Research Article

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Ginsenoside Rp₁, a Ginsenoside Derivative, Blocks Promoter Activation of iNOS and COX-2 Genes by Suppression of an IKK β -mediated NF- κ B Pathway in HEK293 Cells

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Ginsenoside (G) Rp₁ is a ginseng saponin derivative with anti-cancer and anti-inflammatory activities. In this study, we examined the mechanism by which G-Rp₁ inhibits inflammatory responses of cells. We did this using a strategy in which DNA constructs containing cyclooxygenase (COX)-2 and inducible nitric oxide synthase (iNOS) promoters were transfected into HEK293 cells. G-Rp₁ strongly inhibited the promoter activities of COX-2 and iNOS; it also inhibited lipopolysaccharide induced upregulation of COX-2 and iNOS mRNA levels in RAW264.7 cells. In HEK293 cells G-Rp₁ did not suppress TANK binding kinase 1-, Toll-interleukin-1 receptor-domain-containing adapter-inducing interferon- β (TRIF)-, TRIF-related adaptor molecule (TRAM)-, or activation of interferon regulatory factor (IRF)-3 and nuclear factor (NF)- κ B by the myeloid differentiation primary response gene (MyD88)-induced. However, G-Rp₁ strongly suppressed NF- κ B activation induced by I κ B kinase (IKK) β in HEK293 cells. Consistent with these results, G-Rp₁ substantially inhibited IKK β -induced phosphorylation of I κ B α and p65. These results suggest that G-Rp₁ is a novel anti-inflammatory ginsenoside analog that can be used to treat IKK β /NF- κ B-mediated inflammatory diseases.

Keywords: *Panax ginseng*, Ginsenoside Rp₁, Cyclooxygenase 2, Nitric oxide synthase type II, Promoter activity, Nuclear factor- κ B

INTRODUCTION

Nitric oxide (NO) and prostaglandin E₂ (PGE₂) are representative inflammatory mediators produced by

macrophages under inflammatory conditions [1]. These molecules are generated by the activation of induc-

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ible NO synthase (iNOS) and cyclooxygenase-2 (COX-2). Thus, these molecules are primarily controlled by transcriptional and translational regulation by surface receptors such as Toll like receptors (TLR) and their counter adaptor and signaling molecules such as TANK binding kinase (TBK)1, Toll–interleukin-1 receptor-domain-containing adapter-inducing interferon- β (TRIF), TRIF-related adaptor molecule (TRAM), and myeloid differentiation primary response gene (88) (MyD88). The activation of cellular environments finally leads to the functional up-regulation of transcription factors (e.g., nuclear factor [NF]- κ B, interferon regulatory factor [IRF]-3, and activator protein [AP]-1) [2]. Excessive release of NO and PGE₂ is also linked to the onset of serious inflammatory diseases such as rheumatoid arthritis and arteriosclerosis [3]. Inhibition of NO and PGE₂ release, therefore, is considered to be an important therapeutic target.

Ginseng (*Panax ginseng* C. A. Meyer) is an herbal medicine that has long been used to treat diseases such as infection, cancer, arthritis, and atherosclerosis. Recently, numerous scientific studies have identified the functionally active phytochemical components in ginseng and the molecular mechanisms of their therapeutic effects. Furthermore, various disease models have been created to expand the effects of ginseng derived extracts or components [4]. The major pharmacological components of ginseng are thought to be ginseng saponins (called ginsenosides). Recent ginseng researchers have now begun to study the use of individual ginsenosides or specific ginsenoside-rich fractions. For example, it has been reported that ginsenoside (G)-Rg₃ can indirectly suppress tumor growth [5], and it has been launched as an anti-cancer drug. G-F₁ is presently added to in cosmetic biomaterials that are used for their anti-wrinkling effects [6]. G-Rb₁ has also been shown to exhibit anti-arthritis activity [7].

Nonetheless, the development of ginseng-derived ginsenosides as valuable drugs or remedies is now seen as limited because the components are already widely used. To avoid such an application problem, we have tried to prepare novel ginsenoside-derived compounds that improve chemical stability and mass production. Given these goals, G-Rp₁ (Fig. 1) was prepared on a large scale from crude ginsenosides (e.g., G-Rg₃ and G-Rk₁) by means of reduction by hydrogenation [8] and proved to be chemically stable [9]. Through aggressive efforts, it has been demonstrated that G-Rp₁ has strong anti-cancer and anti-inflammatory properties [8,10,11]. Although it is speculated that G-Rp₁ has anti-inflammatory activity, the

Fig. 1. The chemical structure of ginsenoside (G)-Rp₁.

exact inhibitory target of this compound has not yet been identified. In this study, we explored the mechanism by which G-Rp₁ exerts its regulatory effects. To this end, we used a reporter gene assay system in HEK293 cells. Cells were transfected with transcription factor-stimulatory adaptors and signaling molecules.

MATERIALS AND METHODS

Materials

G-Rp₁ (purity, 97%), a racemic mixture of *R*- and *S*-enantiomers in a 1:1.3 ratio, was prepared using established protocols [9]. The stock solution (20 mM) of G-Rp₁ was prepared using 100% dimethyl sulfoxide. Lipopolysaccharide (LPS, *Escherichia coli* 0111:B4), ionomycin A, and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum was obtained from Gibco (Grand Island, NY, USA). All other chemicals were Sigma grade. Phospho-specific or total antibodies to p65 and I κ B α were purchased from Cell Signaling (Beverly, MA, USA).

Cell culture

RAW264.7 cells (the American Type Culture Collection, Rockville, MD, USA) were maintained in complete RPMI1640 medium (supplemented with 100 U/mL of penicillin and 100 μ g/mL of streptomycin, and 10% fetal bovine serum). HEK293 cells (the American Type Culture Collection) were maintained in DMEM medium (supplemented with 100 U/mL of penicillin, 100 μ g/mL of streptomycin, and 10% fetal bovine serum).

Reverse transcription and real-time polymerase chain reaction

Total RNA from LPS-treated-RAW264.7 cells (5×10^6 cells/mL) was prepared by adding TRIzol Reagent (Gibco) according to the manufacturer's protocol [12].

The total RNA solution was stored at -70°C until used. Semi-quantitative real-time (RT) reactions were conducted using MuLV reverse transcriptase. Total RNA (1 µg) was incubated with oligo-dT15 for 5 min at 70°C and then mixed with a 5X first-strand buffer, 10 mM dNTPs, and 0.1 M DTT. The reaction mixture was further incubated for 5 min at 37°C and for 60 min after the addition of MuLV reverse transcriptase (2 U). Reactions were terminated after 10 min at 70°C, and total RNA was depleted by adding RNase H. The polymerase chain reaction (PCR) was conducted with the incubation mixture (2 µl cDNA, 4 µM 5' and 3' primers, a 10X buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.1% Triton X-100], 250 µM of dNTP, 25 mM of MgCl₂, and 1 unit of Taq polymerase [Promega, Madison, WI, USA]). The following incubation conditions were used: a 30 sec denaturation time at 94°C, an annealing time of 30 sec between 55 and 60°C, an extension time of 45 sec at 72°C, and a final extension of 5 min at 72°C. For RT-PCR analysis, one microgram of RNA was submitted to reverse transcription with the Molony murine leukemia virus reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Two microliters of cDNA obtained from each sample were submitted to a qPCR assay using the SYBR green Master mix method (Applied Biosystems, Foster City, CA, USA) in the ABO sequence detection system. The results were normalized with the 18S transcript. The primers (Bioneer, Daejeon, Korea) used in this experiment are indicated in Table 1.

Immunoblotting

Cells (5×10⁶ cells/mL) were lysed in lysis buffer (in mM: 20 TRIS-HCl, pH 7.4, 2 EDTA, 2 EGTA, 50 β-glycerophosphate, 1 sodium orthovanadate, 1 dithiothreitol, 1% Triton X-100, 10% glycerol, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 10 µg/mL pepstatin, 1 benzimidazole, and 2 hydrogen peroxide) for 30 min rotating at 4°C. Lysates were clarified by centrifugation at 16,000 ×g for 10 min at 4°C. Soluble cell lysates were immunoblotted, and phospho- and total levels of IκBα and p65 were visualized as previously reported [13].

Luciferase reporter gene activity assay

HEK293 cells (1×10⁶ /mL) were transfected with 1 µg of plasmids with COX-2 promoter-Luc, iNOS-promoter-Luc, NF-κB-Luc or IFN-β-promoter-Luc in the absence or presence of adaptors or enzyme DNAs as well as β-galactosidase using a PEI method in a 12-well plate. The cells were used for experiments 48 h after transfection. Luciferase assays were performed using

Table 1. Primers used for quantitative real-time and semiquantitative real-time polymerase chain reaction (RT-PCR) analyses

Gene	Primer sequences
<i>For semiquantitative RT-PCR</i>	
IFN-β	F 5'- :CCA CCA CAG CCC TCT CCA TCA ACT AT-3'
	R 5'- CAA GTG GAG AGC AGT TGA GGA CAT C-3'
GAPDH	F 5'-CACTCACGGCAAATTCACGGCAC-3'
	R 5'-GACTCCACGACATACTCAGCAC-3'
<i>For quantitative real-time RT-PCR</i>	
TNF-α	F 5'-TGCCTATGTCTCAGCCTCTTC-3'
	R 5'-GAGGCCATTGGGAAGTCTCT-3'
COX-2	F 5'-GGGAGTCTGGAACATTGTGAA-3'
	R 5'-GCACATTGTAAGTAGGTGGACTGT-3'
iNOS	F 5'-GGAGCCTTTAGACCTCAACAGA-3'
	R 5'-TGAACGAGGAGGGTGGTG-3'
GAPDH	F 5'- CAATGAATACGGCTACAGCAAC-3'
	R 5'- AGGGAGATGCTCAGTGTGG-3'

IFN-β, interferon-β; TNF-α, tumor necrosis factor-α; COX-2, cyclooxygenase-2; iNOS, inducible nitric oxide synthase; F, forward; R, reverse.

the Luciferase Assay System (Promega) [14,15]. Briefly, the transfected cells treated with G-Rp₁ were lysed in the culture dishes with reporter lysis buffer. Lysates were centrifuged at maximum speed for 10 min in an Eppendorf microcentrifuge. Ten µL of the supernatant fraction were incubated with 50 µL of luciferase substrate, and the relative luciferase activity was determined with a Luminoskan Ascent system (Thermo Labsystems, Helsinki, Finland). Luciferase activity was normalized to β-galactosidase activity.

Statistical analysis

Student's *t*-test and one-way ANOVA were used to determine the statistical significance of differences between values for the various experimental and control groups. Data expressed as means±standard errors are taken from at least three independent experiments each performed in triplicate (Figs. 2, 3A, 3B, 4, and 5A). The rest of the data are representative of three different experiments with similar results. A *p*-value of 0.05 or less were considered to be statistically significant.

RESULTS AND DISCUSSION

G-Rp₁ (Fig. 1) has been shown to inhibit the production of IL-1β in LPS-activated macrophages in a dose

Fig. 2. Effect of ginsenoside (G)-Rp₁ on the promoter activity of inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 genes. (A,B) HEK293 cells co-transfected with the plasmid constructs iNOS-Luc or COX-2-Luc (1 μg/mL each) and β-gal (as a transfection control) were treated with G-Rp₁ (20 μM) in the presence or absence of phorbol-12-myristate-13-acetate (PMA, 100 nM)/ionomycin A (1 μM) for 12 h. Luciferase activity was determined by luminometry. (C) RAW264.7 cells (5×10⁶ cells/mL) pre-treated with G-Rp₁ for 1 h were stimulated in the absence or presence of lipopolysaccharide (1 μg/mL) for 6 h. After preparing mRNA, the levels of iNOS, COX-2, tumor necrosis factor (TNF)-α, and GAPDH were determined by real-time polymerase chain reaction. NOR, normal. **p*<0.05 and ***p*<0.01 compared to control.

dependent manner [16]. In addition, it has been reported that β1-integrin (CD29)-mediated cell-cell adhesion, which is frequently seen in inflammatory responses [17], can be suppressed by this compound. Therefore, in this study, we aimed to investigate the anti-inflammatory mechanism of G-Rp₁. To do this, we used a reporter gene assay to examine the ability of this compound to suppress transcriptional activation of iNOS and COX-2, major inflammatory genes.

Transcriptional activation was set up by transfection of luciferase gene-containing DNA constructs with iNOS and COX-2 promoters. Indeed, transfection of each DNA increased its promoter activity, as assessed by luciferase activity, up to 6 fold upon stimulation with PMA and ionomycin A (Fig. 2). Interestingly, G-Rp₁ strongly inhibited their promoter activities up to

90% at 20 μM (Fig. 2A, B), without affecting cell viability up to 40 μM (data not shown). Additionally, this compound dose-dependently suppressed the mRNA expression of iNOS, COX-2, and tumor necrosis factor (TNF)-α induced by LPS stimulation of RAW264.7 cells (Fig. 2C), suggesting that G-Rp₁ works properly. The observed results were similar to a previous report in which G-Rp₁ diminished IL-1β production at the transcriptional level [16].

Transcriptional control by G-Rp₁ was shown to be mediated by suppression of NF-κB activation [18]. In addition, AP-1 and IRF-3 have been suggested to play a critical role in the expression of iNOS and COX-2 genes by direct or indirect activation. Therefore, we next evaluated the involvement of these transcription factors in G-Rp₁-mediated inhibition of transcriptional activation.

Fig. 3. Effect of ginsenoside (G)-Rp₁ on interferon regulatory factor-3 activation and interferon (IFN)-β expression. (A,B) HEK293 cells co-transfected with the plasmid constructs IFN-β-promoter-Luc (1 μg/mL each) and β-gal (as a transfection control) were treated with G-Rp₁ (20 μM) in the presence or absence of DNA constructs for Toll-interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF) or TANK binding kinase (TBK)1 for 12 h. Luciferase activity was determined by luminometry. (C) RAW264.7 cells (5×10⁶ cells/mL) pre-treated with G-Rp₁ for 1 h were stimulated in the absence or presence of LPS (1 μg/mL) for 1 h. After preparing mRNA, the levels of IFN-β and GAPDH were determined by semiquantitative real-time polymerase chain reaction. LPS, lipopolysaccharide.

First, we tested whether G-Rp₁ modulated an IFN-β-mediated indirect pathway in which IFN-β produced by the TRIF/TBK1/IRF-3 pathway can stimulate, autonomously, the production of PGE₂ and NO in macrophages or other inflammatory cells. As Fig. 3 shows, however, G-Rp₁ did not suppress IRF-3 activation, assessed by luciferase activity assay using an IRF-3 binding DNA sequence that contained the IFN-β-promoter construct with a luciferase gene. In particular, upregulation of IFN-β promoter activity by stimulation with TRIF, a major adaptor molecule involved in TLR4 signaling, or TBK1, a major signaling enzyme to activate IKKε and counter transcription factor IRF-3, was not attenuated by G-Rp₁ treatment (Fig. 3). Moreover, G-Rp₁ did not suppress mRNA expression of IFN-β (Fig. 3C), a major outcome of IRF-3 activation [19], suggesting that G-Rp₁ is not involved in blocking the TRIF/TBK1/IRF-3 pathway for IFN-β production. Furthermore, G-Rp₁

did not block the luciferase activity that was mediated by activation of AP-1 stimulated by PMA [20-22] and co-transfection with other stimulatory molecules such as TRIF and TBK1 (data not shown).

Recently, we reported that G-Rp₁ suppresses LPS-mediated NF-κB activation in RAW264.7 cells. Therefore, we investigated whether G-Rp₁ can block NF-κB-mediated promoter activity that had been indirectly increased by various adaptors and signaling molecules in HEK293 cells. Several stimuli and proteins that had been cotransfected into HEK293 cells were found to trigger NF-κB activation and subsequently increase luciferase activity [21]. In particular, PMA and TNF-α are regarded as good inducers, while MyD88, TRIF, TBK1, and IKKβ are considered to be strong activating proteins of the NF-κB pathway. Indeed, under our conditions, cotransfection of or treatment with these molecules enhanced NF-κB-mediated luciferase activity. However,

Fig. 4. Effect of ginsenoside (G)-Rp₁ on nuclear factor (NF)-κB activation induced by adaptor molecules. (A,B,C, and D) HEK293 cells co-transfected with the plasmid constructs NF-κB-Luc (1 μg/mL each) and β-gal (as a transfection control) were treated with G-Rp₁ in the presence or absence of DNA constructs containing TANK binding kinase (TBK)1, Toll-interleukin-1 receptor-domain-containing adapter-inducing interferon-β (TRIF), TRIF-related adaptor molecule (TRAM) or myeloid differentiation primary response gene (88) (MyD88) for 12 h. Luciferase activity was determined by luminometry.

G-Rp₁ treatment did not block MyD88-, TRIF-, TRAM-, and TBK1-mediated NF-κB activation and their subsequent luciferase activities (Fig. 4), although there seems to be an inhibitory pattern when HEK293 cells were treated with 20 μM G-Rp₁ under TBK1 co-transfection conditions (Fig. 4A). However, since a higher concentration (40 μM) of G-Rp₁ failed to show NF-κB inhibitory activity, we concluded that G-Rp₁ is not able to strongly suppress NF-κB activation that is mediated by such adaptor molecules. Unlike the latter results, G-Rp₁ strongly inhibited IKKβ-induced NF-κB activation in HEK293 cells (Fig. 5A). In agreement with this, G-Rp₁ suppressed the phosphorylation of IκBα and p65 (Fig. 5B), suggesting that IKKβ is the true target of G-Rp₁ in its NF-κB suppressive activity. Indeed, G-Rp₁ strongly

down-regulated the release of NF-κB-mediated inflammatory gene products. For example, G-Rp₁ suppressed the production of other inflammatory mediators such as NO (down to 42±2% at 40 μM) and TNF-α (53±2% at 40 μM) [16]. This compound strongly blocked LPS-mediated IL-1β release [16]. The importance of NF-κB in inflammatory responses was also confirmed by treatment with parthenolide and BAY 11-7082, two strong and selective NF-κB inhibitors with distinct anti-inflammatory properties [21]. Therefore, we speculated that NF-κB itself or its activation pathway induced by LPS or other inflammatory signals may be the critical target of G-Rp₁.

Recently, the anti-inflammatory feature of ginsenosides or their structural analogs such as compound K,

Fig. 5. Effect of ginsenoside (G)-Rp₁ on nuclear factor (NF)-κB activation induced by by IκB kinase (IKK)β. (A) HEK293 cells co-transfected with the plasmid constructs NF-κB-Luc (1 μg/mL each) and β-gal (as a transfection control) were treated with G-Rp₁ in the presence or absence of DNA constructs containing IKKβ for 12 h. Luciferase activity was determined by luminometry. (B) HEK293 cells (5×10⁶ cells/mL) transfected with IKKβ (1 μg/mL) were incubated with G-Rp₁ for 1 h. After preparing the nuclear fraction, the phosphorylation or total levels of IκBα and p65 were determined by phospho-specific or total antibodies. **p*<0.05 and ***p*<0.01 compared to control.

G-Rh₁, G-Rb₁, G-Rb₂, G-Rh₂, G-Re, and 20(S)-protopanaxatriol have been revealed [23]. The main anti-inflammatory effects of these compounds are to suppress NO generation, TNF-α release, and PGE₂ production both at transcriptional and translational levels [24]. As we reported previously, the inhibitory target of these compounds is mostly found to be the NF-κB pathway, not the activation of glucocorticoid receptor [16,25]. There was no correlation between the structures of these compounds and their anti-inflammatory activities. However, a unique carbohydrate moiety at the R1 position of ginsenosides seems to be crucial for strong anti-

inflammatory activity [17,26]. Similar structure-activity relationships were found for G-Rp₁ and its structural analogs including G-Rp₂, G-Rp₁, and G-Rp₃, as reported previously [17,26].

Recently, we demonstrated that G-Rp₁ is available after oral administration since this compound strongly blocked melanoma cell metastasis [10]. Moreover, when this compound was orally administered, it was chemically stable and identifiable in blood (data not shown). Furthermore, G-Rp₁ is a novel patentable ginsenoside derivative. Acute toxicity of this compound was found only at doses greater than 2.5 g/kg. These points open

the possibility of developing G-Rp₁ as a novel ginsenoside-derived drug with anti-cancer and anti-inflammatory properties. Continued studies will be focused on exploring the pharmacological value of G-Rp₁ by performing extensive pre-clinical studies.

In conclusion, we found that G-Rp₁ inhibits promoter activities of iNOS and COX-2 as well as the mRNA levels of these genes. In particular, this compound down-regulates NF- κ B-mediated luciferase activity that is mediated by IKK β , but not by other adaptor and signaling molecules. In agreement with these findings, G-Rp₁ strongly diminished the phosphorylation of I κ B α and p65. Since G-Rp₁ is known to be safe and orally effective [10], our results suggest that G-Rp₁ can be used as a novel drug for the treatment of inflammatory diseases.

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