Anti-Inflammatory Effects of Gomisin N, Gomisin J, and Schisandrin C Isolated from the Fruit of Schisandra chinensis

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Schiandra chinensis is a well-known Chinese traditional medicine for the treatment of hepatic disease. In this study, we investigated whether the nine major compounds of Schiandra chinensis could be applied to suppress lipopolysaccharide (LPS)-induced inflammatory responses in murine macrophages (Raw 264.7 cells). Among the nine lignans, three, gomisin J, gomisin N, and schisandrin C, were found to reduce nitric oxide (NO) production from LPS-stimulated Raw 264.7 cells. These three lignans showed low cytotoxic effects in Raw 264.7 cells. Pre-treatment of Raw 264.7 cells with gomisin J, gomisin N, or schisandrin C reduced the expression of mRNA and the secretion of pro-inflammatory cytokines. These inhibitory effects were found to be caused by blockage of p38 mitogen-activated protein kinase and the secretion of pro-inflammatory cytokines. These lignans showed low cytotoxic effects in Raw 264.7 cells. Among the nine lignans, three, gomisin J, gomisin N, and schisandrin C, were found to reduce nitric oxide (NO) production from LPS-stimulated Raw 264.7 cells. These three lignans showed low cytotoxic effects in Raw 264.7 cells. Pre-treatment of Raw 264.7 cells with gomisin J, gomisin N, or schisandrin C reduced the expression of mRNA and the secretion of pro-inflammatory cytokines. These inhibitory effects were found to be caused by blockage of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinases 1 and 2 (ERK 1/2), and c-Jun N-terminal kinase (JNK) phosphorylation.

Key words: Schiandra chinensis; gomisin J; gomisin N; schisandrin C; inflammation

Schiandra chinensis is generally used as a tonic medicine in Korea and it is a medicinal plant used in Chinese medicine. Infusions, decoctions, tinctures, and extracts from different parts of schisandra include large percentages of ether oils, resin, trace amounts of vitamin C, tannins, and staining materials, and large amounts of lipid soluble compounds.1) Moreover, Schisandra chinensis fruits contain a variety of pharmacologically active lignans. These compounds have various pharmacological activities, including detoxifying, antioxidant, anticancerogenic, antihepatotoxic, and anti-inflammatory activities.1)

The inflammatory response is connected to and requires many reagents; especially pro-inflammatory enzymes and cytokines, which are indicators of inflammatory activity. The value of NO, one of the pro-inflammatory enzymes, is shown by inflammatory levels in the inflammatory response.2,3) NO is generated enzymatically by synthases (NOS) and is formed by inducible NOS (iNOS) in macrophages. iNOS-induced NO is involved in various biological processes, including inflammation4,5) and autoimmune diseases.6,7) Cyclooxygenase-2 (COX-2), also a pro-inflammatory enzyme, produces prostaglandin E2 (PGE2) by converting arachidonic acid into prostaglandins (PGs). COX-2 is generally induced by pro-inflammatory cytokines (TNF-α, IL-1, IL-6, interferon, etc.) or LPS.8-10) In normal cells, COX-2 is not increased, but it is highly expressed by macrophages during the inflammatory response.

Pro-inflammatory cytokines, such as TNF-α, IL-1β, and IL-6, are mainly produced in macrophages activated by gram negative bacteria-derived lipopolysaccharide.11-13) In particular, TNF-α plays important roles in the promotion of the inflammatory response, which in turn causes many clinical problems associated with autoimmune disorders such as rheumatoid arthritis, Crohn’s disease, psoriasis, and asthma.14-16) IL-1β is also important in the regulation of the inflammatory response, since it increases the expression of adhesion factors on endothelial cells enabling transmigration of leukocytes. It is also associated with hyperalgesia and fever.17) IL-6, on the other hand, is often induced together with the proinflammatory cytokines TNF-α and IL-1 in many inflammatory conditions. Circulating IL-6 plays an important role in the induction of acute phase reactions.18)

Recent study has evaluated the effects of schisandrin on plasma nitrite concentrations in LPS-treated mice.19) Schisandrin also inhibited α-carrageenan-induced paw edema and acetic acid-induced vascular permeability in mice. Furthermore, it had a preventive effect against LPS-induced sepsis. In an in vitro study, it showed anti-inflammatory activities by inhibiting NO production, PGE2 release, COX-2, and iNOS expression in a Raw 264.7 cell line. However, there are no reports on the anti-inflammatory effects of gomisin J, gomisin N, or schisandrin C, the major lignans isolated from the fruit of Schiandra chinensis. Moreover, the molecular anti-inflammatory mechanism has not yet been studied. In this study, we investigated to determine whether the major compounds of Schiandra chinensis can be applied to suppress LPS-induced inflammatory responses in murine macrophages.

References
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Abbreviations: COX-2, cyclooxygenase-2; ELISA, enzyme-linked immunosorbent assay; ERK1/2, extracellular signal-regulated kinases 1 and 2; IL, interleukin; JNK, c-Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; NO, nitric oxide; PGs, prostaglandins; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF, tumor necrotic factor
Materials and Methods

Extraction and isolation of lignans. Fruit of *S. chinensis* was purchased from a Kyungdong oriental herbal market in Seoul, Korea, in December 2006. Identification of *S. chinensis* was performed at the Wild Vegetable Experiment Station, Gangwon ARES (by Ahn, Soo Young), and a voucher specimen (SC-001) was stored at the Natural Products Research Center, KIST Gangneung Institute (Gangneung, Korea). Schisandra lignans (Fig. 1) were purified as previously described.20) Fruits of *S. chinensis* (250 g) were extracted 3 times with MeOH by sonication for 1 h. After filtration, the MeOH extract was evaporated and extracted with *n*-hexane and CH$_2$Cl$_2$ consecutively. The *n*-hexane fraction (10 g) was chromatographed on an RP-18 column ([4:5/C20cm, 5:5–9:1 MeOH: water, v/v]) to yield Fr. 1–8. Fr. 4 was subjected to semi-preparative HPLC using a YMC J-sphere ODS column ([20/C250mm, 4μm, YMC, Kyoto, Japan]). Schisandrol A, gomisin J, schisandrol B, tigloylgomisin H, and angeloylgomisin H were separated with MeCN-0.1% TFA in H$_2$O isocratic (50:50 in 50 min, 10 mL/min, 254 nm). Schisandrin A, γ-schisandrin, and gomisin N were obtained by semi-preparative HPLC using a YMC Hydrosphere C18 column (20 × 250 mm, 5 μm, YMC) with MeCN-0.1% TFA in H$_2$O isocratic (70:30 in 45 min, 10 mL/min, 254 nm) from Fr. 7. Schisandrin C was finally purified by re-crystallization of Fr. 8. Isolated compounds were identified by $^1$H- and $^{13}$C-NMR spectroscopy.

Reagents and cells. A mouse macrophage cell line, Raw 264.7, was obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco’s Modified Eagle’s Medium (DMEM, Hyclone Laboratories, Logan, UT) supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin (100 U/ml)/streptomycin (100 μg/ml) (Gibco BRL, Grand Island, NY). LPS, dexamethasone, and Griess reagent were from Sigma-Aldrich (St. Louis, MO). Monoclonal antibodies against IκB, p-38, phosphor-p-38, ERK 1/2, phosphor-ERK 1/2, JNK, phospho-JNK, γ-actin, and peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell viability. Cell viability was measured by the WST-1-based colorimetric assay (Takara Bio, Ohtsu, Japan), which relies on the ability of living cells to reduce a tetrazolium salt to a soluble colored formazan product. Raw 264.7 cells at 5 ¥ 10⁴ cells/well were cultured in flat-bottom 96-well plates in triplicate for 24 h. The various concentrations of samples were treated with and without LPS (1 μg/ml). After 24 h of culture, WST-1 reagent was added to both Raw 264.7 cells and blank samples, and the mixture incubated at 37 °C under 5% CO$_2$ for 3 h. The level of the dye formed was then measured using a Bio-rad spectrophotometer at the wavelength of 450 nm. The blank value without cells was subtracted from each experimental value as background.
Statistics. Statistical analyses of data were performed by Student’s t-test to determine statistical significance. Values are given as mean ± S.D.

Results

Cell viability and NO assay of the RAW264.7 cells

To analyze the potential anti-inflammatory activities of lignans RAW264.7 cells were used since they can produce NO on stimulation with LPS. The cells were treated with LPS (1 μg/ml) for 24 h after treatment for 1 h with and without lignans. The control (cell-only) group had neither LPS nor lignans. Dexamethasone (10 μM) was used as a positive control for inhibition of NO production. The cell culture media were harvested and the NO levels were quantified using the Griess reagent. When LPS was added to the RAW264.7 cells, NO production increased dramatically, to 25.7–27.1 μM, from the basal level of 0–0.53 μM and gomisin J, gomisin N, and schisandrin C reduced the NO production in a dose-dependent manner (Fig. 2). Schisandrol A, schisandrol B, tigloylgomisin H, angelylgomisin H, schisandrin A, and γ-schisandrin did not reduce NO production (data not shown). Therefore, the cytotoxicity measurement was effective only in the lignans, gomisin J, gomisin N, and schisandrin C. The cytotoxic effects of these three compounds were evaluated by WST-1 assay after the cells were incubated for 24 h in the presence of LPS. As shown in Fig. 3, there was no evidence of cytotoxicity in any of the lignans, and all concentrations were tested in the WST-1 assay. Thus the inhibitory effects of gomisin J, gomisin N, and schisandrin C at all concentrations on NO production cannot be attributed to the cytotoxic effects. Dexamethasone was used as a positive control, because it has inhibitory effects on LPS-induced macrophage activation.

Effects of lignans on the mRNA and protein levels of pro-inflammatory cytokines

Since the lignans were found to be potent inhibitors of NO production, we further investigated their effects on pro-inflammatory cytokine release by RT-PCR and ELISA. To examine mRNA expression, Raw 264.7 cells were pretreated with gomisin J, gomisin N, and schisandrin C, and then stimulated with LPS (1 μg/ml) for 6 h. As shown in Fig. 4, all three lignans (20 μM) significantly decreased the mRNA levels of IL-1β, IL-6, and TNF-α in the LPS-activated cells. Notably, the

Fig. 2. Effects of Each Compound on the NO Production of the LPS-Stimulated Raw 264.7 Cells.

Raw 264.7 cells were treated with various concentrations (2.5, 5, 10, and 20 μM) of gomisin J, gomisin N, and schisandrin C for 1 h and then the cells were stimulated with 1 μg/ml of LPS. After 24 h, the culture supernatants were subjected to nitrite assay. Dexamethasone at 10 μM was used as a positive control. The values shown are the means ± SDs for three independent experiments. *p < 0.05; **p < 0.01 as compared to the cells treated with LPS.
mRNA levels of IL-1\beta, IL-6, and TNF-\alpha were decreased by each lignan to the level of the LPS-untreated control.

To examine whether the lignans reduced the production of proinflammatory cytokines, Raw 264.7 cells were pretreated with 20 \( \mu \)M of gomisin J, gomisin N, and schisandrin C for 1 h and then LPS (1 \( \mu \)g/ml) was added. The total cellular RNA of each Raw 264.7 cell was isolated after 6 h of LPS treatment, and the expression levels of IL-1\beta, IL-6, and TNF-\alpha were analysed transcriptionally by RT-PCR. In all experiments, the \( \beta \)-actin transcription level was used as a control. Dexamethasone at 10 \( \mu \)M was used as a positive control. The experiment was repeated 3 times, with similar results.

**Effect of lignans on the activation of p38 MAPK, ERK 1/2, and JNK**

To determine whether the anti-inflammatory activities of gomisin J, gomisin N, and schisandrin C are mediated through the MAP kinase pathway, we examined the effects of these three lignans on LPS-induced phosphorylation of p38 MAPK, ERK 1/2, and JNK by Western-blot analysis. Raw 264.7 cells were pretreated with 20 \( \mu \)M lignan for 1 h and stimulated with 1 \( \mu \)g/ml of LPS for 15 and 30 min. The total lysates were then probed with phosphospecific antibodies for p38 MAPK, ERK 1/2, and JNK. As shown in Fig. 6A, stimulation with LPS alone increased the phosphorylation of p38 MAPK, ERK 1/2, and JNK, but, gomisin J, and schisandrin C treatment reduced the level of phosphorylated p38 MAPK in LPS-stimulated Raw 264.7 cells. The inhibitory effect of gomisin J on p38 MAPK phosphorylation was sustained until 30 min after stimulation. More significantly, schisandrin C even reduced phosphorylated ERK 1/2 to the level of untreated.
control. Although phosphorylation of p38 MAPK and ERK 1/2 was not affected by gomisin N treatment, LPS-induced phosphorylation of JNK was reduced by pretreatment with gomisin N. Pretreatment of gomisin J and schisandrin C also reduced the level of phosphorylated JNK. But, no inhibitory effects on JNK phosphorylation due to gomisin J, gomisin N, or schisandrin C were observed at 30 min after stimulation. The amounts of non-phosphorylated p38 MAPK, ERK 1/2, and JNK were unaffected by either LPS or LPS plus lignans treatment. These results suggest that the anti-inflammatory activity of gomisin J, gomisin N, and schisandrin C was caused by inhibition of the LPS-induced phosphorylation of p38 MAPK, ERK 1/2, and JNK.

**Effects of lignans on IκB-α degradation**

NF-κB is known to transactivate inflammatory enzymes and cytokines and to be activated when IκB-α is degraded. To determine the mechanism of lignan mediated inhibition of the production of NO and pro-inflammatory cytokines, we examined to determine whether lignans inhibit LPS-induced degradation of IκB-α in Raw 264.7 cells by western blot with anti-IκB-α antibody. As shown in Fig. 6B, LPS-induced IκB-α degradation was not blocked by pre-treatment with gomisin J, gomisin N, or schisandrin C. Therefore, although we need more experiments, we cautiously suppose that IκB degradation for the activation of NF-κB is not directly involved in the anti-inflammatory effects of gomisin J, gomisin N, or schisandrin C.

**Discussion**

Monocytes and macrophages play important roles in both innate and adaptive immunity in vertebrate animals. The host defense activities of these cells are shown by phagocytic activity and the secretion of immune stimulatory mediators. But, these activities of macrophages are involved in many inflammatory diseases. Therefore, development of a drug that can suppress macrophage activity is considered a valuable approach to the treatment of inflammatory diseases.

Many lignan components, which are known as antioxidants and for their hepatitis healing activities, have been isolated from *Schisandra chinensis* Bail.21–24) Recently evidence has indicated that lignans from *Schisandra chinensis* have the capacity to suppress the inflammatory response induced by bacterial components such as LPS.19,25,26) Guo et al. (2008) reported that schisandrin inhibited NO production, prostaglandin E2 release, COX-2, and inducible nitric oxide synthase expression, and that these effects were due to inhibition of NF-κB, JNK, and p38 MAPK activities in a Raw 264.7 macrophage cell line.19) These results are in agreement with ours, because gomisin J, gomisin N, and schisandrin C are also *Schisandra chinensis* Bail derived lignans, and they inhibited NO production by Raw 264.7 cells. However, our results indicate for the first time that the three lignans gomisin J, gomisin N, and schisandrin C suppress NO production from activated macrophages. Especially, lignans with S-biphenyl configuration, gomisin J, gomisin N, and schisandrin C showed more portent inhibitory activity than R-biphenyl configuration-group lignans (schisandrol A, schisandrol B, tigloylgomisin H, angeloylgomisin H, schisandrin A, and γ-schisandrin) on NO production in Raw 264.7 cells.

Pro-inflammatory cytokines, including TNF-α, IL-1, IL-6, and IFN-γ, play critical roles in inflammatory and autoimmune diseases.26,27) These cytokines are mainly produced in LPS-activated macrophages.11–13) Kang et al.28) reported that Schisandra fructus water extract suppressed phorbol 12-myristate 13-acetate plus A23187...
(calcium ionophore)-induced TNF-α and IL-6 production in the mast cell line (HMC-1). These findings strongly support our conclusion that Schisandra chinensis has specific activity in suppressing the production of pro-inflammatory cytokines. In our results, the inhibitory effects of gomisin J, gomisin N, and schisandrin C on IL-1β, IL-6, and TNF-α mRNA expression as well as protein secretion were also observed when macrophages were pretreated with the three lignans. This indicates that the cytokine production inhibitory activities of gomisin J, gomisin N, and schisandrin C occur at the transcription level. TNF-α was recently identified as a main mediator in inflammatory diseases, because TNF-α is able to stimulate the production of pro-inflammatory cytokines. Therefore, production of IL-6 and IL-1β is correlated with the amount of TNF-α in the culture supernatant of macrophages. Notably, IL-1β has directly opposed activities on the immune system. The first activity is an immunological defense action, because it activates T3 cells and promotes the maturation and clonal expansion of B cells. On the other hand, the second activity is that IL-1β plays an important part in inflammatory diseases, including bacterial infections, autoimmune disorders, noninfectious hepatitis, asthma, and graft-versus-host disease. IL-6 is also classified as a pro-inflammatory cytokine. It is released from T cells and macrophages in response to IL-1, TNF-α, and LPS. Generally, IL-6 stimulates the immune response to trauma, especially from burns or other tissue damage that leads to inflammation. Taken together, TNF-α, IL-1β, and IL-6 have powerful effects on the inflammatory process, and therefore specific inhibition of these cytokines is considered important for the treatment of inflammatory diseases.

p38 MAPK, ERK, and JNK are included in MAP kinases. Generally, phosphorylation of these kinases is induced by stress and bacterial endotoxin within 10–30 min, and they are involved in cell differentiation and proliferation of mammalian cells. Several studies have claimed that MAP kinase is involved in the production of LPS-induced inflammatory mediators from macrophages. A previous study reported that the anti-inflammatory activity of lignans derived from Schisandra chinensis occurs inhibiting phosphorylation of p38 MAPK and JNK. ERK is also involved in the expression of various inflammatory inducers, including NO, COX, and cytokines. Therefore, ERK are target molecules in anti-inflammatory medicine. In our experiments, the anti-phosphorylation activity of lignans was seen within 30 min. Phosphorylated p38 was reduced by gomisin J and schisandrin C, and phosphorylated ERK was reduced by pretreatment with schisandrin C. Also, LPS-induced phosphorylation of JNK was reduced by gomisin N. Although broader timing and various concentrations of lignans were not demonstrated, we suggest that the MAP kinase inhibitory activities of gomisin J, gomisin N, and schisandrin C are implicated in anti-inflammatory activities.

Generally, NF-κB, a mammalian transcription factor that regulates various genes, involves inflammatory responses, including the expression and production of NO, COX-2, and pro-inflammatory cytokines in macrophages. In this study, whether or not lignans inhibit the activation of macrophages by preventing IκB degradation was determined. Gomisin J, gomisin N, and schisandrin C were indicated not to prevent LPS-induced IκB degradation. These results support those of previous studies, which reported that LPS strongly increased IκB degradation for activation of the NF-κB but did not clearly prevent degradation of IκB with schisandrin isolated from Schisandra chinensis. IκB degradation is an indicator in the measurement of NF-κB activity, because the dissociation of IκB means translocation of active NF-κB to the nucleus, but IκB degradation is just one indicator, and it is not absolute. Further studies using the electrophoretic mobility shift assay or western blot analysis of RelA with nuclear extract are required to figure out whether the NF-κB signal is involved in the immunosuppressive activities of lignans.

In summary, our results indicate that gomisin J, gomisin N, and schisandrin C have anti-inflammatory activities in activated macrophages. That is, we identified that these three lignans are potent inhibitors of the LPS-induced NO and production of pro-inflammatory cytokines by gene expression. The inhibitory activities of gomisin J, gomisin N, and schisandrin C were found to be caused by blockage of p38 MAPK, ERK, and JNK phosphorylation in the macrophages. We concluded that Schisandra chinensis-derived lignans have the potential to prevent inflammation.

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**References**