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Suppression of iNOS expression by fucoidan is mediated by regulation of p38 MAPK, JAK/STAT, AP-1 and IRF-1, and depends on up-regulation of scavenger receptor B1 expression in TNF-α- and IFN-γ-stimulated C6 glioma cells☆

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Abstract

In neurodegenerative disorders, activated glial cells overproduce nitric oxide (NO), which causes neurotoxicity. Inducible NO synthase (iNOS) is a potential therapeutic target in neurodegenerative diseases. Here, we examined the action of fucoidan, a high-molecular-weight sulfated polysaccharide, on tumor necrosis factor-α (TNF-α)- and interferon-γ (IFN-γ)-induced NO production in C6 glioma cells. Fucoidan suppressed TNF-α- and IFN-γ-induced NO production and iNOS expression. In addition, fucoidan inhibited TNF-α- and IFN-γ-induced AP-1, IRF-1, JAK/STAT and p38 mitogen-activated protein kinase (MAPK) activation and induced scavenger receptor B1 (SR-B1) expression. Blocking of SR-B1 did not reverse the inhibitory effect of fucoidan on TNF-α- and IFN-γ- stimulated NO production. However, inhibition of SR-B1 expression by siRNA increased iNOS expression and p38 phosphorylation in TNF-α- and IFN-γ-stimulated C6 cells.

Overall, p38 MAPK, AP-1, JAK/STAT and IRF-1 play an important role in the inhibitory effect of fucoidan on TNF-α- and IFN-γ-stimulated NO production, and intracellular SR-B1 expression may be related to the inhibition of iNOS expression by fucoidan via regulation of p38 phosphorylation. The present results also suggest that fucoidan could be a potential therapeutic agent for treating inflammatory-related neuronal injury in neurological disorders.

Keywords: Fucoidan; iNOS; p38; SRB1; Glia

1. Introduction

β-Amyloid accumulation triggers Alzheimer's disease (AD) pathogenesis via two mechanisms: induction of neuronal apoptosis and stimulation of glia-mediated inflammation, which also results in neuronal cell death [1,2]. Activated glial cells are a source of nitric oxide (NO) that is largely produced by inducible NO synthase (iNOS) in response to a series of proinflammatory cytokines, including TNF-α, IFN-γ and interleukin-1β (IL-1β) overproduced in the brains of AD patients [3,4]. Since NO contributes to neuronal toxicity, inhibition of iNOS expression may be a promising treatment for AD [5].

In AD, there is strong expression of the scavenger receptor (SR) in association with senile plaques. SR has two simultaneous functions: the clearance of beta-amyloid via phagocytosis and mediation of β-amyloid–stimulated inflammatory signaling [6–9]. SRs participate in the removal of many foreign substances and waste materials in the body with ligand specificity and a variety of receptor molecules [10–12]. According to structural characteristics, scavenger receptors are categorized into Class A SRs, including SR-A1, SR-A2 and MARCO; Class B SRs, including SR-B1 and CD36; and Drosophila Class C scavenger receptor (Class C SRs) [13].

Fucoidan is a polyanionic macromolecule composed predominantly of sulfated fucose and is found in various species of the edible brown seaweeds [14]. Fucoidan is a ligand of the Class A scavenger receptor (SR-A) and has anticoagulant, antithrombotic, antitumor, antiviral, anticomplement and anti-inflammatory activities [15–18]. SR-A is directly involved in cell signaling and cytokine production [19–22]. Nakamura et al. [23] showed that fucoidan stimulation of iNOS expression in RAW264.7 cells is mediated via the Class A scavenger receptor. However, another study found that blocking SR-A with antibodies did not inhibit the effect of fucoidan, suggesting that SR-A1 is not involved in fucoidan effect [24]. Moreover, TNF-α production and phosphorylation of tyrosine-containing proteins were

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similarly induced by fucoidan treatment in both wild-type and SR-A-deficient macrophages [25]. Fucoidan was also known to bind to Class B scavenger receptors [12,26]. Thus, the precise role of each scavenger receptor in response to fucoidan remains a matter of debate.

TNF-α and IFN-γ, which are proinflammatory cytokines, are overproduced in AD brains. Blasko et al. [27,28] suggested that this combination stimulates the production of amyloid-beta peptide, which modulates glial inflammatory responses. In this study, we examined the effects and cellular signaling and molecular mechanisms of fucoidan on iNOS–NO system activity in C6 cells treated with a combination of TNF-α and IFN-γ.

2. Materials and methods

2.1. Chemicals and antibodies

Unless otherwise indicated, all chemicals were purchased from Sigma (St. Louis, MO, USA). TNF-α, IFN-γ and an antibody against iNOS were purchased from BD Transduction Laboratories (Lexington, KY, USA). Antibodies against ERK1/2, p38, JNK, JAK, STAT, phospho-ERK1/2, phospho-p38, phospho-JNK, phosphor-JAK and phosphor-STAT were purchased from Cell Signaling Technology (Beverley, MA, USA). Antibody against SR-B1 was purchased from Novus Biologicals (Littleton, CO, USA). Anti-rat SR-BI monoclonal antibody that neutralizes the activity of SR-B1 was kindly provided by Dr. Y. Nakanishi (Kanazawa University, Ishikawa, Japan) [29]. The ERK inhibitor, U0126, and p38 inhibitor, SB203580, were purchased from Calbiochem (La Jolla, CA, USA).

2.2. Cell culture

The rat glioma cell line (C6) was purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's Modified Eagle's Medium (Gibco Co., Grand Island, NY, USA) containing 10% fetal bovine serum and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin) and incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.3. Nitric oxide measurement

C6 glial cells were cultured with IFN-γ, TNF-α or a combination of TNF-α and IFN-γ in the absence or presence of fucoidan for 24 h. Nitric oxide was measured as nitrite released. Briefly, 100 μl of the supernatant was removed from each well and placed into an empty 96-well plate. After adding 100 μl of Griess reagent to each well, the absorbance was measured at 550 nm using a Molecular Device microplate reader (Molecular Device, Menlo Park, CA, USA). NO_2^- concentrations were calculated from a $NaNO_2$ standard curve and are indicative of the amount of NO production. Griess reagent was prepared by mixing 1 part 0.1% naphthylethylene diamine dihydrochloride in distilled water with 1 part 1% sulfanilamide in 5% concentrated H_3PO_4.

2.4. Reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from C6 cells treated with TNF-α and IFN-γ for 24 h using Trizol. The total amount of harvested RNA was measured using a UV/Vis spectrophotometer at 260 nm. One microgram of the total RNA was reverse transcribed using SuperScript II (Invitrogen Life Technologies, Carlsbad, CA, USA). Polymerase chain reaction (PCR) was then performed in a final volume of 20 μl containing 1 μl of template, 0.25 μl of Taq DNA polymerase (Takara, Seoul, Korea) and 20 nmol of each primer. The PCR cycler was programmed for 20 cycles for β-actin, iNOS and SR-B1. Each cycle was held at 94°C for 30 s, 60°C for 30 s and 72°C for 60 s. The PCR products were separated on a 1.2% agarose/ethidium bromide gel, which was then photographed under UV illumination. The single-stranded cDNA was amplified by PCR using the following specific primers for iNOS, β-actin and SR-B1:

iNOS sense — 5′-CTTCCTGGACATCACTACCC-3′,
iNOS antisense — 5′-CTGCTGCCAGAAACTTCGGA-3′;
β-actin sense — 5′-AGCCATGTACGTAGCCATCC-3′,
β-actin antisense — 5′-CTCTCAGCTGTGGTGGTGAA-3′;
SR-B1 sense — 5′-ACCATTCATGACACCCGAAT-3′;
SR-B1 antisense — 5′-CTCTGTTCGAACCACAGCAA-3′.

2.5. Western blot analysis

Cells in six-well plates were washed with D-PBS and lysed with homogenization buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 1 mM phenylmethylsulphonyl fluoride). Protein concentrations were measured using the DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Whole lysates (20 μg) were resolved on a 10% SDS-polyacrylamide gel, transferred to an Immobilon polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL, USA) and probed with the appropriate antibodies. Blots were then developed using an enhanced chemiluminescence (ECL) kit (Amersham). In all immunoblotting experiments, the blots were reprobed with an anti-β-actin antibody as a control for protein loading.

2.6. Electrophoretic mobility shift assay

C6 cells (1×10^6 cells/ml) were seeded on 10-cm plates (10 ml/plate) and treated with reagents for 6 h. The cells were collected by trypsin-EDTA and suspended in buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenyl-methyl-sulfonyl-fluoride, 0.1% NP-40). The cells were then incubated on ice for 15 min and centrifuged at 1500×*g* for 10 min at 4°C. The nuclear pellets were resuspended in 30 μl of buffer C (20 mM HEPES, pH 7.9, 0.2 M NaCl, 1.5 mM $MgCl_2$, 0.2 M EDTA, 25% glycerol, 1 mM phenyl-methyl-sulfonyl-fluoride) and kept on ice for 15 min with intermittent agitation. The samples were subjected to centrifugation at 13,000×*g* for 10 min at 4°C, and the supernatant was collected and stored at −70°C. The protein concentrations were measured using a Bio-Rad protein assay kit. Electrophoretic mobility shift assays (EMSAs) were performed using a LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL, USA). Briefly, the oligonucleotides, 5′-AGTTGAGGGGACTTTCCCAGGC-3′, 5′-CGCTTGATGACTCAGCCGGAA-3′ and 5′-TTAT-GAAAGTGAAATATTGAC-3′, which contain the consensus DNA binding sites for NF-κB, AP-1 and IRF-1, respectively, were labeled with biotin using a Biotin 3′-End DNA Labeling Kit (Pierce). The binding reaction mixtures (20 μl final volume) containing the labeled oligonucleotide probes (20 fmol), binding buffer [100 mM Tris, 500 mM KCl, 10 mM dithiothreitol, 10 mM EDTA, 50 ng/μl Poly (dIdC), pH 7.5] and 6 μg of nuclear cell lysate were incubated for 25 min at room temperature. The protein–DNA complexes were subsequently separated on 6% polyacrylamide gels and transferred electrophoretically to nylon membranes (Boehringer Mannheim Biochemica, Mannheim, Germany) for chemiluminescence band detection. The binding specificity was examined using competition experiments, where a 100-fold excess of unlabeled oligonucleotide with the same sequence was added to the reaction mixture before adding the biotin-labeled oligonucleotide.

2.7. Cell transfection and RNA interference

Transient transfection was performed using psuperNeoGFP (Oligoengine, Seattle, WA, USA). According to the manufacturer's instructions, C6 cells (1×10^6 cells/ml) were seeded in six-well plates 24 h before transfection. Appropriate mixtures of Lipofectamine (Invitrogen) with plasmid containing shRNA sequence targeting SR-B1 were added to each well containing DMEM. The shRNA sequence used against murine SR-B1 was 5′-GCTCGGCGTTATCATGATT-3′. Nontargeting shRNA served as a control DNA sequence with no matches upon BLAST search. After 48 h of incubation, the medium was removed and cells were harvested for experiments. NO production and iNOS expression were measured as mentioned earlier.

2.8. Statistical analysis

Each experiment was repeated three times, and the results of one representative experiment are shown. The results were expressed as means±S.E.M. and analyzed via ANOVA. A statistical probability of $P<.05$ was considered significant.

3. Results

3.1. Fucoidan inhibits NO and iNOS production in TNF-α- and IFN-γ-stimulated C6 cells

C6 cells stimulated with both TNF-α (50 ng/ml) and IFN-γ (500 U/ml) released large amounts of nitrite into the culture medium in a time-dependent manner (Fig. 1A). TNF-α in combination with IFN-γ synergistically increased NO production as compared to TNF-α or IFN-γ alone. The increased NO release was accompanied by induction of iNOS mRNA and protein (Fig. 1B). Fucoidan inhibited the formation of NO in a concentration-dependent manner (Fig. 1C). Fucoidan also inhibited the induction of iNOS by TNF-α and IFN-γ, as shown by immunoblotting (Fig. 1D). Fucoidan (50 μg/ml) almost completely inhibited both NO and iNOS production in TNF-α- and IFN-γ-stimulated cells. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay showed that fucoidan did not affect cell viability at these concentrations (data not shown).

3.2. Fucoidan suppresses the activation of p38MAPK in TNF-α- and IFN-γ-stimulated NO production

The MAP kinase pathways control the production of inflammatory mediators, such as NO. Treatment with TNF-α and IFN-γ

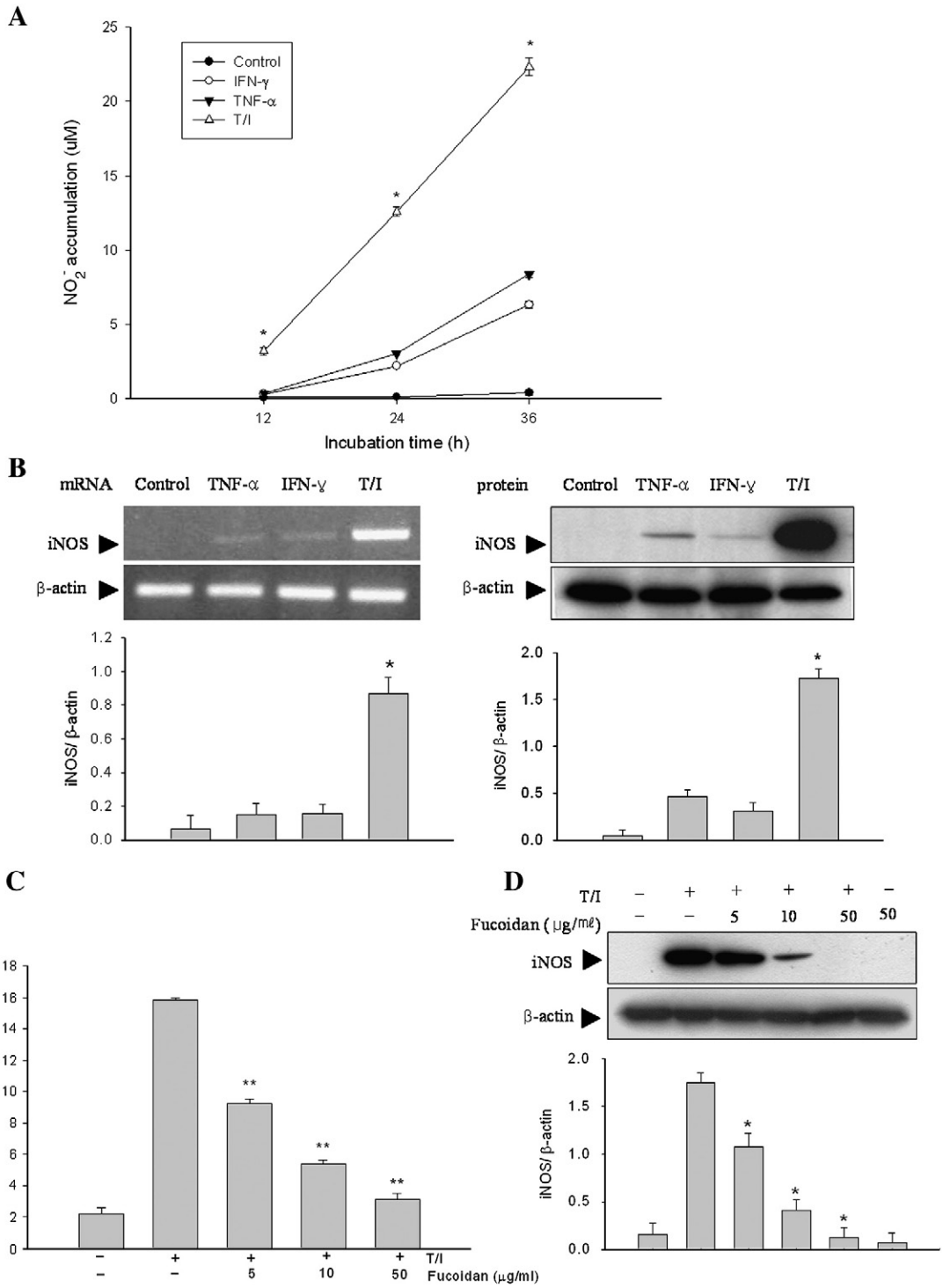


Fig. 1. Effects of fucoidan on TNF-α- and IFN-γ-induced NO production and iNOS expression in C6 cells. (A) Cells were treated with TNF-α (50 ng/ml), IFN-γ (500 U/ml) or TNF-α (50 ng/ml) and IFN-γ (500 U/ml) (T/I) for various times. The culture supernatants were collected and the nitrite levels were measured by the Griess method. (B) Cells were treated with TNF-α (50 ng/ml), IFN-γ (500 U/ml), or TNF-α (50 ng/ml) and IFN-γ (500 U/ml) (T/I) for 24 h. The levels of iNOS mRNA and protein expression were determined by RT-PCR analysis and Western blotting, respectively. β-Actin mRNA and protein levels were also measured as an internal control. In some experiments, the cells were incubated with T/I in the absence or presence of various concentrations of fucoidan for 24 h. The culture supernatants were collected and the nitrite levels measured by the Griess method (C), and the iNOS protein levels were determined by Western blot assay using an anti-iNOS antibody (D). The β-actin protein levels were measured as an internal control. In (A) and (C), data are expressed as mean±S. E.M. of three independent experiments performed in triplicate. In (B) and (D), the results are representative of three separate experiments. *Significantly different from either TNF-α, IFN-γ treatment (A and B) or both (C and D); $P<.05$.

clearly increased activated p38 mitogen-activated protein kinase (MAPK) after 10, 30 and 90 min. This treatment also slightly increased ERK1/2 phosphorylation after 10, 30 and 90 min, as well as JNK phosphorylation within 10 min. Fucoidan (50 μg/ml) inhibited the induction of p38 MAPK and ERK1/2 but not JNK (Fig. 2A).

Pretreatment with SB203580 (a p38 MAPK inhibitor) at 10 μM for 1 h suppressed TNF-α- and IFN-γ-stimulated NO production by 39%,

whereas U0126 (an ERK MAPK inhibitor) had no effect (Fig. 2B). SB203580 also inhibited the expression of iNOS (Fig. 2C). Thus, fucoidan inhibits p38 MAPK in TNF-α- and IFN-γ-induced production of NO.

3.3. Fucoidan inhibits the activation of AP-1, JAK/STAT and IRF-1 in TNF-α- and IFN-γ-stimulated C6 cells

The murine iNOS promoter contains several transcription factor binding sites, including NF-κB, AP-1, IRF-1 and STAT-1 sites, to produce iNOS gene expression. TNF-α and IFN-γ treatment for 4 h increased the DNA binding activities of NF-κB, AP-1 and IRF-1 in electrophoretic mobility shift assays. Fucoidan (50 μg/ml) inhibited AP-1 and IRF-1 activation, but not NF-κB activity (Fig. 3A).

We also examined the effect of fucoidan on both the phosphorylation of JAK2 and STAT1, which are associated with iNOS expression by IFN-γ stimulation, and the expression of IRF-1, a downstream transcription factor of Stat1 in the IFN-signaling pathway. The phosphorylation levels of JAK2 and STAT1 were elevated by TNF-α and IFN-γ treatment, as were levels of IRF-1 protein. Fucoidan treatment decreased this induction of JAK2 and STAT1 phosphorylation and IRF-1 expression (Fig. 3B). The degree of IRF-1 activation caused by TNF-α and IFN-γ treatment appears greater than that of AP-1 activation, implying that activation of IRF-1 plays a critical role in the production of NO in the TNF-α- and IFN-γ-stimulated C6 cells. Thus, fucoidan treatment blocks iNOS–NO induction by TNF-α and IFN-γ treatment through down-regulating p38 MAPK, AP1, JAK2, STAT1 and IRF-1.

3.4. SR-B1 signaling is not involved in the inhibitory effect of fucoidan on TNF-α- and IFN-γ-stimulated iNOS production

Fucoidan is a ligand for scavenger receptor A, and the effects of fucoidan on iNOS production in RAW264.7 cells are mediated by scavenger receptor A [23]. However, the role of SR-B1 in fucoidan activity is unclear. Therefore, the present study was undertaken to

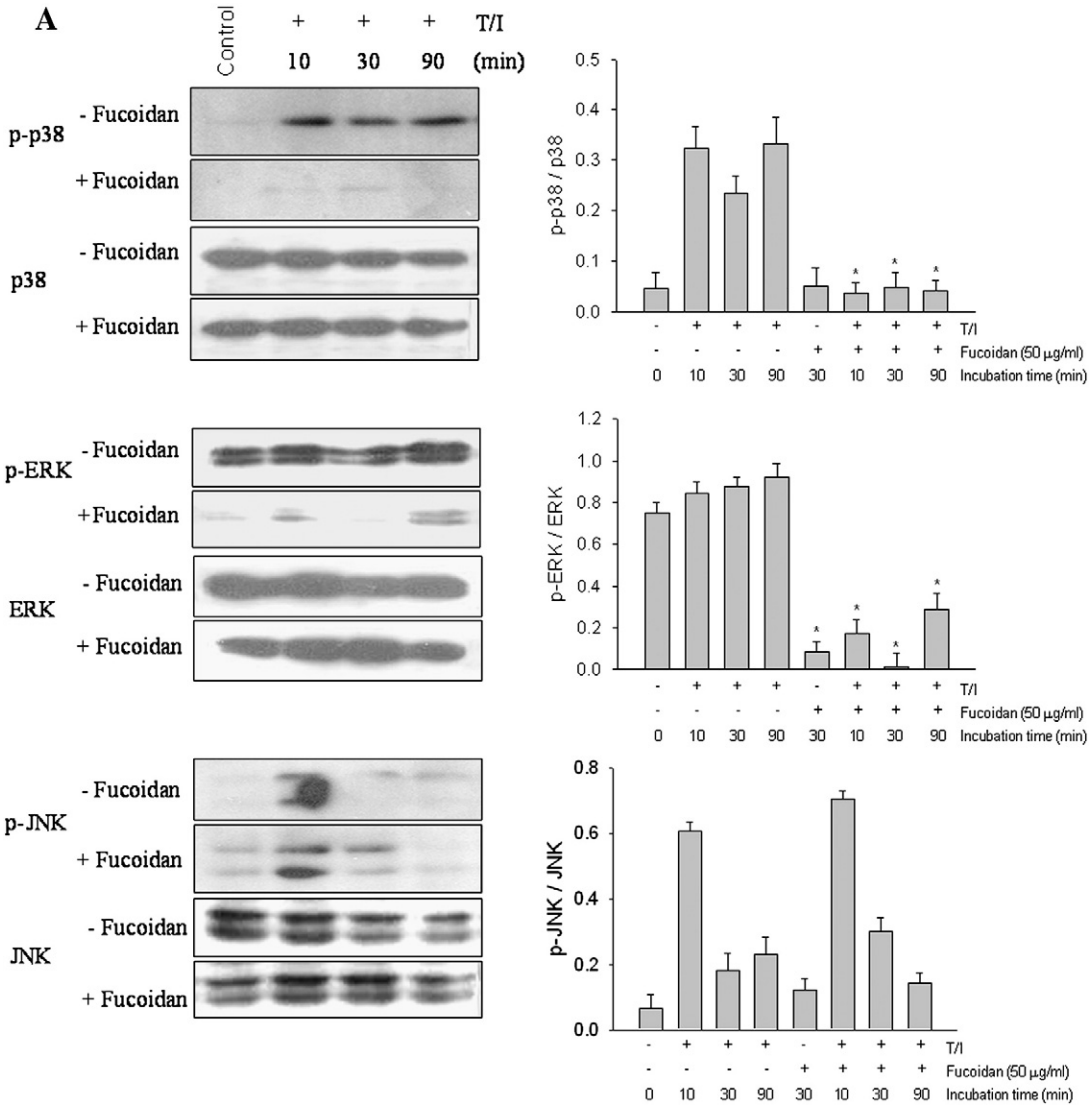


Fig. 2. Effect of fucoidan on MAPK activation in TNF-α- and IFN-γ-stimulated C6 cells. (A) Cells were treated with TNF-α (50 ng/ml) and IFN-γ (500 U/ml) (T/I) in the presence or absence of fucoidan (50 μg/ml) for the indicated times. Whole-cell lysates were analyzed by Western blot using the appropriate antibodies. (B) Cells were pretreated with the indicated concentrations of the p38 inhibitor SB203580 or the ERK inhibitor U0126 for 1 h, followed by incubation with T/I for 24 h. The culture supernatants were collected and the nitrite levels were measured by the Griess method. (C) Whole-cell lysates were prepared and subjected to Western blotting using antibodies specific against iNOS. The data in (B) are expressed as mean±S.E.M. of three independent experiments performed in triplicate. The data shown are representative of three independent experiments with similar results. Densitometry results shown in parallel represent the mean±S.E.M. of three independent experiments. *Significantly different from T/I treated; $P<.05$.

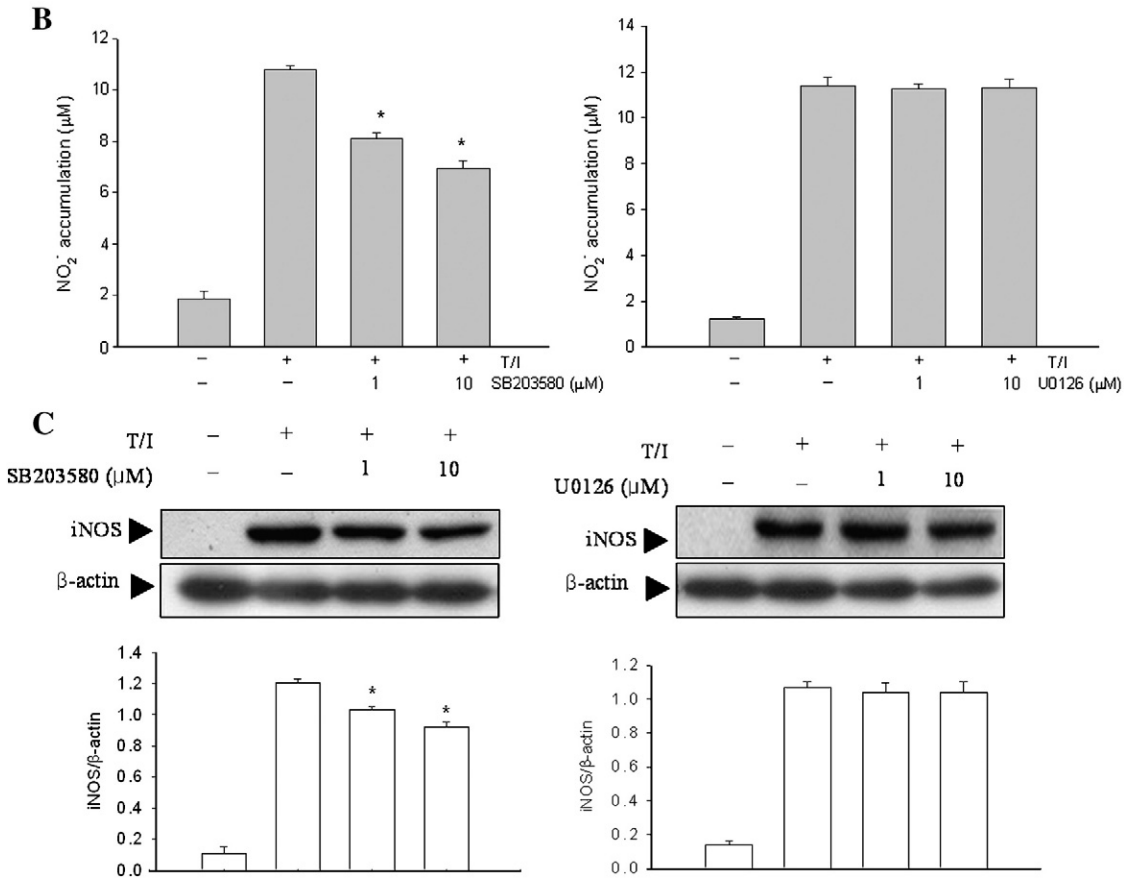


Fig. 2 (*continued*).

examine whether SR-B1 is involved in the effects of fucoidan. As shown in Fig. 4A, treatment with SR-B1 blocking antibody for 1 h did not block the inhibitory effect of fucoidan. Pretreatment with the SR-B1 ligands, high-density lipoprotein (HDL), low-density lipoprotein (LDL) or phosphatidylserine liposomes (PS) for 1 h did not block fucoidan activity either (Fig. 4B). These data suggest that SR-B1 signaling is not required for the inhibitory effect of fucoidan on NO production in TNF-α- and IFN-γ-stimulated cells.

3.5. SR-B1 expression is required for the inhibitory effect of fucoidan on iNOS production and might be related to p38 phosphorylation

We next considered the possibility that SR-B1 expression mediates the inhibitory effect of fucoidan on NO production. To test this hypothesis, we treated cells with a combination of TNF-α and IFN-γ in the absence or presence of fucoidan. Western blotting revealed that TNF-α and IFN-γ treatment decreased SR-B1 expression, whereas reduced SR-B1 expression was restored by treatment with fucoidan. In addition, fucoidan alone increased the expression of SR-B1 (Fig. 5A). Thus, fucoidan may inhibit iNOS–NO activity by increasing SR-B1 production. Transfection of an RNAi directed against SR-B1 blocked SR-B1 protein expression and increased iNOS–NO activity as compared with cells transfected with a nontargeting control shRNA (Fig. 5B and C). These data suggest that endogenous SR-B1 expression plays a role in the iNOS–NO system. Reducing SR-B1 expression also increased phosphorylation of p38 MAPK alone (Fig. 6A), but did not significantly affect p38 phosphorylation downstream of TNF-α and IFN-γ stimulation (Fig. 6B). This may be due to overactivation of p38 MAP kinase by TNF-α and IFN-γ stimulation, which could override the block by an RNAi to SR-B1. As shown in Fig. 6B, fucoidan treatment decreased the levels of p38 phosphorylation in SR-B1 shRNA-transfected cells stimulated with TNF-α and IFN-γ. Thus, SR-B1 expression is required for the inhibition of NO production by fucoidan, and this process is mediated by p38 MAPK.

4. Discussion

Although various biological activities of fucoidan have been previously demonstrated, the underlying mechanism of fucoidan remains poorly understood. Under normal conditions, the immune system provides protection against pathogens, but immune over-activation, such as uncontrolled expansion of inflammatory responses, might cause tissue damage and loss of function in brain injury [1]. Further investigation of fucoidan may point to new strategies in therapeutic design for neuronal diseases related to overactivation of the immune system. NO acts as an intracellular messenger and regulates cellular functions, such as vasorelaxation and inflammation, as well as eliminates pathogens and tumor cells. However, overproduction of NO contributes to numerous pathological processes, including injury of the brain and spinal cord [30]. NO production in excess of physiological thresholds in neurodegenerative disorders, including Alzheimer's disease, is a consequence of glial cell activation by inflammatory cytokines, predominantly via up-regulation of TNF-α and IFN-γ [3,4,28]. Since glia-associated NO production plays a crucial role in the induction of tissue injury in inflammatory diseases, prevention of iNOS expression represents an important therapeutic goal. In this study, fucoidan inhibited NO production and iNOS expression induced by TNF-α plus IFN-γ. Fucoidan does not

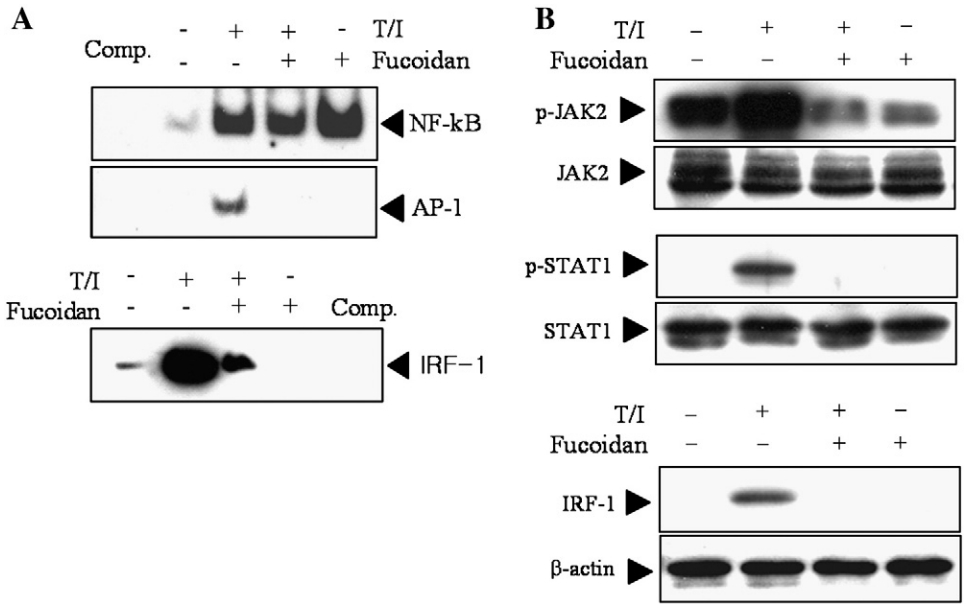


Fig. 3. Effect of fucoidan on NF-κB, AP-1 and IRF-1 activation, and JAK2 and STAT1 phosphorylation, and IRF-1 expression in C6 cells. (A) Cells were treated with TNF-α (50 ng/ml) and IFN-γ (500 U/ml) (T/I) in the presence or absence of fucoidan (50 μg/ml) for 4 h. Nuclear extracts were prepared and assayed for NF-κB, AP-1 or IRF-1 by EMSA on 6% polyacrylamide gels using DIG-labeled double-stranded oligonucleotides containing NF-κB, AP-1 or IRF-1 consensus sequences. (B) Cells were treated with T/I in the presence or absence of fucoidan (50 μg/ml) for 30 min and 24 h, respectively. Whole-cell lysates were analyzed by Western blot using the appropriate antibodies. The data shown are representative of three independent experiments with similar results.

affect the stability of expressed iNOS mRNA, implying that fucoidan acts presumably by inhibiting the transcription of iNOS. β-Actin mRNA levels remained unchanged, thereby excluding nonspecific actions of fucoidan on gene expression. Therefore, the inhibition of NO production is attributed to the suppression of iNOS mRNA transcription.

Many pathways are implicated in transmitting extracellular signals to the nuclei for iNOS gene expression [31]. The MAPK cascades are involved in cytokine- and lipopolysaccharide-mediated iNOS induction in primary glial cells [32,33]. Here, we show that treatment with TNF-α and IFN-γ results in increased iNOS–NO system activity, and this activation was mediated by p38 MAPK, which is in keeping with the notion that the p38 MAPK pathway plays an important role in the transcriptional activation of the iNOS gene [34]. Furthermore, fucoidan significantly inhibited phosphorylation of p38 MAPK and the enhancement of iNOS–NO activity induced by TNF-α plus IFN-γ. In addition, a p38 MAPK inhibitor blocked the increase in iNOS–NO system activity induced by treatment with TNF-α and IFN-γ. Thus, fucoidan may work through blocking p38 MAPK in the TNF-α- and IFN-γ-induced NO production pathway.

Activation of MAPK leads to activation of transcription factors, which are important molecular targets for pharmacological intervention and drug development because they regulate multiple genes involved in the control of immune and inflammatory responses. The promoter region of iNOS contains numerous consensus sequences for transcription factors, including NF- κB, AP-1, STAT and IRF-1 [35]. Treatment with TNF-α in combination with IFN-γ increased the DNA binding activity of NF-kB, AP-1 and IRF-1, and fucoidan inhibited both

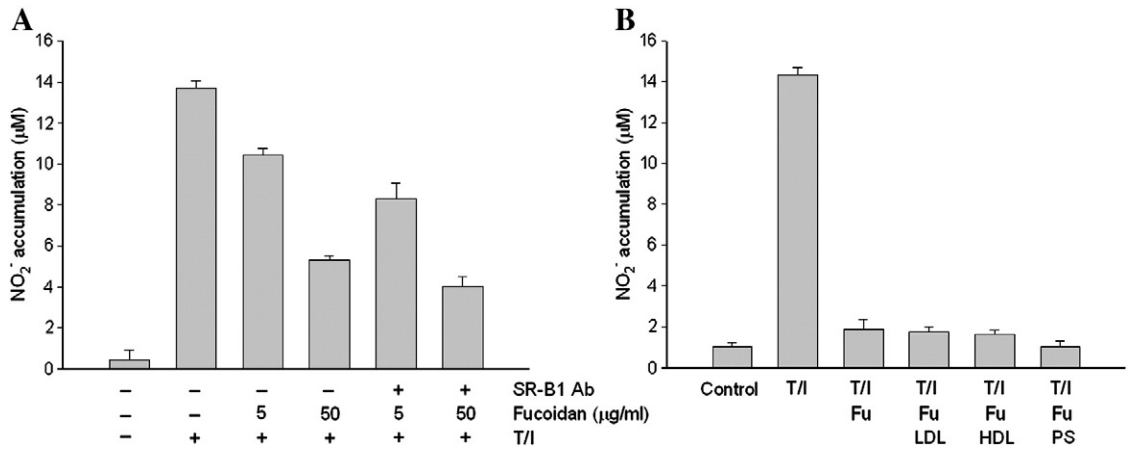
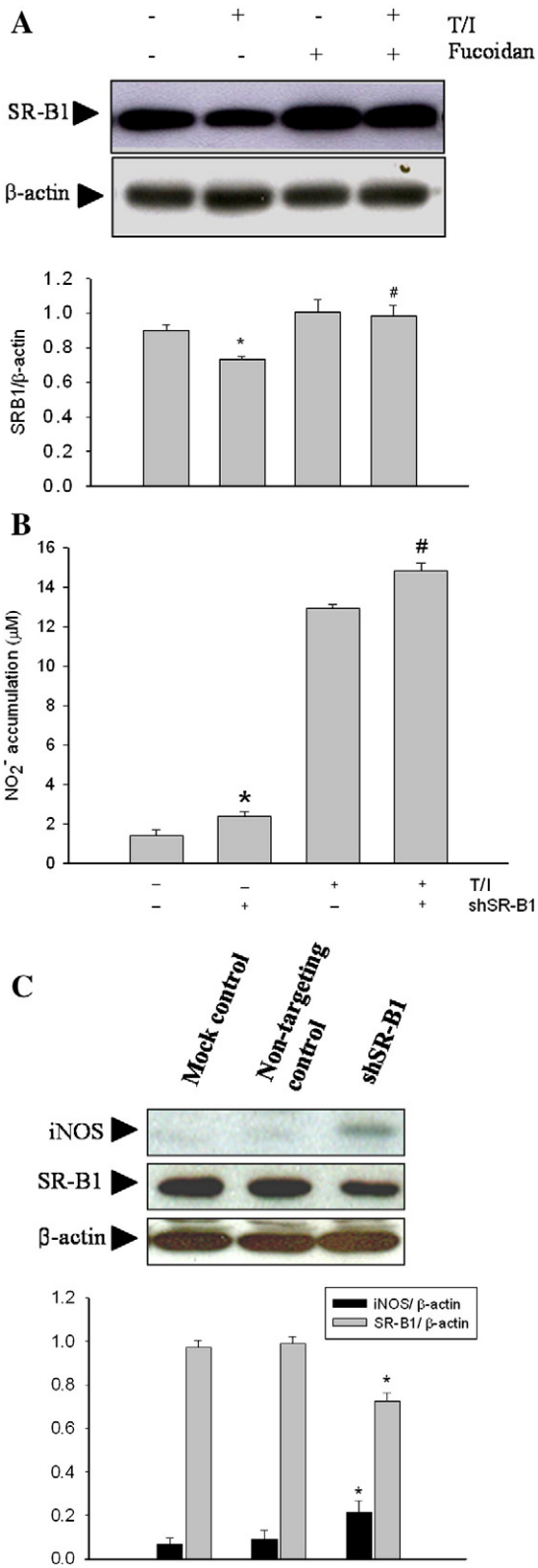


Fig. 4. Lack of SR-B1 signaling involvement in the inhibitory effect of fucoidan on NO production in TNF-α- and IFN-γ-stimulated C6 cells. (A) Cells were pretreated with 50 μg/ml SR-B1 blocking antibody for 1 h, followed by incubation with TNF-α (50 ng/ml) and IFN-γ (500 U/ml) (T/I) in the presence or absence of fucoidan (5 and 50 μg/ml) for 24 h. The culture supernatants were collected and the nitrite levels were measured by the Griess method. (B) Cells were pretreated with HDL (50 μg/ml), LDL (50 μg/ml) or phosphatidylserine liposomes (PS) (20 μg/ml) for 1 h, followed by incubation with TNF-α (50 ng/ml) and IFN-γ (500 U/ml) (T/I) in the presence or absence of fucoidan (50 μg/ml) for 24 h. The culture supernatants were collected and the nitrite levels were measured by the Griess method. Data are expressed as mean±S.E.M. of three independent experiments performed in triplicate.

AP-1 and IRF-1 activation without affecting NF-κB activity. Moreover, treatment of C6 cells with fucoidan decreased STAT1 phosphorylation and IRF-1 expression. Overall, our findings indicate that fucoidan inhibits the combination-induced phosphorylation of p38 MAPK, leading to inhibition of AP-1, STAT-1 and IRF-1 activation.



It has been known that the primary signaling of cytokines including TNF-α and IFN-γ occurs through the JAK/STAT pathway, which involves sequential receptor recruitment and activation of the receptor-associated protein tyrosine kinases JAK1 and JAK2 and subsequent tyrosine phosphorylation [36]. JAK2 is auto-phosphorylated and phosphorylated JAK2 then trans-phosphorylated JAK1. In addition, treatment with TNF-α in combination with IFN-γ increased JAK2 and STAT1 phosphorylation [37]. Many studies have demonstrated that cell surface receptors including TNF-α/IFN-γ receptor commonly become internalized after ligand binding [38,39]. Based on these findings, it is plausible that fucoidan could interfere with the activation of TNF-α/IFN-γ receptors or induce TNF-α/IFN-γ receptors internalization. In this study, fucodian suppressed JAK2 tyrosine phosphorylation, suggesting that the inhibition occurs at least in part upstream of STATs in the JAK/STAT pathway. In contrast, inhibitors of receptor internalization did not affect the inhibitory effect of fucodian on NO production (data not shown), indicating that fucoidan might not be able to induce receptor internalization.

Scavenger receptors are expressed on various cell types and cell lines, such as myeloid cells (macrophages and dendritic cells) and certain endothelial cells. SR-A (Class A receptors) is the best characterized. Unlike most cell-surface receptors, SR-A exhibits a broad ligand binding specificity, including modified lipoproteins, polynucleotides and polysaccharides. It has been recently suggested that fucoidan binds SR-A and induces NO production via SR-A-mediated signal transduction in macrophages [23]. However, these findings do not rule out the possibility that other unidentified signaling molecules or pathways likely contribute to NO production.

SR-B1 is a multifunctional receptor capable of binding a wide array of native and modified lipoproteins [10–12]. Its primary function is promoting the selective uptake of cholesteryl esters from HDL, and SR-B1 functions as a binding site for atherogenic lipoproteins, including native LDL and modified LDL. In addition, SR-B1 mediates the activation of endothelial NO synthase production by HDL [40,41]. However, the exact role of SR-B1 in the response to fucoidan remains to be elucidated. The present data demonstrate that neither a blocking antibody against SR-B1 nor SR-B1 ligands reversed the effects of fucoidan. SR-B1 expression results in NO synthesis by eNOS in human microvascular endothelial cells [42]. In addition, we show that fucoidan restored the reduced SR-B1 expression after treatment with TNF-α and IFN-γ to inhibit iNOS–NO activity. siRNA-mediated depletion of SR-B1 significantly increased iNOS–NO activity, implying that endogenous SR-B1 expression plays a role in the iNOS–NO system. Furthermore, treatment with fucoidan resulted in decreased levels of p38 phosphorylation in SR-B1 shRNA-transfected cells stimulated with TNF-α and IFN-γ. Taken together, SR-B1 expression is required for the inhibition of NO production by fucoidan and this process is mediated by p38 MAPK to regulate the activity of the iNOS–NO system.

Fig. 5. Requirement of SR-B1 expression for the inhibitory effect of fucoidan on the iNOS–NO system. (A) C6 cells were treated with TNF-α (50 ng/ml) and IFN-γ (500 U/ml) (T/I) in the presence or absence of fucoidan (50 μg/ml) for 24 h, respectively. Whole-cell lysates were analyzed by Western blot using anti-SR-B1 antibodies. β-Actin protein levels were measured as an internal control. (B) C6 cells were transfected with nontargeting control or SR-B1-specific shRNA, then stimulated with T/I for 24 h. The culture supernatants were collected and the nitrite levels were measured by the Griess method. (C) C6 cells were transfected with mock control, nontargeting control or SR-B1-specific shRNA, then stimulated with T/I for 24 h. The whole-cell lysates were analyzed by Western blot using anti-iNOS antibodies. β-Actin protein levels were measured as an internal control. In (B), data are expressed as mean±S.E.M. of three independent experiments performed in quintuplicate. The data shown are representative of three independent experiments with similar results. Densitometry results shown in parallel represent the mean±S.E.M. of three independent experiments. *Significantly different from control (no treatment). #Significantly different from T/I treated; $P<.05$.

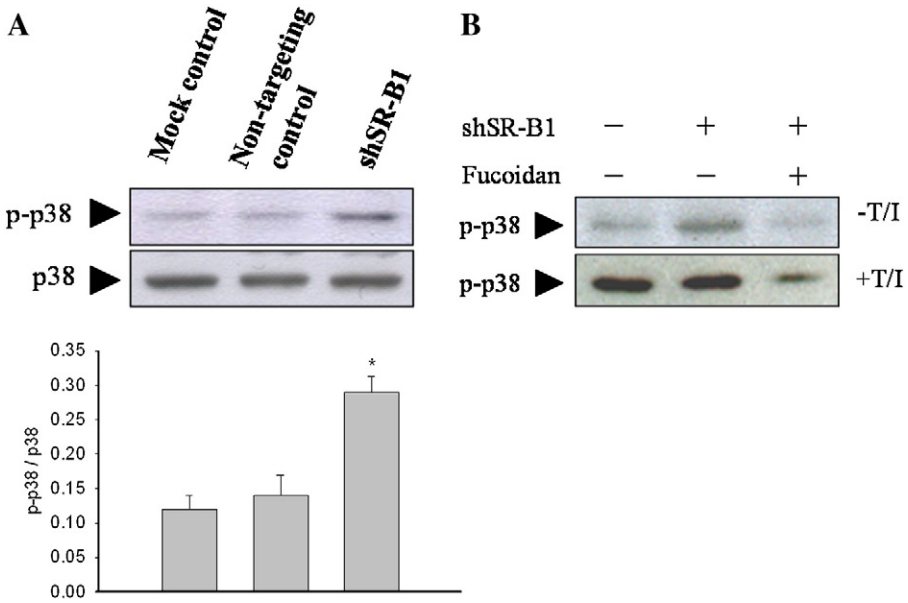


Fig. 6. SR-B1-dependent p38 MAPK activation in response to fucoidan. (A) C6 cells were transfected with mock control, nontargeting control or SR-B1-specific shRNA for 2 days, and whole-cell lysates were analyzed by Western blot using a phospho-specific p38 antibody. The blot was reprobed with a p38 antibody to ensure for equal protein loading. (B) C6 cells were transfected with either nontargeting control or SR-B1-specific shRNA, then stimulated with fucoidan (50 μg/ml) in the presence or absence of TNF-α (50 ng/ml) and IFN-γ (500 U/ml). Whole-cell lysates were analyzed by Western blot using a phospho-specific p38 antibody. The data shown are representative of three independent experiments with similar results. Densitometry results shown in parallel represent the mean±S.E.M. of three independent experiments. *Significantly different from control (no treatment); $P<.05$.

In conclusion, fucoidan inhibited the production of NO induced by TNF-α and IFN-γ in C6 glioma cells. This action resulted from the suppression of p38 MAPK, AP-1, JAK2, STAT1 and IRF-1 activation. In addition, the present results indicate that the inhibitory effects of fucoidan on iNOS–NO system activity are regulated by intracellular SR-B1 expression in TNF-α- and IFN-γ-stimulated C6 cells, and SR-B1 expression is associated with p38 MAPK. Thus, these findings provide an integrated mechanism extending previous hypotheses and implicate fucoidan as a potential therapeutic for the treatment of neurodegenerative diseases.

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