Differential effects of ginsenosides on NO and TNF-α production by LPS-activated N9 microglia

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Abstract

Ginsenosides, the main active components of ginseng, have been reported to exert neuroprotective effects in the central nervous system. In this report, the effects of ginsenoside-Rd and -Rb2, two protopanaxadiols, and ginsenoside-Rg1 and -Re, two protopanaxatriols, on the production of nitric oxide (NO) and TNF-alpha (TNF-α) by lipopolysaccharide (LPS)-activated N9 microglial cells were studied. All ginsenosides studied potently suppressed TNF-α production in LPS-activated N9 cells. Ginsenoside-Rg1 and -Re, but not ginsenoside-Rb2 and -Rd, inhibited the production of NO in LPS-activated N9 cells. Ginsenosides inhibited the phosphorylation of c-Jun NH2-terminal kinase (JNK), c-Jun and extracellular signal-regulated kinase (ERK). The findings herein show that the inhibition of LPS-induced ERK1/2 and JNK activation may be a contributing factor to the main mechanisms by which ginsenosides inhibits RAW264.7. To clarify the mechanistic basis for its ability to inhibit TNF-α and NO induction, the effect of ginsenosides on transcription factor NF-κB protein level was also examined. These activities were associated with the down-regulation of inhibitor κB (IκB). These findings suggest that the inhibition of LPS-induced NO formation and TNF-α production in microglia by ginsenosides is due to its inhibition of NF-κB, which may be the mechanistic basis for the anti-inflammatory effects of ginsenosides. The significant suppressive effects of ginsenosides on proinflammatory responses of microglia implicate their therapeutic potential in neurodegenerative diseases accompanied by microglial activation.

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1. Introduction

Microglia is the main resident immunocompetent and phagocytic cell in CNS [1]. Activation of microglia could induce the release of some neurotoxic substances such as nitric oxide (NO) and TNF-α; which mediates inflammatory processes in CNS [2,3]. Presence of activated microglia has been demonstrated in pathological lesions in several neurological diseases including Alzheimer’s disease (AD), Parkinson’s disease (PD) [4], multiple sclerosis (MS) [5], and acquired immune deficiency syndrome dementia [6]. The root of Panax ginseng C.A. Meyer (Ginseng) has been used in traditional Chinese medicine for treatment
Fig. 1. The chemical structures of the ginsenosides used in the experiments.

Ginsenoside Rg1  glc  H  o-glc
Ginsenoside Re   glc  H  o-rham(1-2)glc
Ginsenoside Rb2  ara(1-6)glc  glc(1-2)glc  H
Ginsenoside Rd   glc  glc(1-2)glc  H

Fig. 2. The effects of ginsenosides on NO production induced by LPS in N9 cells. N9 cells were treated with ginsenosides and LPS (1 μg/ml) simultaneously for 48 h. The results are expressed as the percentage values taking LPS treatment group as 100%. Data were presented as the means±SEM of three separate experiments. ###P<0.001 as compared to the control group (cultured in medium alone), *P<0.05, **P<0.01, ***P<0.001 as compared to groups treated with LPS alone, as examined with Dunnett’s t test.

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of a wide variety of ailments. The major active ingredients of ginseng are ginseng saponins, or called as ginsenosides. Chemically, ginsenosides are triterpene saponins. According to the structure of the non-sugar (aglycon) part of the molecule, ginsenosides have been classified into three groups: protopanaxadiol-type ginsenosides, protopanaxatriol-type ginsenosides, and oleanolic acid-type saponins. Studies have demonstrated that ginseng extracts or ginsenosides, in addition to their actions on the CNS and the cardiovascular system, also exhibit immunomodulatory activities [7–9].

Since activated microglia play an important pathological role in neurodegenerative diseases and ginseng has been used in the treatment of such diseases as part of the traditional Chinese medicine, the present studies tested the effects of 4 ginseng saponins, ginsenoside-Rb2, -Rd, -Rg1 and -Re, belonging to the protopanaxadiol and protopanaxatriol groups respectively (Fig. 1), on TNF-α and NO release by LPS-activated N9 microglial cells. It was found that selected ginsenosides suppressed microglial activation by LPS.

2. Materials and methods

2.1. Materials

Ginsenoside-Rb2, -Re, -Rd, and -Rg1 were isolated by the Department of Chemistry for Nature Products, Shenyang Pharmaceutical University. Ginsenoside-Rb2, -Rd and -Re were dissolved in PBS. Ginsenoside-Rg1 was dissolved initially in dimethyl sulfoxide (DMSO) and was diluted with PBS for experiments. DMSO at the highest concentration possibly present in experimental conditions (0.1%) was not toxic to cells. LPS (E5:055) and L-NAME were purchased from Sigma (St. Louis, MO, USA). Thiazolyl blue (MTT) was purchased from Sino-American Biotechnology Company (Beijing, China). mTNF-α was purchased from Diaclone (Besancon Cedex, France). Iscove’s modified Dulbecco’s medium (IMDM) was from Gibco BRL (Grand Island, USA). Fetal bovine serum (FBS) was purchased from TBD Biotechnology Development (Tianjin, China).

2.2. Cell culture

The murine N9 microglial cell line was a kind gift from Dr. P. Ricciardi-Castagnoli (Universita Degli Studi di Milano-

Fig. 3. The effects of ginsenosides on TNF-α release by LPS in N9 cells. N9 cells were treated with ginsenosides and LPS (1 μg/ml) simultaneously for 48 h. Data were presented as the means ± SEM of two separate experiments. ***P<0.001 as compared to the control group, *P<0.05, **P<0.01, ***P<0.001 as compared to groups treated with LPS alone, as examined by Dunnett’s t test.
Bicocca, Milan, Italy). The cells were grown in IMDM supplemented with 5% heat-inactivated FBS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5×10⁻⁵ M 2-mercaptoethanol [10].

2.3. Cell viability

Cell viability was evaluated by the MTT reduction assay [11]. The cells were incubated with MTT (0.25 mg/ml) for 3 h at 37 °C. The formazan crystals in the cells were solubilized with a solution containing 50% dimethylformamide and 20% sodium dodecyl sulfate (pH 4.7). The level of MTT formazan was determined by measuring its absorbance at the wavelength of 490 nm with a SPECTRA (shell) Reader (TECAN, Austria).

2.4. TNF-α production

Cells at density of 3×10⁴ cells/well were plated onto 96-well microtiter plates. Ginsenosides with LPS (1 μg/ml) were added to the culture medium of N9 microglial cells for 48 h. The levels of mTNF-α induced by LPS in N9 microglial cells were measured by using an ELISA kit according to the manufacturer’s instructions (Diaclone, France).

2.5. Nitrite assays

Accumulation of nitrite (NO₂⁻) in the culture media, an indicator of NO synthase activity, was measured by Griess reaction [12]. Cells at density of 3×10⁴ cells/well were plated

![Fig. 4. The effects of ginsenosides on ERK, JNK and c-Jun phosphorylation induced by LPS in N9 cells. N9 cells were treated with different concentrations of ginsenosides for 30 min followed by LPS (1 μg/ml) for 30 min. The cells were then lysed and measured for ERK1/2, JNK and c-Jun phosphorylation by Western blotting.]
onto 96-well microtiter plates. Ginsenosides with or without LPS (1 μg/ml) were added to the culture medium of N9 microglial cells for 48 h. Fifty microliter culture supernatants were mixed with 50 μl Griess reagent (part I: 1% sulphanilamide; Part II: 0.1% naphthylethylene diamide dihydrochloride and 2% phosphoric acid) at room temperature. Fifteen minutes later, the absorbance was determined at 540 nm using the SPECTRA (shell) Reader. Nitrite concentration was calculated with reference to a standard curve of sodium nitrite.

2.6. Western blotting

Cells at density of $5 \times 10^5$ cells/cm$^2$ were plated into 25 cm$^2$ flasks. N9 cells were treated with ginsenosides for 30 min followed by LPS (1 μg/ml) for 30 min. Western blot analysis was performed as previously described [13], with modifications. Briefly, cells were washed with ice-cold PBS and solubilized with lysis buffer. The protein concentration in the supernatant of the lysate was measured by BCA protein assay (Pierce, Rockfold, IL). The cell lysate containing 30 μg proteins was electrophoresed by 12% SDS-PAGE, and transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech). The membranes were soaked in blocking buffer (5% skimmed milk) and then incubated overnight with primary antibodies against phosphorylated ERK1/2 (1:200) and total ERK1/2 (1:500), phosphorylated JNK (1:1000), NF-κB (1:1000), phosphorylated IκBα (1:1000), phosphorylated c-Jun (1:1000) (note: all used primary antibodies in the experiment were purchased from Santa Cruz Biotech) followed by horseradish peroxidase conjugated secondary antibodies. The protein of interest was detected by enhanced chemiluminescence reagents from Amersham (Arlington Heights, IL).

2.7. Data analysis

Results were expressed as mean±SEM of three experiments (or replicate experiments) performed in triplicate samples. One-way ANOVA followed by Dunnett’s $t$-test were used for statistical analyses (SPSS 12.0 software, SPSS Inc., USA).

3. Results

3.1. The effects of ginsenosides on LPS-induced NO production

As a first step, the cytotoxic activity of ginsenosides on N9 microglial cell in the absence and presence of LPS was assessed using MTT assays. Co-treatment of both unstimulated and stimulated N9 cells with ginsenoside-Rg1, -Re, -Rb2 and -Rd did not affect the cell viability (data not shown).

In unstimulated N9 cells only small amounts of NO$_2^-$ (3.1±0.2 μM) could be detected in medium. Pretreatment of unstimulated cells with ginsenosides over 48 h did not result in any change in the production of NO$_2^-$ (data not shown). However, stimulation of N9 cells with LPS resulted in a marked increase in NO$_2^-$ production (28.3±7.8 μM). Ginsenoside-Rg1 (0.01–10 μM) and -Re (10, 100 μM), which belong to the protopanaxatriol group, decreased the NO$_2^-$ production. However, ginsenoside-Rb2 and -Rd, which belong to protopanaxadiol group, did not show any effect. In the parallel experiments, L-NAME, a NO synthase inhibitor, significantly suppressed NO production (Fig. 2).

3.2. The effects of ginsenosides on LPS-induced TNF-α secretion by microglial cells

Unstimulated N9 cells produced low amount of TNF-α. However, in LPS-stimulated N9 cells a significant increase in

![Fig. 5. The effects of ginsenosides on IκBα phosphorylation and NF-κB induced by LPS in N9 cells. N9 cells were treated with different concentrations of ginsenosides for 30 min followed by LPS (1 μg/ml) for 30 min. The cells were then lysed and measured for IκBα phosphorylation and NF-κB by Western blotting.](image-url)
the secretion of TNF-α was observed. Co-treatment of N9 cells with ginsenosides significantly decreased LPS-induced TNF-α secretion (Fig. 3).

3.3. The effects of ginsenosides on ERK, JNK, and c-Jun phosphorylation induced by LPS

Because ginsenosides selectively inhibited the production of NO and TNF-α in LPS-activated N9 microglial cells, the potential mechanisms by which ginsenosides exerted their effects were then explored, by examining the capacities of ginsenosides to regulate phosphorylation of ERK, JNK, and c-Jun, the crucial regulators of cell functions. In N9 microglial cells, ginsenosides treatment for 30 min significantly reduced the levels of ERK, JNK and c-Jun phosphorylation induced by LPS (Fig. 4A, B). Ginsenosides by themselves had no significant effects on ERK, JNK and c-Jun phosphorylation (data not shown). These results indicated that ginsenosides were capable of interrupting LPS signaling cascade in microglial cells, leading to the inhibition of NO and TNF-α release.

3.4. The effects of ginsenosides on NF-κB and IκBα phosphorylation induced by LPS

The subsequent experiments were designed to elucidate the role of NF-κB in the process of LPS stimulated N9 microglial cells, as the LPS-mediated NF-κB activation is correlated with the phosphorylation of IκBα and subsequent degradation. Here, NF-κB and phosphorylation of IκBα protein levels were detected by an immunoblot analysis. As shown in Fig. 5, the amount of NF-κB levels were increased by treatment with LPS for 30 min, and after 30 min period of treatment with ginsenosides, studied ginsenosides could effectively decreased the NF-κB protein levels. To confirm the results of inhibition on expression of NF-κB, the phosphorylation of IκBα was also assessed. The results showed that the phosphorylation of IκBα was also inhibited by ginsenosides in LPS-induced N9 microglial cells. This suggests that ginsenosides inhibit NO and TNF-α production via the down-regulation of phosphorylation of IκBα and thus prevent NF-κB activation.

4. Discussion

Improper up-regulation of NO and TNF-α is associated with the pathogenesis of inflammatory disease in the CNS. Previous studies have shown that protein kinase C (PKC), protein kinase A (PKA), CaMPK and mitogen activated protein kinases (MAPKs) are involved in LPS-induced signal transduction leading to TNF-α production and iNOS expression in microglia [14] and peripheral blood mononuclear cells [15]. The production of TNF-α is crucial for the synergistic induction of NO synthesis in LPS-stimulated macrophages [16,17]. However, the present study showed that all ginsenosides tested have significant inhibitory effects on LPS-induced TNF-α secretion in microglia, although they belong to different structure groups of ginsenosides. Interestingly, only ginsenoside-Rg1 and -Re, two protopanaxatriol, but not ginsenoside-Rb2 and -Rd, two protopanaxadiol, inhibited LPS-induced NO production in microglia. These results suggest that different ginsenosides may selectively regulate NO and TNF-α productions by microglial cells. Therefore they may have distinct pharmacological significance in the treatment of inflammatory disease in the CNS.

As shown in Fig. 1, the difference between protopanaxadiol-type ginsenosides and protopanaxatriol-type ginsenosides is the number of hydroxyl moieties. It is assumed that the number of hydroxyl moieties in the ginseng triterpene saponins would be one of the crucial factors to differentiate their effect on LPS-induced NO production in microglia. On the other hand, the number and type of sugars in the ginseng saponins may determine their potency on suppression of both TNF-α and NO production.

It is known that ginsenosides can be metabolized by human intestinal bacteria into their metabolites either protopanaxadiol or protopanaxatriol, then are absorbed into the blood. Although ginsenosides are scarcely detectable in the brain, probably due to the difficulty for penetrating the blood–brain barrier, many studies have demonstrated their central activities by peripheral administration in vivo experiments [18,19]. The present studies did not test the possible effects of the metabolites of these ginsenosides in these parameters. However, studies have demonstrated that the metabolites of these ginsenosides also possess potential biological activities. Compound K, a metabolite of Rb2, and Rd promote T cell proliferation, strength the NK cell activity [20,21]. Oh et al. have found that 20(S)-protopanaxatriol (PPT), one of the major ginsenoside metabolites, blocks the increase in LPS-induced iNOS and COX-2 expressions through inactivation of nuclear factor-kappa B by preventing I-kappa B alpha phosphorylation and degradation [22]. Recently results have shown that compound K transformed from Rb2 is effective against inflammation [23]. Therefore, the present results suggest that both ginsenosides and their metabolites exert biological activities and in certain situations they might share some common activities.

Meanwhile, it should be noticed that ginsenosides did not show good dose-dependent effect on the inhibition of TNF-α and NO production in the present study. This phenomenon is also observed in other studies by using ginsenosides [24–26]. The complexity and diversity of metabolites result in difference of biological activities should be also considered. Changes of pH, temperature,
amount of enzyme and concentrations of ginsenosides might affect the transformation rate of ginsenosides. However, the exact mechanisms that determine the activities of ginseng saponins require further investigation.

ERK1/2 and JNK activities contributing to the up-regulation of iNOS expression in macrophages cells have been reported [24,25]. The ERK and JNK pathways are identified to be of major relevance for the LPS-mediated expression of iNOS. All ginsenosides studied reduced LPS-induced phosphorylations of ERK1/2, JNK and c-Jun, the key molecules in the signaling cascade of LPS. It is interesting to note that with increasing concentrations, ginsenosides appeared to have more potent inhibitory effect on phosphorylations of ERK, JNK and c-Jun induced by LPS. At the higher concentrations, all ginsenosides completely abolished LPS-induced ERK, JNK and c-Jun phosphorylations in N9 cells. The promoter region of the murine gene encoding iNOS contains two NF-κB binding sites, located 55 and 971 bp upstream of the TATA box [26]. The binding of the potentially relevant transcription factor, NF-κB, to the κB sites has been shown to be functionally important for iNOS induction by LPS. The NF-κB family of transcription factors regulates the expression of many genes, including the iNOS gene, which is involved in immune and inflammatory responses. NF-κB is located in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, IκB [27]. External stimuli for macrophage activation increase the phosphorylation of IκBα, leading to its degradation and the simultaneous activation and translocation of NF-κB to the nucleus for binding to its cognate DNA binding site in the regulation region of a variety of genes. The capacity of ginsenosides to inhibit ERK1/2, JNK and c-Jun phosphorylation mediated by LPS in N9 cells suggests a broader and complex pattern of the regulatory role of ginsenosides in microglial activation.

The present study demonstrated for the first time that ginsenosides with divergent structure have different effects on the production of TNF-α and NO by LPS-activated N9 microglial cells. Both protopanaxatriol-type ginsenosides-Rg1, -Re and protopanaxadiol-type ginsenosides-Rb2, -Rd inhibited LPS-induced TNF-α production and ERK1/2, JNK and c-Jun phosphorylations. Moreover, ginsenoside-Rg1 and -Re also inhibited LPS-induced NO production. The data demonstrated that ginsenosides inhibited the LPS-induced production of TNF-α and NO production in N9 cells by blocking NF-κB activation. Because NF-κB is one of the critical transcription factors that regulates the transcription of many genes associated with inflammation, the inhibition of this transcription factor by ginsenosides suggest that propanaxatriol-type ginsenosides might have better potential as leading compounds for the prevention or treatment of inflammation-related neurodegenerative diseases in CNS.

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