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Effect of Ginsenoside Rd on Nitric Oxide System Induced by Lipopolysaccharide Plus TNF-α in C6 Rat Glioma Cells

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Effects of ginsenosides on nitric oxide (NO) production induced by lipopolysaccharide plus TNF-α (LNT) were examined in C6 rat glioma cells. Among several ginsenosides, ginsenoside Rd showed a complete inhibition against LNT-induced NO production. Ginsenoside Rd attenuated LNT-induced increased phosphorylation of ERK. Among several immediate early gene products, only Jun B and Fra-1 protein levels were increased by LNT, and ginsenoside Rd attenuated Jun B and Fra-1 protein levels induced by LNT. Furthermore, LNT increased AP-1 DNA binding activities, which were partially inhibited by ginsenoside Rd. Our results suggest that ginsenoside Rd exerts an inhibitory action against NO production via blocking phosphorylation of ERK, in turn, suppressing immediate early gene products such as Jun B and Fra-1 in C6 glioma cells.

Key words : Ginsenoside Rd, Lipopolysaccharide, TNF-α, Nitric oxide, C6 glioma cell, ERK, Jun B, Fra-1

INTRODUCTION

Nitric oxide (NO) is a short-lived molecule that mediates a wide range of biologic effects. Its biological effects include vasorelaxation (Palmer *et al.*, 1987), neuro-transmission (Garthwaite, 1991), inhibition of platelet aggregation (Radomski *et al.*, 1987), as well as microbial and tumor cell killing (Nathan, 1992). The enzyme responsible for NO synthesis, nitric oxide synthase (NOS), converts L-arginine to L-citrulline and NO (Marletta, 1993). In the brain, three genes encode NO synthase isoforms, with significant differences in their regulation. Neuronal and endothelial NOS are constitutively expressed in astrocytes and in subpopulation of neurons. Their activity is predominantly regulated via intracellular calcium/calmodulin signals in response to hormone or neurotransmitter stimulation (Bredt and Snyder, 1990; Busse and Mulsch, 1990). A third type of NOS [an inducible NOS (iNOS)], which presents in microglia and astrocytes, is regulated at the transcriptional level in response to cytokines and lipopolysaccharide (LPS), and does not require calcium for its activity (Jaffrey and Snyder, 1995; Nathan, 1992). Although NO produced by iNOS accounts for the bacterial and tumoricidal properties of microglia, it is a particular importance in the pathophysiologies of inflammatory neurological diseases including demyelinating disorders (e.g. multiple sclerosis, experimental allergic encephalopathy, and X-adrenoleukodystrophy) and in ischemia and traumatic injuries associated with infiltrating microglia and the production of proinflammatory cytokines (Koprowski *et al.*, 1993; Merrill *et al.*, 1993; Bo *et al.*, 1994; Cross *et al.*, 1994; Mitrovic *et al.*, 1994; Hooper *et al.*, 1997). It is now increasingly clear that glial cells in the central nervous system also produce NO in response to the induction of iNOS by bacterial LPS and series of cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ). Astrocytes in the healthy brain do not express iNOS; however, after the ischemic, traumatic, neurotoxic, or inflammatory damages, the activated astrocytes express iNOS in rodent and human (Galea *et al.*, 1992; Hu *et al.*, 1995; Pahan *et al.*, 1997a,b, 1998).

Several lines of evidence have demonstrated that ginsenosides play important role in the regulation of NO synthesis. In some studies, ginsenosides exert activating actions in the regulation of NOS system. For example,

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Friedl *et al* (2001) have reported that ginseng extracts stimulates NO synthesis in murine macrophages (RAW 264.7 cells). In addition, ginsenosides increase NO production in aorta, kidney, and bronchial smooth muscle (Kang *et al.*, 1995; Gillis, 1997; Tamaoki *et al.*, 2000). However, ginsenosides also show inhibitory actions in the regulation of NOS. In support of this suggestion, Kim *et al* (1998) have shown that ginsenosides Rb1 and Rg3 exert inhibitory actions against glutamate-induced neurotoxicity by attenuating NO overproduction in cultured rat cortical cells. Furthermore, Park *et al* (1996) have shown that ginsenoside Rh1 or Rh2 markedly reduce NO production from IFN-γ plus LPS-treated macrophages. However, the role of ginsenosides in the regulation of NO synthesis induced by LPS plus TNF-α (LNT) in C6 rat glioma cells has not been well characterized. Thus, the present study was designed to examine the effects of several ginsenosides on NO synthesis. We now report that ginsenoside Rd effectively inhibits NO production and iNOS mRNA expression induced by LNT. Moreover, Jun B and Fra-1 appear to be involved in the regulatory effect of ginsenoside Rd on LNT-induced NO production and iNOS gene expression in C6 rat glioma cells.

MATERIALS AND METHODS

Drugs and chemicals

LPS was purchased from Sigma chemicals (MI, USA). TNF-α was purchased from R&D systems (Woongbee Meditech, Seoul, Korea). Ginsenosides Rb1, Rb2, Rc, Rd, Re and Rg1 were obtained from Korea Ginseng and Tobacco Research Institute (Taejon, Korea).

C6 glioma cell culture

C6 rat glioma cells obtained from Korean Cell Line Bank (Seoul, Korea) were maintained in DMEM/F-12 medium containing 10% fetal bovine serum and 2 mM gentamicin. Cells were plated on 25 cm^2 culture flask (Falcon, Franklin, NJ). The cultures were incubated at 37°C in 5% CO_2, and after 1 day, the medium was completely changed to a fresh culture medium. At 70% confluency, the cells were incubated with serum free DMEM/F-12 medium (DMEM-base medium for nitrite assay) for 24 h prior to the incubation with LPS and TNF-α.

Nitrite assay

Synthesis of NO was determined by an assay of the culture supernatant for nitrite, a stable reaction product of NO with molecular oxygen. Briefly, 100 μL of culture supernatant was allowed to react with 100 μL of Griess reagent (Sigma Chemicals) (Feinstein *et al.*, 1994) and incubated at the room temperature for 15 min. The optical density of the assay samples was measured spectrophotometrically at 570 nm. Fresh culture media served as the blank in all the experiments. Nitrite concentration was calculated from a standard curve derived from the reaction of $NaNO_2$ in the assay.

Isolation of total RNA and proteins

Total cellular RNA was extracted from C6 rat glioma cells using a rapid guanidine thiocyanate-water saturated phenol/chloroform extraction procedure and subsequent precipitation with acidic sodium acetate (Chomczynski and Sacchi, 1987). Total cellular RNA in the aqueous phase was precipitated with ice-cold isopropyl alcohol. Isolated RNA samples were subjected to spectrophotometric analysis at 260 nm and 280 nm. The separated organic layer was extracted twice with an equal volume of sterilized (Millipore) water and proteins were precipitated by adding 2 volumes of absolute ethanol to the water-extracted organic phase. The protein pellets were washed twice with cold absolute ethanol and dried. The dried pellets were dissolved in a denaturing buffer (6 M guanidium chloride, 20 mM Tris-HCl [pH 8.0], and 1 mM EDTA). The protein samples were dialyzed against a renaturing buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 5 mM $MgCl_2$, 0.4 mM phenylmethylsulfonyl fluoride, and 20% glycerol) at 4°C. The concentration of protein was determined with the Coomassie blue protein assay reagent (Pierce Chemical Co., Rockford, IL) using bovine serum albumin (BSA) as the standard.

Preparation of DIG-labeled cRNA probes

The cRNA probes for iNOS (Galea *et al.*, 1994) and cyclophilin (Danielson *et al.*, 1988) were synthesized in vitro from linearized expression vectors which contained SP6 or T7 viral promoter. One μg of linearized plasmid was mixed with RNA labelling mixture that containing ATP, CTP, GTP and Dig-labeled-UTP, transcription buffer, and SP6 or T7 RNA polymerase. After incubation at 37°C for 2 hrs, the mixture was co-incubated with DNase I (RNase free) at 37°C for 15 min, precipitated in ethanol containing lithium chloride at 70°C for 30 min, and washed with 70% chilled ethanol.

Non-isotopic Northern blot analysis

Isolated RNA samples were dissolved in 40-50 mL water and 400×diluted RNA solution was subjected to spectrophotometric analysis at 260 and 280 nm. Ten microgram of total RNA and equal volume of RNA loading buffer (50% glycerol, 1 mM EDTA) were denatured in 65°C for 10 min and subsequently cooled on ice. The denatured RNA samples were electrophoresed on 1% agarose-formaldehyde gels under 60-70 V and transferred to nylon Hybond-N hybridization membrane sheets

(Amersham, Buckinghamshire, England). After UV cross-linking, the membranes were pre-hybridized at 68°C for at least 1 h in prehybridization buffer (5×SSC, 50% formamide, 0.02% SDS, 0.1% sodium *N*-lauroylsarcosine, and 2% blocking reagent). The digoxigenin (DIG)-labeled iNOS probe was added to prehybridization buffer containing 50% formamide. The membranes were incubated overnight at 68°C in a shaking water bath, and washed twice for 10 min per wash in 2×SSC and 0.1% SDS at room temperature. Then, the membranes were washed twice for 15 min per wash 0.1×SSC and 0.1% SDS. After equilibrating the membranes in Buffer I (100 mM maleic acid [pH 7.5] and 150 mM NaCl) for 1 min, the membranes were gently agitated in Buffer II (1% blocking reagent in Buffer I) for 30-60 min. The membranes were hybricized with the diluted anti-DIG-alkaline phosphatase (1:10,000 [75 mU/mL]) in Buffer II for 30 min. After washing the membranes twice for 15 min per wash in 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 50 mM $MgCl_2$) for 2 min. Diluted CSPD (Boehringer Mannheim) (1:100 dilution in Buffer III) was spread over the surface of the membranes. After incubation of the membranes at 37°C for 15-20 min, the membranes were exposed to Hyperfilm-ECL (Amersham) for detection of the chemiluminescent signal. For rehybridization, blots were washed for 20 min at room temperature in sterilized (Millipore) water. The membranes were washed for overnight at 65°C in 50 mM Tris-HCl (pH 8.0), 50% dimethylformamide and 1% SDS to remove the hybridized probe. And rehybridized to the DIG-labeled rat cyclophilin cRNA probe, a gene encoding peptidyl-prolyl cis-trans isomerase which is constitutively expressed in most mammalian tissues with the exception of skeletal muscle (Danielson *et al.*, 1988; Takahashi *et al.*, 1989).

Western blot analysis

The concentration of protein was determined with the Coomassie blue protein assay reagent (Pierce chemical Co., Rockford, IL) using bovine serum albumin (BSA) as a standard. Cellular protein (50 μg) was mixed with an equal volume of SDS loading buffer (20% glycerol, 100 mM tris-HCl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.1% bromophenol blue), boiled for 3 min, and separated by electrophoresis in 12% polyacrylamide gels as described (Laemmli, 1970). A pre-stained rainbow protein mixture (Amersham Co., Arlington Heights, IL) was used as the molecular weight standard. Proteins were transferred from acrylamide gel onto nitrocellulose membrane accoriding to the previous published procedures (Towbin *et al.*, 1979). Electro-transferred to polyvinylidene difluoride filters were first blocked with blocking buffer (3% skim milk, 1% BSA, 10 mM Trizma base [pH 8.0], and 150 mM NaCl) and then incubated with antisera against Fra-1 (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA), Jun B (1:1000) (Santa Cruz Biotechnology), and ERK/phospho-ERK (1:1000) (New England Biolabs, Beverly, MA) in blocking buffer for 4 h at room temperature. Filters were then washed 3 times with Tris-buffered saline containing 0.3% Tween-20 (TBST; 10 mM Trizma base [pH 8.0], 150 mM NaCl, and 0.3% Tween 20) for 5 min and then incubated with the goat anti-rabbit donkey IgG-horseradish peroxidase conjugate (1:5000) in blocking buffer at room temperature for 1 h. After washing the filters with TBST for 10 min (3 times), ECL-plus solution (Amersham Life Science Co., England) were added. Then, the membranes were exposed to Hyperfilm-MP (Amersham) for detection of light emission.

Preparation of nuclear extracts and Non-isotopic electrophoretic mobility-shift assay

Nuclear extract from stimulated or unstimulated C6 cells (1×10^7 cells) were prepared using the method of Dignam *et al.* (1983) with slight modification. Cells were harvested, washed twice with ice-cold TBS, and lysed in 400 μL of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotonin, 5 μg/mL pepstatin A, and 5 μg/mL leupeptin) containing 0.1 % Nonidet P-40 for 15 min on ice, vortexed vigorously for 15 s, and centrifuged at 5,000×g for 5 min. The pellet nuclei were washed with buffer A without Nonidet P-40, and resuspended in 40 μL of buffer B (20 mM HEPES (pH 7.9), 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 5 μg/mL aprotonin, 5 μg/mL pepstatin A, and 5 μg/mL leupeptin). After 30 min on ice, lysates were centrifuged at 15,000×g for 15 min. Supernatant containing the nuclear proteins were diluted with modified buffer C (20 mM HEPES (20 mM HEPES (pH 7.9), 20 % (v/v) glycerol, 0.05 M KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol) and stored at 70°C until use.

The AP-1 (5'-CGC-TTG-ATG-ACT-CAG-CCG-GAA-3'; Santa Cruz Biotechnology) were annealed by incubation an equal molar concentration of each single-stranded oligonucleotide in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 200 mM NaCl at 95°C for 10 min, and then the mixture was allowed to cool to the room temperature. The DNA-binding assay was performed according to the instructions in the manual provided with the DIG-Gel Shift Kit (Boehringer Mannheim). Binding reactions were carried out at the room temperature for 20 min and reaction mixtures contained 30 μg of nuclear protein, 20 mM HEPES (pH 7.6), 30 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.2% Tween 20, 50 mg/mL poly (dI-dC), and approximately 0.3 pmol of specified probe labeled with DIG-ddUTP using terminal deoxynucleotidyl

transferase (Boehringer Mannheim). Protein-DNA complexes were separated from protein-free DNA by non-denaturing electrophoresis in 5% polyacrylamide gels. Gels were run at the room temperature in 89 mM Tris (pH 8.3), 89 mM boric acid, and 2 mM EDTA at a constant voltage (8 V/cm), and electroblotted onto positively charged nylon membranes. The membranes were baked at 80°C for 15 min, washed with 0.3% Tween 20 in Buffer I, and hybridized with the diluted anti-DIG-alkaline phosphatase (1:10,000 (75 mU/mL)) in Buffer II for 30 min. After two washes for 15 min with 0.3% Tween 20 (in Buffer I), the membranes were equilibrated in Buffer III (100 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 50 mM $MgCl_2$) for 2 min. The method for detection of chemiluminescence was identical to the method used for the non-isotopic Northern blot analysis.

Statistical analysis

All values shown in the Fig. 1A and B of NO production were expressed mean±standard error of the mean (S.E.M.) of *n* determinations, obtained at least three independent experiment days. The results were examined by a Student's unpaired *t*-test. *P* values of less than 0.05 were considered significant.

RESULTS

Effects of ginsenosides on NO production and iNOS gene expression induced by LPS plus TNF-α

Effect of ginsenosides on NO production induced by LPS plus TNF-α (LNT) was examined in C6 rat glioma cells. As shown in Fig. 1A, LPS (500 ng/mL) plus TNF-α (20 ng/mL) treatment for 6 h causes secretion of nitrite from C6 rat glioma cells. This selected time was chosen on the basis of preliminary studies and previous publication (Lee *et al.*, 2003). Among several ginsenosides, 30 min pretreatment with ginsenoside Rd (50 μM) showed a complete inhibition against NO production induced by LNT. Furthermore, as shown in Fig. 1B, NO production induced by LNT was concentration-dependently inhibited by ginsenoside Rd (0 to 50 μM). In a Northern blot

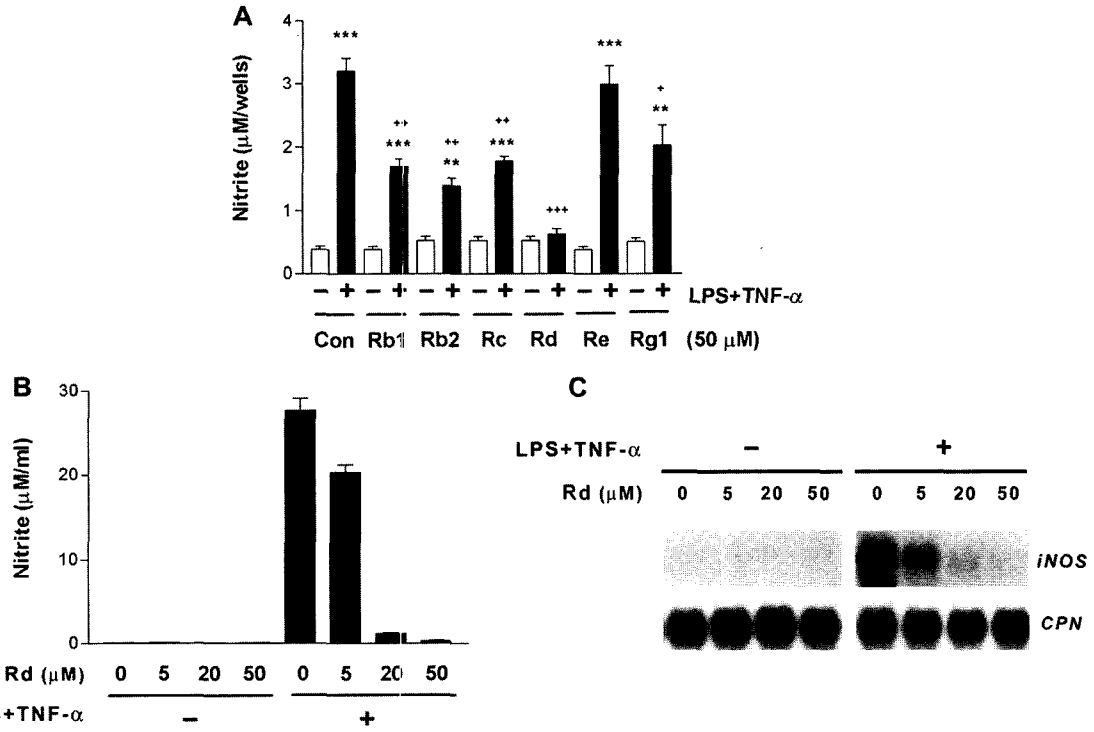

Fig. 1. Effects of ginsenosides on nitric oxide production and inducible nitric oxide synthase (iNOS) mRNA expression induced by lipopolysaccharide (LPS) plus tumor necrosis factor-α (TNF-α) in the C6 glioma cells. (A) Nitrite concentrations were measured in culture media using the Griess reaction at 6 hr after combination treatment of LPS plus TNF-α. Various ginsenosides (Rb1, Rb2, Rc, Rd, Re, Rg1) were pretreated for 30 min prior to treatment of LPS plus TNF-α at the dose of 50 μM. (B) Various doses (0 to 50 μM) of ginsenoside Rd were pretreated during 30 min, and then, nitrite concentrations were measured at 6 h after co-treatment of LPS plus TNF-α. (C) Northern blot analysis for inducible nitric oxide synthase (iNOS) mRNA was performed at 6 h after the combination treatment of LPS and TNF-α. Various doses (0 to 50 μM) of ginsenoside Rd were pretreated for 30 min prior to LPS plus TNF-α treatment. The constitutively expressed mRNA encoding cyclophilin (CPN) was used as an internal loading control. Final concentration was as follows: LPS, 500 ng/mL; TNF-α, 20 ng/mL. The vertical bars in graphs indicate the S.E.M. (***P* < 0.01, and ****P* < 0.001; compared to the untreated group, +*P* < 0.05, ++*P* < 0.01, and +++*P* < 0.001; compared to the LPS plus TNF-α-treated control group; n=3 independent experiments).

analysis, treatment with C6 rat glioma cells with the same concentration of LNT for 6 h increased iNOS gene expression. Pretreatment for 30 min with ginsenoside Rd inhibited iNOS mRNA level induced by LNT in a dose-dependent manner (Fig. 1C).

Effects of ginsenoside Rd on phosphorylation of ERK induced by LPS plus TNF-α

Effect of ginsenoside Rd on phosphorylation of ERK induced by LPS plus TNF-α (LNT) was examined in C6 rat glioma cells. As shown in Fig. 2, LPS (500 ng/mL) plus TNF-α (20 ng/mL) treatment for 6 h increased phosphorylation of ERK protein and ginsenoside Rd attenuated phosphorylation of ERK protein induced by LNT. However, LNT treatment for 6 h did not affect the total ERK protein level Fig. 2).

Effects of ginsenoside Rd on Jun B and Fra-1 proteins levels and AP-1 DNA binding activity induced by LPS plus TNF-α

As shown in Fig. 3, the stimulation with LPS (500 ng/mL) plus TNF-α (20 ng/mL) for 3 h increased Jun B protein levels, which reached at maximal level at 6 h. The stimulation with LPS plus TNF-α (LNT) increased Fra-1 protein levels at all time course (3 h and 6 h). Ginsenoside Rd attenuated Jun B and Fra-1 protein levels induced by LNT. Furthermore, LNT increased AP-1 DNA binding activity (Fig. 4) and ginsenoside Rd partially inhibited AP-1 DNA binding activity induced by LNT.

DISCUSSION

The results of the present study suggest that ginsenoside Rd shows an inhibitory action against LNT-induced NO production and iNOS mRNA expression in C6 rat glioma cells. In addition, the inhibitory action of Rd against

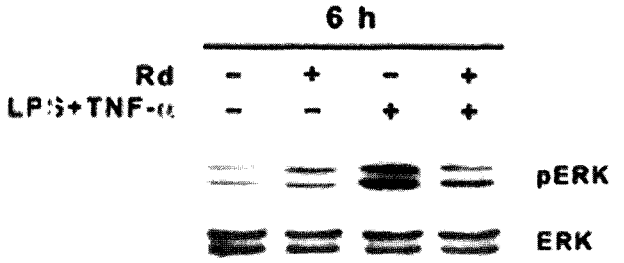

Fig. 2. Effect of ginsenoside Rd on phosphorylation of ERK induced by lipopolysaccharide (LPS) plus tumor necrosis factor-α (TNF-α) in C6 rat glioma cells. Western blot analysis was performed at 6 h after treatment with LPS (500 ng/mL) plus TNF-α (20 ng/mL). Ginsenosides Rd was pretreated for 30 min prior to treatment of LPS plus TNF-α at the dose of 50 μM. Fifty μg of total cellular protein was used for determination of phospho-ERK and total ERK protein level. Polyclonal antibodies against phospho-ERK 1/2 (p44/p42) and total ERK (p44/p42) were used at a 1:1000 dilution.

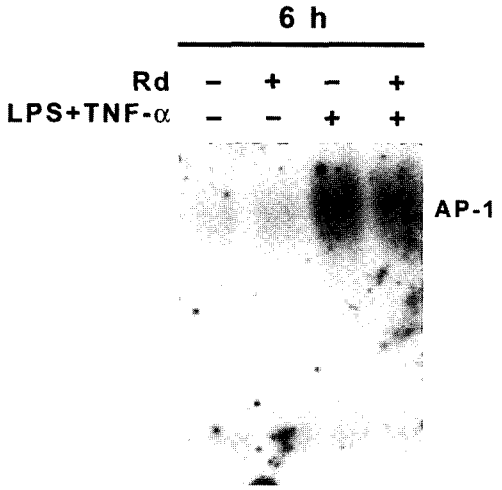

Fig. 4. Effect of ginsenoside Rd on AP-1 DNA binding activity induced by lipopolysaccharide (LPS) plus tumor necrosis factor-α (TNF-α) in C6 rat glioma cells. At 6 h after LPS (500 ng/mL) plus TNF-α (20 ng/mL) stimulation, the AP-1 DNA binding activities were examined using electrophoretic mobility-shift analysis as described in MATERIAL AND METHODS. Ginsenosides Rd was pretreated for 30 min prior to treatment of LPS plus TNF-α at the dose of 50 μM. Fifty μg of nuclear protein was used for determination of AP-1 DNA binding activity.

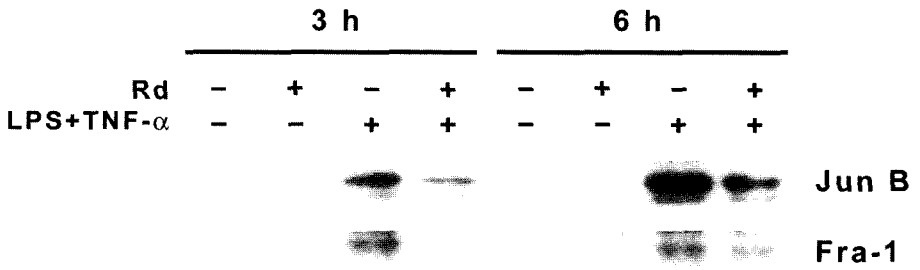

Fig. 3. Effects of ginsenoside Rd on Jun B and Fra-1 proteins levels induced by lipopolysaccharide (LPS) plus tumor necrosis factor-α (TNF-α) in C6 rat glioma cells. Western blot analysis was performed at 3 h and 6 h after treatment with LPS (500 ng/mL) plus TNF-α (20 ng/mL). Ginsenosides Rd was pretreated for 30 min prior to treatment of LPS plus TNF-α at the dose of 50 μM. Fifty μg of total cellular protein was used for determination of Jun B and Fra-1 protein level. Polyclonal antibodies against Jun B and Fra-1 were used at a 1:1000 dilution.

LNT-induced response might be mediated via decreasing phosphorylation of ERK protein induced by LNT. Furthermore, ginsenoside Rd appears to inhibit LNT-induced iNOS mRNA expression by decreasing the activation AP-1 (Fra-1 and Jun B) transcription factors.

A combination of LNT increases NO production via iNOS mRNA expression at 6 h in C6 glioma cells. This finding is in line with the several previous studies using the same cells (Feinstein *et al.*, 1994; Lee *et al.*, 2003) as well as the primary cultured rat astrocytes (Simmons and Murphy, 1994). In the present study, we clearly showed a new finding that ginsenoside Rd showed an inhibitory action against LNT-induced NO production and iNOS mRNA expression in C6 glioma cells. However, there s a controversy for the role of ginsenoside in the regulation of NO synthesis. For example, it has been reported that ginsenosides or ginseng extracts can exert activating actions in the regulation of NOS system in murine macrophage (Friedl *et al.*, 2001), aorta (Kang *et al.*, 1995), kidney (Gillis, 1997), and bronchial smooth muscle (Tamaoki *et al.*, 2000). On the contrary, ginsenosides may show inhibitory actions in the regulation of NOS in cultured rat cortical cells (Kim *et al.*, 1998) or in murine peritoneal macrophage (Park *et al.*, 1996). Taken together with our present result, it can be partially explained by that the differential mechanisms may be involved in the regulation of NO synthesis by ginsenosides in a cell- or tissue-specific way. However, it is not ruled out that the structural differences of each ginsenoside may cause this discrepancy.

The roles of ERK must be considered in the regulation of iNOS expression, since this kinase can be activated by LPS plus IFN-γ in a mouse macrophage cell line performed in previous studies (Chan *et al.*, 2001). The phosphorylation of ERK protein was increased by LNT. This result is in line with other study that phosphorylation of ERK protein is reported to be involved selectively in iNOS mRNA in primary cultured rat astrocytes but not in mouse (Bhat *et al.*, 1998). The present study was performed to investigate the possible role of ERK protein in ginsenoside Rd action against the iNOS mRNA expression and NO production. And we found that ginsenoside Rd attenuated the phosphorylation of ERK protein induced by LNT. This result suggests that phosphorylation of ERK protein may be important for the regulatory action of Rd in iNOS mRNA expression and NO production induced by LNT in C6 glioma cells.

We found in the present study that AP-1 transcription factors, such as Fra-1 and Jun B, were increased by LNT in C6 glioma cells. Induction of AP-1 proteins, such as Fra-1 and Jun B, may cause an induction of iNOS mRNA through interacting with AP-1 domain located in iNOS promoter regions. Furthermore, AP-1 DNA binding activity was increased by LNT. Ginsenoside Rd attenuated Jun B and Fra-1 protein levels as well as AP-1 DNA binding activity induced by LNT. Currently, in the supershifted assay, we have reported that supershifted bands are observed in the C6 cells coincubated with antibody against Fra-1 or Jun B (Lee *et al.*, 2003). These results are supported by several studies that promoter region of iNOS gene contains AP-1 domain (Zhang *et al.*, 1998; Angel and Karin, 1991; Schreck *et al.*, 1991; Thanos and Maniatis, 1995). Thus, it may conclude that ginsenoside Rd inhibits LNT-induced iNOS mRNA expression and NO production by attenuating induction of AP-1 transcriptional factors such as Jun B and Fra-1.

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