Red ginseng extract regulates differentiation of monocytes to macrophage and inflammatory signalings in human monocytes

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Abstract This study was aimed to investigate the effect of red ginseng extract (RGE) on monocyte to macrophage differentiation and inflammatory signalings in THP-1 human monocytes. In HPLC analysis, RGE contained saponin level of 516 µg/mg (extract) with 14 ginsenosides. RGE effectively suppressed the monocyte-to-macrophage differentiation induced by phorbol 12-myristate 13-acetate (PMA) by inhibiting the THP-1 cell adhesion. This result is evidenced by the down-regulation of cluster of differentiation molecule β (CD11 β) and CD36. RGE significantly reduced translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (78%), while cytosolic NF- κ B was increased (53%), compared with LPS group. In addition, RGE significantly increased the protein abundance of nuclear factor (erythroid-derived 2)-like 2 (Nrf2) and its target protein, hemoxygenase-1 (HO-1), but, Kelch-like ECH-associated protein 1 (KEAP1), a negative regulator of Nrf2, was greatly decreased by RGE. Furthermore, RGE effectively

mediated the regulation of Nrf2 level in nucleus and cytoplasm of THP-1.

Keywords Human monocyte (THP-1) · Red ginseng extract (RGE) · Monocyte-to-macrophage differentiation · Inflammatory signaling

Introduction

Red ginseng (RG) has traditionally been used for numerous health benefits in the East Asia countries including Korea (Han et al., 2005). Red ginseng is generally obtained from the processes consisting of steaming with high temperature and then drying, resulting in red color (Kim et al., 2000). Such process has been known to change compositions and levels of ginseng saponins, enhancing its biological activity (Kim et al., 2000). RG has been reported to exert various beneficial effects such as the increase of body flow and vital energy, anti-diabetes, immunomodulation, anti-inflammation (Kim et al., 2000; Saba et al., 2018).

Chronic inflammation has become a not-to-be-ignored issue in the recent society, as it can lead to various severe diseases such as diabetes, cardiovascular and pulmonary diseases (Chen et al., 2017).

Inflammation is a complex immune-system-mediated response which protects the body from pathological invasion or tissue damage (Chen et al., 2017). However, prolonged inflammatory responses are harmful to the body by damaging healthy organs or cells with enhanced levels of inflammatory biomarkers (Panichi et al., 2012). Activated by a certain intracellular stimuli, such as lipopolysaccharide (LPS), the body increased the inflammatory leukocytes such as monocyte and macrophages, from which pro-inflammatory gene expression is up-regulated. Macrophage

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plays a crucial role in inflammation by mediating the recruitment of additional inflammatory cytokines and interleukins. The expression of inflammatory cytokines is controlled by nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B), the pro-inflammatory dimeric transcription factor. It moves into the nucleus, binds to promoter regions of inflammatory genes, and stimulates their expression, promoting inflammatory responses (Lee et al., 2008; Sung et al., 2012). In recent years, nuclear factor E2-related factor 2 (Nrf2) signaling, an antioxidant machinery, has also been implicated to inflammation (Kang et al., 2018; Kobayashi et al., 2016). The up-regulation of Nrf2 and its target gene, hemoxygenase-1 (HO-1), a rate-limiting enzyme in heme catabolism, has been known to have cytoprotective effect, and suppress the inflammatory responses (Kobayashi et al., 2016).

Monocytes play an important role in the vascular inflammation by acting at the very first inflammatory response (Kobayashi et al., 2016). In particular, monocytes differentiate into macrophages which then attaches to the epithelial cells of blood vessel walls, eventually being able to roll and migrate towards to the inflammation site (León and Ardavin, 2008). The lasting differentiation and activation of this monocyte implicated to the increase of cardiovascular inflammatory diseases (Yang et al., 2014). THP-1 human monocytes are the acute monocytic leukemia cell line which differentiate into macrophages by an induction of phorbol 12-myristate 13-acetate (PMA) and lipopolysaccharide (LPS)-mediated stimulation of the cells have been known to promote inflammatory factors (Park et al., 2007). Many studies reported anti-inflammatory effects of RGE (Baek et al., 2016; Hong et al., 2016), but the effect of RGE on inflammatory signaling and monocyte-to-macrophage differentiation in human monocyte (THP-1) has been limited. Hence, this study addressed the regulatory effect of RGE on the monocyte-to-macrophage differentiation and two inflammatory machineries (Nrf2 and NF- κ B signalings) which govern the inflammatory responses using THP-1 cells.

Materials and methods

Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Gaithersburg, MD, USA). RPMI 1640 medium was purchased from WELGNE Inc. (Daegu, Korea) Fetal bovine serum (FBS), Penicillin–streptomycin (PS), phosphate-buffered saline (PBS) were obtained from Hyclone (Logan, UT). EZ-Cytox Assay Kit was purchased from DoGenBio co. (Seoul, Korea). *Escherichia coli* lipopolysaccharide (LPS), and Phorbol 12-myristate

13-acetate (PMA) were purchased from Sigma-Aldrich Chemical co. (St Louis, MO, USA). Trypsin-ethylenediaminetetraacetic acid (EDTA) Solution (0.25%) was obtained from WELGENE Inc. (Daegu, Korea). Trypan Blue Solution (0.4%) was purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibodies for the cluster of differentiation molecule β (CD11 β), CD36, and NF- κ B proteins were purchased from Cell Signaling Technology, Inc. (Danvers, MA, U.S.A.). Nrf2, KEAP1 and Heme oxygenase-1 (HO-1), Lamin B, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, U.S.A.). All other chemicals were purchased from Sigma-Aldrich Chemical Co.

Cell culture

THP-1 human monocytes obtained from the American Type Culture Collection (ATCC, TIB-71) were cultured in RPMI-1640 supplemented with 10% FBS, 1% PS, and 0.05 mM 2-mercaptoethanol. The cells were seeded at 2×10^5 cells/well in a 60 mm dish. When the confluence reached 60%, the cells were differentiated by adding 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h. Differentiated cells were stimulated by LPS (1 μ g/mL) to induce inflammatory responses. Various concentrations of RGE were pretreated 2 h before stimulation of PMA or LPS (Supplement Fig 1). THP-1 cells were maintained at 37 °C under 5% CO₂ and 95% humidity.

THP-1 cell adherence test

THP-1 monocytes were pretreated with different concentrations of RGE (0–50 μ M), 2 h before 100 ng/mL PMA stimulation, and the cells were incubated for additional 48 h. Then the cells were washed three times using PBS to remove non-adherent cells. Adherent macrophages were observed using microscope, detached with 0.25% Trypsin–EDTA solution. Detached cells were stained with diluted Trypan Blue solution (0.2%). Total cells were counted using hemocytometer and microscope (Leica DM2500, Wetzlar, Germany).

Cell viability assay

To analyze the cytotoxic effects of RGE on THP-1 cells, EZ-Cytox assay was performed for water-soluble tetrazolium salt. Cells were seeded at 2×10^4 cells per well in a 96 well plate. After 24 h-incubation, various concentrations of RGE or vehicle, RGE with 1 μ g/mL LPS (RGE + LPS), and RGE with PMA (100 ng/mL) (RGE + PMA), were added, and incubated for additional 48 h. These samples or vehicle (DMSO) were treated to the

culture at 0.25%. THP-1 cells were treated with 12-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium (WST-1) reagent at 37 °C for 1 h. The formazan dye was solubilized and its absorbance was determined at 450 nm (Spectra Max M3; Molecular Devices).

Sample preparation

Red ginseng extract (RGE) was prepared using 70% ethanol from red ginseng which was obtained from The Korean Society of Ginseng (Daejeon Korea). Five gram of red ginseng was added to 70% ethanol (100 mL), and extracted with reflux and boiling for 2 h. The extract was filtered using whatman No. 1 to remove insoluble debris, after which filtered extract was concentrated using evaporator. The concentrate was lyophilized to be stored and dissolved in dimethyl sulfoxide (DMSO) to be used as a sample.

HPLC analysis

GRE-derived ginsenosides was analyzed using HPLC system (Agilent Technologies 1260 Infinity, Santa Clara, USA) equipped with Imtakt Cadenza CD-C18 column (4.6 mm × 75 mm, 3 µm, Portland, OR, USA). The ginsenosides were isolated by gradient system of solvent A (10% acetonitrile) and solvent B (90% acetonitrile) at 40 °C. The solvent A/B ratios in the gradient mode were follows: an initial ratio of 95/5 for 0–3 min, a linear increase to 90/10 at 20 min, a linear decrease to 75/25 at 50 min, and a final ratio of 95/5 at 60 min. The flow rate and injected volume were 1.3 mL/min and 5 µL, respectively. The samples were measured at 203 nm using UV detector. Ginsenosides used as standards are Rb1, Rb2, Rc, Rd, Re, Rf, F2, Rh1(s), Rh2(s), Rk1, Rg1, Rg2(s), Rg2(r), Rg3(s), Rg3(r), Rg5, CK (compound K).

Western blotting

Protein extracts (50 µg) were separated at 10% sodium dodecyl sulfate poly acrylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene difluoride (PVDF) membrane overnight. Primary antibody (1:1000) was probed overnight and secondary antibody (1:5000) was applied to the membrane for 2 h. Multiple washes were conducted with Tris Buffered Saline with Tween 20 (TBST) buffer, between and after the incubation. Then the protein bands were visualized by enhanced chemiluminescence (ECL) solution and LAS 4000 imager (Fuji, New York, NY, USA). Visualized protein bands were quantified by ImageJ program (NIH, National Institutes of Health, Bethesda, MD, USA).

Preparation of cytosolic and nuclear fractions

To analyze the translocation of two different transcription factors, Nrf2 and NF-κB, cytosolic and nuclear fractions were separated as follows: cultured THP-1 macrophages were washed twice with PBS, prior to the addition of buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.9), 50 mM NaCl, 0.5 M sucrose, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycophosphate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 4 µg/mL aprotinin, and 2 µg/mL pepstatin A]. The cell resuspensions were collected into 1.5 mL Eppendorf tubes with scrapers. The cells were lysed by pipetting on ice for more than 50 times and centrifuged at 500 × *g* for 10 min. The supernatants (cytoplasmic fraction) were collected and store at − 80 °C for the analysis. The cell pellets were gently re-suspended in buffer B [10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM ethylene glycol bis (2-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 1 mM PMSF, 4 µg/mL aprotinin, and 2 µg/mL pepstatin A]. The cell suspensions were centrifuged at 500 × *g* for additional 5 min and the supernatants were discarded. Buffer C [0.1% NP-40, 10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM PMSF, 4 µg/mL aprotinin, and 2 µg/mL pepstatin A] was added to the cell pellet and were vortexed at highest speed for 10 min. The cell lysates were centrifuged at 20,000 × *g* for 10 min at 4 °C. The obtained supernatants (nuclear fraction) were stored at − 80 °C for following analysis.

Real-time PCR

Cultured cells were washed twice with PBS. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.) according to the manufacturer's protocol. cDNA was synthesized using LaboPass cDNA synthesis kit (CosmoGenetech Co., Ltd., Seoul, Korea) from 1 µg of total RNA. Synthesized cDNA was mixed with targeted primers and SYBR Green PCR Master Mix (Applied Biosystems). Reactions were executed in triplicate for each pair of primers using AriaMx Real-Time PCR system (Agilent Technologies). Primers used in this study were followings: TNF-α (Forward-AGCCCCAGTCTGTATCCTT, Reverse-CTCCCTTTCAGAACTCAGG), IL-6 (Forward-CTGGGGATGTCTGTAGCTCA, Reverse-CTGTGAAGTCTCCTCTCCGG), IL-1β (Forward-ACTCATTTGTGGCTGTGGAGA, Reverse-TTGTTTCATCTCGGAGCCTGT), MCP-1 (Forward-GCTGCTACTCATTACAGC, Reverse-CTTCTTGGCCTTGTTTCCC), GAPDH (Forward-CTGCGACTTCAACAGCAACT, Reverse-GAGTTGGGATAGGGCCTCTC).

Flow cytometry

GRE-treated PMA differentiated macrophages (1×10^6 cells/well) were harvested from 6 well plates and washed twice in PBS before treatment with specific fluorochrome (FITC) conjugated antibody against CD36 [CD36 monoclonal antibody, FITC, Thermo Fisher Scientific (Waltham, MA, USA)]. Antibodies were used at a 1:1000 dilution. Cells were then washed twice in PBS by centrifugation, supernatant was discarded and cells were resuspended in 400 μ L PBS, and analyzed using a FACScalibur flow cytometer (BD Biosciences, Oxford, UK) according to the manufacturer's instructions.

ELISA analysis

PMA-treated THP-1 cells were treated with GRE in various concentrations for 2 h, after which LPS was treated for 24 h. Culture media were collected, and IL-6 and MCP-1 were analyzed by ELISA according to the manufacturer's protocol (BD Biosciences, and Cayman Chemical respectively).

Statistical analysis

All results were analyzed from three independent experiments and expressed as mean $\pm$ SEM using SPSS software (SPSS Inc., Chicago, IL, U.S.A). One-way ANOVA and Tukey's multiple range test were used to compare differences among groups. Differences with $p < 0.05$ were considered as significant.

Results and discussion

Saponin analysis from RGE

HPLC analysis showed RGE contained 15 kinds of ginsenosides; Rb1, Rb2, Rc, Rd, Re, Rf, F2, Rg3(s), Rg1, Rg2(s), Rg2(r), Rg3(r), Rg5, Rh1(s), and Rk1. However, CK and Rh2 were not detected in RGE (Fig. 1 and Table 1). The combined level of these ginsenosides was 516.82 μ g/mg, occupying more than 50% of total RGE (Table 1). Rb1 was the most abundant ginsenoside (130.15 μ g/mg), contributing to 25.5% of total ginsenosides, while Rg1 was the lowest ginsenoside 12.84 μ g/mg (Table 1). This result was similar to a study showing that Rb1, Rc, and Rg1 were the most abundant ginsenosides in ginseng main root (Kang and Kim, 2016), except for Rg1. The difference of Rg1 was shown to be due to the different extraction method. Rb1, a representative ginsenoside, was recognized to be responsible for the most of biological activities of ginseng (Ahmed et al., 2016). A recent study

introduced Rb1 as a major ginsenoside for neuroprotection (Ahmed et al., 2016). RGE included red ginseng-specific ginsenosides such as Rg2, Rg3, Rh1, which are produced or elevated by heat stimulation during red ginseng process (Keum et al., 2000; Kim et al., 2000). These ginsenosides have been known to show anti-tumor and hypotensive effect (Keum et al., 2000; Kim et al., 2000). Therefore, RGE is shown to have various saponins which are able to play pharmacological effects.

Effect of RGE on cell viability

Various concentrations of RGE (6.25, 12.5, 25, 50, 100, 200, 300, 400 and 500 μ g/mL) were tested on viability of THP-1 cells, with or without PMA (100 ng/mL) or LPS (1 μ g/mL) (Fig. 2A). RGE only and co-treatment of RGE and PMA didn't show any cytotoxicity until concentration of RGE 500 μ g/mL. Co-treatment of RGE and LPS on differentiated THP-1 macrophage did not show any significant cytotoxicity until RGE 400 μ g/mL (Fig. 2A). The data indicated that RGE less than 200 μ g/mL did not affect the viability of THP-1 monocytes or macrophages. Thus, subsequent experiments were performed within a non-toxic range of RGE; 0 ~ 100 μ g/mL.

Effect of RGE on monocyte-to-macrophage differentiation

To determine the effect of RGE on monocyte-to-macrophage differentiation, cell adherence assay was conducted using trypan blue assay. As shown in Fig. 2B, adherence of cells was decreased by the treatment of RGE. Adherent cell numbers were decreased by 40% in high dose of RGE (100 μ g/mL) (Fig. 2B). Protein expression level of cluster of differentiation 11 β (CD11 β) and CD36, which are the surface markers of differentiation to macrophage, were greatly induced by PMA treatment (Fig. 2B, C). This PMA-induced increase of CD11 β and CD36 was decreased with RGE treatment. High dose of RGE (100 μ g/mL) reduced the protein levels of CD11 β and CD36 by 80% and 47%, respectively, compared to PMA group. In addition, in flow cytometry analysis, PMA obviously increased CD36 expressed cells (49.9–84.7%), but GRE (100 μ g/mL) treatment significantly decreased the CD36-stained cells by 10% (Fig. 2E, F). The results suggested that RGE effectively inhibited PMA-induced monocyte-to-macrophage differentiation by downregulating CD11 β and CD36 in THP-1 macrophages. In the response to stimuli, monocytes can be recruited from the blood stream to specific place and thereby differentiate into macrophages. Macrophages play an important role in innate and adapted immunity via phagocytosis and cytokine production (Gordon and Martinez, 2010; Wynn et al., 2013). It is associated with the

Fig. 1 HPLC analysis of RGE. Chromatogram of RGE-derived ginsenosides

Table 1 Ginsenoside composition from red ginseng extract

No.	Ginsenosides	Ginsenoside content (μg/mg, extract)
1	Rg1	12.84
2	Re	27.66
3	Rf	34.47
4	Rh1(s) + Rg2(s)	15.32
5	Rg2(r)	18.90
6	Rb1	130.15
7	Rc	57.75
8	Rb2	44.51
9	Rd	17.25
10	F2	44.41
11	Rg3(s)	36.06
12	Rg3(r)	15.84
13	CK	— ^a
14	Rg5	32.59
15	Rk1	29.06
16	Rh2(s) + Rh2(r)	—
Total	516.81	

^aNot detected

homeostasis including tissue remodeling or metabolic regulation (Gordon and Martinez, 2010). Macrophage has been differentiated into two types of phenotypes according to differentiation circumstances. M1 type is generally involved in the inflammatory responses via production of pro-inflammatory cytokines and reactive oxygen species (ROS), while M2 is associated with immune modulation and tissue remodeling via limited inflammatory production of anti-inflammatory markers (Gordon and Martinez, 2010; Wynn et al., 2013). However, high concentration of PMA (50 ng/mL) or IFN- γ have been known to shift macrophage towards activated M1 state (Gordon and Martinez, 2010;

Wynn et al., 2013). This continuous activation of macrophage is implicated to the pathogenesis of chronic diseases including atherosclerosis, diabetes, and obesity (Wynn et al., 2013). Therefore, inhibitory effect of RGE on monocyte-to-macrophage differentiation suggested that RGE can suppress the adverse shift of THP-1 into inflammatory macrophage. However, current study did not address the effect of RGE on shift of THP-1 into M2 macrophage. Since induction into M2 macrophage was also an important aspect for anti-inflammatory effect of RGE, RGE would be examined on the shift of THP-1 into M2 macrophage in the future.

Effect of RGE on NF- κ B signaling

NF- κ B is a transcription factor which is playing an important role in mediating several pro-inflammatory responses in the cell (Lee et al., 2008). Since the production of inflammatory cytokines from THP-1 was resulted from transactivation of NF- κ B into nucleus, the effect of RGE on nuclear NF- κ B level was examined. LPS markedly increased NF- κ B level in nucleus, but cytosolic NF- κ B was significantly reduced (Fig. 3A). LPS-induced increase of NF- κ B was significantly suppressed by RGE in a dose-dependent manner (Fig. 3A, B). RGE reduced nuclear NF- κ B by 78%, but increased cytosolic protein by 54%, compared with LPS-only group (Fig. 3B). This result indicated that RGE can effectively suppress inflammatory responses by inhibiting translocation of NF- κ B into nucleus. Transactivation of NF- κ B is dependent on the phosphorylation of I κ B α in cytoplasm (Scheinman et al., 1995). I κ B α forms the complex with NF- κ B in rested state of cells, but inflammatory stimuli causes the release of NF- κ B from the complex with the phosphorylation of I κ B α . Then, NF- κ B is transported into nucleus to promote

Fig. 2 Effect of RGE on THP-1 cell viability and monocyte to macrophage differentiation. Cell viability of THP-1 cells was assessed by WST assay (A). THP-1 monocytes were treated with different concentrations of RGE 2 h prior to the PMA (100 ng/mL) stimulation for 48 h. The effect of various concentrations of RGE on cell adherence was determined by trypan blue assay (B). Protein

abundance of CD11β and CD36 were examined by Western blot (C) and quantified by ImageJ software (D). CD36-stained cells were analyzed in flow cytometry (E, F). The values are presented as the mean and standard error of the mean (mean ± SEM). Different letters indicate statistically significant difference among the groups ($p < 0.05$). CON no treatment, PMA only PMA treatment

expression of inflammatory genes (Scheinman et al., 1995). RGE effectively suppressed phosphorylation of IκBα (Fig. 3C, D). This result showed that RGE-mediated inhibition of translocation of NF-κB was due to the suppression of IκBα phosphorylation. The inhibitory effect of ginseng

extract or saponins on NF-κB translocation has been studied on several cell lines (Yang et al., 2017; Yayah et al., 2012). A recent study showed that Rg3 suppresses radiation-induced NF-κB activation and IκBα phosphorylation in lung cancer cell (Wang et al., 2015). On the other

Fig. 3 Effect of RGE on phosphorylation of IκBα and nuclear translocation of NF-κB on THP-1 cells. Protein levels of NF-κB (nuclear and cytosol) and phosphor-IκBα were analyzed by Western blot (A), and the results were quantified by ImageJ software (B). The

values are presented as the mean and standard error of the mean (mean ± SEM). Different letters indicate statistically significant difference among the groups ($p < 0.05$). CON no treatment, LPS only LPS treatment

hand, compound K, a derivative of Rb1, was reported to activate the translocation of NF-κB into nucleus (Yang et al., 2017). These results supported by our data showing ginsenosides composition in which Rb1, Rb2, and Rc are abundant, while compound K was not detected in RGE. These results indicated that various ginsenosides can be responsible for the inhibition of NF-κB activation, and each ginsenoside, possibly, may differentially regulate NF-κB as seen in compound K and Rg3. Thus, NF-κB-targeted composition of red ginseng saponins would be established in further studies.

Effect of RGE on Nrf2-KEAP1 signaling

Nuclear factor-erythroid 2 p45-related factor 2 (Nrf2)/kelch-like ECH-associated protein 1 (KEAP1) signaling has originally been known to play a protective role against electrophilic or oxidative stresses in cells (Loboda et al., 2016). Recent years, this signaling has also been closely involved in inflammation (Zhong et al., 2013). In particular, hemeoxygenase-1 (HO-1), a target protein of Nrf2, has been shown to exert anti-inflammatory effect via the suppression of reactive oxygen species (ROS) production, which can trigger inflammatory responses in cells (Tafari et al., 2016). In this study, the effect of RGE on Nrf2-KEAP1 signaling was determined. LPS induced the downregulation of Nrf2 and HO-1 compared with normal

control; 50% and 62%, respectively (Fig. 4A, B). LPS-induced decrease of Nrf2 and HO-1 protein levels was effectively reversed by RGE. High dose of RGE (100 μg/mL) up-regulated the protein levels of Nrf2 and HO-1, by approximately 2.8-fold and 4.0-fold, respectively, compared with LPS-only group (Fig. 4B). In contrast, protein content of KEAP1, a negative regulator of Nrf2 nuclear translocation, showed a significant decrease in RGE-treated groups; each RGE groups (25, 50, and 100 μg/mL) reduced KEAP1 proteins by 1.6-fold, 2.4-fold, and 3.9-fold, respectively compared with LPS-only group (Fig. 4A, B). This result showed that RGE regulates Nrf2-KEAP1 signaling to activate HO-1 in human THP-1 macrophage cells. The activation of Nrf2's target gene, HO-1, is dependent on the Nrf2 translocation into nucleus (Bak et al., 2016; Li et al., 2010). In general, ROS or electrophiles has been known to stimulate Nrf2-KEAP1 signaling, and Nrf2 is dissociated with the complex with KEAP1 in cytoplasm, then, KEAP1 undergoes proteasomal ubiquitination to be degraded. The free Nrf2 moves into nucleus to bind to promoter region of antioxidant response genes to promote their gene expression (Loboda et al., 2016). Most Nrf2 protein was located to cytoplasm rather than nucleus in LPS-only group (Fig. 4C, D), indicating that LPS-induced inflammatory stimulation suppressed the translocation of Nrf2 into nucleus. LPS-induced inhibition of Nrf2 translocation into nucleus was greatly reversed by RGE

Fig. 4 Effect of RGE on Nrf2 signaling on THP-1 cells. Protein abundance of Nrf2, KEAP1, and HO-1 were analyzed by Western blot (A), and quantified by ImageJ software (B). Nuclear Nrf2 was examined by Western blot (C), and quantified by ImageJ software

(D). The values are presented as the mean and standard error of the mean (mean $\pm$ SEM). Different letters indicate statistically significant difference among the groups ($p < 0.05$). CON no treatment, LPS only LPS treatment

Fig. 5 Effect of RGE on inflammatory cytokines on THP-1 cells. mRNA expressions of the indicated cytokines were analyzed by real-time PCR (A). IL-6 and MCP-1 production was determined by ELISA (B). The values are presented as the mean and standard error of the

mean (mean $\pm$ SEM). Different letters indicate statistically significant difference among the groups ($p < 0.05$). CON no treatment, LPS only LPS treatment

treatment. The nuclear Nrf2 in the 100 µg/mL RGE group was hugely increased by showing a-47-fold elevated protein level than LPS-only group, whereas cytosolic Nrf2 protein abundance was 41% lower than the LPS group (Fig. 4C, D). These results indicated that RGE promotes translocation of Nrf2 into nucleus, and RGE-mediated increase of nuclear Nrf2 promoted the expression of HO-1. Several studies reported the relationship of ginseng and

Nrf2 signaling (Bak et al., 2016; Li et al., 2010). Li et al. showed that anti-oxidative stress of ginseng was mediated by Nrf2 activation in cardiomyocytes (Li et al., 2010). Since various ginsenosides, which were also contained in RGE, regulated Nrf2-HO-1 (Ning et al., 2018; Shi et al., 2018), the ginseng-mediated activation of Nrf2-KEAP1 system was considered to be due to the combined effect of various ginseng-derived ginsenosides. Therefore, red

ginseng-derived ginsenosides compositions targeting Nrf2-KEAP1 would also be established with NF- κ B signaling-targeted ginsenosides in the future.

Effect of RGE on inflammatory cytokines

For inflammatory cytokines, LPS treatment markedly induced the expression of pro-inflammatory cytokines (Fig. 5). This LPS-induced upregulation of pro-inflammatory cytokine mRNAs was effectively suppressed by RGE in a dose-dependent manner. High dose of RGE decreased the expression levels of the inflammatory cytokines, IL-1 β , IL-6, TNF- α , and MCP-1 mRNAs, by 99%, 99%, 71%, and 96% respectively (Fig. 5). This result showed that RGE can inhibit inflammatory response by downregulating pro-inflammatory cytokines. This data was also correlated to the RGE-mediated differential regulation on NF- κ B/Nrf2 translocation into nucleus. RGE-mediated activation and suppression of Nrf2 and NF- κ B, respectively, on the translocation into nucleus was shown to be interplayed for anti-inflammation (Wardyn et al., 2015). A recent study showed that the interaction of NF- κ B and its target genes for inflammatory responses was interrupted by the Nrf2 activation (Kang et al., 2018). In reality, p-65, a NF- κ B subunit, and Nrf2 were shown to compete for binding to transcriptional co-activator CREB-binding protein (CBP)-p300 complex (Sun et al., 2009). The formation of Nrf2-CBP complex was reported to be regulated by p-65 expression level; p-65 overexpression or downregulation decrease or increase, respectively, the complex formation (Sun et al., 2009). In addition, HO-1 enzymatic metabolites such as carbon monoxide and biliverdin were shown to inhibit NF- κ B or its target inflammatory molecules (Belcher et al., 2006). Collectively, RGE seemed to play an anti-inflammatory effect via crosstalk of NF- κ B and Nrf2 signalings. Detailed effects of RGE on crosstalk the Nrf2 and NF- κ B requires further study.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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