Inhibition of TNF-α-Mediated NF-κB Transcriptional Activity by Dammarane-Type Ginsenosides from Steamed Flower Buds of Panax ginseng in HepG2 and SK-Hep1 Cells

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Abstract

Panax ginseng is a medicinal herb that is used worldwide. Its medicinal effects are primarily attributable to ginsenosides located in the root, leaf, seed, and flower. The flower buds of Panax ginseng (FBPG) are rich in various bioactive ginsenosides, which exert immunomodulatory and anti-inflammatory activities. The aim of the present study was to assess the effect of 18 ginsenosides isolated from steamed FBPG on the transcriptional activity of NF-κB and the expression of tumor necrosis factor-α (TNF-α)-stimulated target genes in liver-derived cell lines. Noticeably, the ginsenosides Rk3 and Rs4 exerted the strongest activity, inhibiting NF-κB in a dose-dependent manner. SF and Rg6 also showed moderately inhibitory effects. Furthermore, these four compounds inhibited the TNF-α-induced expression of IL8, CXCL1, iNOS, and ICAM1 genes. Consequently, ginsenosides purified from steamed FBPG have therapeutic potential in TNF-α-mediated diseases such as chronic hepatic inflammation.

Key Words: NF-κB inhibitory activity, Panax ginseng flower buds, Tumor necrosis factor-α, Hepatocyte derived cells

INTRODUCTION

Nuclear factor-κB (NF-κB) plays an important role in immune and inflammatory responses by regulating genes encoding pro-inflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes (Li and Verma, 2002; Gasparini and Feldmann, 2012). Therefore, NF-κB pathway inhibitors act as anti-inflammatory compounds (Nam, 2006; Luqman and Pezzuto, 2010). Although inflammation is a basic response to injury or infection, the harmful effects of inflammation can cause a variety of chronic diseases including arthritis, fibrosis, and cancer. Inflammatory responses also play important roles in the development of liver disease in both humans and animals. Hepatitis, which can be either acute or chronic, results from inflammation of the liver, and is characterized by the presence of hepatic inflammatory cells. Chronic hepatitis is associated with a high risk of hepatic carcinoma (Berasain et al., 2009). At the molecular level, free radicals and aldehydes produced during chronic hepatitis can induce deleterious gene mutations and posttranslational modifications in cancer-associated genes (Kawanishi et al., 2006). Other inflammatory products, including cytokines, growth factors, and transcription factors such as nuclear factor-κB (NF-κB), regulate the expression of cancer-related (both tumor suppressor genes and oncogenes) and key inflammatory genes, such as interleukin-8 (IL-8), chemokine (C-X-C motif) ligand-1 (CXCL-1), inducible nitric oxide synthase (iNOS), and intercellular adhesion molecule-1 (ICAM-1) (Lentsch and Ward, 2000; Elsharkawy and Mann, 2007; Holt et al., 2008; Farinati et al., 2010).

Panax ginseng (PG) Meyer (Araliaceae), a traditional herbal drug in Oriental medicine, is used to treat a variety of diseases (Hofseth and Wargovich, 2007; Ernst, 2010; Vuksan et al., 2010). Ginsenosides are the major active components of PG. Although roots are considered to be the best source of PG, leaves also contain high concentrations of ginsenosides (Tung et al., 2009; Liu et al., 2010; Tung et al., 2010a). Therefore, ginseng leaves could function as a supplementary source of pharmacologically active ginsenosides (Christensen, 2009; Wang et al., 2009).

Traditionally, PG root is air-dried, yielding white ginseng, or steamed at 100°C to produce red ginseng. Steamed ginseng is believed to be more pharmacologically effective than air-dried ginseng. The differences in the biological effects of
air-dried and steamed ginseng are attributed to significant changes to ginsenosides during steaming (Baek et al., 1996). However, the anti-inflammatory effects of steamed PG from flowers have not been assessed.

Our previous studies focused on identifying the bioactive constituents in steamed FBPG, leading to the identification of one dammarane-type saponin, ginsenoside SF, and 17 known saponins, including ginsenoside Rh₄, ginsenoside Rk₁, ginsenoside F₁, (20E)-ginsenoside F₄, ginsenoside Rg₂, pseudoginsenoside RC, ginsenoside Rg₁, ginsenoside Rg₃, ginsenoside Rg₆, 6α-acetyl-ginsenoside Rg₁, ginsenoside Rb₁, ginsenoside Re, ginsenoside Rb₂, ginsenoside Re, viraginsenoside R₅, ginsenoside Mb, and ginsenoside F₃ (Tung et al., 2010b). Some of these compounds possess antioxidant and anticancer activities, and inhibit LPS-stimulated IL-12 production (Tung et al., 2010c, 2010d, 2011).

In this study, the effects of 18 ginsenosides, isolated from steamed FBPG, on TNF-α-induced NF-κB transcriptionsal activity in human hepatocyte-derived cells (HepG2 and SK-Hep1) were evaluated using an NF-κB-luciferase assay. Their effects on iNOS promoter activity, and the expression of NF-κB target genes, including interleukin-8 (IL-8), chemokine (C-X-C motif) ligand-1 (CXCL-1), inducible nitric oxide synthase (iNOS), and intercellular adhesion molecule-1 (ICAM-1), were evaluated by RT-PCR in TNF-α-stimulated cells.

MATERIALS AND METHODS

Chemical and sample preparation
Ginsenosides were isolated from the steamed flower buds of *Panax ginseng* (FBPG) as identified in our previous reports (Tung et al., 2010b). Apigenin, potently inhibited the transcriptional activity of NF-κB (Funakoshi-Tago et al., 2011), was the product of Sigma-Aldrich. All other chemicals and reagents were of analytical grade. The tested ginsenosides and apigenin (Sigma-Aldrich) were dissolved in DMSO.

Cell lines and culture
HepG2 and SK-Hep1 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 10 μg/ml streptomycin, at 37°C and 5% CO₂.

Human TNF-α was purchased from ATgen (Seoul, Korea).

Cell toxicity assay
Cell-Counting Kit (CCK)-8 (Dojindo, Kumamoto, Japan) was used to analyze the effect of compounds on cell toxicity according to the manufacturer's instructions. Cells were cultured overnight in 96-well plate (~1×10⁴ cells/well). Cell toxicity was assessed after the addition of compounds on dose-dependent manner. After 24 h of treatment, 10 μl of the CCK-8 solution was added to triplicate wells, and incubated for 1 h. Absorbance was measured at 450 nm to determine viable cell numbers in wells.

NF-κB and iNOS-luciferase assay
Human hepatocarcinoma HepG2 and SK-Hep1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 10 μg/ml streptomycin at 37°C and 5% CO₂. The luciferase vector was first transfected into cells. After a limited amount of time, the cells were lysed, and luciferin, the substrate of luciferase, was introduced into the cellular extract along with Mg²⁺ and an excess of ATP. Under these conditions, luciferase enzymes expressed by the reporter vector could catalyze the oxidative carboxylation of luciferin. Cells were seeded at 2×10⁵ cells per well in a 12-well plate and grown. After 24 h, cells were transfected with inducible NF-κB or iNOS firefly luciferase reporter and constitutively expressing Renilla reporter. After 24 h of transfection, medium was changed to assay medium (Opti-MEM+0.5% FBS+0.1 mM NEAA+1 mM sodium pyruvate+100 units/ml penicillin+10 μg/ml streptomycin) and cells were pretreated for 1 h with either vehicle (DMSO) and compounds, followed by 1 h of treatment with 10 ng/ml TNF-α for 20 h. Unstimulated cells were used as a negative control (−), apigenin was used as a positive control. Dual Luciferase assay was performed 48 h after transfection, and promoter activity values are expressed as arbitrary units using a Renilla reporter for internal normalization.

RNA preparation and reverse transcriptase polymerase chain reaction (RT-PCR)
RNA Preparation and Reverse Transcriptase Polymerase Chain Reaction (RT-PCR): Total RNA was extracted using Easy-blue reagent (Intron Biotechnology, Seoul, Korea). Approximately 2 μg total RNA was subjected to reverse transcription using Moloney murine leukemia virus (MMLV) reverse transcriptase and oligo-dT primers (Promega, Madison, WI, USA) for 1 h at 42°C. PCR for synthetic cDNA was performed using a Taq polymerase pre-mixture (TaKaRa, Japan). The PCR products were separated by electrophoresis on 1% agarose gels and stained with EtBr. PCR was conducted with the following primer pairs: iNOS sense 5’-TCATCCGCTATGCTGGCTAC-3’, iNOS antisense 5’-CTAGGGTTCAGCGCATTG-3’, ICAM-1 sense 5’-CTGCAGAT- CAGTGACCATC-3’, ICAM-1 antisense 5’-GTCAGTTTCCCCGGACAA-3’, IL-8 sense 5’-GGGTCTTGTTAGGTTGCC-3’, IL-8 antisense 5’-TCTGGATCTTTGGATGC-3’, CXCL-1 sense 5’-AGGGAGATTACCCCAAGAAC-3’, CXCL-1 antisense 5’-5’-TACACGTGGGATGCAGA3’, β-actin sense 5’-TCACCCACACTCTGGCCATCG-3’, and β-actin antisense 5’-CACGGAGAATCCTTCTGGGAAGATG-3’. HepG2 and SK-Hep1 cells were pretreated in the absence and presence of compounds for 1 h, then exposed to 10 ng/ml TNF-α for 6 h. Total mRNA was prepared from the cell pellets using Easy-blue. The levels of mRNA were assessed by RT-PCR.

Statistical analysis
Unless otherwise stated, all experiments were performed with triplicate samples and repeated at least three times. All results are expressed as the mean ± S.E.M. Data was analyzed by one-factor analysis of variance (ANOVA). Upon observation of a statistically significant effect, the Newman-Keuls test was performed to determine the difference between the groups. A p value *(<0.05) and **(<0.01) were considered to be significant.

RESULTS
Ginsenosides inhibit NF-κB activity in hepatocyte-derived cell lines
To identify novel NF-κB inhibitors from the steamed flower
buds of *Panax ginseng* (FBPG), 18 dammarane-type ginsenosides were evaluated using the NF-κB reporter system. To determine non-toxic concentrations, HepG2 cells were treated with 0.1, 1, and 10 μM of each compound, and cell viability was assessed by MTS assay. No compounds were significantly cytotoxic at up to 10 μM, suggesting that NF-κB inhibition was not toxic (data not shown). HepG2 cells were then pre-treated with different ginsenosides at concentrations ranging from 0.01 to 10 μM for 1 h, and induced with TNF-α for 20 h. Rk₃ and Rs₄ significantly inhibited TNF-α-induced NF-κB transcriptional activity, with IC₅₀ values of 14.24 ± 1.30 and 12.44 ± 2.01 μM, respectively (Table 1, Fig. 1, 2). SF and Rg₆ also reduced NF-κB transcriptional activity, with IC₅₀ values of 33.86 ± 4.14 and 29.34 ± 2.22 μM, respectively (Table 1, Fig. 1, 2). Six additional ginsenosides F₁, Rg₁, Rb₁, and Rb₂ (20E)-ginsenoside F₄, and pseudoginsenoside RC₁ moderately inhibited the transcriptional activity of NF-κB with IC₅₀ values of 42.51 ± 1.97, 45.47 ± 3.64, 61.22 ± 3.69, 37.46 ± 5.01, 89.62 ± 10.64, and 98.24 ± 7.61 μM, respectively (Fig. 2). Apigenin, the positive control, potently inhibited the transcriptional activity of NF-κB, with an IC₅₀ of 1.64 ± 0.19 μM. These results suggest that the ginsenosides SF, Rk₃, Rg₆, and Rs₄ inhibited TNF-α-induced NF-κB transcriptional activity in HepG2 cells.

To confirm that these compounds inhibited NF-κB activation in HepG2 cells, their effects on TNF-α-induced NF-κB transcriptional activity in SK-Hep1 hepatocyte-derived cells were evaluated using the

![Chemical structures of the ginsenosides SF, Rk₃, Rg₆, and Rs₄.](image-url)

**Table 1.** Inhibitory effects of ginsenosides SF, Rk₃, Rg₆, and Rs₄ on the TNF-α-induced NF-κB transcriptional activity and iNOS promoter activity in HepG2 and SK-Hep1 hepatocyte-derived cells

| Ginsenoside or Compound | IC₅₀ (μM)* | HepG2 | SK-Hep1 |
|-------------------------|-----------|-------|---------|
| SF                      | 33.86 ± 4.14 | 13.07 ± 0.64 | 18.17 ± 0.69 |
| Rk₃                     | 14.24 ± 1.30 | 9.83 ± 0.06 | 15.32 ± 0.29 |
| Rg₆                     | 29.34 ± 2.22 | 19.45 ± 0.55 | 25.12 ± 1.04 |
| Rs₄                     | 12.44 ± 2.01 | 10.01 ± 1.21 | 11.98 ± 0.85 |
| Apigenin**              | 1.64 ± 0.19 | 4.45 ± 0.24 | 3.60 ± 0.21 |

*Results are the means ± SEM of three independent experiments performed in triplicate.

**Positive control.** Apigenin
NF-κB reporter system. Cells were pretreated with the four ginsenosides at concentrations ranging from 0.01 to 10 μM for 1 h, and then induced with TNF-α for 20 h. SF, Rk3, Rg6, and Rs4 significantly inhibited TNF-α-induced NF-κB transcriptional activity, with IC50 values of 10.17 ± 0.69, 15.32 ± 0.29, 25.12 ± 1.04, and 11.98 ± 0.85 μM, respectively, consistent with the data from HepG2 cells (Table 1, Fig. 3). As expected, apigenin potently inhibited NF-κB transcriptional activity, with an IC50 of 3.60 ± 0.21 μM. These data suggest that the ginsenosides SF, Rk3, Rg6, and Rs4 inhibit NF-κB-induced NF-κB transcriptional activity in HepG2 and SK-Hep1 cells.

Effect of ginsenosides on NF-κB target genes expression

NF-κB regulates several genes involved in immunity, inflammation, and cell proliferation, as well as those that result in the negative feedback of NF-κB signaling (Gasparini and Feldmann, 2012). Therefore, we assessed expression of NF-κB target genes that play an important role in the inflammatory response, including IL-8 (cytokine), CXCL-1 (chemokine), ICAM-1 (migration), and iNOS (inflammatory inducible enzyme), in HepG2 and SK-Hep1 cells treated with ginsenosides (Fig. 4). Consistent with the inhibition of NF-κB, SF, Rk3, Rg6, and Rs4 all inhibited the induction of IL8, CXCL1, iNOS, and ICAM1 mRNA significantly in a dose-dependent manner, suggesting that these compounds reduced the transcription of these genes. Importantly, the expression of the housekeeping protein β-actin was unchanged by ginsenosides.

The ginsenosides SF, Rk3, Rg6, and Rs4 also decreased TNF-α-induced iNOS promoter activity, with IC50 values ranging from 6 to 20 μM (Table 1, Fig. 5). These data suggest that the dammarane-type ginsenosides isolated from steamed FBPG suppress TNF-α-induced NF-κB transcriptional activity via the inhibition of iNOS gene transcription.

DISCUSSION

The aim of the present study was to identify novel inhibitors of NF-κB, a transcription factor that is a major target in drug discovery due to its causative role in inflammation, cancer, and many other diseases. Eighteen ginsenosides were isolated from the flower buds of Panax ginseng (FBPG), and their ability to inhibit NF-κB activation was assessed. Four ginsenosides (22%) inhibited NF-κB activity at a concentration of 10 μM. This high percentage of active compounds was not a surprise, since extracts from ginseng and saponins are recognized as promising NF-κB inhibitors. For example, the NF-κB-inhibiting saponin ginsenosides Rd, Re, and Rp3 are currently being assessed in preclinical and clinical trials (Park et al., 2008; Lee et al., 2012; Wang et al., 2012).

In this study, the ginsenosides SF, Rk3, Rg6, and Rs4 inhibited TNF-α-induced NF-κB promoter activity in hepatocyte-derived HepG2 and SK-Hep1 cells in a dose-dependent man-

http://dx.doi.org/10.4062/biomolther.2013.096
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by modulating gene transcription. They also inhibited the transcription of the NF-κB target genes *IL8*, *CXCL1*, *iNOS*, and *ICAM-1* gene expression in HepG2 and SK-Hep1 hepatocyte-derived cells. HepG2 and SK-Hep1 cells were pretreated with the ginsenosides SF, Rk3, Rg6, and Rs4, or the vehicle (DMSO), for 1 hour, then treated with TNF-α (10 ng/ml) for 6 h. Total mRNA was extracted from the cell pellets using TRIzol reagent. Relative mRNA levels were assessed by RT-PCR. Expression levels are displayed as the ratio of *IL-8*, *CXCL-1*, *iNOS*, and *ICAM-1* signal strength to a reference gene (β-actin), compensating for variations in the RNA concentrations.

In conclusion, we demonstrated that the ginsenosides SF, Rk3, Rg6, and Rs4, isolated from steamed flower buds of *Panax ginseng*, suppressed TNF-α-induced NF-κB activation, which subsequently inhibited the expression of *IL8*, *CXCL1*, *iNOS*, and *ICAM1* in hepatocyte-derived HepG2 and SK-Hep1 cells. Our data demonstrated that these compounds have therapeutic potential as anti-inflammatory agents. However, the detailed mechanism by which they inhibit TNF-α-induced...
NF-κB activation remains to be elucidated.

ACKNOWLEDGMENTS

This study was supported by a grant from the Priority Research Center Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0093815), Republic of Korea.

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