Ginsenoside Rd inhibits the expressions of iNOS and COX-2 by suppressing NF-κB in LPS-stimulated RAW264.7 cells and mouse liver

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Ginsenoside Rd is a primary constituent of the ginseng rhizome and has been shown to participate in the regulation of diabetes and in tumor formation. Reports also show that ginsenoside Rd exerts anti-oxidative effects by activating anti-oxidant enzymes. Treatment with ginsenoside Rd decreased nitric oxide and prostaglandin E2 (PGE2) in lipopolysaccharides (LPS)-challenged RAW264.7 cells and in ICR mouse livers (5 mg/kg LPS; LPS + ginsenoside Rd [2, 10, and 50 mg/kg]). Furthermore, these decreases were associated with the down-regulations of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 and of nuclear factor (NF)-κB activity in vitro and in vivo. Our results indicate that ginsenoside Rd treatment decreases: 1) nitric oxide production (40% inhibition); 2) PGE2 synthesis (69% to 93% inhibition); 3) NF-κB activity; and 4) the NF-κB-regulated expressions of iNOS and COX-2. Taken together, our results suggest that the anti-inflammatory effects of ginsenoside Rd are due to the down-regulation of NF-κB and the consequent expresional suppressions of iNOS and COX-2.

Keywords: Panax ginseng, Ginsenoside Rd, Inducible nitric oxide synthase, Cyclooxygenase-2, Prostaglandin E2

INTRODUCTION

The transcription factor nuclear factor (NF)-κB has been studied by many researchers because of its apparent involvement in the inductions of several genes and diseases [1,2]. NF-κB is considered to play a key role in the production of inflammatory mediators, such as, pro-inflammatory cytokines (tumor necrosis factor-α and interleukin-1), cell adhesion molecules (intercellular cell adhesion molecule-1 and vascular cell adhesion molecule-1), immuno-receptors, and acute phase protein production [3]. In resting cells, NF-κB is localized in the cytoplasm as a heterodimer composed of two polypeptides of 50 kDa (p50) and 65 kDa (p65), which are non-covalently associated with cytoplasmic inhibitory proteins, such as IκB. In response to exposure to lipopolysaccharides (LPS), viral infection, the expressions of certain viral products, or other physiological stimuli, IκB undergoes a series of biological events, namely, rapid phosphorylation in its N-terminal domain by a large

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http://ginsengres.org  pISSN: 1226-8453  eISSN: 2093-4947
multi-kinase complex, poly-ubiquitination, and degradation by 26S proteasome, which allows translocation of NF-kB heterodimer to the nucleus [4]. Having reached the nucleus NF-kB activates the transcription of several inflammatory enzymes, such as cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS), by interacting with kB sites in their promoter regions. Furthermore, nitric oxide (NO) derived from iNOS and prostaglandin E₂ (PGE₂), which is synthesized by COX-2, plays a pivotal role in the pathogenesis of acute and chronic inflammation [5]. Studies in a number of cell and animal models have shown that iNOS inhibitors prevent the development of a number of diseases, including experimental allergic encephalomyelitis, atherosclerosis, cancer, inflammatory bowel syndrome, and transplantation rejection [6-8]. Moreover, the inappropriate activation or up-regulation of COX-2 is a characteristic of the majority inflammatory diseases [9], and therefore, the attenuation of the abnormal up-regulations of iNOS and COX-2 is considered a strategy for the treatment and prevention of inflammation conditions and related diseases.

Ginseng (the root of Panax ginseng Meyer, Araliaceae) has been used as a herbal remedy in eastern Asia for thousands of years. In traditional oriental medicine, the roots of ginseng are used to treat dyspepsia, as an aphrodisiac, and to enhance resistance to stressors, such as anxiety and fatigue. The ginsenosides are a component of ginseng preparations, and to date several have been isolated, identified, and characterized, namely, Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rh1, and Rh2. Furthermore, ginsenosides have been reported to have anti-diabetic [10] and anti-tumor [11] activities and anti-oxidative properties [12,13]. However, the cellular and molecular mechanisms responsible for the anti-inflammatory properties of ginsenosides are not well understood. In the present study, we investigated the mechanism responsible for the anti-inflammatory effect of ginsenoside Rd in RAW264.7 cells and ICR mice.

MATERIALS AND METHODS

Materials

The Korean red ginseng extract used was manufactured by the Korea Ginseng Corporation (Seoul, Korea). LPS (from Escherichia coli 0111:B4, CAS registry no. L2630) was obtained from Sigma (St. Louis, MO, USA), 2,7-dichlorodihydrofluorescein diacetate from Molecular Probes (Eugene, OR, USA), and Immobilon-P transfer membranes from Millipore (Bedford, MA, USA). Antibodies for iNOS and COX-2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and enhanced chemiluminescence Western blotting detection reagents were from Amersham Life Science (Arlington Heights, IL, USA). The radio-nucleotide [γ-32P]-ATP was obtained from Amersham (Bucks, UK). All other materials were of the highest grades commercially available. All other compounds were from Sigma unless otherwise stated.

Animals

Male, specific-pathogen free ICR mice (weight 30 to 32 g, 6 wk of age) were purchased from Hyochang Science (Daegu, Korea). Animals were housed in a controlled environment (24°C, 50% to 60% RH) and provided with standard rodent chow and water. This study complied with the Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources (ISBN 0-309-05377-3).

Experimental groups

Thirty-six ICR mice were assigned to one of the following six groups: 1) a saline injected group (the control group, n=6); 2) a 5 mg/kg LPS group (n=6); 3) three ginsenoside Rd (at 2, 10, or 50 mg/kg) plus 5 mg/kg LPS groups (ginsenoside+LPS groups, n=6); and 4) a 10 μM dexamethasone plus 5 mg/kg LPS group (the dexamethasone group, n=6). Saline, LPS, ginsenoside, and dexamethasone were administered intraperitoneally and ginsenoside and dexamethasone were administered 2 h prior to LPS treatment. Mice were dissected 4 h after LPS treatment.

Cell culture

RAW264.7 cells (a murine macrophage cell line) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown at 37°C in Dulbecco’s-modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 233.6 mg/mL of glutamine, 72 mg/mL penicillin-streptomycin, 0.25 mg/mL of amphotericin B, and 10% heat-inactivated fetal bovine serum (FBS) in a humidified 5% CO₂/95% air atmosphere.

Purification of ginsenosides

Nine fractions of ginsenosides were isolated as described by Kitagawa et al. [14]. Briefly powdered red ginseng (the steamed, dried, powdered roots of P. ginseng produced by the Korean Ginseng and Tobacco Research Institute, Daejeon, Korea) was refluxed in methanol. The extract so obtained was suspended in water and
extracted with n-butanol (saturated with water), and the butanol extract was evaporated in vacuo. The powder obtained (total ginsenosides) was dissolved in methanol and extracted with diethylether by stirring. Thus, repeated column chromatography of the ether-soluble portion using a Bondapek C18 column eluted with methanol-water (1:1-7:3) followed by a silica gel column eluted with CHCl3, methanol-water (10:1) provided ginsenosides Rh1 and Rh2. The methanol-soluble portion was subject to silica gel column chromatography eluted with CHCl3-methanol-water (65:35:10, the low phase) and n-butanol-ethylacetate-methanol-water (4:2:1:1, the low phase), followed by Bondapek C18 column chromatography eluted with methanol-water (1:1-7:3) to give ginsenoside Rd. The purities of each of the ginsenosides obtained were determined using melting points, optical rotations, and pos, fast atom bombardment mass spectrometry (FAB-MS). Melting points were determined using a Fisher-John unit, optical rotations using a Jasco DIP-370 Instrument, and pos FAB-MS spectra were obtained using a VG-VSEQ spectrometer (type EBqQ).

**Measurement of nitrite (NO) levels**

RAW264.7 cells were seeded in 96-well plates at a density of 2.0×10^4 cells/well. Cells were treated with ginsenoside for 1 h and then incubated for 18 h in DMEM containing 1% FBS with or without 100 ng/mL LPS. Nitrite levels in media were determined using a modification of the procedure described by Hwang et al. [16]. Briefly, 100 µL aliquots of cell culture supernatants were reacted with 100 µL of Griess reagent (0.1% [w/v] naphthylethylendiamide dihydrochloride in H2O and 1% [w/v] sulfanilamide in 5% phosphoric acid), and then absorbance was read at 550 nm using an ELISA reader GENios (Tecan Instruments, Salzburg, Austria).

**Measurement of prostaglandin E2 production in macrophages**

PGE2 production was determined using a modification of the procedure described by Hwang et al. [16]. Briefly, RAW264.7 cells were seeded in 96-well plates at a density of 1.5×10^4 cells/well, treated with ginsenosides for 1 h, and then incubated for 18 h in DMEM containing 1% FBS with or without 100 ng/mL of LPS. Amount of PGE2 in culture media were determined using a specific Enzyme Immunoassay kit (EIA, Amersham Pharmacia Biotech, UK).

**Western blotting**

Western blotting was carried out as described previously [17]. Homogenized samples were boiled for 5 min with a gel-loading buffer (0.125 M Tris-HCl, pH 6.8, 4% SDS, 10% 2-mercaptoethanol and 0.2% bromophenol blue) in ratio of 1:1. Total protein-equivalents for each sample were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using 10% acrylamide gels as described by Laemml [18], and transferred to polyvinylidene fluoride membrane at 15 V for 1 h in a semi-dry transfer system. The membrane was immediately placed into blocking buffer (1% non-fat milk) in 10 mM Tris, pH 7.5, 100 mM NaCl, and 0.1% Tween 20. The blot was allowed to block at room temperature for 1 h. The membrane was incubated with specific primary antibody (COX-2 or iNOS) at 25°C for 3 h, and followed by a horse radish peroxidase-conjugated anti-rabbit antibody (Santa Cruz, 1:10,000) or anti-goat antibody (Santa Cruz, 1:10,000) at 25°C for 1 h. Antibody labeling was detected using West-zol Plus and chemiluminescence FluorochemTMSP (Alpha Innotech Corporation, San Leandro, CA, USA). Pre-stained protein markers were used for molecular weight determinations.

**Electrophoretic mobility shift assay**

Electrophoretic mobility shift assay (EMSA) method was used to characterize the binding activities of NF-kB in nuclear extracts [19]. NF-kB oligonucleotide was 5'-GAGAGGCAAGGGGATTCCCTTAGTTAGGA-3' [20]. Protein-DNA binding assays were performed with 10 µg of nuclear protein. To minimize salt on binding, the concentration of salt was adjusted to the same level in all samples. Unspecific binding was blocked by using 1 µg of poly(dl-dC)poly(dl-dC). The binding medium contain 5% glycerol, 1 mM MgCl2, 50 mM NaCl, 0.5 mM ethylenediaminetetraacetic acid (EDTA), 2 mM dithiothreitol, 1% nonyl phenoxypolyethoxylethanol-40, and 10 mM Tris, pH 7.5. In each reaction, 20,000 cpm of radiolabeled probe was included. Samples were incubated at room temperature for 20 min, and the nuclear protein-32P-labeled oligonucleotide complex was separated from free 32P-labeled oligonucleotide by electrophoresis through a 5% native poly-acrylamide gel in a running buffer containing 50 mM Tris, pH 8.0, 45 mM borate, and 0.5 mM EDTA. After separation was achieved, the gel was vacuum dried for autoradiography and exposed to Fuji X-ray film at -80°C for 1 to 2 d.

**Tissue preparation**

One gram of liver tissue was homogenized in 10 mL of homogenizing buffer (50 mM potassium phosphate buffer containing 1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 µM pepstatin, 80 mg/L trypsin inhibi-
tor, pH 7.4) and centrifuged at 900 g at 4°C for 15 min. The supernatants so obtained were then re-centrifuged at 12,000 g at 4°C for 15 min to yield a sedimented mitochondrial fraction and cytosol fraction. All fractions were stored at -80°C until required.

**Measurement of NO$_2^-$ plus NO$_3^-$ levels in vivo**

Total NO$_2^-$ plus NO$_3^-$ (NOx) levels were measured using an NO-analyzing system (ENO-20; Eicom Corp., Kyoto, Japan). Samples were deproteinized by adding an equal volume of methanol, and then centrifuged at 12,000 g for 10 min at 4°C to avoid column occlusion by macromolecules. Nitrite and nitrate were then separated on a polystyrene polymer column, and nitrate was reduced to nitrite by passing its fraction through a cadmium column. The eluate was then mixed with Griess reagent, and absorbance of the purple dye was measured at 540 nm using a flow-through spectrophotometer. Concentrations of nitrite and nitrate were determined using a computer system (Power Chrom; Eicom, Kyoto, Japan), which automatically measured the areas of absorbance peaks. The minimal detectable concentrations of nitrite and nitrate using this method were both about 0.01 μM [21].

**Statistical analysis**

All values in figures are expressed as means±SE (n=6). The analysis was conducted using one-way ANOVA's post-hoc test, and p-values of <0.05 were considered statistically significant.

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**RESULTS**

**Screening of ginsenosides Rb1, Rb2, Rc, Rd, Re, Rf, Rg1, Rh1, and Rh2 for nitric oxide suppression in RAW264.7 cells**

NO is a signaling molecule that plays a critical role in vascular smooth muscle relaxation, reduction of platelet aggregation and adhesion [22]. On the other hand, NO also is synthesized at inflammatory sites by iNOS with nicotinamide adenine dinucleotide phosphate and oxygen as substrates. iNOS is induced in response to LPS, interferon-g, and a variety of pro-inflammatory cytokines [23]. We examined the effects of ginsenoside on NO production in RAW264.7 cells stimulated by LPS, and dexamethasone, a well-known inhibitor of iNOS and COX-2, was used as a positive control. Ginsenosides Rd, Rc, and Rb2 (Fig. 1) were found to be the effective components of ginseng; about 40% inhibition of NO production was achieved versus LPS treatment alone (Fig. 2). As shown in Fig. 2, the nitrite level in culture medium was markedly increased from 2.05±0.05 μM to 10.08±0.29 μM 18 h after treatment with LPS. Nine ginsenosides were screened to test their anti-inflammatory effects. In subsequent experiments, we decided to use ginsenoside Rd, because little is known of its effects.

**Inhibitory effects of ginsenoside Rd on NO$_2^-$ plus NO$_3^-$ levels and inducible nitric oxide synthase expression activity**

In order to determine whether ginsenoside Rd in-

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**Fig. 1.** Structures of ginsenosides.
hibits iNOS expression in vitro, we investigated iNOS activities after adding ginsenoside Rd to LPS-stimu-

lated RAW264.7 cells. NOx levels in culture medium were significantly elevated from 5.170±0.002 μM to 12.81±0.85 μM 18 h after treatment with LPS, but after treatment with ginsenoside Rd (at 5, 50, and 100 μM), dose-dependent decreases in nitrite levels were observed in cultured cells, peaking at 100 mM of the ginsenoside Rd treatment (Fig. 3A).

iNOS protein expressions in LPS-stimulated macrophages were examined by Western blotting. Densitometer scans of respective blots showed that RAW264.7 cells did not express detectable levels of iNOS protein when incubated in medium alone for 18 h (Fig. 3B), whereas iNOS expression increased dramatically in cells treated with LPS treatment (100 ng/mL) for 18 h. Furthermore, LPS-induced iNOS expression was significantly abolished when ginsenoside Rd was added at a concentration of 100 μM.

Inhibitory effects of ginsenoside Rd on prostaglandin E2 and cyclooxygenase-2 protein levels

COX is a key enzyme in the generation of prostanoids (PGs) and other eicosanoids from arachidonic acid [24],...
The anti-inflammatory effects of ginsenoside Rd and two COX isozymes, COX-1 and COX-2, have been identified. In most tissues, COX-1 is considered the constitutive isoform that catalyzes the synthesis of PGs. On the other hand, COX-2 is an inducible isoform and is expressed after an inflammatory stimulus, such as LPS, inflammatory cytokines, growth factors, or tumor promoters. Furthermore, COX-2 is responsible for the production of large amounts of pro-inflammatory PGE2 [25]. In this study, the effects of ginsenoside Rd on PGE2 production and COX-2 expression were investigated.

Fig. 4B shows that LPS-mediated COX-2 expression in RAW264.7 cells was effectively inhibited by ginsenoside Rd at 50 μM, as determined by densitometry. Furthermore, 100 μM of ginsenoside Rd was more effective than 10 μM of dexamethasone. We next examined PGE2 levels. Fig. 4A shows that ginsenoside Rd markedly suppressed PGE2 production in LPS-stimulated RAW264.7 cells. In fact, PGE2 levels were inhibited by 69% to 93% by 50 to 100 μM of ginsenoside Rd, whereas dexamethasone at 10 μM inhibited PGE2 production by 83%.

**Effects of ginsenoside Rd on lipopolysaccharides-induced nuclear factor-κB activation in macrophage cell line**

A report showed that NF-κB can be activated by LPS in macrophage cell lines [26]. Since NF-κB plays an important role in the expressions of iNOS and COX-2, we examine whether NF-κB activity is modulated by ginsenoside Rd treatment in LPS-induced cells. A DNA binding assay was used to assess the ability of ginsenoside Rd to reduce the ability of active NF-κB to bind to a radiolabeled oligonucleotide containing κB DNA elements using EMSA. Nuclear extracts from LPS-stimulated cells were incubated with increasing concentrations of ginsenoside Rd for 30 min at 37°C (Fig. 5). The binding of NF-κB to DNA in the presence of LPS only was markedly increased in a small proportion of macrophages. However, ginsenoside Rd inhibited NF-κB binding to DNA dose-dependently and significantly inhibited NF-κB binding to DNA at a concentration of 50 μM. This result demonstrates that NF-κB DNA binding can be inhibited by ginsenoside Rd in a dose-dependent manner.
Inhibitory effects of ginsenoside Rd on NOx levels and on the expressions of inducible nitric oxide synthase, and cyclooxygenase-2 in vivo

We performed in vivo experiments to confirm the results obtained in vitro. Total NOx levels in mouse liver were determined using an NO-analyzer, as described in the Materials and Methods section. Mice were injected with three different concentrations of ginsenoside Rd (2, 10, and 50 mg/kg) and 2 h later were injected with LPS (5 mg/kg). Mice were sacrificed 4 h after LPS treatment. Total NOx levels in liver were much increased in LPS-injected mice, but pretreatment with ginsenoside Rd dose-dependently inhibited NOx level increases (Fig. 6A). Furthermore, whereas naïve mice showed no detectable iNOS protein in liver, iNOS protein was highly expressed in LPS (5 mg/kg) treated mice. Pretreatment with ginsenoside Rd markedly reduced LPS-induced iNOS expression (Fig. 6B). In addition, we examined whether ginsenoside Rd also affects COX-2 expression. The levels of COX-2 protein were examined by immunoblot analysis using a specific anti-COX-2 antibody. In LPS (5 mg/kg) treated mouse livers, COX-2 was highly expressed, whereas pretreatment with ginsenoside Rd markedly reduced LPS-induced COX-2 expression (Fig. 6C). Taken together, these in vivo results confirmed the observed in vitro effects of ginsenoside Rd on iNOS and COX-2.

DISCUSSION

Studies show that nitric oxide is a principle mediator of a wide range of toxic oxidative reactions, such as the initiation of lipid peroxidation, the inhibition of mitochondrial respiratory chain enzymes, the inhibition of membrane sodium/potassium ATP-ase activity, and the inactivation and oxidative modifications of proteins [27]. Furthermore, all of these toxic effects are related to acute and chronic inflammation [28,29]. In our previously studies, we also found that nitric oxide contributes to vascular inflammation [30,31], and thus, the excessive expression of iNOS, a precursor of NO, is likely to be implicated in the pathogenesis of many inflammatory diseases. Recently, natural occurring phytochemicals have been shown to reduce the undesirable expression of iNOS [32,33], which suggests that suitable phytochemicals could be used to treat inflammation. Similarly, the present study shows that ginsenoside Rd reduces NO production by inhibiting iNOS expression in LPS-activated murine macrophages and in vivo.

COX-2 expression has been reported to weaken antioxidant capacity in mouse macrophages [34,35], and oxidative stress is known to regulate and exacerbate inflammation. Therefore, we used RAW264.7 cells in this study to evaluate the effect of ginsenoside Rd on the transcriptional activities of COX-2. Our results show that COX-2 activity and levels of its by-product PGE₂ are decreased by ginsenoside Rd.

NF-κB activation is known to be associated with the up-regulations of the expressions of iNOS and COX-2 mediated by LPS. To elucidate the molecular actions of ginsenoside Rd, we examined its ability to inhibit the production of iNOS and COX-2. EMSA revealed that the activity of NF-κB binding to the consensus sequence of κB was inhibited by ginsenoside Rd in LPS-activated RAW264.7 cells.

Several other studies have attempted to elucidate the inhibitory effects of ginsenoside on iNOS expression and NO production in vitro and in vivo. For example, Park et al. [36] suggested that ginsenoside Rb1 modulates NO and PGE₂ biosynthesis in RAW264.7 cells induced by LPS, and in a later study found that in mice, ginsenoside Rh1 possesses anti-inflammatory activity due to its ability to inhibit the expressions of iNOS and COX-2 [37]. Ginsenoside Rh6 has been reported to have an inhibitory effect on NO production in C6 rat glioma cells [38], and recently, was shown to attenuate oxidative stress [39]. These results support the view that ginsenoside Rh1 inhibits the pro-inflammatory mediators, iNOS and COX-2.

The above findings raise the question as to the nature of any potential pathway of initiation by ginsenoside Rd. In our previous study, we found that ginsenosides Rh1
and Rh2 mediate the nuclear translocation of glucocorticoid receptor (GR) and to the differentiation of teratocarcinoma stem cells [40], which suggests that the GR signal cascade provides a means whereby ginsenoside Rd could act therapeutically.

Based on our findings, we conclude that the anti-inflammatory property of ginsenoside Rd is due to the expression inhibitions of iNOS and COX-2. These effects of ginsenoside Rd were further supported by its effectiveness to modulate pro-inflammatory NF-κB activity. iNOS and COX-2 are known to play pivotal roles in the pathogenesis of acute and chronic inflammation, and thus, the inhibition of the abnormal up-regulations of iNOS and COX-2 provides a molecular basis for the therapeutic effect of ginsenoside Rd on inflammation and inflammatory diseases. Furthermore, ginsenoside Rd inhibited PGE2- and NO-triggered inflammatory responses in vitro and in vivo, and since the aberrant over-expressions of COX-2 and iNOS are implicated in the pathogenesis of various inflammation-related diseases, the results of this study indicate that ginsenoside Rd may have applications for the treatment of inflammatory disorders.

**ACKNOWLEDGEMENTS**

This work was supported by a grant in 2011 from the
Korean Society of Ginseng and by a grant of the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea (A090582). This study was financially supported by the 2012 Post-Doc Development Program of Pusan National University.

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