Sci. Bull. (2015) 60(8):773–784
DOI 10.1007/s11434-015-0773-4

www.scibull.com
www.springer.com/scp

Article

Life & Medical Sciences

Anti-inflammatory activity of ginsenosides in LPS-stimulated RAW 264.7 cells

Sungeun Ahn · Muhammad Hanif Siddiqi · Hae-Yong Noh · Yu-Jin Kim · Yeon-Ju Kim · Chi-Gyu Jin · Deok-Chun Yang

Received: 10 November 2014 / Accepted: 11 February 2015 / Published online: 8 April 2015

Abstract Ginsenosides, the main active constituents of *Panax ginseng* Meyer (*P. ginseng*), have potential therapeutic effects. All tested ginsenosides except ginsenoside F1 have previously been reported in inflammation studies using the RAW 264.7 macrophage cell line. We examined the anti-inflammatory effects of single sugar moiety ginsenosides such as compound K (CK), Rh2, Rh1, and F1 that were isolated from *P. ginseng* through in silico docking studies. We investigated their biological activity predictions, including absorption, distribution, metabolism, excretion, and toxicity and PASS properties, on the suppression of NF-κB, followed by in vitro validation in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells. The molecular docking results of our study showed that all treated ginsenosides are non-toxic and may be drug-like molecules. The molecular binding interactions of these ginsenosides with the active residues of NF-κB noticeably support their anti-inflammatory activity. CK and Rh1 significantly reduced the production of nitric oxide, cyclooxygenase-2 (COX-2), and pro-inflammatory cytokines such as prostaglandin E2 and tumor necrosis factor alpha (TNF-α) in a dose-dependent manner. Real-time PCR and Western blot analyses further confirmed that protopanaxadiols (PPDs) and protopanaxatriols (PPTs) inhibitory effects may have been due to the down-regulation of TNF-α, inducible nitric oxide synthase, COX2, nuclear factor kappa B (NF-κB), and I kappa B kinase. The expression of co-stimulatory molecules such as ROS was also inhibited by CK and Rh1 in a dose-dependent manner. Furthermore, activation of NF-κB in LPS-stimulated RAW 264.7 macrophages was significantly suppressed by CK and Rh1. Taken together, these results provide evidence that PPD- and PPT-type ginsenosides including CK and Rh1 may exhibit strong anti-inflammatory effects by inhibiting pro-inflammatory mediators through down-regulation of NF-κB.

Keywords Ginsenosides · Protopanaxadiol · Protopanaxatriol · Inflammation · NF-κB/IKK · RAW 264.7

1 Introduction

Inflammation is a complex biological process involving the response of several vascular tissues to injurious stimuli, such as damaged cells, irritants, and pathogens. Inflammation plays a critical role in various human diseases, including cancer, neurological disorders, metabolic syndrome, cardiovascular disease, inflammatory bowel disease, arthritis, and infectious diseases [1–3]. In the pathogenesis of inflammation, the overexpression of different cytokines such as COX-2, inducible nitric oxide synthase (iNOS), PGE2, and TNF-α produced by activated macrophages plays an important role in the induction of acute and chronic inflammatory diseases [4, 5]. Lipopolysaccharide (LPS), a well-known component of the cell wall of gram-negative bacteria, stimulates a number of signaling pathways, including NF-κB/IKK signaling in macrophages [6]. NF-κB plays a significant role in the pathogenesis and regulation of inflammatory responses [7].

Electronic supplementary material The online version of this article (doi:10.1007/s11434-015-0773-4) contains supplementary material, which is available to authorized users.

S. Ahn · M. H. Siddiqi · H.-Y. Noh · Y.-J. Kim · Y.-J. Kim · C.-G. Jin · D.-C. Yang (✉)
Ginseng Genetic Bank, Graduate School of Biotechnology, College of Life Science, Kyung Hee University, Suwon 449-701, Korea
e-mail: dcyang@khu.ac.kr

During normal conditions, NF-κB dimers (p50 and p65 subunits) exist in the cytoplasm, complexed with the inhibitor IκB. After cell activation, specific kinases phosphorylate IκB, leading to its degradation and the release of NF-κB from IκB. This allows translocation of NF-κB into the nucleus, where it binds to specific promoter regions of target genes and induces different transcription factors for inflammatory disease, such as iNOS and COX-2 [8, 9]. Therefore, NF-κB is considered to play a vital role in the up-regulation of several inflammatory mediators such as pro-inflammatory cytokines, including interleukin-1 beta (IL-1β) and TNF-α [10]. Thus, agents that restrain the activation of the IKK/NF-κB complex could potentially be used to manage inflammatory disorders [11, 12]. As NF-κB activation is commonly involved in the pathogenesis of inflammatory diseases, it is a common target in the development of anti-inflammatory drugs. Many therapeutic agents have been studied for anti-inflammatory activity, but are associated with diverse side effects. Thus, the discovery of anti-inflammatory therapeutic agents that down-regulate NF-κB and pro-inflammatory cytokines with few side effects could be very beneficial.

Korean ginseng (*Panax ginseng* Meyer) is the most common traditional medicinal plant and has been used for the treatment of several diseases for over 2000 years. The active components of ginseng are collectively known as ginsenosides. On the basis of structure, these ginsenosides are divided into two main groups, the protopanaxadiols (PPD) and the protopanaxatriols (PPT). These ginsenosides have several pharmacological and biological effects, including anticancer, anti-diabetic, anti-osteoporosis, and anti-inflammatory activities [13]. However, the combined comparison of PPD- and PPT-type ginsenosides with single sugar moiety from *P. ginseng* in inflammatory diseases is unknown. We aimed to identify effective ginsenoside(s) that can play a preventive and protective role against inflammatory disorders, with the goal of developing a ginsenoside-containing anti-inflammatory diet. NF-κB inhibitory activity became our key focus for preliminary screening. As a part of our ongoing screening project of ginsenosides to assess anti-inflammatory properties, we selected single sugar molecules from *P. ginseng* such as Rh2, CK (PPD), Rh1, and F1 (PPT; Fig. 1) and studied their anti-inflammatory effects by using an in silico docking study followed by in vitro validation in LPS-stimulated RAW 264.7 cells.

2 Materials and methods

2.1 Computational study

2.1.1 Selection of compounds/ligands preparation

The set of tested ginsenosides were collected from *P. ginseng* plants based on pharmacological activity, mainly anti-inflammatory behavior. The list of PPD- and PPT-type ginsenosides includes Rh2, CK, Rh1, and F1. The structures of the given ginsenosides were collected from our own in-house database. The two-dimensional structure (2D) of these ginsenosides was drawn with the ChemSketch program (http://

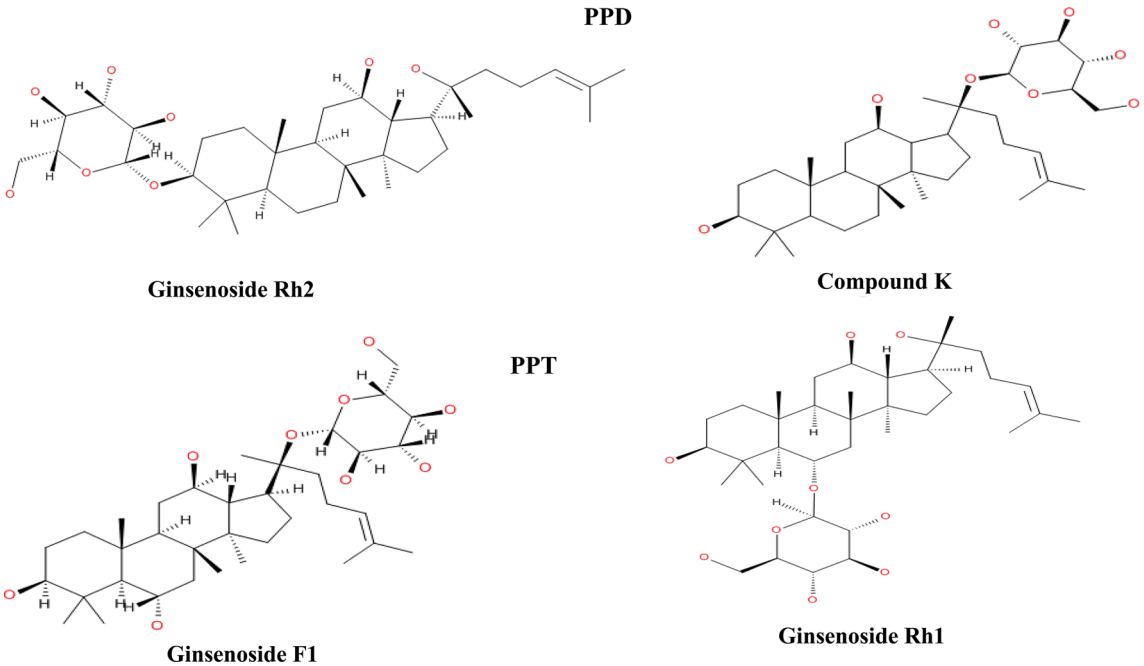

Fig. 1 Structures of PPD- and PPT-type ginseng saponin

www.acdlabs.com; Advanced Chemistry Development, Inc. Toronto, ON, Canada). The drawn 2D structure is in the .mol format, and the three-dimensional (3D) structure is in the .pdb format and was drawn through Discovery Studio 3.5 visualizer (DS 3.5) (DS, http://www.accelrys.com; Accelrys, Inc., SanDiego, CA, USA). The standard drugs indomethacin and dexamethasone, which act on the NF-κB/IKK signaling pathway in inflammatory diseases, were used as control ligands [14]. All compounds were energy-minimized using the PyRx program [15].

2.1.2 NF-κB preparation and molecular docking interaction studies

The 3D structure of NF-κB was obtained from the protein data bank (PDB ID: 1NFK) with a resolution of 3.0 Å. Water molecules, ligands, and other hetero atoms were removed, and hydrogen atoms were added [16, 17]. Energy minimization was performed by using the 200 steps of steepest descent [18], followed by the conjugate gradient method on the PyRx program [15, 19].

2.1.3 Pharmacokinetic (ADMET) properties prediction

Predicting pharmacokinetic properties based on chemical structure is an important task in drug design. Therefore, the ADMET properties were evaluated for the selected ginsenosides from a molecular docking simulation using Qikprop software version 3.0 through the Schrödinger program (http://www.schrodinger.com). The collected ginsenosides PPD and PPT were introduced into Maestro GUI wizard and neutralized before calculating properties. This is an essential step, because neutralized ginsenosides CK/Rh2 (PPD) and Rh1/F1 (PPT) can individually produce both pharmacokinetically and physicochemically relevant properties. Similarly, Qikprop also gives a range for comparing a particular molecule's activities with those of 95 % of recognized drugs. Hepatotoxicity was estimated using the ADMET element accessible from the DS 3.5 program. ADMET values such as aqueous solubility, serum protein binding, logP for octanol/water, blood brain barrier (BBB) level, hepatotoxicity, CYP2D6 inhibition probability, and human oral absorption in the GI system (%) were computed for all ginsenosides.

2.1.4 In silico biological activity prediction

We determined the kind of pharmacological and biological properties present in the selected PPD- and PPT-type ginsenosides based on their chemical structure formula by using PASS (prediction of activity spectra for substances) (http://www.pharmaexpert.ru/passonline/). This online computational technique was employed to predict different biological activities of the tested ginsenosides. This method produced a list of biological activities along with the values for the probability of active (*Pa*) and probability of inactive (*Pi*) states. The accuracy of biological activity was carefully measured based on *Pa* values >0.7, while a $Pa < 0.7$ suggested a smaller chance of biological activity.

2.2 In vitro study

2.2.1 Chemicals and reagents

All selected PPD (CK, Rh2)- and PPT (Rh1, F1)-type ginsenosides were kindly provided by the Ginseng Genetic Bank of Kyung Hee University. RPMI-1640 cell culture medium, phosphate-buffered saline (PBS), fetal bovine serum (FBS), and penicillin–streptomycin (P/S) were from WelGENE Inc. (Daegu, Korea). LPS, sodium nitrite ($NaNO_2$) solution, NG-monomethyl-L-arginine (L-NMMA), dexamethasone, and Griess reagent were obtained from Sigma–Aldrich Co., St. Louis, MO, USA. Primary antibodies for NF-κB and IKK and secondary antibody were from Santa Cruz, CA, USA. Other materials used were commercial products of the highest grade available.

2.2.2 Cell culture

Murine macrophage cell lines (RAW 264.7) were obtained from the Korean Cell Line Bank (Seoul, South Korea), and passages 5–10 were seeded at a density of 5×10^3 cells/well in a 96-well microplate in RPMI-1640 medium containing 10 % (V/V) fetal bovine serum (FBS) and 1 % (V/V) P/S. Cells were incubated at 37 °C in a humidified atmosphere containing 5 % CO_2 and 95 % air for 24 h.

2.2.3 Cell viability assay

To determine the toxic effects of the treated PPD- and PPT-type ginsenosides on cell growth and viability, cells were treated with various concentrations of PPD- and PPT-type ginsenosides (0.01–200 μmol/L) for 72 h. After 3 days of treatment, cell viability was measured with an MTT assay. 10 μL MTT solution (5 mg/mL) was added to each well, and the plates were incubated for 3–4 h; then, the formazan was dissolved in DMSO, and the absorbance of each well was determined by absorbance at 570 nm on a ELISA reader (Bio-Tek Instruments, Inc., Vinooski, VT, USA).

2.2.4 Measurement of nitrite levels

To determine levels of nitrite in the cultured cells, RAW 264.7 cells were seeded as mentioned above and pretreated with 1 μg/mL LPS with various concentrations of PPD and PPT ginsenosides (0.01–10 and 0.1–100 μmol/L,

respectively) or L-NMMA for 48 h. We used Griess reagents to determine nitrite levels in the media. Briefly, an equal volume (100 μL) of supernatant was mixed with Griess reagent, and absorbance was measured at 540 nm against a standard sodium nitrite curve using a microplate reader (Bio-Tek Instruments, Inc., Vinooski, VT, USA).

2.2.5 Measurement of intracellular reactive oxygen species (ROS)

To determine the level of intracellular ROS, RAW 264.7 cells were plated in 24-well plates at a density of 5×10^3 cell per well, supplemented with RPMI-1640, 10 % (V/V), FBS and 1 % (V/V) P/S, and incubated for 48 h at 37 °C in a humidified incubator (5 % CO_2 and 95 % air). After 48 h, the cells were stimulated with 1 μg/mL of LPS in the presence or absence of PPD and PPT ginsenosides incubated for 48 h at 37 °C. After 48 h, the medium was removed, and cells were washed with PBS and stained with 10 μmol/L DCF-DA [20] for 30 min in dark conditions. ROS generation was determined by fluorescence using a microplate reader (Bio-Tek Instruments, Inc., Vinooski, VT, USA) at 485-nm excitation and 535-nm emission.

2.2.6 Measurement of PGE2 and TNF-α

To measure PGE2 and TNF-α synthesis, RAW 264.7 cells were cultured as they were for the ROS assay. PGE2 and TNF-α production was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Quantikine/Mouse; R&D Systems) (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm, with subtraction measured at 540 nm by ELISA reader (Bio-Tek Instruments, Inc., Vinooski, VT, USA). Concentrations of PGE2 and TNF-α concentration in each well were then calculated from their respective standard curves.

2.2.7 Reverse transcription polymerase chain reaction (RT-PCR)

Macrophage RAW 264.7 cells were stimulated with LPS (1 μg/mL) in the presence or absence of PPD and PPT. Total RNA was isolated by harvesting the whole-cell lysate using Trizol LS reagents (Invitrogen, Carlsbad, CA, USA) according to the protocol supplied by the manufacturer. Total RNA (2 μg) was used to synthesize first-stranded cDNA using the Thermo Scientific cDNA synthesis kit (Onebio, Lithuania, EU) according to the manufacturer's instructions. For amplification of β-actin, TNF-α, iNOS, and COX-2, the following primers were used: β-actin, 5′ACTCTTCC AGCCTTCCCTCC3′ (forward) and 5′CGTACAGGTC TTTGCGGATG3′ (reverse); TNF-α, 5′AGGGGAAATGA GAGACGCAA3′ (forward), 5′TTCCCCATCTCTTGCCA CAT3′ (reverse); iNOS, 5′ACCCAAGGTCTACGTTCA GG3′ (forward), 5′CGCACATCTCCGCAAATGTA3′ (reverse); and COX-2, 5′CCTGAGCATCTACGGTTTGC3′ (forward), 5′ACTGCTCATCACCCCATTCA3′ (reverse). For PCR amplification, the following conditions were used: 94 °C for 5 min for one cycle and then 94 °C for 1 min, 56 °C for 30 s, and 72 °C for 1 min for 30 cycles. The amplified PCR products were subjected to 0.8 % agarose gel electrophoresis at 100 V for 25 min. The relative gene expression levels as compared with the endogenous reference β-actin were calculated using the delta cycle threshold (*C*t) method [21].

2.2.8 Western blot analysis

RAW 264.7 cells were seeded at a density of 5×10^3 cells/well in a 6-well plate. After 24 h of incubation, the cells were stimulated with LPS (1 μg/mL) with or without various concentrations of PPD or PPT (0.01–10 or 0.1–100 μmol/L, respectively) for 48 h. The 5 μmol/L dexamethasone was used as an anti-inflammatory positive control. After the indicated period of incubation, the cells were washed three times with PBS and proteins were extracted from cells in ice-cold PRO-PREPTM protein extraction solution (iNtRON Biotechnology, Seongnam-Si, Korea) according to the provided protocol. Then, the whole-cell lysate was transferred to a micro-centrifuge tube and centrifuged at 15,000 *g* at 4 °C for 20 min. The protein concentration of the resulting supernatant was assessed with the Bio-Rad protein assay kit according to the given instructions. Cellular proteins were then boiled for 3–5 min, and 30 mg of total protein per lane was separated by 8 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (125 V, 120 min). The proteins in the gel were transferred onto a nitrocellulose membrane (ATTO, Japan, Clear Blot Membrane-p, AE-6667), and after 1.5-h incubation, the membrane was blocked with 5 % skim milk and probed overnight with a 1:1,000 dilution of anti-NF-κB or a 1:2,000 dilution of rabbit poly-clonal anti-IKK. After five washes (5 min/wash) with TPBS, the membranes were incubated with the goat anti-mouse horseradish peroxidase-conjugated secondary antibodies (1:10,000 dilution) for 2 h at room temperature (RT), and the blot was detected using an enhanced chemiluminescence system (Amersham Biosciences Inc., Piscataway, NJ) and exposed to X-ray film (Fuji Photo Film Co. Ltd).

2.2.9 Statistical analysis

The results are representative of at least four independent experiments and are expressed as the mean ± SE.

Comparison of the control and treatment groups was performed using ANOVA variance analysis, and the statistical significance was analyzed by Duncan's test. Differences of $*P < 0.05$ were considered statistically significant.

3 Results

3.1 ADMET properties of tested ginsenosides

All selected PPD- and PPT-type ginsenosides from *P. ginseng* were first evaluated for drug-like behavior based on their chemical descriptors. The pharmaceutically relevant properties of the prepared ginsenosides Rh2, CK, Rh1, and F1 were generated using the Qikprop module accessible from the Schrödinger program (http://www.schrodinger.com). We tested the blood brain barrier (BBB) level (QPlogBB: standard range −3.0 to 1.2) to determine the ability of the molecule to pass through this barrier. Other parameters such as molecular weight, human oral absorption in the gastrointestinal tract (QP (%): standard range <25 % is poor and >80 % is high), serum protein binding (QPlogKhsa: standard range −1.5 to 1.5), CYP2D6 inhibition probability (standard 0 is non-inhibitor and 1 is inhibitor), and octanol/water partition coefficient (QPlogPo/w: acceptable range −0.2 to 6.5) were examined for selected ginsenosides. As toxicity prediction is a vital task in natural compound research [22], we predicted hepatotoxicity properties (0 is non-toxic and 1 is toxic) for tested ginsenosides using the ADMET module available from DS 3.5. The detailed pharmacokinetic (ADMET) values from the tested ginsenosides are shown in Table S1. These results clearly demonstrate that ginsenosides Rh2, CK, Rh1, and F1 act as bioactive compounds that possess anti-inflammatory activity and might be potential therapeutic agents for inflammatory disease.

3.2 PASS prediction

PASS, which calculates the biological property spectrum based on the chemical structure formula of a molecule, showed significant biological targets for the selected PPD- and PPT-type ginsenosides. This program was applied to the multilevel neighborhoods of atoms (MNA) properties and was used to estimate the targets from the original chemical structure formula. The biological target active molecules can be identified based on values of *Pa* close to 1 and values of *Pi* close to 0 [23]. The analysis of ginsenosides showed probable biological activity with the anti-inflammatory, immunostimulant, and transcription factor NF kappa B inhibitor. The predicted targets for the selected PPD- and PPT-type ginsenosides along with *Pa* and *Pi* values are described in Table S2.

3.3 Molecular docking result

The molecular docking program is mostly used for the precise calculation of protein–ligand interactions at the molecular level. This was carried out for NF-κB with selected PPD- and PPT-type ginsenosides along with indomethacin and dexamethasone as the control drugs for validation in AutoDock. Specific active site residues were kept flexible, along with the ligand. The molecular docking interaction results for each ginsenoside are listed in Table S3. The given ginsenosides were screened on the basis of their binding energy as well as the hydrogen bonding ability with active site residues of NF-κB. PPD-type ginsenosides (CK and Rh2) interact with the NF-κB residues Tyr57, Lys144, Cys59, and Lys145, with a binding affinity of −7.58 and −7.89 kcal/mol, respectively (Fig. 2a, b). The probable binding orientation of the NF-κB vital active site Tyr57 residue with CK and Rh2 involved one hydrogen bond between the Tyr57 O group and the OH group of CK, and between the Cys59 O group and the OH group of Rh2. Similarly, PPT-type ginsenosides (F1 and Rh1) interact with the Thr143, Lys145, and Lys146 residues of NF-κB with a binding affinity of −8.3 and −7.92 kcal/mol, respectively (Fig. 2c, d). Interestingly, all these hydrogen bond interactions were observed in the single sugar molecules of the selected ginsenosides. However, interactions between indomethacin and dexamethasone (control drugs) with NF-κB showed less binding energy (−6.84 and −6.6 kcal/mol, respectively (Fig. 2e, f)). Our results showed that among the PPD- and PPT-type ginsenosides, CK and Rh1 are involved in two and four hydrogen bond interactions with NF-κB active site residues with strong binding energy (−8.3 and −7.58 kcal/mol).

3.4 Effect of ginsenosides on macrophage toxicity

Prior to evaluating the anti-inflammatory effect of these single sugar molecules in PPD (Rh2, CK)- and PPT (Rh1, F1)-type ginsenosides, we investigated their cytotoxic effect on cell growth and viability in RAW 264.7 macrophage cells with an MTT assay. The tested ginsenosides did not affect normal cell growth at concentrations up to 10 μmol/L (PPD) and 100 μmol/L (PPT) (Fig. 3a, b). However, at 100 and 200 μmol/L, both PPD-type compounds showed a pronounced cytotoxic effect on cell viability, compared to PPT; based on these results, the rest of our experiments were carried out at concentrations of 0.01–10 μmol/L PPD (Rh2, CK) and 0.1–100 μmol/L PPT (Rh1, F1).

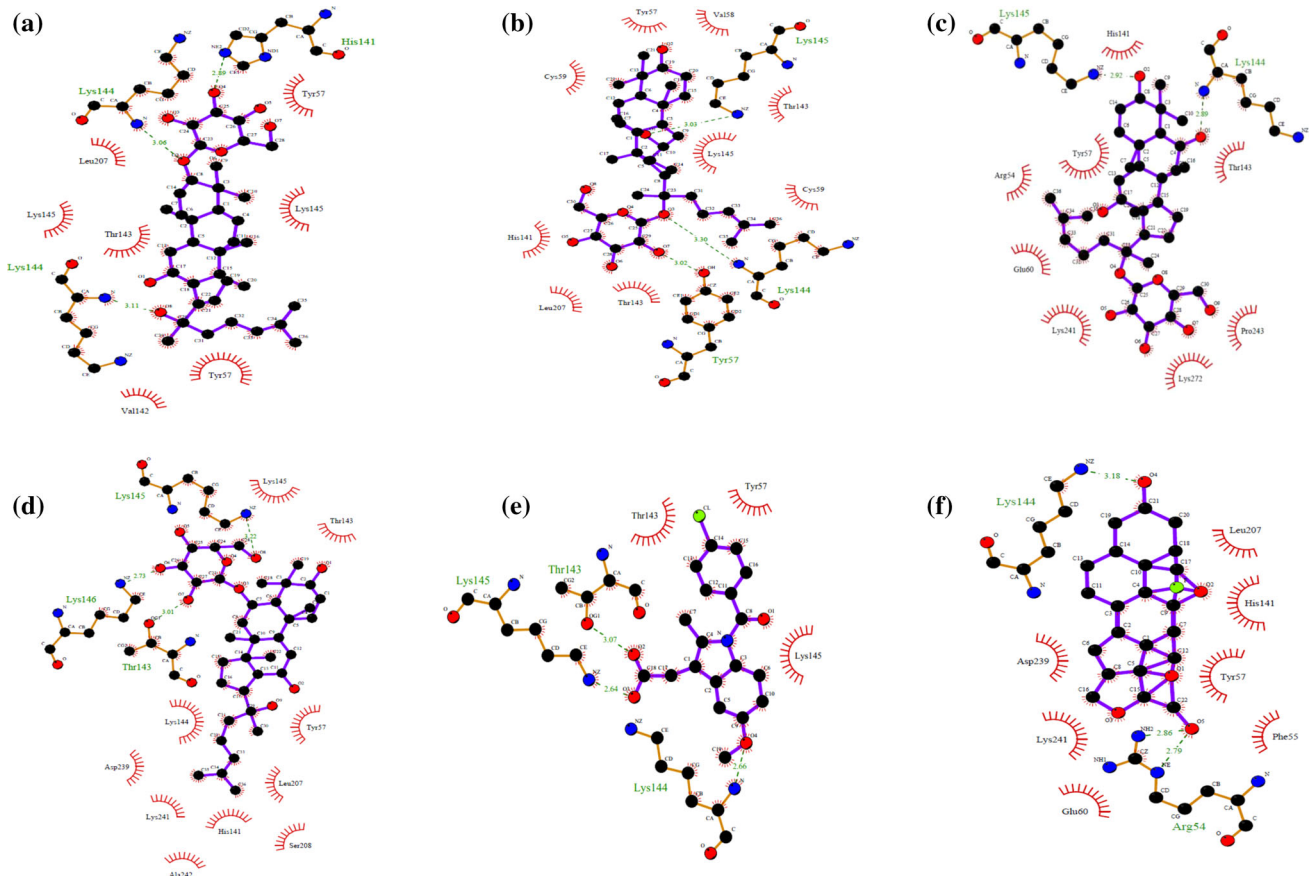

Fig. 2 LIGPLOT of the NF-κB complexed with **a** CK, **b** Rh2, **c** F1, **d** Rh1, **e** indomethacin, **f** dexamethasone (H bond in green)

3.5 Inhibition of LPS-induced NO production by PPT- and PPD-type ginsenosides

Nitric oxide (NO) is one of the most important pro-inflammatory mediators. Thus, inhibition of the over-production of this agent is an attractive target in drug discovery for inflammatory disease. We compared the anti-inflammatory effects of the selected ginsenosides on the inhibition of NO production in LPS-stimulated RAW 264.7 cells. In this study, L-NMMA was used as a positive control for suppression of NOS synthesis. Treatment of RAW 264.7 cells with LPS induced NO production; in contrast, the production of LPS-induced NO was considerably decreased by both PPD and PPT in a dose-dependent manner (Fig. 3c, d). Both PPD- and PPT-type compounds exhibited strong, dose-dependent inhibition of LPS-induced NO production; however, analysis of our data strongly suggested that CK and Rh1 showed more significant reductions in LPS-induced NO production at 0.1 μmol/L (82.63 % and 76 %, respectively). LPS can induce iNOS production [11], so we determined the mRNA expression of iNOS in LPS-stimulated RAW 264.7 cells. Inhibition by both PPD- and PPT-type compounds was similar. As shown in Fig. 6c, d, the ginsenosides Rh1 and CK significantly suppressed LPS-induced mRNA expression of iNOS, which was similar to their inhibition of NO production in RAW 264.7 cells. This suggests that Rh1 and CK could have the same mechanism of action on mRNA expression of iNOS in RAW 264.7 cells.

3.6 The inhibitory effects of PPD- and PPT-type ginsenosides on LPS-induced ROS production in RAW 264.7 cells

To investigate the inhibitory effects of PPD- and PPT-type ginsenosides on LPS-induced ROS generation, RAW 264.7 macrophage cells were treated with various concentrations of tested ginsenosides in the presence or absence of LPS for 48 h, and the amount of ROS production in the culture supernatant was examined by the DCF-DA method. Treatment of RAW 264.7 cells with LPS promptly augmented intracellular ROS production, as determined by using DCF-DA, which was effectively attenuated by treatment with both PPD- and PPT-type ginsenosides, especially by Rh1 and CK (Fig. 4).

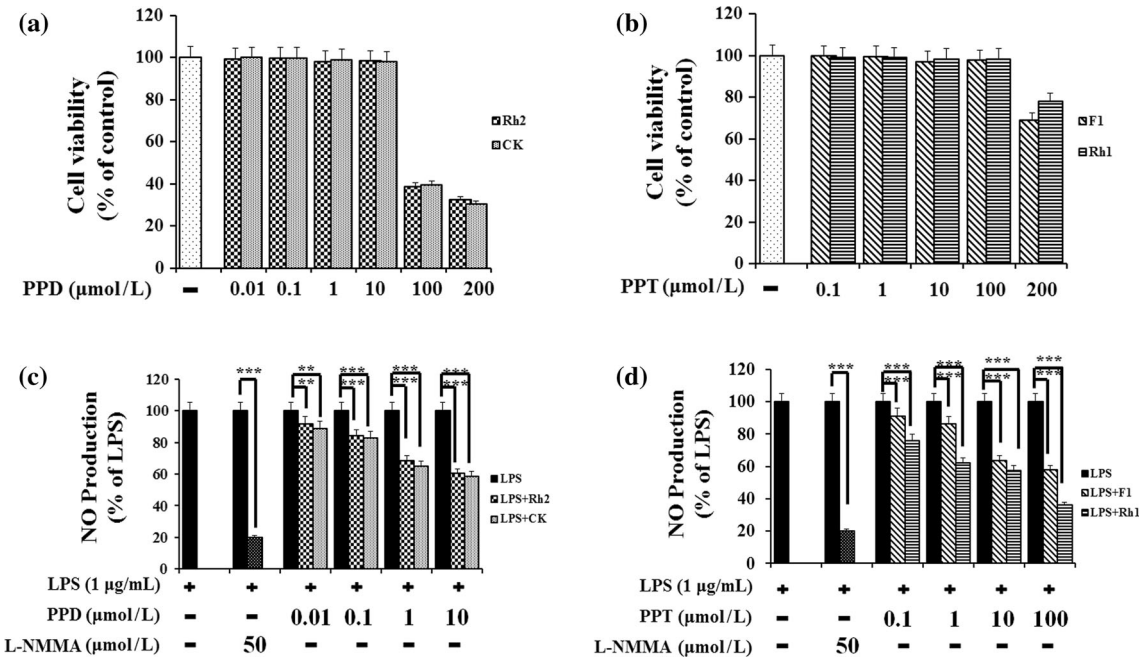

Fig. 3 Effects of PPD- and PPT-type ginsenosides on cell viability (**a**, **b**) and NO (**c**, **d**), respectively. In the cell viability assay, RAW 264.7 cells were incubated for 72 h with various concentrations of PPD (0.01–200 μmol/L) and PPT (0.1–200 μmol/L). Cell viability was determined, as described in the materials and methods. In case of the nitrite production assay, the cells were pretreated with indicated concentrations of PPD and PPT for 1 h and then stimulated with LPS (1 μg/mL) for 48 h. The concentration of nitrite was determined as described in the experimental. Data shown represent the mean values of three experiments ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to the group treated with LPS alone

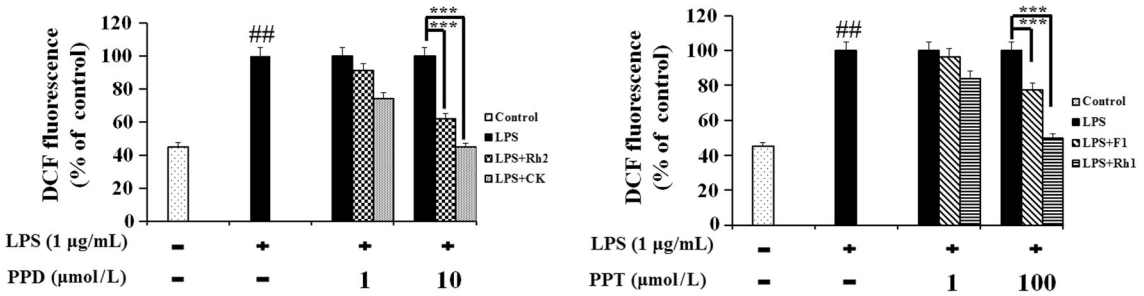

Fig. 4 Effects of PPD- and PPT-type ginsenosides on the ROS production in RAW 264.7 macrophages. Cells were pretreated with PPD and PPT for 1 h and then stimulated with LPS (1 μg/mL) for 48 h. The absorbance of ROS was measured as described in the materials and methods. Data shown represent the mean values of three experiments ± SD. $^{\#\#}P < 0.01$ when compared with control versus LPS; $^{***}P < 0.001$ as compared to the group treated with LPS alone

3.7 PPD- and PPT-type ginsenosides suppress production of PGE2 and TNF-α in LPS-stimulated macrophage RAW 264.7 cells

The effects of CK, Rh2 (PPD) and Rh1, F1 (PPT) on the levels of PGE2 and TNF-α in the culture supernatant of RAW 264.7 cells were measured after 48-h treatment with 1 μg/mL LPS with or without PPD- and PPT-type ginsenosides. PGE2 and TNF-α production by both PPD- and PPT-type ginsenosides was evaluated with dose ranges of 0.1–10 and 0.1–100 μmol/L, respectively, through enzyme immunoassay (ELISA). LPS treatment (1 μg/mL) resulted in a significant activation of PGE2 and TNF-α production compared with untreated cells (Fig. 5a–d). Moreover, pretreatment with PPD and PPT prominently inhibited LPS-induced PGE2 and TNF-α production. L-NMMA was used as a positive control for inhibition of PGE2 and TNF-α production (Fig. 5a–d).

3.8 Effects of PPD- and PPT-type ginsenosides on regulation of inflammatory genes in LPS-stimulated RAW 264.7 cells

We also measured the effects of Rh1, F1, CK, and Rh2 on mRNA expression levels of TNF-α, iNOS, and COX-2 in RAW 264.7 cells. As shown in Fig. 6a–f, mRNA

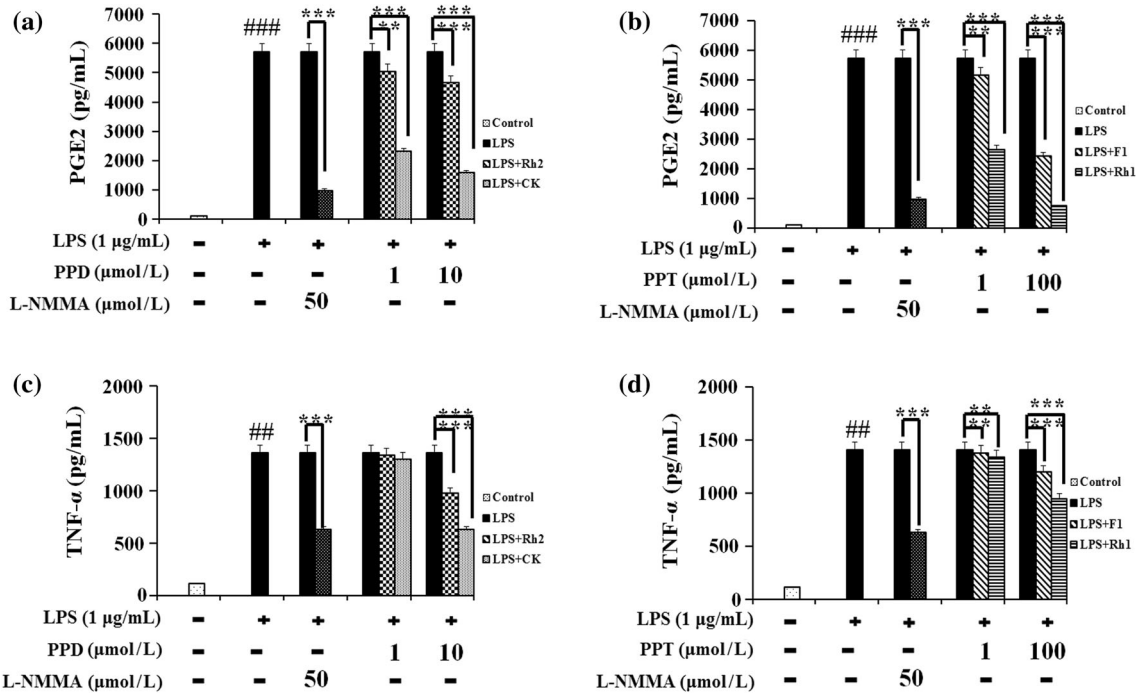

Fig. 5 Effects of PPD- and PPT-type ginsenosides on the production of PGE2 and TNF-α. RAW 264.7 cells were pretreated with PPD and PPT for 1 h and then stimulated with LPS (1 μg/mL) for 48 h. The concentrations of PGE2 (**a**, **b**) and TNF-α (**c**, **d**) were determined as described in the materials and methods. Data shown represent the mean values of three independent experiments ±SD. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with control LPS; $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to the group treated with LPS alone

expression of TNF-α, iNOS, and COX-2 was markedly up-regulated in RAW 264.7 cells stimulated with LPS, while treatment with both PPD- and PPT-type ginsenosides dramatically inhibited the overproduction of TNF-α, iNOS, and COX-2 mRNA. CK and Rh1 significantly decreased the LPS-induced mRNA increase in inflammatory genes. However, LPS as well as our tested compounds did not affect mRNA expression of the housekeeping gene β-actin (Fig. 6a–f).

3.9 PPD- and PPT-type ginsenosides negatively regulate the NF-κB activation by inhibition of IKK complex activation

The NF-κB and IKK signaling complex is a key regulator of inflammatory responses induced by LPS and pro-inflammatory cytokines [24]. IL-1β, iNOS, COX-2, and NO expression is regulated mainly at the transcriptional level through NF-κB/IKK signaling [25, 26]. Thus, we measured the effects of the PPD- and PPT-type ginsenosides on the transcriptional regulation of NF-κB induced by LPS using RAW 264.7 cells through immunoblotting analysis. As shown in Fig. 7, the protein levels of NF-κB and IKK were undetectable in RAW 264.7 cells without LPS stimulation. Stimulation with LPS alone markedly increased the translocation of NF-κB to the nucleus in macrophages. CK and Rh1 significantly down-regulated LPS-induced NF-κB translocation in a dose-dependent manner (Fig. 7). NF-κB remains basally inactive in the cytosol due to the interaction with IκB; in response to inflammatory inducers such as LPS, IκB is phosphorylated and degraded, allowing translocation of NF-κB to the nucleus [10]. Thus, we studied the inhibitory effects of PPD- and PPT-type ginsenosides on the stimulation and subsequent degradation of IκB. When the RAW 264.7 cells were treated with LPS, IκB phosphorylation was markedly increased, while CK and Rh1 significantly decreased IKK phosphorylation at 10 and 100 μmol/L (Fig. 7).

4 Discussion

Inflammation is one of the important biological defense mechanisms of the human body in response to injurious external stimuli such as chemical toxins and microbial infections. The inflammatory process is characterized by regulation of numerous pro-inflammatory cytokines as well as activation of different signaling pathways, including the NF-κB/IKK pathway [25, 27]. Leukocytes abolish injurious microorganisms and dead cells, inhibiting the spread of the annoyance (irritation) and allowing for repair of the damaged tissue. However, constant and excessive

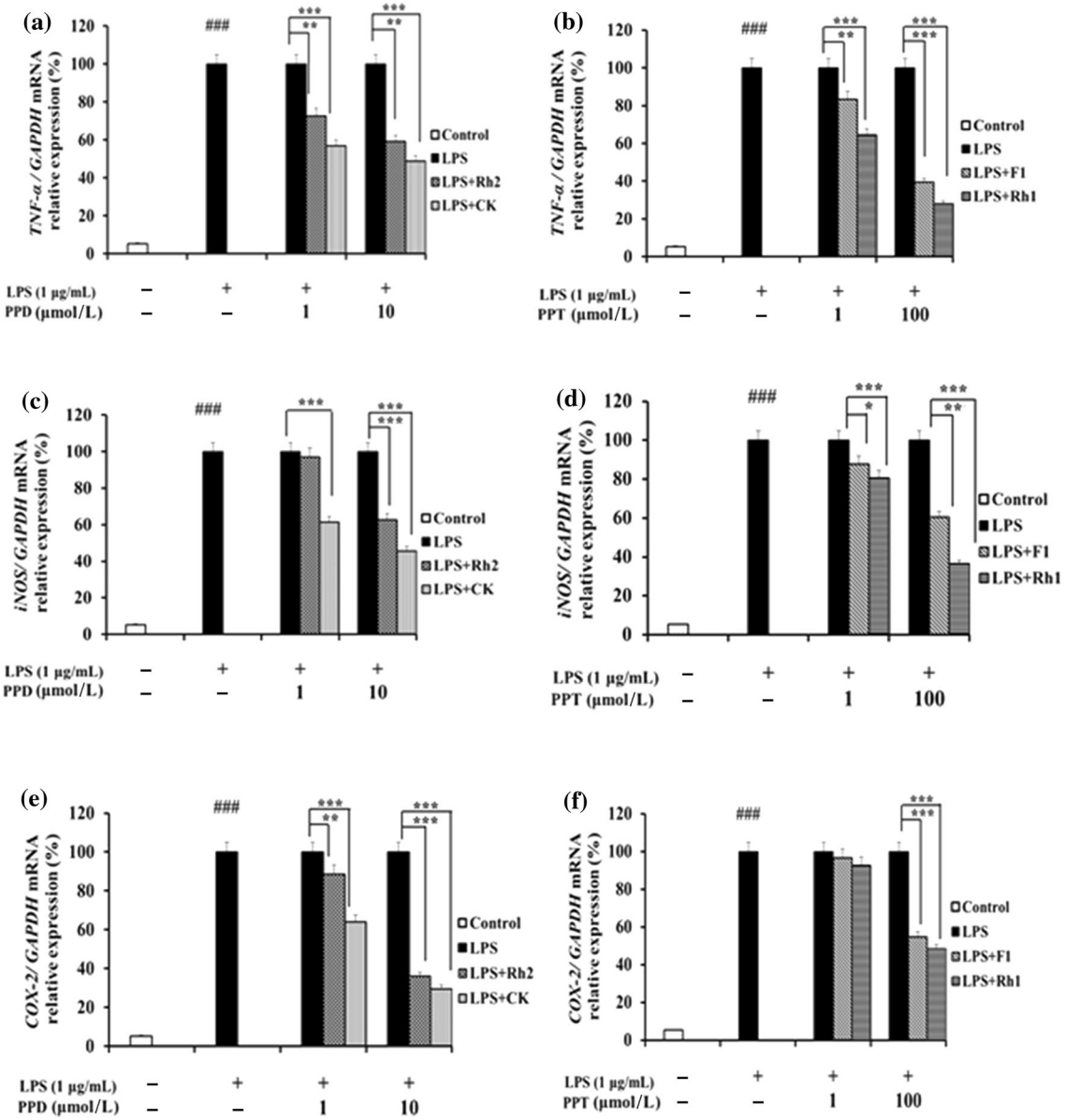

Fig. 6 Effects of PPD- and PPT-type ginsenosides on the expression of *TNF-α,* iNOS and *COX-2* mRNA expression level. RAW 264.7 cells were pretreated with the indicated concentrations of PPD and PPT for 1 h and then stimulated with LPS (1 μg/mL) for 48 h. Subsequently, total RNAs were extracted, and the mRNA expression levels *TNF-α*, *iNOS*, and *COX-2* were determined by RT-PCR analysis and compared with those of β-actin. The data shown are representative of mean values of three independent experiments ± SD. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ when compared with control versus LPS; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ as compared to the group treated with LPS alone

inflammation initiates a variety of pathological disorders. Several inflammatory mediators including nitric oxide and pro-inflammatory cytokines are crucially involved in the initiation of inflammatory diseases. Macrophages are the essential element of the human immune system. During inflammation, macrophages generate pro-inflammatory cytokines such as PGE2 and TNF-α, in addition to other important inflammatory mediators, such as iNOS and COX-2, to defend the body from tissue damage or infection [28]. These pro-inflammatory agents play a significant role in the pathogenesis of acute and chronic inflammatory disorders through the NF-κB/IKK signaling pathway. Many studies have proved that COX-2 and iNOS cytokines are greatly up-regulated during inflammation. The pathological condition of inflammation is activated by a complex mechanism that is caused by microbial antigens, such as LPS [29]. LPS directly activates macrophages, which release inflammatory agents such as PGE2, NO, TNF-α, ILs, and leukotrienes [30]. *Panax ginseng* (family Araliaceae), normally known as Korean ginseng, is one of the most

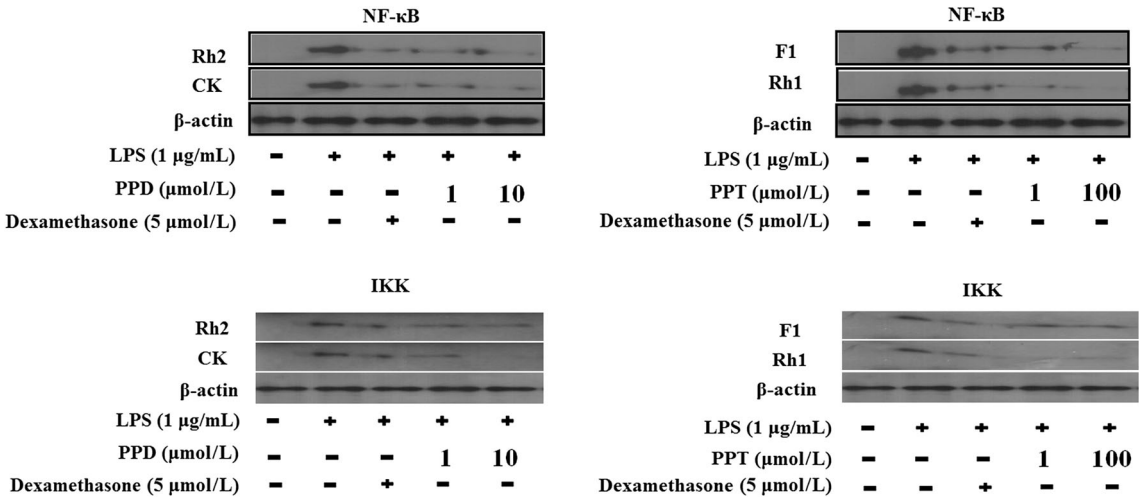

Fig. 7 Effects of PPD- and PPT-type ginsenosides on LPS-induced NF-κB and IKK in RAW 264.7 cells cultures stimulated with LPS (1 μg/mL) in the absence or presence of PPD- and PPT-type ginsenosides. Whole-cell lysates were subjected to Western blot analysis using antibodies against NF-κB and IKK and compared with those of β-actin

important medicinal plants. Its active compounds, such as ginsenosides, have several pharmacological beneficial effects. Poor oral absorption is a major drawback in the clinical use of high molecular weight ginsenosides, which limits their therapeutic usage. Most ginsenosides are orally administered, with their mechanisms naturally interacting with intestinal microflora in the gastrointestinal (GI) tract. These ginsenosides might be metabolized before they are absorbed from the GI tract. Various studies suggested that different low-polarity ginsenosides such as Rg3(S), Rg5/Rk1, and Rh3 contain different pharmacological and biological activities including anti-dermatitis, memory-enhancing, and neuroprotective activities [31, 32]. Similarly, Tawab et al. suggested that after the oral administration of ginseng extract to humans, different PPD- and PPT-type ginsenosides such as CK, F1, and Rh1 were noticed in the blood and urine [33].

So far, few ginsenosides (high and lower molecular weight) have been evaluated for their anti-inflammatory effects. We compared the biological effect of PPD- and PPT-type ginsenosides isolated from *P. ginseng* that contain a single sugar molecule such as Rh2, CK, Rh1, and F1, and assessed their anti-inflammatory effect in silico and in LPS-stimulated RAW 264.7 cells as an in vitro model. We first investigated the inhibitory influence of PPD- and PPT-type ginsenosides on the production of NF-κB through in silico docking studies, which allowed us to measure ADMET properties including aqueous solubility (accepted range is between 6.5 and 0.5), serum protein binding (accepted range is between −1.5 and 1.5), logP for octanol/water (accepted range is between −0.2 and 6.5), blood brain barrier (BBB) level (accepted range is between −3.0 and 1.2), hepatotoxicity (accepted range is between 0 and 1; non-toxic is 0 and toxic is 1), CYP2D6 inhibition probability (accepted range non-inhibitor is 0 and inhibitor is 1), and human oral absorption in the gastrointestinal tract (accepted range <25 % poor and >80 % high; Table S1). The findings of our ADMET analysis demonstrated that among the docked ginsenosides, CK and Rh1 have strong binding affinity with NF-κB compared to indomethacin and dexamethasone (control drugs) (Fig. 2a, d). Moreover, the values of predicted PPD- and PPT-type ginsenosides fall in the accepted groups of 95 % of known drugs. Our docking results might support ginsenosides as potential anti-inflammatory therapeutic agents. These computational calculation results indicated that CK and Rh1 are orally bioactive compounds that might be used effectively for treatment of targeted disease. Based on the chemical structure formula, we analyzed the biological activity spectrum with PASS analysis software. Our PASS data showed substantial biological targets for ginsenosides Rh1 and CK. Results of our PASS data analysis of tested PPD- and PPT-type ginsenosides showed that Rh1 and CK may act as anti-inflammatory agents, immunostimulants, and transcription factor NF-κB inhibitors. The estimated targets from ginsenosides along with their *Pa* and *Pi* values are given in Table S2. Furthermore, in order to identify the biological interactions of natural products, the molecular interaction levels of NF-κB protein and PPD- and PPT-type ginsenosides were evaluated using the molecular docking method. The NF-κB crystal structure was obtained with the docking simulation using the AutoDock program, and the binding interactions were confirmed by their hydrogen bond development and binding energy to the critical active residues of NF-κB and PPD- and PPT-type ginsenosides. Docking results showed that compared to controls

(indomethacin and dexamethasone), ginsenosides Rh1 and CK strongly bind with NF-κB through four and three hydrogen bonds to the active site residues of NF-κB with −8.3 and −7.58 kcal/mol binding affinities, respectively (Table S3). A graphical representation of PPD- and PPT-type ginsenosides interacting with the active residues of NF-κB is shown in Fig. 1. These hypotheses were further validated through in vitro study using RAW 264.7 cells. In vitro analysis showed that CK and Rh1 significantly down-regulated LPS-induced NF-κB as well as the subsequent initiation of pro-inflammatory mediators, including NO, PGE2, TNF-α, and ROS.

Pro-inflammatory cytokines, such as NO, PGE2, TNF-α, and ROS may have important roles in regulating inflammation and immune mechanisms [33]. We evaluated the anti-inflammatory ability of PPD- and PPT-type ginsenosides by evaluating their inhibitory effects on NO, ROS, PGE2, and TNF-α production in LPS-stimulated RAW 264.7 cells. The released nitrite production was assessed by the Griess method. In the culture supernatant, an equal volume of Griess reagent was used as an indicator for NO production. When the RAW 264.7 cells were stimulated with LPS, the nitrite content was significantly augmented (Fig. 3c, d). However, this LPS-induced NO production was significantly suppressed by CK and Rh1 at 10 and 100 μmol/L, respectively. ROS and PGE2 production induced by LPS in RAW 264.7 cells was markedly decreased at 10 and 100 μmol/L by CK and Rh1, respectively (Figs. 4, 5a, b). TNF-α overexpression may be crucial for the synergistic induction of NO synthesis in LPS-stimulated RAW 264.7 cells [34, 35]; we found that CK and Rh1 significantly inhibit TNF-α production in RAW 264.7 cell stimulated by LPS compared to the LPS-treated cells (Fig. 5c, d).

NF-κB and IKK are important transcription factors that are activated by various stimuli such as LPS, TNF-α, and IFN-γ. Upon stimulation, NF-κB translocates into the nucleus, where it contributes to the overexpression of many pro-inflammatory genes such as iNOS and COX-2 [12]. Preventing NF-κB activity through overexpression of Iκβα suppresses both inflammatory response and tissue damage in rheumatoid arthritis [36, 37]. We showed that CK and Rh1 have significant anti-inflammatory effects in RAW 264.7 cells. As shown in Fig. 1, the dissimilarity among the PPD and PPT types is due to the number of hydroxyl and glucose groups attached to the C-3, C-6 and C-20 positions. Therefore, it is presumed that the number of OH and sugar moieties in minor ginsenosides from the *P. ginseng* could be fundamental anti-inflammatory elements. Several other studies indicated that production of these pro-inflammatory cytokines, as well as iNOS and COX-2, is dependent on overexpression of NF-κB in inflammatory disorders [12]. We determined that the blocking of pro-inflammatory cytokines including iNOS and COX-2 production by CK and Rh1 might result from inhibition and blocking of NF-κB activation in LPS-stimulated RAW 264.7 cells. RT-PCR analysis revealed that Rh1 and CK significantly decreased LPS-induced mRNA expression levels of TNF-α, iNOS, and COX2 at 10 and 100 μmol/L, respectively (Fig. 6).

To detect the processes involved in the suppression of NF-κB activity by the tested ginsenosides, we evaluated the anti-inflammatory effects of Rh2, CK (PPD), Rh, and F1 (PPT) on NF-κB/IKK signaling pathways. The phosphorylated IκB degraded due to the stimulation with LPS, which allows translocation of NF-κB to the nucleus, where it stimulates several pro-inflammatory cytokines such as iNOS and COX-2 [38]. Therefore, down-regulation of NF-κB is the most crucial target for improvement and treatment of numerous inflammatory disorders. We therefore studied the anti-inflammatory effects of PPD- and PPT-type ginsenosides on down-regulation of NF-κB/IKK signals. Immunoblotting analysis showed that CK and Rh1 down-regulate the protein expression level of NF-κB and IKK in a dose-dependent manner (Fig. 7). Findings of our study are in agreement with the previous results, suggesting that 20 (S)-protopanaxatriol, one of ginsenoside metabolites, and ginsenoside Rh1 inhibit iNOS and COX2 production through inactivation of NF-κB in RAW 264.7 cells [24]. This pattern was also found in the case of PGE2 and TNF-α production.

In summary, our results reveal that CK and Rh1 have strong anti-inflammatory action on the suppression of LPS-induced NO, ROS, PGE2, and TNF-α production in RAW 264.7 cells through down-regulation of the NF-κB/IKK signaling pathway. These findings on the anti-inflammatory action of ginsenosides Rh1 and CK and their underlying mechanisms of action will improve our understanding of the molecular targets of inflammation. Based on our study, Rh1 and CK may be potential therapeutic agents for the prevention and treatment of various inflammatory diseases. However, further in vivo study is required to determine how these ginsenosides inhibit the overexpression of inflammatory mediators.

Acknowledgments This work was supported by a post-doctoral fellowship grant from the Kyung Hee University in 20120351. The ginseng samples used in this study were provided by Kyung Hee University.

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Baldwin AS Jr (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. Annu Rev Immunol 14:649–683
2. Chen YQ, Ghosh S, Ghosh G (1998) A novel DNA recognition mode by the NF-kappa B p65 homodimer. Nat Struct Biol 5:67–73

3. Yamamoto Y, Gaynor RB (2004) I kappa B kinases: key regulators of the NF-kappa B pathway. Trends Biochem Sci 29:72–79
4. Laskin DL, Pendino KJ (1995) Macrophages and inflammatory mediators in tissue injury. Annu Rev Pharmacol Toxicol 35:655–677
5. Guzik TJ, Korbut R, Adamek-Guzik T (2003) Nitric oxide and superoxide in inflammation and immune regulation. J Physiol Pharmacol 54:469–487
6. Guha M, Mackman N (2001) LPS induction of gene expression in human monocytes. Cell Signal 13:85–94
7. Zhang G, Ghosh S (2001) Toll-like receptor-mediated NF-kappa B activation: a phylogenetically conserved paradigm in innate immunity. J Clin Investig 107:13–19
8. Xie QW, Kashiwabara Y, Nathan C (1994) Role of transcription factor NF-kappa B/Rel in induction of nitric oxide synthase. J Biol Chem 269:4705–4708
9. Barnes PJ, Karin M (1997) Nuclear factor-kappa B: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 336:1066–1071
10. Karin M, Ben-Neriah Y (2000) Phosphorylation meets ubiquitination: the control of NF-[kappa] B activity. Annu Rev Immunol 18:621–663
11. De Bosscher K, Vanden Berghe W, Vermeulen L et al (2000) Glucocorticoids repress NF-kappa B-driven genes by disturbing the interaction of p65 with the basal transcription machinery, irrespective of coactivator levels in the cell. Proc Natl Acad Sci USA 97:3919–3924
12. Yamamoto Y, Gaynor RB (2001) Therapeutic potential of inhibition of the NF-kappa B pathway in the treatment of inflammation and cancer. J Clin Investig 107:135–142
13. Pan MH, Lai CS, Dushenkov S et al (2009) Modulation of inflammatory genes by natural dietary bioactive compounds. J Agric Food Chem 57:4467–4477
14. Bosscher KD, Berghe WV, Haegeman G (2003) The interplay between the glucocorticoid receptor and nuclear factor-κB or activator protein-1: molecular mechanisms for gene repression. Endocr Rev 24:488–522
15. Digital Briefs (2009) New software and websites for the chemical enterprise. Chem Eng News 87:48
16. Muller CW, Rey FA, Sodeoka M et al (1995) Structure of the NF-kappa B p50 homodimer bound to DNA. Nature 373:311–317
17. Maestro. 7.0 (2004) Schrodinger, LLC, Portland
18. Sterck HD (2013) Steepest descent preconditioning for nonlinear GMRES optimization. Numer Linear Algebra Appl 20:453–471
19. Barzilai JJ, Borwein M (1988) Two-point step size gradient methods. IMA J Numer Anal 8:141–148
20. Lautraite S, Bigot-Lasserre D, Bars R et al (2003) Optimization of cell based assays for medium through screening of oxidative stress. Toxicol Vitro 17:207–220
21. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta *CT*) method. Methods 25:402–408
22. Mohan CG, Gandhi T, Garg D et al (2007) Computer-assisted methods in chemical toxicity prediction. Mini-Rev Med Chem 7:499–507
23. Poroikov VV, Filimonov DA, Ihlenfeldt WD et al (2003) PASS biological activity spectrum predictions in the enhanced open NCI database browser. J Chem Inf Comput Sci 43:228–236
24. Bonizzi G, Karin M (2004) The two NF-kappa B activation pathways and their role in innate and adaptive immunity. Trends Immunol 25:280–288
25. Karin M, Cao Y, Greten FR et al (2002) NF-kappa B in cancer: from innocent bystander to major culprit. Nat Rev Cancer 2:301–310
26. Jun CD, Choi BM, Kim HM et al (1995) Involvement of protein kinase C during taxol-induced activation of murine peritoneal macrophages. J Immunol 154:6541–6547
27. Corriveau CC, Danner RL (1993) Antiendotoxin therapies for septic shock. Infect Agents Dis 1:44–52
28. Shapira L, Soskolne WA, Houri Y et al (1996) Protection against endotoxic shock and lipopolysaccharide induced local inflammation by tetracycline: correlation with inhibition of cytokine secretion. Infect Immun 64:825–828
29. Janeway CA Jr, Medzhitov R (2002) Innate immune recognition. Annu Rev Immunol 20:197–216
30. Bao HY, Zhang J, Yeo SJ et al (2005) Memory enhancing and neuroprotective effects of selected ginsenosides. Arch Pharm Res 28:335–342
31. Shin YW, Bae EA, Kim DH (2006) Inhibitory effect of ginsenoside Rg5 and its metabolite ginsenoside Rh3 in an oxazolone-induced mouse chronic dermatitis model. Arch Pharm Res 29:685–690
32. Tawab MA, Bahr U, Karas M et al (2003) Degradation of ginsenosides in humans after oral administration. Drug Metab Dispos 31:1065–1107
33. Mukaida N (2000) The roles of cytokine receptors in diseases. Rinsho Byori 48:409–415
34. Song SB, Tung NH, Quang TH et al (2012) Inhibition of TNF-alpha-mediated NF-kappa B transcriptional activity in HepG2 cells by dammarane-type saponins from *Panax ginseng* leaves. J Ginseng Res 36:146–152
35. Tilg H, Wilmer A, Vogel W et al (1992) Serum levels of cytokines in chronic liver diseases. Gastroenterology 103:264–274
36. Li Q, Verma IM (2002) NF-kappa B regulation in the immune system. Nat Rev Immunol 2:725–734
37. Shibata W, Maeda S, Hikiba Y et al (2007) Cutting edge: the I kappa B kinase (IKK) inhibitor, NEMO-binding domain peptide, blocks inflammatory injury in murine colitis. J Immunol 179: 2681–2685
38. Surh YJ, Chun KS, Cha HH et al (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-kappa B activation. Mutat Res 480–481: 243–268