and real-time PCR for inflammation-related factors utilizing GBE. As a result, NO expression was suppressed by GBE. iNOS, COX-2, and NF-κB p65 protein expression levels were inhibited in a concentration-dependent manner. mRNA expressions of pro-inflammatory factors TNF-α, IL-1β, IL-6, MCP-1, and PTGES2 were significantly suppressed. Besides, monocytes collected by MCP-1 are differentiated into macrophages and stimulated by inflammatory cytokine (TNF-α, IL-1β, IL-6) and COX that are involved in the inflammatory response, thereby inhibiting PTGES2 involved in PGE_2 expression.

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