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Schizandra chinensis Alkaloids Inhibit Lipopolysaccharide-Induced Inflammatory Responses in BV2 Microglial Cells

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Abstract − *Schizandra chinensis* (*S. chinensis*) exhibits a harmless, 'adaptogen-type' effect leading to improvements in mental performance and learning efficacy in brain. Activated microglia contributes to neuronal injury by releasing neurotoxic products, which make it important to regulate microglial activation to prevent further cytological as well as functional brain damage. However, the effect of *S. chinensis* on microglial activation has not been examined yet. We have investigated the effects of four compounds (Gomisin A, Gomisin N, Schizandrin and Schizandrol A) from *S. chinensis* on lipopolysaccharide (LPS)-induced microglial activation. In this study, BV2 microglial cells were activated with LPS and the microglial activation was assessed by up-regulation of activation markers such as nitric oxide (NO), reactive oxygen species (ROS), and matrix metalloproteinase-9 (MMP-9). The results showed that all four compounds significantly reduced the intracellular level of ROS, the release of NO and MMP-9 as well as LPS-induced phosphorylation of ERK1/2. These results strongly suggested that *S. chinensis* may be useful to modulate inflammation-mediated brain damage by regulating microglial activation.

Keywords: *Schizandra chinensis*, Microglia, Activation, Nitric oxide (NO), Reactive oxygen species (ROS), Matrix metalloproteinase-9 (MMP-9)

INTRODUCTION

Unlike the old belief that brain is an "immune previleged organ", recent experimental results suggested that the inflammation in the central nervous system (CNS), i.e. neuroinflammation, is linked to neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Hensley *et al*., 1995; McGeer and McGeer, 2004a). In this process, microglial activation plays a pivotal role in the regulation of neuroinflammatory phenotypes. For example, highly activated microglial cells have been found in the substantia nigra of PD patients (Banati *et al*., 1998) as well as in the vicinity or within the amyloid-beta plaques of Alzheimer's disease patients (Bornemann *et al*., 2001). Activated microglial cells generate high levels of reactive oxygen species (ROS), nitric oxide (NO), and matrix metalloproteinase-9 (MMP-9) (Darley-Usmar *et al*., 1995; McGeer and McGeer, 2004b; del Zoppo *et al*., 2007).

The intracellular reduction-oxidation state is important in maintaining homeostasis and proper cellular functions. However, when this balance is lost, excessive ROS production induces oxidative stress, which contributes to cellular damage through the modulation of factors such as NF-κB and AP-1 that regulate gene expression (Pham *et al*., 2004; Gloire *et al*., 2006).

Due to its cytotoxic activity, nitric oxide (NO) has also been implicated in a large number of pathological processes (Bruhwyler *et al*., 1993). The cytotoxicity of NO is increased by reaction with superoxide anion ($O_2^{\cdot -}$) to form the highly reactive peroxynitrite ($ONOO^-$) (Beckman *et al*., 1990). Several researchers including us have reported that

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NO from cytokine-stimulated microglia or astrocytes potentiated NMDA-mediated or glucose deprivation-induced neural/glial toxicity (Hewett *et al*., 1994; Choi and Kim, 1998).

Matrix metalloproteinases (MMPs) are zinc-dependent proteolytic enzymes that are involved in the remodeling of the extracellular matrix (ECM) in a variety of physiological and pathological processes (Yong *et al*., 1998). Normally, there is a careful balance between cell division, matrix synthesis and matrix degradation, which is under the control of cytokines, growth factors and cell matrix interactions. But under pathological conditions, this balance is altered (Yu *et al*., 1997). The role of extracellular proteolysis in inflammatory demyelination, originally hypothesized as a mechanism for myelin degradation, is increasingly recognized as a pathogenetic step and as a target for therapy in human demyelinating diseases (Cuzner and Opdenakker, 1999).

Mitogen-activated protein kinase (MAPK) pathways are important signal transducing cascades that transmit extracellular signals to an appropriate response (Chang and Karin, 2001). The extracellular signal-regulated kinases 1 and 2 (ERK1/2), one of the MAPK family, are generally regarded as being related to cell survival, proliferation, and differentiation (Xia *et al*., 1995; Segal and Greenberg, 1996). Recent reports from several groups including us, however, suggested that the activation of ERK1/2 also plays crucial roles in brain inflammation including microglial activation (Zhao *et al*., 2007; Lu *et al*., 2007; Park *et al*., 2007).

Schizandra chinensis (*S. chinensis*) is widely used in traditional oriental medicine. *S. chinensis* extracts show hepatoprotective effect, improve eye vision in darkness, increase stamina, physical performance and working capacity, particularly in tiredness, and is recommended for treatment of patients in depressive states (Panossian and Wagner, 2005). *S. chinensis* has many effective components for brain function including Gomisin A, Gomisin N, Schizandrin and Schizandrol A. Gomisin A showed inhibitory effects on the activity of acetylcholinesterase enzyme (AChE) (Hung *et al*., 2007). Schizandrin inhibited the $Fe^{(2+)}$-cystein-induced peroxidative damages of brain mitochondria (Xue *et al*., 1992). Schizandrol A exerted inhibitory effects on the central nervous system (CNS) through regulation of dopamine systems (Zhang and Niu, 1991).

Due to the essential role of neuroinflammatory processes in the progression or etiology of neurodegenerative diseases, the development of anti-neuroinflammatory agents has strong impacts on the therapeutic management of diseases including Alzheimer's disease and Parkinson's diseases. Several compounds or mixture of compounds derived from natural products have drawn much attention in regard to the possible use as a therapeutic agent against those diseases.

Using BV2 microglial cells, which have been shown to mimic numerous primary microglial responses (Blasi *et al*., 1990; Bocchini *et al*., 1992; Murphy *et al*., 1998), we have investigated the effects of four compounds (Gomisin A, Gomisin N, Schizandrin and Schizandrol A) from *S. chinensis* on lipopolysaccharide (LPS)-induced microglial activation.

MATERIALS AND METHODS

Materials

All four *S. chinensis* compounds (Fig. 1) used in this study, i.e. Gomisin N, Gomisin A, Schizandrol A and Schizandrin were dissolved in 0.01% DMSO (Sigma, St. Louis, MO). These compounds were kindly supplied by the National Center for Standardization of Herbal Medicine (Seoul, Korea). Lipopolysaccharide (LPS) (026:B6) was purchased from Sigma (St. Louis, MO). Glucose-free DMEM and fetal bovine serum were from Gibco BRL (Grand Island, NY). All other reagents were obtained from Sigma (St. Louis, MO).

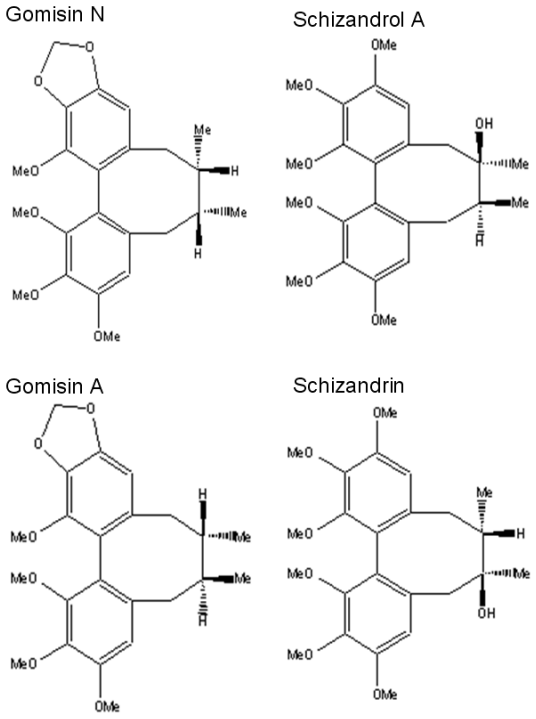

Fig. 1. Chemical structure of Gomisin A, Schizandrol A, Gomisin N and Schizandrin.

Cell culture and treatment

The murine BV-2 cell line was maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified incubator under 5% CO_2. Confluent cultures were passed by trypsinization. For experiments, cells were washed twice with warm DMEM and then treated in serum-free medium. Cells were pretreated with *S.chinensis* compounds 20 min prior to LPS stimulation. After 9 h, ROS production was determined by DCF-DA methods as described below. NO production and iNOS expression was determined 24 h after LPS stimulation.

Measurement of ROS production

BV2 microglial cells were loaded with 2,7-dihydrodichlorofluorescein diacetate (DCF-DA, 5 μg/ml) in PBS for 10 min and then rinsed with the same solution. After a 30-min incubation at room temperature, the fluorescence of DCF was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm (slit widths 20 and 25 nm, respectively) using a fluorescence microplate reader (TECAN, Austria). DCF-DA diffuses through cell membranes and is subsequently deacetylated enzymatically by intracellular esterases to the non-fluorescent DCF-H. Oxidants including $ONOO^-$ effectively convert DCF-H to the highly fluorescent DCF (Possel *et al*., 1997). Fluorescence intensities were corrected for autofluorescence (i.e., fluorescence of cells not loaded with DCF-DA).

Determination of NO

NO production from the immunostimulated cells was determined by measuring nitrite, a stable oxidation product of NO, as described previously (Green *et al*., 1990). In brief, nitrite levels were determined by adding the Griess reagent (mixing equal volumes of 0.1% napthylethylenediamine dihydrochlroride and 1% sulfanilamide in 5% phosphoric acid). After 10 min, the absorbance at 540 nm was determined using an UV spectrophotometer (Beckman DU-650, Fullerton, CA).

Gelatin zymography

Zymography was performed for semi-quantitative analysis of gelatinase levels secreted into the culture medium as described by Gottschall *et al*. (1995), which was slightly modified by us (Lee *et al*., 2003). In brief, samples were mixed with 5 μl of 4×SDS sample buffer (8% w/v SDS, 40% glycerol, 200 mM Tris-HCl, pH 6.8, and 0.02% bromphenol blue) in the absence of a reducing agent and resolved by electrophoresis at 180 V for 1 h on 8% polyacrylamide gel containing 0.1% SDS and gelatin at a final concentration of 1 mg/ml. Thereafter, gels were washed twice in 2.5% Triton X-100 for 30 min each to remove the SDS and then incubated for 24 h at 37°C in reaction buffer (20 mM Tris-HCl, 166 mM $CaCl_2$, pH 7.6). After staining the gel with 0.1% Coomassie Brilliant Blue R-250, gelatinolytic activities were visualized as a clear band in the uniformly stained background. The molecular weight of the gelatinase was estimated by comparing the migration distance of the clear bands with the distance migrated by markers of known molecular weight (Gibco BRL, Grand Island, NY).

Western blot

The activation of ERK1/2 was determined by Western blot using antibodies specific for phosphorylated, activated forms of ERK1/2. Cells were treated with LPS and/or compounds from *S. chinensis* in serum free DMEM and were lysed with 100 μl of 2×sample buffer (4% w/v SDS, 20% glycerol, 200 mM DTT, 0.1 M Tris-HCl, pH 6.8, and 0.02% bromophenol blue). The samples were fractionated by 10% SDS-PAGE and electrotransferred to nitrocellulose (NC) membrane. The NC membrane was blocked with 5% Blotto and then incubated at room temperature for 2 h with mAb against pERK1/2 (Cell Signaling Technology, Beverly, MA, USA) which was diluted at 1:3,000 in 5% Blotto (Santa Cruz Biotechnology Inc., Santa Cruz, CA). After three 10 min washes with PBS containing 0.2% Tween-20 (PBS-T), the NC membranes were incubated with peroxidase-labeled goat anti-mouse IgG at room temperature for 2 h. After extensive washing with PBS-T, the membranes were developed by enhanced chemiluminescence (Amersham, Buckinghampshire, UK). As loading controls, Western blot was performed using antibodies against β-actin in 1:30,000 dilution (Sigma).

Semiquantitative RT-PCR

Total RNA was extracted from BV2 microglial cells using Trizol reagent (GibcoBRL, Grand Island, NY). Reverse transcription was performed for 60 min at 45°C with 2 μg of total RNA using *Maxime* RT PreMix Kit (iNtRON Biotechnology, Seoul) according to the manufacturer's protocol. Oligo $(dT)_{15}$ was used as a primer for this reaction. The samples were then heated at 94°C for 5 min to terminate the reaction. The cDNA obtained from 0.5 μg total RNA was used as a template for PCR amplification of MMP-9, iNOS (accession number U03699) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession number M17701) mRNA as described previously (Lee *et al*., 2003). The following Primers were used for amplification reaction:

for MMP9,

forward primer 5'-TAAGGTATTCAGTTACTCCTAC-TGGAA-3'
reverse primer 5'-CCTCTCTAGCACACATGCACTT-3' for iNOS,
forward primer 5'-CAAGAGTTTGACCAGAGGACC-3'
reverse primer 5'-TGGAACCACTCGTACTTGGGA-3' for GAPDH,
forward primer 5'-TCCCTCAAGATTGTCAGCAA-3'
reverse primer 5'-AGATCCACAACGGATACATT-3'

PCR mixture contained 1 pmol of each forward and reverse primer and 5 μl of each cDNA and *Maxime* PCR PreMix Kit (i-Taq). For amplification, the following PCR incubation times were used: 94°C for 5min followed by 28 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1 min and continued by an final extension step at 72°C for 10 min. The amplified PCR products were electrophoresed and analysed on 1% agarose-gel. For comparison, PCR reaction for housekeeping gene, GAPDH, was also performed. The expected size of the amplified DNA fragments was 654 bp for iNOS, 324 bp for MMP9, and 308 bp for GAPDH .

Transient transfection and MMP-9 promoter reporter assay

BV2 microglial cells were co-transfected with MMP-9 promoter-luciferase reporter plasmid and pCMV-β-galactosidase reporter plasmid using Lipofectamine 2000 reagent as reported previously (Kim *et al*., 2007). Next day, BV2 microglial cells were pretreated with *Schizandra chinensis* compounds (20 μM) and then treated with LPS (0.2 μg/ml) in serum-free DMEM condition. Luciferase and β-galactosidase activities were assayed by using the luciferase and β-galactosidase enzyme assay system (Promega, Madison, WI), respectively. Luciferase activity was normalized with the β-galactosidase activity in the cell lysates.

Statistical analysis

Data are expressed as the mean ± standard deviation (S.D.) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Student's *t*-test. Data with values of $p < 0.05$ were generally accepted as statistically significant.

RESULTS

S. chinensis compounds reduced ROS generation in LPS-stimulated microglia

The chemical structure of four compounds used in this study was depicted in Fig. 1. To investigate whether *S. chinensis* compounds affect ROS generation, BV2 microglial cells were pre-treated with each *S. chinensis* compounds (20 μM) and immunostimulated with LPS (0.2 μg/ml) for 9 hrs. The concentration (20 μM) was pre-determined, which did not cause a significant effect on cell viability that was determined by MTT reduction assay (data not shown).

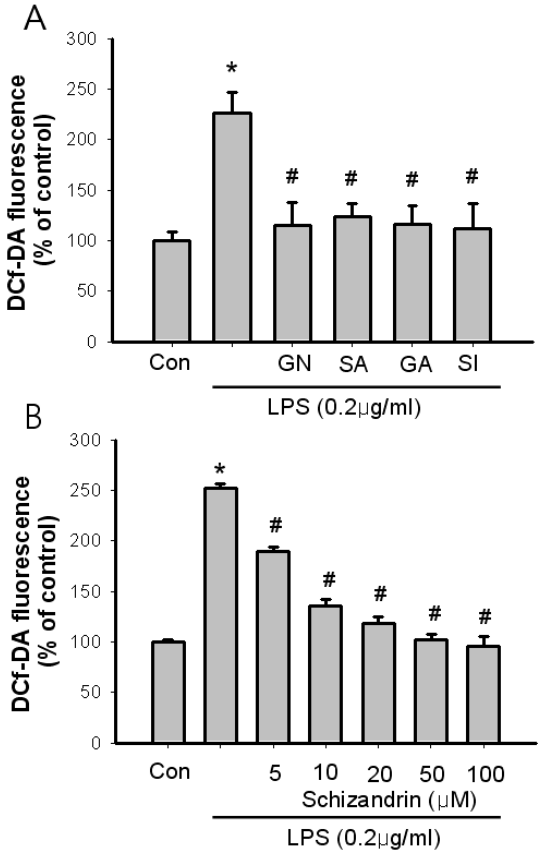

Fig. 2. Inhibitory effects of *S. chinensis* compounds on ROS generation in BV2 microglial cells. BV2 cells were pretreated with each 20 μM compounds from *S. chinensis* (A) or different concentration of Schizandrin (B) followed by 0.2 μg/ml LPS treatment. Then, cells were incubated with 2,7-dihydrodichlorofluorescein diacetate for 30 min. The fluorescence of DCF was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm using a fluorescence microplate reader. Results are the mean ± standard deviation (S.D.) of three independent experiments. Asterisk (*) indicates significant induction compared with non-treated control. # indicates significant difference from LPS-stimulated group ($p < 0.05$).

BV2 cells exposed to LPS showed a significant increase (approximately two-fold) in intracellular ROS level, and this increase was returned to near basal level by treatment with *S. chinensis* compounds, i.e. Gomisin A, Gomisin N, Schizandrin and Schizandrol A (Fig. 2A). The concentration-response relationship of Schizandrin (Fig. 2B) showed that even at 5 μM concentration, Schizandrin significantly inhibited LPS-induced increase in ROS production in rat primary astrocytes.

S. chinensis compounds inhibited LPS-induced NO production

Next, we determined the level of NO, which plays important roles in the modulation of neuroinflammatory processes in the brain. NO production was assayed by measuring the levels of a stable NO metabolite, nitrite in the conditioned medium. All four compounds of *S. chinensis* decreased LPS-induced NO production (Fig. 3A). Gomisin A, Gomisin N, Schizandrin and Schizandrol A effectively inhibited NO production by 20-40%. The potency of inhibition was in the order of Gomisin N < Schizandrol A < Gomisin A < Schizandrin.

Inhibition of iNOS expression by *S. chinensis*

To determine whether the inhibitory effect of *S. chinensis* compounds on NO production was due to the decrease in the cytosolic iNOS protein level, BV-2 microglial cells were treated with *S. chinensis* compounds and/or with LPS, and the levels of iNOS protein and mRNA were detected by Western blot and RT-PCR, respectively. As shown in Fig. 3, all four compounds of *S. chinensis* decreased LPS-induced iNOS mRNA (Fig. 3B) and protein expression (Fig. 3C) suggesting that the inhibition of LPS-induced NO production by *S. chinensis* was mediated by the inhibition of iNOS expression.

The extent of inhibition was relatively weak with Gomisin N and there was no statistical difference in mRNA and protein level albeit there was a tendency of decreased expression by Gomisin N. Likewise, the level of mRNA was not significantly different with Schizandrol A although there was a clear tendency of inhibition of iNOS mRNA expression by Schizandrol A (Fig. 3B).

S. chinensis compounds inhibited LPS-induced MMP-9 expression

In addition to ROS and RNS, proteases including MMPs

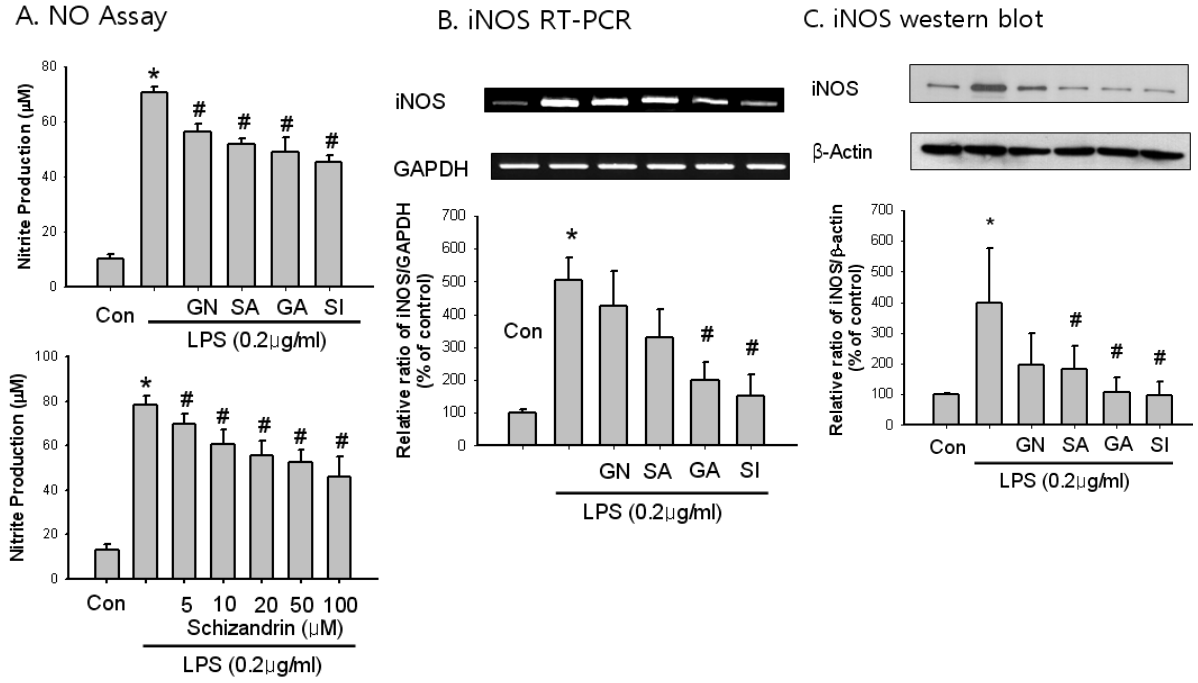

Fig. 3. *S. chinensis* compounds reduced NO production by the inhibition of iNOS expression. BV2 cells were pretreated with each 20 μM compounds from *S. chinensis* or different concentrations of Schizandrin followed by 0.2 μg/ml LPS treatment. (A) Cultured medium was collected, and nitrite level was determined by Griess reaction. (B) Treated cells were collected, and RT-PCR assays against iNOS mRNA were performed. (C) Western blot for iNOS. Lower panel shows quantitative data. Results are the mean ± standard deviation (S.D.) of three independent experiments. Asterisk (*) indicates significant induction compared with non-treated control. # indicates significant difference from LPS-stimulated group ($p < 0.05$).

and tPA is induced after immunological insult condition in the brain, which regulates many different neuropathological processes including extracellular matrix digestion, regulation of cell migration and regeneration. To investigate the effect of *S. chinensis* compounds on MMP-9 expression, BV2 microglial cells were treated with *S. chinensis* compounds and/or with LPS. The activity of MMP-9 and MMP-2 was determined by gelatin zymography. LPS induced the expression of MMP-9 and pre-treatment with four *S. chinensis* compounds decreased LPS-induced MMP-9 expression (Fig. 4, 5). LPS treatment did not affect MMP-2 activity as we reported previously (data not shown, Lee *et al*., 2003). The potency of the inhibition was in the order of Gomisin N < Schizandrol A < Gomisin A < Schizandrin, which is similar to the NO inhibition experiments (Fig. 3A). RT-PCR was also performed to determine the effect of *S. chinensis* compounds on MMP-9 mRNA level. Fig. 5B showed that pre-treatment with each of the four *S. chinensis* compounds decreased the LPS-induced MMP-9 mRNA expression suggesting that the regulation of MMP-9 activity by *S. chinensis* compounds is mediated by transcriptional control of MMP-9 gene expression. The regulation of MMP-9 gene transcription was further confirmed by MMP-9 promoter-luciferase activity assay. As shown in Fig. 5A, LPS stimulated MMP-9 promoter-luciferase activity and pre-treatment of each of the four *S. chinensis* compounds decreased LPS-induced MMP-9 promoter luciferase activity to control level.

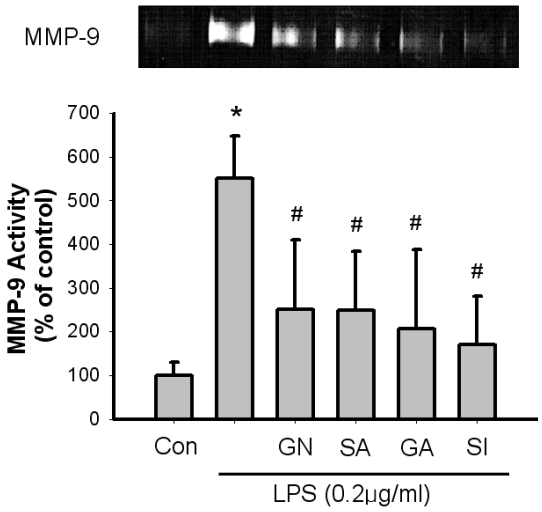

Fig. 4. MMP-9 Activity was reduced by all four *S. chinensis* compounds. BV2 cells were pretreated with each 20 μM compounds of *S. chinensis* followed by 0.2 μg/ml LPS treatment. Then, conditioned medium was collected and zymography was performed as indicated in materials and methods. Upper panel shows representative figure, and lower panel shows quantitative data. Results are the mean ± standard deviation (S.D.) of three independent experiments. Asterisk (*) indicates significant induction compared with non-treated control. # indicates significant difference from LPS-stimulated group ($p < 0.05$).

Inhibition of ERK1/2 phosphorylation by *S. chinensis*

Since production of numerous inflammatory mediators including NO and MMP-9 were ERK1/2 MAPK dependent in glial cells, we investigated whether *S. chinensis* compounds inhibit ERK1/2 activation. Fig. 6 showed that 30 min incubation with 0.2 μg/ml LPS activated ERK1/2,

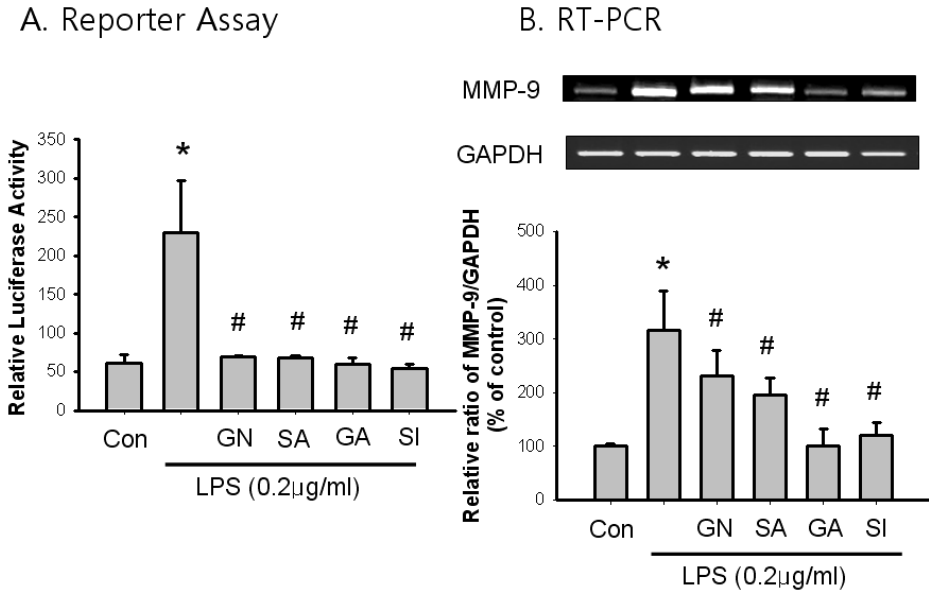

Fig. 5. Inhibition of MMP-9 expression by *S. chinensis* compounds. BV2 cells were pretreated with each 20 μM compounds from *S. chinensis* followed by 0.2 μg/ml LPS treatment. (A) MMP-9 reporter assay was carried out using Promega kit as described in materials and methods. (B) MMP-9 mRNA level was detected using RT-PCR. Results are the mean ± standard deviation (S.D.) of three independent experiments. Asterisk (*) indicates significant induction compared with non-treated control. # indicates significant difference from LPS-stimulated group ($p < 0.05$).

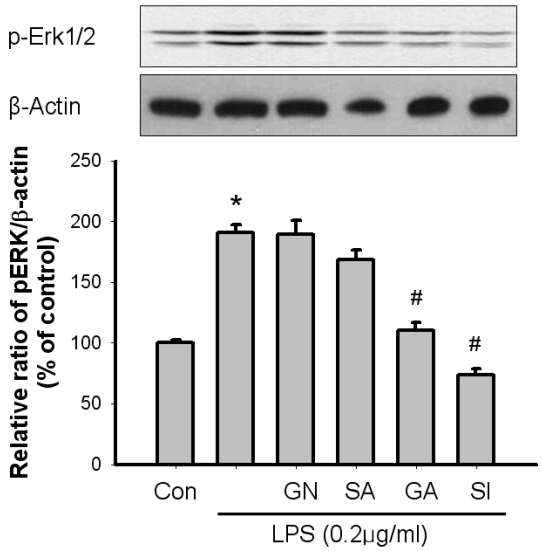

Fig. 6. Effects of *S. chinensis* compounds on LPS-induced phosphorylation of ERK1/2. BV2 cells were pretreated with each 20 μM compounds from *S. chinensis* followed by 0.2 μg/ml LPS treatment. After 2 h, Western blot against pERK1/2 were performed. Results are the mean ± standard deviation (S.D.) of three independent experiments. Asterisk (*) indicates significant difference compared with non-treated control. # indicates significant difference from LPS-stimulated group ($p < 0.05$).

which is consistent with previous reports (Lee *et al*., 2003) and the increased activity was significantly suppressed by pre-incubation with *S. chinensis* compounds (20 μM, respectively) for 20 min. Again, the potency of inhibition of ERK1/2 phosphorylation was in the order of Gomisin N < Schizandrol A < Gomisin A < Schizandrin. Gomisin N and Schizandrol A tend to inhibit ERK1/2 phosphorylation although there is no statistical significance. The weak inhibitory effects of Gomisin N and Schizandrol A on ERK1/2 phosphorylation were consistent with the weak inhibitory effects of these compounds on NO production as well. There was no change in the level of total ERK2 (data not shown) or β-actin (Fig. 6) as determined by Western blot using antibodies specific against total ERK2 or β-actin.

DISCUSSION

In this report, we demonstrated that four compounds (Gomisin A, Gomisin N, Schizandrin and Schizandrol A) from *S. chinensis* suppressed microglial activation. All four compounds from *S. chinensis* significantly decreased LPS-induced production of inflammatory mediators such as ROS (Fig. 2), NO (Fig. 3), iNOS (Fig. 3) and MMP-9 (Fig. 4, 5) as well as the activation of ERK1/2 (Fig. 6) in LPS-stimulated BV2 microglial cells. These compounds can be classified as lignans and have two methylated phenol groups in their core structure, which is a recurrent structural feature in many polyphenol compounds with anti-oxidant activity. In general, Schizandrin or Schizandrol A has stronger anti-inflammatory activity compared with Gomisin N suggesting that the addition of dioxolane ring structure diminishes the anti-inflammatory activity. However, care should be given not to over-simplify the anti-inflammatory structural features of these compounds, which needs detailed study and interpretation in the future.

We used LPS to immunologically stimulate BV2 microglial cells. Many researchers including us used LPS stimulation as a general model system for immunological stimulation of glial cells including microglia and astrocytes. LPS-stimulation induced strong and consistent immunological activation of glial cells through its action on TLR4, which might be related to the inflammatory burden of neurodegenerative diseases such as Alzheimer's disease.

Although microglial activation is one of the body's normal responses against brain injury, inflammatory mediators released from activated microglia may induce neuronal injury, leading to neurodegenerative diseases. It has been reported that toxic molecules released by activated neuroglial cells might be involved in the pathogenesis of various neurodegenerative diseases such as Parkinson's disease (PD), Alzheimer's disease (AD) and HIV-associated dementia (Gonzalez-Scarano and Baltuch, 1999; McGeer and McGeer, 1995; Minghetti and Levi, 1998). Thus, many researchers have focused on the use of natural products to control the production of neuroinflammatory regulators, especially from microglia (Fiebich *et al*., 2005; Jung *et al*., 2007; Lau *et al*., 2007). The present study demonstrated that some compounds from *S. chinensis* suppressed microglial activation. Although *S. chinensis* has many effective compounds regulating brain function, there has been no report regarding the possible anti-neuroinflammatory action. In one study, anti-inflammatory activity of Gomisin A was reported (Yasukawa *et al*., 1992), but in this case, it was performed in the model of TPA-induced skin tumor formation in mice, and the other two studies were performed in the hepatotoxicity model (Ohkura *et al*., 1990; Yamada *et al*., 1993).

Excessive ROS production may induce inflammatory response and it has been reported that antioxidants inhibit NF-κB activation and suppress the expression of inflammatory cytokines including NO (Tsai *et al*., 1999; Liang *et al*., 1999). Additionally, Lin *et al*. reported that over-expression of catalase attenuates ROS production and subsequently inactivates AP-1 in endothelial cells (Lin

et al., 2004). The strong antioxidant effects of *S. chinensis* compounds may, at least in part, explain the anti-inflammatory effects in microglial cells.

In the present study, *S. chinensis* compounds inhibited LPS-induced iNOS activity and NO production (Fig. 3) in BV2 microglial cells. It has been reported that NO modulates COX-2 activity in a cGMP-independent manner, and NO plays a critical role in the release of PGE2 by the direct activation of COX-2 (Salvemini *et al*., 1993). Considering the essential role of COX-2 in inflammatory cascade, investigating the role of *S. chinensis* compounds on COX-2 activity in BV2 microglial cells may provide further evidence of the anti-inflammatory action of these compounds. Consistent with the above notion, Schizandrin has been reported to reduce LPS-induced COX-2 expression in RAW 264.7 macrophageal cell line (Guo *et al*., 2008).

Additionally, exposure to endogenous and exogenous stimulators including LPS induced iNOS and triggered deleterious cellular responses inducing inflammation, sepsis, and stroke (Nakashima *et al*., 2003; Semmler *et al*., 2005). Therefore, the inhibitory effect of *S. chinensis* for NO and iNOS can have wide-spreading effects in the regulation of neuroinflammatory response of LPS-stimulated brain as in the case of modulation of ROS production.

In this study, the extent of inhibition of NO production (Fig. 3A) was less prominent compared with that of iNOS protein expression (Fig. 3C). The differential regulation of other subtypes of NOS such as eNOS or delayed inhibition of iNOS protein expression compared to NO accumulation may provide additional insights into the exact mechanism of NO regulation by *S. chinensis* compounds, which should be investigated further in the future study.

In this study, LPS-induced NO production as well as iNOS expression was inhibited by *S. chinensis* compounds. However, constitutive NO production was not inhibited by these compounds (data not shown) suggesting that NO inhibition is not mediated by the diirect inhibition of NOS activity.

Increased MMP-9 activity has been reported in the cerebrospinal fluid (CSF) of MS patients, and this protease activity has generally been thought to be reflective of the inflammatory process (Gijbels *et al*., 1994; Kieseier *et al*., 1999). Increased level of MMP-9 release from microglia may worsen the severity of neuroinflammatory diseases. It can be postulated that the inhibitory effect of *S. chinensis* against MMP-9 production (Fig. 4, 5), as found in this study, may provide anti-inflammatory effect in brain, which might be beneficial in several neuropathological conditions including MS. However, it has been also suggested that some MMPs, upregulated following CNS injury, may contribute to the recovery of the CNS function (Yong *et al*., 1998). Care should be given to determine whether the inhibitory effect of *S. chinensis* for MMP-9 production would be beneficial or detrimental. In this regard, determining the time window as well as the localization of MMP-9 induction after brain injury may have profound impact in devising best way to prevent neural damage in neuroinflammatory condition. The use of *S. chinensis* compounds may have beneficial effects in brain injury by modulating MMP-9 induction.

Activation of ERK1/2 plays crucial role in the regulation of inflammatory phenotype, not only in iNOS and MMP induction but also in other parameters of inflammatory responses. For example, Zhao *et al*. showed that PGE2 release was dependent on microglial activation and ERK1/2 phosphorylation, and also showed COX-2 expression in microglia was reduced by MEK1/2 inhibition (Zhao *et al*., 2007). Considering the inhibitory role of *S. chinensis* for ERK1/2 activation (Fig. 6), it would be also interesting to examine whether *S. chinensis* compounds inhibit LPS-induced PGE2 and/or COX-2 in microglial cells.

In summary, for the first time, we showed that four compounds (Gomisin A, Gomisin N, Schizandrin and Schizandrol A) from *S. chinensis* suppressed microglial activation through the inhibition of the intracellular level of ROS, the release of NO and MMP-9, the phosphorylation of ERK1/2 which were induced by LPS. Because it is almost without doubt that the inhibition of microglial activation is a critical target for regulation of neuroinflammatory phenotype in brain, the results from the present study suggest that the four compounds from *S. chinensis* play a protective role in the neuroinflammatory condition by inhibiting the production of toxic inflammatory mediators in microglia. *S. chinensis* has anticholinesterase activity (Hung *et al*., 2007), which may be helpful in ameliorating the memory deficits in Alzheimer's disease patients. Whether anti-inflammatory effects of natural compounds from *S. chinesis* contribute to the recovery of functional outcome in brain insults condition, is an open question we eager to answer in the near future.

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