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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 Rk_3 and Rs_4 exerted the strongest activity, inhibiting NF-κB in a dose-dependent manner. SF and Rg_6 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

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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 Rh_4, ginsenoside Rk_3, ginsenoside F_1, (20*E*)-ginsenoside F_4, ginsenoside Rg_2, pseudoginsenoside RC_1, ginsenoside Rg_6, ginsenoside Rs_4, ginsenoside Rg_1, 6'-acetyl-ginsenoside Rg_1, ginsenoside Rb_1, ginsenoside Rc, ginsenoside Rb_2, ginsenoside Re, vinaginsenoside R_4, ginsenoside Mb, and ginsenoside F_4 (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 transcriptional 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 37ºC and 5% CO_2. 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 (~1×10^4 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 Eagles' medium (DMEM) (Invitrogen, Carlsbad, CA, USA) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 10 μg/ml streptomycin at 37ºC and 5% CO_2. 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 Mg^{2+} 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 2×10^5 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, Madi-son, WI, USA) for 1 h at 42ºC. 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'-CTCAGGGTCACGGCCATTG-3', *ICAM-1* sense 5'-CTGCAGACAGTGACCATC-3', *ICAM-1* antisense 5'-GTCCAGTTTCCCGGACAA-3', *IL-8* sense 5'-GGGTCTGTTGTAG-GGTTGCC-3', *IL-8* antisense 5'-TCTGGATCCTGGCTAGCA-GA-3', *CXCL-1* sense 5'-AGGGAATTCACCCCAAGAAC-3', *CXCL-1* antisense 5'-5'-TAACTATGGGGGATGCAGGA-3', β-actin sense 5'-TCACCCACACTGTGCCCATCTACG-3', and β-actin antisense 5'-CAGCGGAACCGCTCATTGCCAATG-3'. HepG2 and SK-Hep1 cells were pretreated in the absence and presence of compounds for 1 hr, 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

Table 1. Inhibitory effects of ginsenosides SF, Rk_3, Rg_6, and Rs_4 on the TNF-α-induced NF-κB transcriptional activity and iNOS promoter activity in HepG2 and SK-Hep1 hepatocyte-derived cells

Ginsenoside or Compound	IC_{50} (μM)*			
	HepG2		SK-Hep1	
	NF-κB	iNOS	NF-κB	iNOS
Ginsenoside SF	33.86 ± 4.14	13.07 ± 0.64	18.17 ± 0.69	11.26 ± 0.87
Ginsenoside Rk_3	14.24 ± 1.30	9.83 ± 0.06	15.32 ± 0.29	6.02 ± 0.37
Ginsenoside Rg_6	29.34 ± 2.22	19.45 ± 0.55	25.12 ± 1.04	10.36 ± 0.69
Ginsenoside Rs_4	12.44 ± 2.01	10.01 ± 1.21	11.98 ± 0.85	6.92 ± 0.65
Apigenin**	1.64 ± 0.19	4.45 ± 0.24	3.60 ± 0.21	3.63 ± 0.17

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

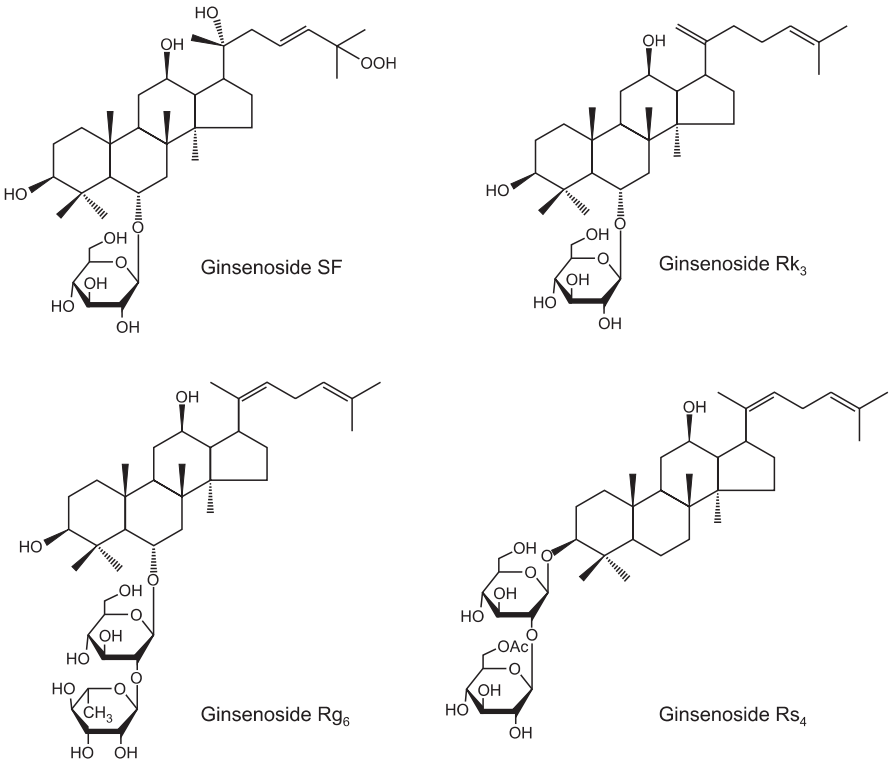

Fig. 1. Chemical structures of the ginsenosides SF, Rk_3, Rg_6, and Rs_4.

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 pretreated with different ginsenosides at concentrations ranging from 0.01 to 10 μM for 1 h, and induced with TNF-α for 20 h. Rk_3 and Rs_4 significantly inhibited TNF-α-induced NF-κB transcriptional activity, with IC_{50} values of 14.24 ± 1.30 and 12.44 ± 2.01 μM, respectively (Table 1, Fig. 1, 2). SF and Rg_6 also reduced NF-κB transcriptional activity, with IC_{50} values of 33.86 ± 4.14 and 29.34 ± 2.22 μM, respectively (Table 1, Fig. 1, 2). Six additional ginsenosides F_1, Rg_1, Rb_1, and Rb_2, (20*E*)-ginsenoside F_4, and pseudoginsenoside RC_1 moderately inhibited the transcriptional activity of NF-κB with IC_{50} 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). Finally, ginsenosides Rh_4, Rg_2, Rc, Re, Mb, F_4, 6'-acetyl-ginsenoside Rg_1, and vinaginsenoside R_4 had no effect (Fig. 2). Apigenin, the positive control, potently inhibited the transcriptional activity of NF-κB, with an IC_{50} of 1.64 ± 0.19 μM. These results suggest that the ginsenosides SF, Rk_3, Rg_6, and Rs_4 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 cells were evaluated using the

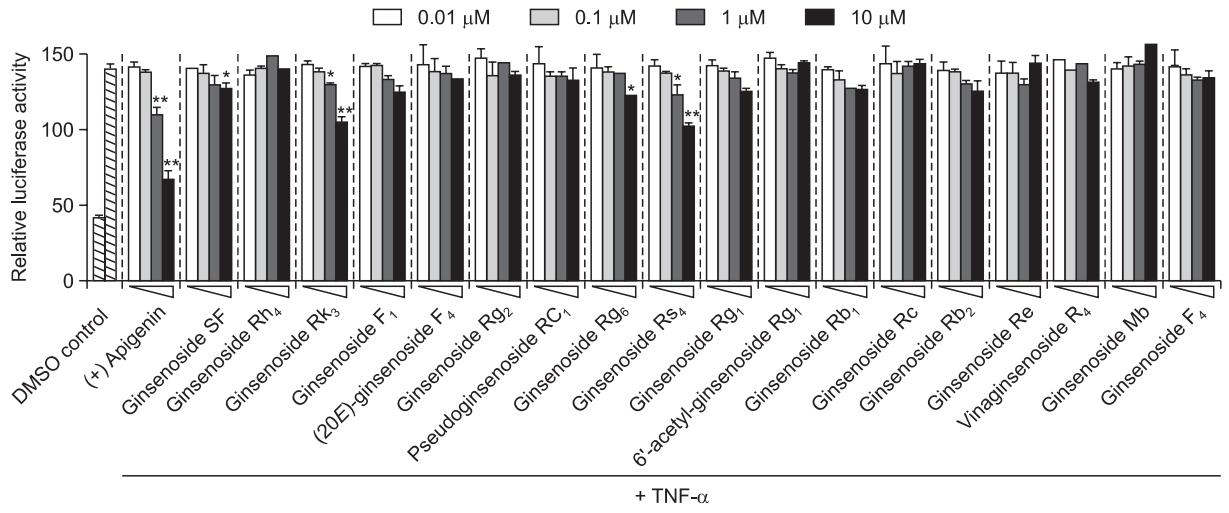

Fig. 2. Effect of 18 ginsenosides on TNF-α-stimulated NF-κB transcriptional activity in HepG2 cells. Cells transiently transfected with pNF-κB-Luc were pretreated for 1 h with either vehicle (DMSO) or compounds, then treated with TNF-α (10 ng/ml). Unstimulated cells acted as a negative control. Cells were harvested and assayed for luciferase activity. Apigenin was used as a positive control (+). Results are expressed as the mean luciferase activity ± S.E.M. (n=3). Statistical significance is indicated as **p*<0.05, ***p*<0.01.

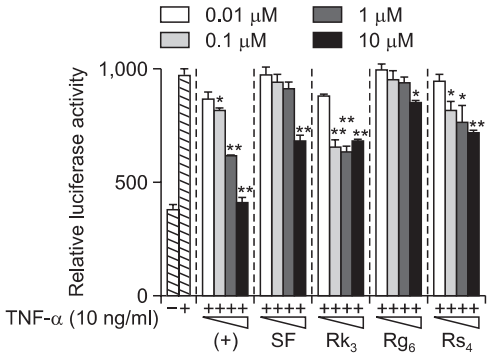

Fig. 3. Effect of ginsenosides SF, Rk_3, Rg_6, and Rs_4 on TNF-α stimulated NF-κB transcriptional activity in SK-Hep1 cells. Cells transiently transfected with pNF-κB-Luc were pretreated for 1 h with either vehicle (DMSO) or compounds, then treated with TNF-α (10 ng/ml). Unstimulated cells acted as a negative control. Cells were harvested and assayed for luciferase activity. Results are expressed as the mean luciferase activity ± SEM (n=3). Statistical significance is indicated as **p*<0.05, ***p*<0.01.

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, Rk_3, Rg_6, and Rs_4 significantly inhibited TNF-α-induced NF-κB transcriptional activity, with IC_{50} 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 IC_{50} of 3.60 ± 0.21 μM. These data suggest that the ginsenosides SF, Rk_3, Rg_6, and Rs_4 inhibited TNF-α-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, Rk_3, Rg_6, and Rs_4 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, Rk_3, Rg_6, and Rs_4 also decreased TNF-α-induced iNOS promoter activity, with IC_{50} 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 TNF-α-induced 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 Rp_1 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, Rk_3, Rg_6, and Rs_4 inhibited TNF-α-induced NF-κB promoter activity in hepatocyte-derived HepG2 and SK-Hep1 cells in a dose-dependent man-

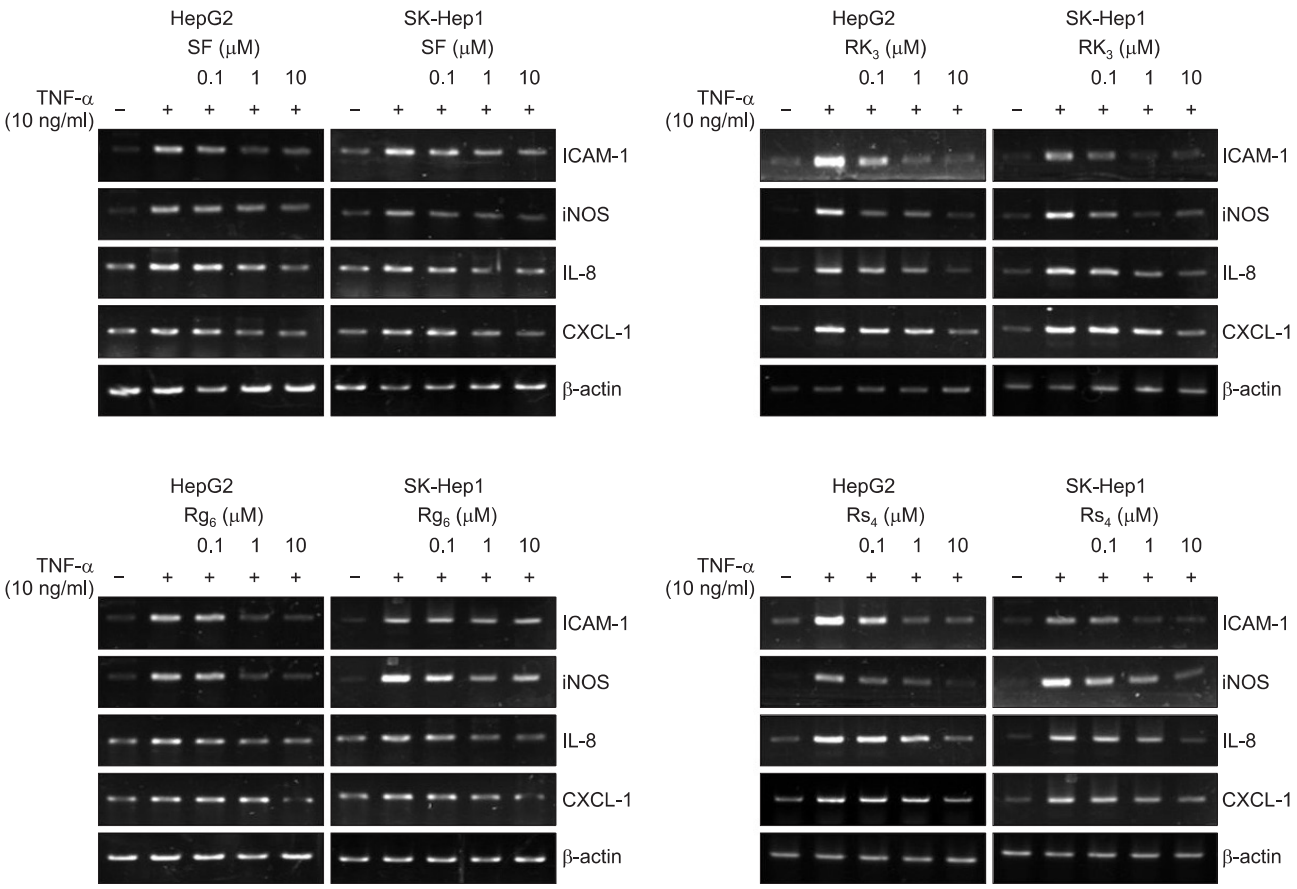

Fig. 4. Effect of ginsenosides SF, Rk_3, Rg_6, and Rs_4 on *IL-8*, *CXCL-1*, *iNOS*, and *ICAM-1* gene expression in HepG2 and SK-Hep1 hepatocyte-derived cells. HepG2 and SK-Hep1 cells were pretreated with the ginsenosides SF, Rk_3, Rg_6, and Rs_4, or the vehicle (DMSO), for 1 hour, then treated with 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.

ner by modulating gene transcription. They also inhibited the transcription of the NF-κB target genes *IL8*, *CXCL1*, *iNOS*, and *ICAM1*, as demonstrated by reduced mRNA expression in TNF-α-treated HepG2 and SK-Hep1 cells. To our knowledge, this is the first report of these effects. Of the remaining compounds, ginsenosides F_1, Rg_1, Rb_1, Rb_2, (20*E*)-ginsenoside F_4, and pseudoginsenoside RC_1 exerted only small inhibitory effects, with IC_{50} values of 42.51, 45.47, 61.22, 37.46, 89.62, and 98.24 μM, respectively. Furthermore, ginsenosides Rh_4, Rg_2, Rc, Re, Mb, F_4, 6'-acetyl-ginsenoside Rg_1, and vinaginsenoside R_4 had no effect.

We demonstrated previously that ginsenosides F_1, Rg_1, Rb_1, and Rb_2, isolated from the air dried leaves of *Panax ginseng*, moderately inhibited NF-κB in HepG2 cells (Song *et al*., 2012). Furthermore, ginsenoside Rc and Re had no effects, consistent with this study.

We also reported that the ginsenoside Rg_6 potently inhibited LPS-induced IL-12 production by 82% in bone marrow-derived dendritic cells (Tung *et al*., 2011). Consistent with this, ginsenoside Rg_6 also exerted anti-complement effects, with an IC_{50} of 174 μM, in antibody-sensitized sheep erythrocytes (EA) as indicator cells (Lee *et al*., 2011). Ginsenoside Rk_3 exerts cardioprotective effects against hypoxia-reoxygenation injury via the phosphatidylinositol 3-kinase (PI3K)/AKT and mitogen-activated protein kinase (MAPK) signaling pathways in H9c2 cardiomyocytes (Sun *et al*., 2013). In addition, ginsenoside Rk_3 and Rs_4 exert anti-proliferative effects, with IC_{50} values of 187 and 20 μM, respectively, in human hepatocarcinoma cells (Kim *et al*., 1999; Toh *et al*., 2011). Inhibiting NF-κB by blocking TNF-α resulted in apoptosis in transformed hepatocytes, and inhibited hepatocarcinogenesis. Therefore, the NF-κB signaling pathway may have pro-carcinogenic effects, and so may be a potential target for novel anti-tumor agents (Mantovani *et al*., 2008). Based on our data, we suggest that the ginsenosides SF, Rk_3, Rg_6, and Rs_4 may act as NF-κB pathway inhibitors that could be applied as targeted therapies for conditions such as inflammatory hepatic cancer and inflammatory hepatitis.

In conclusion, we demonstrated that the ginsenosides SF, Rk_3, Rg_6, and Rs_4, 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

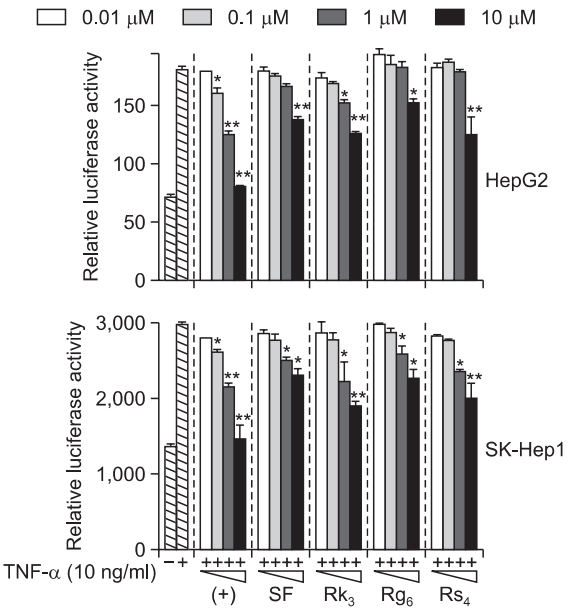

Fig. 5. Effect of ginsenosides SF, Rk_3, Rg_6, and Rs_4 on TNF-α-stimulated iNOS promoter activity. HepG2 and SK-Hep1 cells transiently transfected with iNOS-Luc were pretreated for 1 h with either vehicle (DMSO) or compounds, then treated with TNF-α (10 ng/ml). Unstimulated HepG2 and SK-Hep1 cells acted as a negative control. Cells were harvested and assayed for luciferase activity. Results are expressed as the mean luciferase activity ± S.E.M. (n=3). Statistical significance is indicated as **p*<0.05, ***p*<0.01.

NF-κB activation remains to be elucidated.

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