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Differential Regulation by Fucoidan of IFN-γ-Induced NO Production in Glial cells and Macrophages

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ABSTRACT

Fucoidan has shown numerous biological actions; however, the molecular bases of these actions have being issued. We examined the effect of fucoidan on NO production induced by IFN-γ and the molecular mechanisms underlying these effects in two types of cells including glia (C6, BV-2) and macrophages (RAW264.7, peritoneal primary cells). Fucoidan affected IFN-γ-induced NO and/or iNOS expression both in macrophages and glial cells but in a contrast way. Our data showed that in C6 glioma cells both JAK/STAT and p38 signaling positively regulated IFN-γ-induced iNOS, which were inhibited by fucoidan. In contrast, in RAW264.7 cells JAK/STAT is a positive regulator whereas p38 is a negative regulator of NO/iNOS production. In RAW264.7 cells, fucoidan enhanced p38 activation and induced TNF-α production. We also confirmed the dual regulation of p38 in BV-2 microglia and primary peritoneal macrophages. From these results, we suggest that fucoidan affects not only IFN-γ-induced NO/iNOS production differently in brain and peritoneal macrophages due to the different roles of p38 but the effects on TNF-α production in the two cell types. These novel observations including selective and cell-type specific effects of fucoidan on IFN-γ-mediated signaling and iNOS expression raise the possibility that it alters the sensitivity of cells to the p38 activation. J. Cell. Biochem. 111: 1337–1345, 2010.

KEY WORDS: iNOS; p38; IFN-γ; MACROPHAGE; GLIA; FUCOIDAN

NO is a messenger molecule involved in many physiological and pathological processes within cells and tissues. NO is synthesized by NO synthases, which are classified into two groups. The constitutively expressed forms, including neuronal NOS (nNOS) and endothelial NOS (eNOS), and inducible form (iNOS) which is expressed in many kinds of cells including macrophages, glial cells, hepatocytes, and smooth muscle cells in response to various cellular stresses [Kleinert et al., 2003]. Sustained production of NO endows macrophages with cytostatic or cytotoxic activity against viruses, bacteria, fungi, protozoa, and tumor cells [MacMicking et al., 1997]. NO in the brain contributes to both physiologic and pathophysiologic processes. It regulates blood flow and may act as a neurotransmitter or as a neurotoxic agent, depending on the cellular source, amount, and production site. Large amounts of NO released from activated glial cells in brain contribute to oligodendrocyte degeneration in demyelinating diseases and neuronal death during ischemia, trauma, and neurodegenerative diseases [Togo et al., 2004; Duncan and Heales, 2005].

The brown seaweeds, which have been consumed as a food or relish to provide a dietary fiber source, are the most important economic seaweeds cultured in Asia. Fucoidan, which is found in the extracts from brown seaweeds (e.g., *Ascophyllumnodosum* and *Fucus vesiculosus*), is a polyanionic macromolecule composed predominantly of sulfated fucose moieties. In recent years, fucoidan has been extensively studied due to its numerous biological activities including its anticoagulant, antithrombotic, antitumor, antiviral, anti-complement, and anti-inflammatory effects [Baba et al., 1998; Berteau and Mulloy, 2003; Mourão, 2004]. However, the molecular mechanisms underlying these effects of fucoidan are still unclear. The effect of fucoidan on NO and iNOS production has been studied in quiescent and LPS-activated RAW264.7 macrophages, showing both unique and conflicting results [Nakamura et al., 2006; Yang et al., 2006]. However, the effect of fucoidan on inflammatory

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cytokine-induced stimulation on the cells is not known. Also, the effect of fucoidan on NO/iNOS production in neuroglial cells (usually referred to simply as glial cells or glia) has not been much reported.

Previously, we reported the suppressive effects of fucoidan on NO/iNOS production in C6 glioma cells treated with the combination of TNF-α and IFN-γ [Do et al., 2010]. These two well-known pro-inflammatory cytokine combinations have known to produce β-amyloid deposition in brain [Blasko et al., 1999, 2000]. Here, we examined the molecular mechanisms underlying the suppressive effects of fucoidan on NO/iNOS production in C6 cells induced IFN-γ to determine the exact mechanisms of fucoidan in neuroinflammatory circumstance. In addition, we compared these mechanical effects of fucoidan on NO/iNOS production in other macrophage cell types. Investigation on these issues brought us novel observation that fucoidan regulates IFN-γ-induced NO/iNOS production differently in macrophages and glial cells. Therefore, to further determine how different mechanisms are related to the distinct effects of fucoidan on NO/iNOS expression, we used two different cell types of glial cells (C6 glioma and BV-2 microglia) and macrophages (RAW264.7 and primary peritoneal macrophages).

MATERIALS AND METHODS

MATERIALS

Unless otherwise indicated, all chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium, RPMI medium, penicillin, streptomycin, trypsin/ethylene diaminetetra acetic acid (EDTA), and FBS were purchased from Gibco Co. (Grand Island, NY). TNF-α, IFN-γ, and antibody against iNOS were purchased from BD Transduction Laboratories (Lexington, KY). Antibodies against p38, phospho-p38 (Thr180/Tyr182), phospho-JAK2 (Tyr1007/1008), and phospho-STAT1 (Tyr701) were purchased from Cell Signaling Technology (Beverley, MA). Antibody against IRF-1 was purchased from Santa Cruz Biotechnology (CA). STAT1 inhibitor, fludarabine, p38 inhibitor, SB203580, and TNF-α antagonist (catalog number: 654255-MG) were purchased from Calbiochem (La Jolla, CA). SR-A1 blocking antibody was purchased from R&D Systems (Minneapolis, MN).

CELL CULTURES

The C6 rat glioma cells, BV-2 mouse microglia cells, and RAW264.7 mouse macrophages were purchased from the American Type Culture Collection (Manassas, VA). Primary peritoneal macrophages, isolated from both female and male BALB/c mice, were collected 4 days after an intraperitoneal injection of 3 ml of 24 mg/ml solution thioglycolate. C6 and BV-2 cells were cultured in Dulbecco's modified Eagle's medium. RAW264.7 macrophages and mouse peritoneal macrophages were cultured in RPMI medium. The media were supplied with 10% FBS and antibiotics (100 IU/ml of penicillin and 100 μg/ml of streptomycin). The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. For nitric oxide measurement and ELISA, 2×10^4 cells/well were seeded on 96-well plates overnight before treatment. For RT-PCR and Western blot, 4×10^5 cells/well were seeded on six-well plates and cultured overnight before treatment.

FUCOIDAN PREPARATION

Fucoidan extracted from *Fucus vesiculosus* was purchased from Sigma–Aldrich (St. Louis, MO) and dissolved in distilled water. Potential contamination of fucoidan with endotoxin was examined using QCL-1000 Chromogenic LAL end-point assay (Lonza Walkersville, Walkersville, MD) following the manufacturer's manual. The detection limit of the kit was 0.1 EU/ml. Endotoxin level of 100 μg/ml fucoidan preparation was <0.1 EU/ml.

NITRIC OXIDE MEASUREMENT

Nitric oxide was measured as nitrite released from C6 glioma cells and RAW264.7 macrophages. Briefly, 100 μl of supernatants was combined with equal volumes of Griess reagent (1% sulfanilamide, 0.1% naphthalenediamine dihydrochloride, 2.5% phosphoric acid) and incubated at room temperature for 10 min. The absorbance at 540 nm was determined with an E MAX precise microplate reader (Molecular Devices, Eugene, OR), and nitrite concentrations were calculated from a nitrite standard curve.

RNA EXTRACTION

Using Trizol reagent, total RNA was extracted from C6 glioma cells and RAW264.7 macrophages and treated with reagents in six-well plates. A precipitation step using isopropanol plus high salt precipitation solution (0.8 M sodium citrate and 1.2 M NaCl) and a washing step were repeated four times to completely remove fucoidan from the RNA extract. The total amount of harvested RNA was measured using an UV/Vis spectrophotometer at 260 nm.

REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION ANALYSIS

One microgram of the total RNA was reverse transcribed to cDNA using SuperScript II (Invitrogen Life Technologies, Carlsbad, CA). Polymerase chain reaction (PCR) was then carried out 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 was programmed for 20 cycles for β-actin, GAPDH, iNOS, and TNF-α. 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 electrophoresed in a 1.2% agarose/ethidium bromide gel, which was then photographed under UV illumination. The single-stranded cDNA was amplified by PCR using the specific primers for iNOS, β-actin, and GAPDH.

WESTERN BLOT ANALYSIS

Cells in six-well plates were washed by D-PBS and lysed with homogenization buffer (50 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 1 mM phenylmethylsulfonyl fluoride). Protein concentrations were measured using the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Twenty micrograms of each sample was electrophoresed on sodium dodecylsulfate–polyacrylamide gels and transferred to Hybond-ECL nitrocellulose membranes (Amersham Biosciences, Piscataway, NJ). The membranes were blocked with 5% skim milk in Tris-buffered saline/non-fat Tween (TBST) for 1 h. The membranes were incubated with primary Abs for 1 h at room temperature. They were then washed with TBST three times for 5 min and incubated with secondary ALP-conjugated antibodies for 1 h. The membranes were washed again and autoradiography was

carried out using an enhanced chemiluminescence kit (Amersham Bioscience).

ELISA ASSAY

RAW264.7 macrophages were stimulated with various doses of fucoidan and SB203580 for 24 h. Concentrations of TNF-α were determined by sandwich ELISA according to the manufacturer's instructions (BD Pharmingen, San Diego, CA) with lower detection limits of 10 pg/ml.

STATISTICAL ANALYSIS

Each experiment was repeated three times, and the results of a representative experiment are shown. The results are expressed as means ± SEM and were analyzed by ANOVA. A statistical probability of $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ was considered significant.

RESULTS

FUCOIDAN REGULATES IFN-γ-INDUCED NO/iNOS PRODUCTION DIFFERENTLY IN GLIAL CELLS AND MACROPHAGES

In C6 glioma cells, both TNF-α (50 ng/ml) and IFN-γ (500 U/ml) significantly increased the production of NO. In contrast, in RAW264.7 macrophages only IFN-γ (500 U/ml) but not TNF-α (50 ng/ml) induced NO production (Fig. 1A). iNOS protein expression also showed the same pattern (Fig. 1B), suggesting a difference between these two cell types in response to TNF-α. Nitrite accumulation following stimulation with TNF-α and/or IFN-γ for 24 h in the absence or presence of fucoidan (50 μg/ml) was examined. As shown in Figure 1A, in either C6 glioma cells or RAW264.7 macrophages fucoidan did not have an effect on TNF-α-induced NO production. In contrast, fucoidan had opposing actions on IFN-γ-induced NO production in two cell types in which it significantly suppressed NO production in C6 cells but it increased

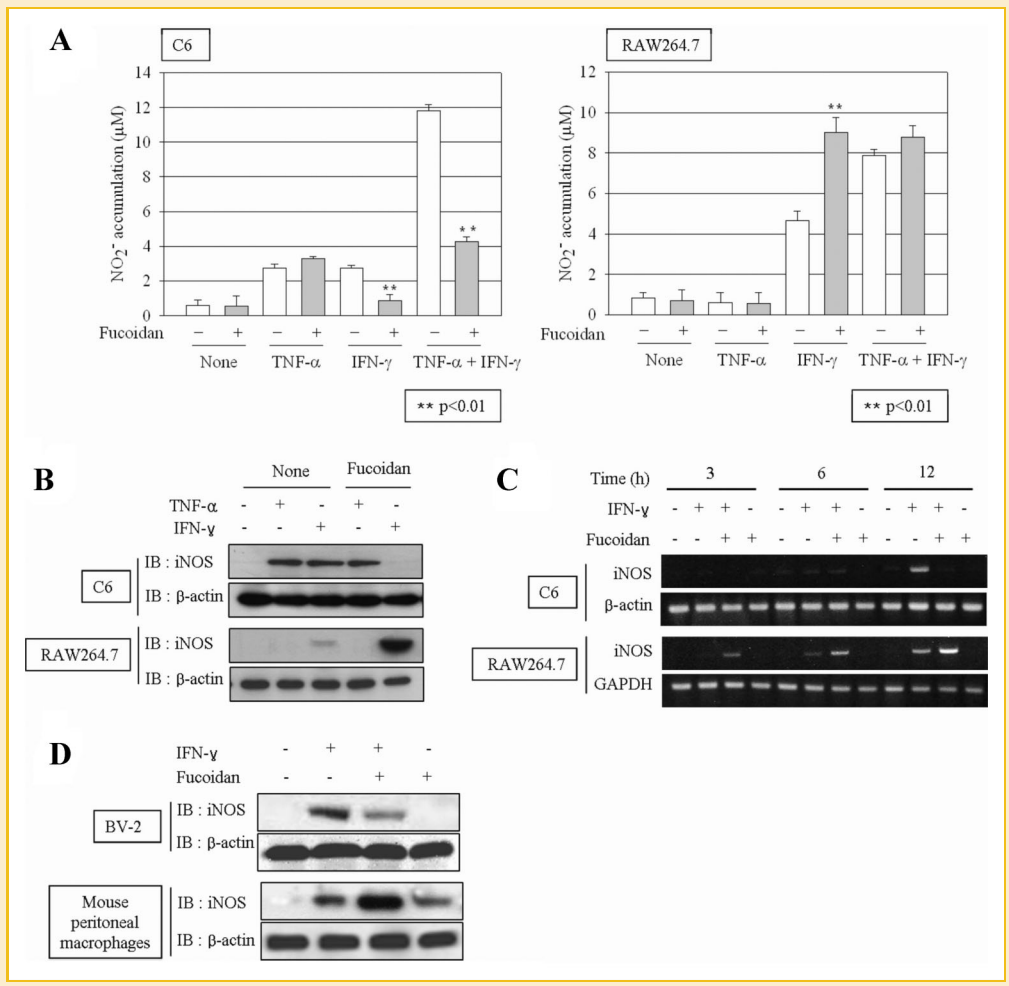

Fig. 1. Distinct effects of fucoidan on IFN-γ-induced NO production and iNOS expression in glial cells and peritoneal macrophages. C6 and RAW264.7 were treated with TNF-α (50 ng/ml), IFN-γ (500 U/ml), or TNF-α (50 ng/ml) plus IFN-γ (500 U/ml), in the absence or presence of fucoidan (50 μg/ml) for 24 h. A: Nitrite level was measured by the Griess method in both the cells. B: iNOS protein levels were determined by Western blot assay using an anti-iNOS antibody. C: The levels of iNOS mRNA expression were determined by RT-PCR analysis at the indicated times. D: BV-2 cells and mouse peritoneal macrophages were treated with IFN-γ (500 U/ml) in the absence or presence of fucoidan (50 μg/ml in mouse peritoneal macrophages and 0.5 μg/ml in BV-2 cells) for 24 h.

NO production in RAW264.7 cells. Fucoidan also suppressed NO production in C6 cells exposed to TNF-α and IFN-γ but had no effect on NO production by RAW264.7 cells exposed to these cytokine combination. iNOS protein expression after 24 h treatment of fucoidan showed a similar pattern as seen with suppressive NO production with IFN-γ-induced C6 and BV-2 cells but hyperinduction in RAW264.7 and primary peritoneal macrophages (Fig. 1B,D). Likewise, fucoidan reduced the expression of iNOS mRNA (first seen at 12 h after IFN-γ exposure) in C6 cells but increased the iNOS mRNA levels at 3, 6, and 12 h in RAW264.7 cells (Fig. 1C). Fucoidan (50 μg/ml) alone did not have any effect on NO/iNOS production in either cell type without cytotoxicity except BV-2 cells (data not shown). In BV-2 cells, 50 μg/ml of fucoidan showed cytotoxicity; therefore, proper concentration has been screened and determined at 0.5 μg/ml of fucoidan for this cell line.

FUCOIDAN REGULATES JAK/STAT ACTIVATION SIMILARLY AND p38 ACTIVATION DIFFERENTLY IN IFN-γ-ACTIVATED C6 AND RAW264.7 CELLS

Having observed that fucoidan affects only IFN-γ-induced NO/iNOS production, but not TNF-α-induced NO/iNOS production, we next elucidated the mechanism by which fucoidan regulates IFN-γ-induced NO/iNOS production in C6 glioma and RAW264.7 macrophages. It is well known that JAK/STAT is the main pathway for transducing IFN-γ activity. Binding of IFN-γ activates the JAK/STAT pathway by sequential inducing JAK2, JAK1, and then STATs tyrosine phosphorylation, which results in the expression of target genes. IFN-γ also activates p38 which may be important in IFN-γ-induced signaling. Thus, we examined the effect of fucoidan on JAK/STAT and p38 phosphorylation in IFN-γ-activated cells. Our data show that in C6, IFN-γ strongly induced the phosphorylation of JAK-2, STAT-1, and p38 over time (from 15 min to 6 h). Fucoidan (50 μg/ml) completely reversed these effects of IFN-γ (Fig. 2A,B). Expression of IRF-1, the downstream effector of STAT-1, which is believed to have important functions in iNOS expression, was increased by IFN-γ treatment and this increase was also abrogated by fucoidan (Fig. 2B). The inhibiting effect of fucoidan on JAK/STAT phosphorylation was much less pronounced in RAW264.7 cells. And, in clear contrast to C6 cells, fucoidan caused an increase in IFN-γ-induced p38 phosphorylation in RAW264.7 cells (Fig. 2C). This increased phosphorylation was most prominent at 30 and 90 min of treatment.

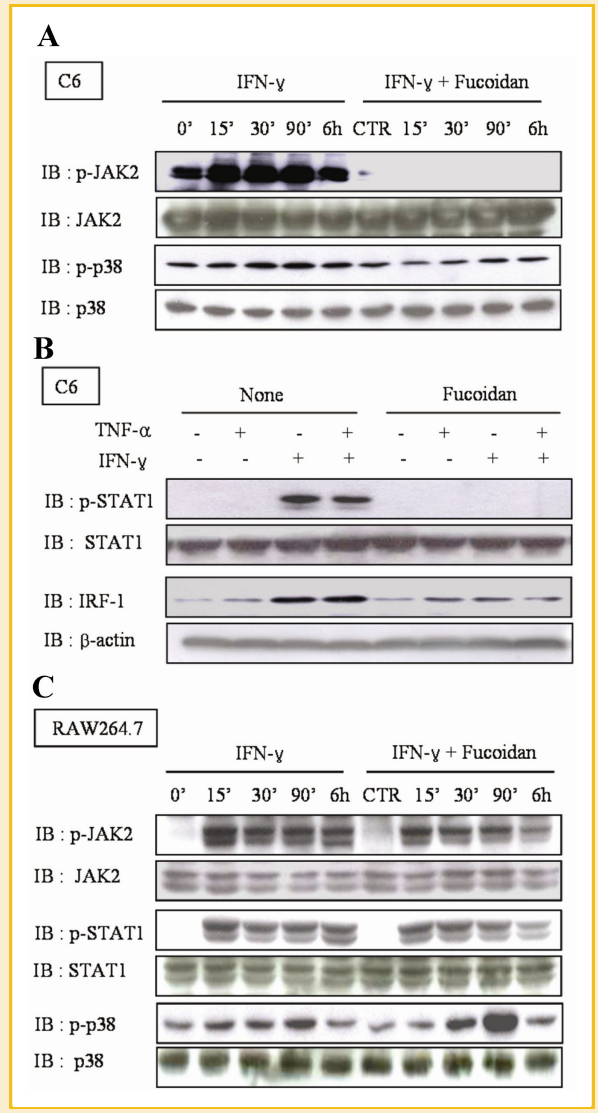

Fig. 2. Effect of fucoidan on JAK/STAT and p38 activation in IFN-γ-treated C6 glioma cells and RAW264.7 macrophages. A: C6 cells were treated with IFN-γ (500 U/ml) in the absence or presence of fucoidan (50 μg/ml) for the indicated times. Control (CTR) represents treatment with fucoidan (50 μg/ml) for 15 min. Whole-cell lysates were then prepared and subjected to Western blotting using antibodies specifically against phospho-JAK2 and phospho-p38. B: C6 cells were treated with TNF-α (50 ng/ml), IFN-γ (500 U/ml), or TNF-α (50 ng/ml) plus IFN-γ (500 U/ml), in the absence or presence of fucoidan (50 μg/ml) for 30 min for phospho-STAT1 detection and for 24 h for IRF-1 detection. C: RAW264.7 were treated with IFN-γ (500 U/ml) in the absence or presence of fucoidan (50 μg/ml) for the indicated times. Control (CTR) represents treatment with fucoidan (50 μg/ml) for 15 min. Whole-cell lysates were then prepared and subjected to Western blotting using antibodies specifically against p-JAK2, p-STAT1, and p-p38.

INVOLVEMENT OF p38 AND JAK/STAT IN IFN-γ-INDUCED NO/iNOS PRODUCTION IN GLIAL CELLS AND MACROPHAGES

Based on the observed effects of fucoidan on IFN-γ-induced NO/iNOS production as well as on IFN-γ-induced JAK/STAT and p38 activation, we next examined the involvement of JAK/STAT and p38 in IFN-γ-induced iNOS expression in C6 and RAW264.7 macrophages. Figure 3 showed that pretreatment with STAT1 inhibitor (fludarabine) at 20 μM reversed IFN-γ-induced iNOS expression in both C6 glioma cells and RAW264.7 macrophages, suggesting that STAT1 is a positive regulator of IFN-γ-induced iNOS expression in both cell types. However, pretreatment with p38 inhibitor (SB203580) at 10 μM inhibited IFN-γ-stimulated iNOS production in C6 glioma cells but increased IFN-γ-stimulated iNOS expression in RAW264.7 macrophages (Fig. 3A,B). We also confirmed these different effects of fucoidan with p38 inhibitor on iNOS expression in BV-2 microglia and peritoneal macrophages (Fig. 3C). These results imply that p38 regulates IFN-γ-induced iNOS expression differently in these two cell types.

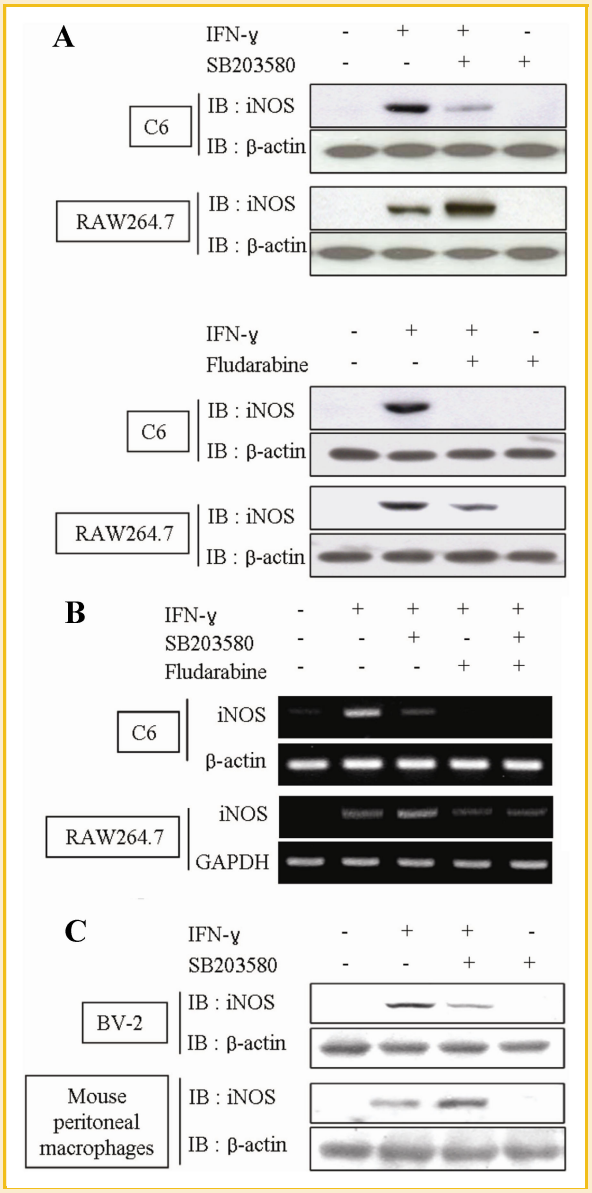

Fig. 3. Involvement of p38 and STAT1 in IFN-γ-induced iNOS expression in glial cells and peritoneal macrophages. C6 cells and RAW264.7 macrophages were pretreated with a p38 inhibitor, (SB203580, 10 μM) or a STAT1 inhibitor (fludarabine, 20 μM) for 1 h, followed by incubation with IFN-γ (500 U/ml) for 24 h. A: Both cell lysates were then prepared and subjected to Western blotting. B: Pretreatment with same concentration of respective inhibitors above followed by incubation with IFN-γ (500 U/ml) for 12 h. The levels of iNOS mRNA expression were determined by RT-PCR analysis. C: BV-2 cells and mouse peritoneal macrophages were pretreated with same concentration of respective inhibitors above followed by incubation with IFN-γ (500 U/ml) for 24 h. Cell lysates were then prepared and subjected to Western blotting.

THE INVOLVEMENT OF TNF-α PRODUCTION IN STIMULATORY EFFECT OF FUCOIDAN ON IFN-γ-INDUCED NO/iNOS PRODUCTION IN MACROPHAGES

Next, we further explored the mechanisms by which fucoidan enhances IFN-γ-induced NO production in RAW264.7 cells. There are some reports that fucoidan can increase TNF-α production [Hsu et al., 2001; Choi et al., 2005; Yang et al., 2008]. From our results (Fig. 1A), C6 and RAW264.7 cells exhibited a synergistic effect of IFN-γ combined with TNF-α on NO production. We, therefore, examined whether fucoidan increases TNF-α production and results in an increase in IFN-γ-induced iNOS production. Fucoidan alone significantly induced TNF-α production in a dose-dependent manner after 24 h of treatment in RAW264.7 cells and peritoneal macrophages (Fig. 4A,E) but not in C6 and BV-2 cells (Fig. 4C,F). In addition, a TNF-α antagonist reversed the stimulatory effect of fucoidan on IFN-γ-induced NO production in RAW264.7 cells but not in C6 cells (Fig. 4B,D), demonstrating that TNF-α mediates the stimulatory effect of fucoidan on IFN-γ-induced NO/iNOS production in this selective cell type.

INVOLVEMENT OF p38 IN THE EFFECT OF FUCOIDAN ON IFN-γ-INDUCED TNF-α AND NO/iNOS PRODUCTION IN RAW264.7 CELLS

We next focused on confirming the involvement of p38 in the effect of fucoidan on IFN-γ-induced TNF-α and NO/iNOS production in RAW264.7 cells. As shown in Figure 5, fucoidan alone increased p38 phosphorylation (p-p38) in a dose-dependent manner (Fig. 5A). The increase in p-p38 was seen as early as 30 min and peaked by 60 min (Fig. 5B). Treatment of fucoidan and IFN-γ increased p-p38, respectively, and their combination had a much stronger expression (Fig. 5C). Although pretreatment with a p38 inhibitor (SB203580) did not affect the level of TNF-α production in fucoidan- or IFN-γ-treated cells, this treatment strongly suppressed the level of TNF-α in cells treated with both fucoidan and IFN-γ (Fig. 5D). These results imply that although p38 is not involved in TNF-α production by fucoidan alone, it is a positive regulator that maintains increased TNF-α level in IFN-γ-stimulated cells by fucoidan. Thus, p38 activation status is a key determinant of TNF-α production in IFN-γ-activated RAW264.7 cells.

Figure 5E shows the dual effects (negative and positive) of p38 on NO/iNOS production in IFN-γ-treated RAW264.7 macrophages by fucoidan. p38 inhibition increased NO production and iNOS expression in the absence of fucoidan. However, in the presence of fucoidan, which enhances p38 activation, the p38 inhibitor had no effect on iNOS expression. These results raise the possibility that fucoidan alters the sensitivity of cells to the p38.

DISCUSSION

Recently, there has been increasing interest in elucidating molecular mechanisms underlying the various biological activities of fucoidan. In this study, we examined the effect of fucoidan on IFN-γ-induced NO/iNOS production and the molecular mechanisms underlying these effects in two types of cells such as glia (C6, BV-2) and macrophages (RAW264.7, peritoneal primary cells). Unexpectedly, fucoidan oppositely regulates IFN-γ-induced NO/iNOS production in glial cells and macrophages. In detail, fucoidan blocked IFN-γ-induced iNOS expression in C6 and BV-2 cells, whereas it increased IFN-γ-induced iNOS expression in RAW264.7 and peritoneal macrophages (Fig. 1). We also showed that these different NO/iNOS production probably occur through a mechanism

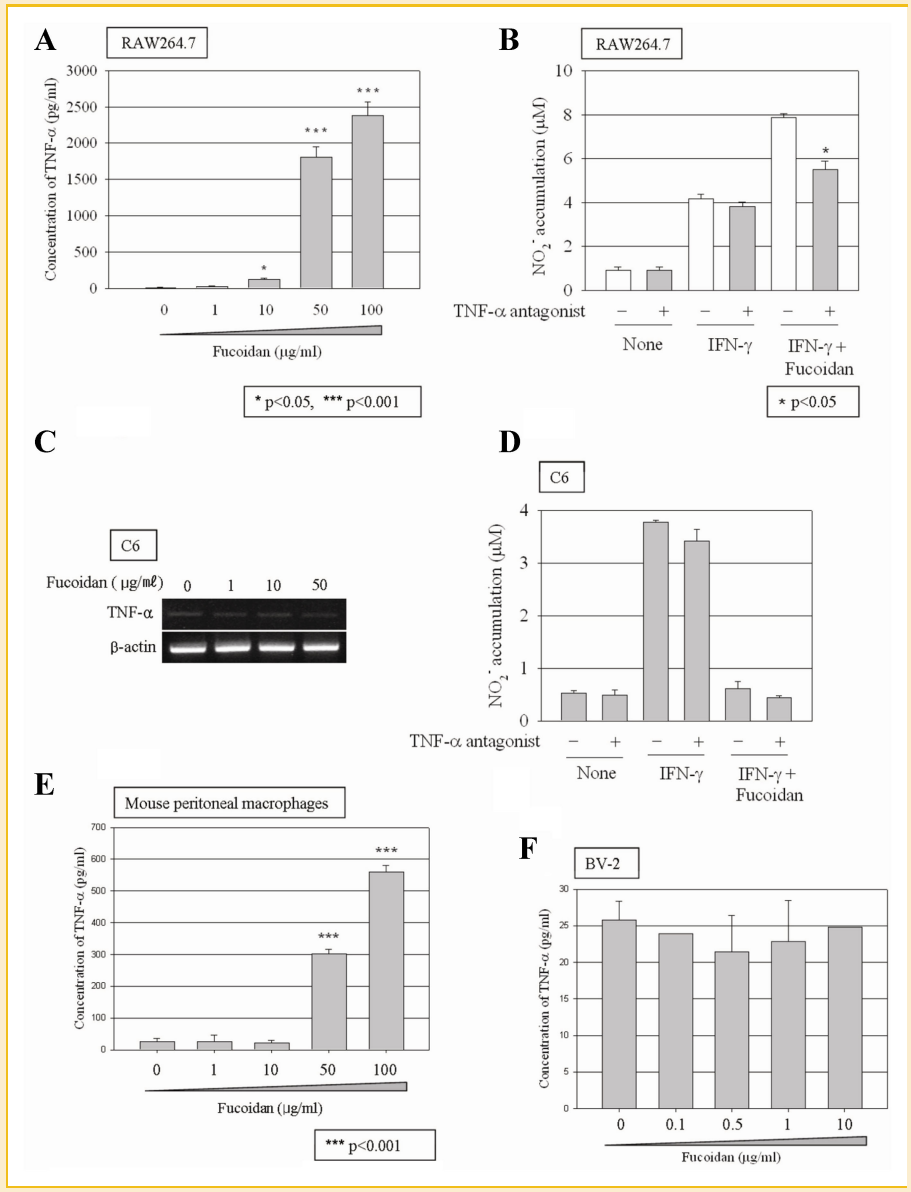

Fig. 4. Involvement of TNF-α production in the effect of fucoidan on IFN-γ-induced NO/iNOS production is different in glial cells and peritoneal macrophages. A: RAW264.7 macrophages, (E) mouse peritoneal macrophages, and (F) BV-2 cells were treated with various doses of fucoidan for 24 h. The cultured supernatants were collected and TNF-α production was examined by ELISA assay. B: RAW264.7 macrophages and (D) C6 glioma cells were pretreated with TNF-α antagonist (20 μM) for 1 h, followed by incubation with IFN-γ (500 U/ml) in the absence or presence of fucoidan (50 μg/ml) for 24 h. Nitrite level was measured by the Griess method. C: C6 cells were treated with various doses for 24 h. TNF-α mRNA expression was determined by RT-PCR analysis. The data shown are representative of three independent experiments with similar results. * and *** indicate $P < 0.05$ and $P < 0.001$, respectively.

associated with the effects of fucoidan on p38 activation in IFN-γ-stimulated cells.

The cytokine IFN-γ plays an essential role in innate and adaptive immunity and one action responsible for its inflammatory function results from excessive induction of iNOS gene expression and NO production. Binding of IFN-γ to its receptor activates gene expression mainly via the JAK/STAT/IRF-1 pathway [Winston et al., 1997]. It is well known that the murine iNOS gene contains IFN-γ response elements (IRE), GAS and ISRE, which are trans-activated in response to the activation of JAK/STAT signaling. It is also well known that the transcriptional induction of iNOS by IFN-γ depends on JAK/STAT and IRF-1.

In both C6 and RAW264.7 cells, IFN-γ increased JAK2, STAT1 phosphorylation and IRF-1 expression. And fucoidan treatment reversed these stimulatory effects of IFN-γ although the effect of fucoidan on RAW264.7 was much weaker than in C6 cells (Fig. 2). Using various specific inhibitors, we confirmed that IFN-γ-induced iNOS expression in both cell types is dependent on JAK/STAT signaling (Fig. 3). From these data, it can be concluded that fucoidan suppresses IFN-γ-induced iNOS expression in C6 glioma cells via

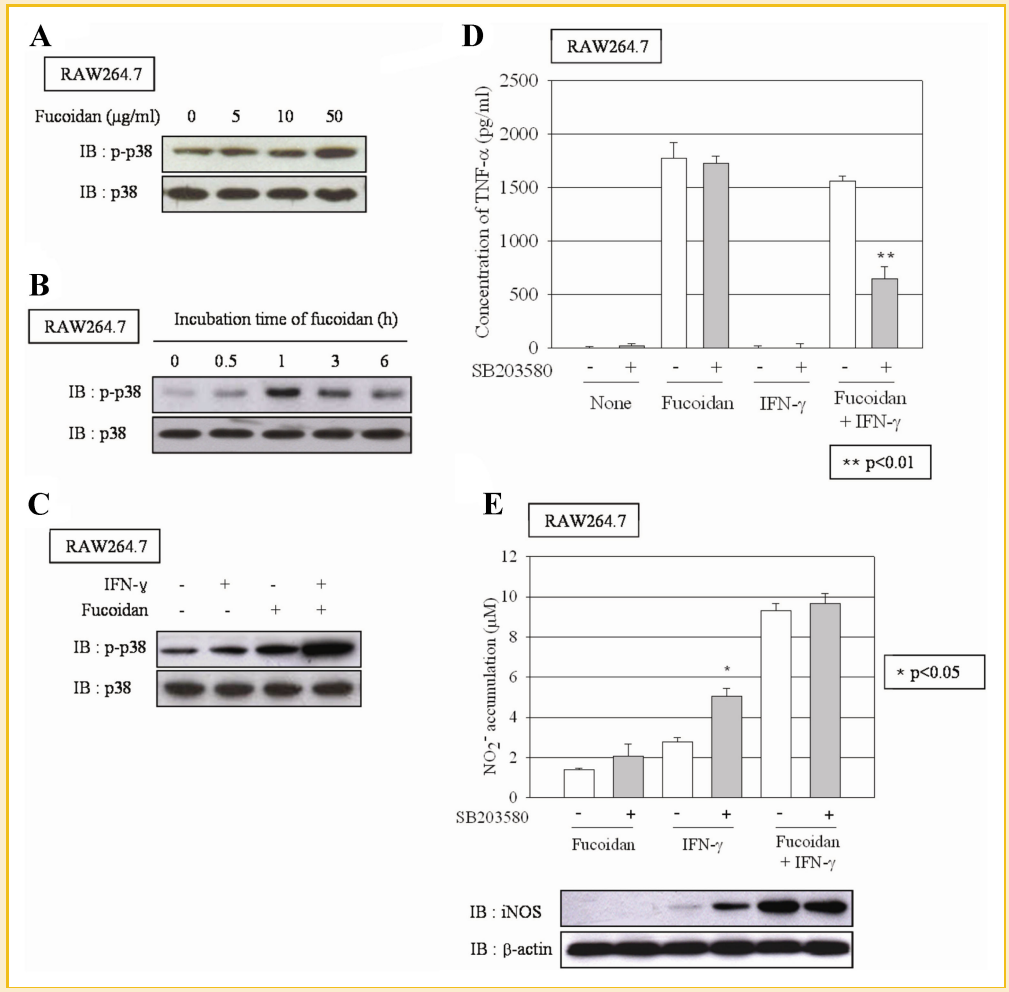

Fig. 5. Involvement of p38 in the effect of fucoidan on IFN-γ-induced TNF-α and NO/iNOS production in RAW264.7 macrophages. A: Cells were treated with various doses of fucoidan for 30 min and (B) with fucoidan at 50 μg/ml for the indicated times. C: Cells were treated with IFN-γ (500 U/ml) in the absence or presence of fucoidan (50 μg/ml) for 30 min. Cell lysates were then prepared for Western blotting using antibodies specifically against p-p38 and p38. D: Cells were pretreated with a p38-specific inhibitor (SB203580, 10 μM) or vehicle (DMSO) for 1 h, followed by incubation with fucoidan (50 μg/ml) and/or IFN-γ for 24 h. TNF-α production was examined by ELISA assay and (E) nitrite level was measured by the Griess method. Also, cell lysates were then prepared and subjected to Western blotting.

JAK/STAT signaling inhibition. We also found that treatment with IFN-γ increased p-p38 in both cell types. However, interestingly, fucoidan suppressed p-p38 activation in C6 glioma cells while it increased this activation in RAW264.7 cells. Using SB203580, a specific inhibitor of p38, we confirmed that p38 upregulates IFN-γ-induced iNOS expression in C6 glioma and BV-2 microglia, whereas it downregulates IFN-γ-induced iNOS expression in RAW264.7 and peritoneal macrophages (Fig. 3). These interesting results mean that the dual effect of p38 on IFN-γ-induced iNOS expression depends on cell states and that there is a difference between glia and macrophages cell types. Won et al. [2004] also have reported that there is a dual effect of cAMP in iNOS expression in C6 glioma and RAW 264.7 cells, which is mediated by differential regulation of p38 MAPK/ATF-2 activation.

TNF-α alone induces NO production in C6 glioma cells but not RAW264.7 cells (Fig. 1). However, there is a strong synergistic effect between TNF-α and IFN-γ for NO production in both cell types. There are few reports that fucoidan treatment has been shown to augment macrophage TNF-α production [Hsu et al., 2001; Choi et al., 2005; Yang et al., 2008]. We, therefore, examined the involvement of TNF-α production in the regulatory effect of fucoidan on IFN-γ-induced iNOS expression. Our results showed that fucoidan strongly increased TNF-α production in macrophages (RAW264.7 and peritoneal macrophages) but not in glia (C6 and BV-2 cells), which was further confirmed by treatment with TNF-α antagonist (Fig. 4). This suggests that TNF-α production plays a key role in the stimulatory effect of fucoidan on IFN-γ-induced NO production in macrophages. Although the effect of fucoidan on TNF-α production in macrophages has been reported, there has been no evidence for the involvement of p38 in TNF-α production caused by fucoidan. In this study, we found that although p38 does not directly regulate fucoidan-induced TNF-α production, it is essential to maintain the level of fucoidan-induced TNF-α production in IFN-γ-treated cells. Therefore, p38 appears to be involved in the dual

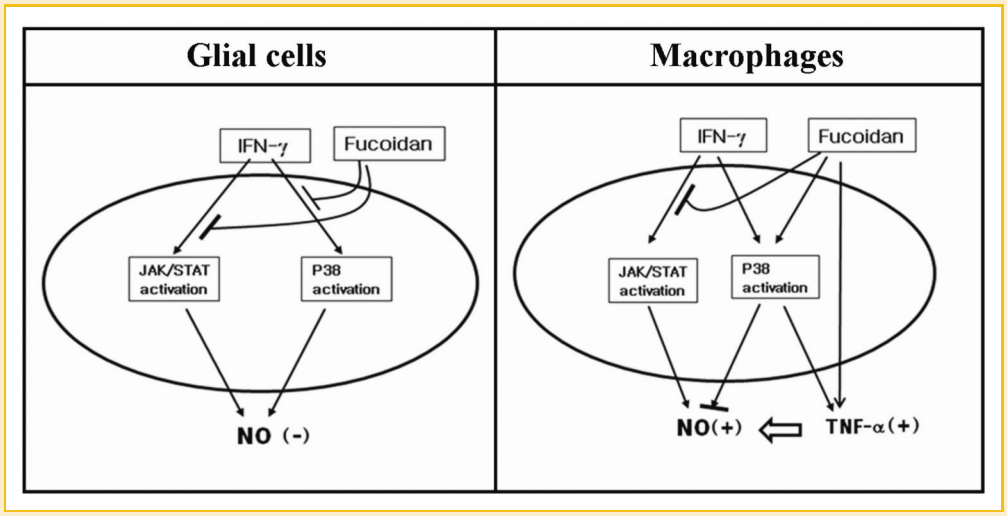

Fig. 6. Different effect of fucoidan on IFN-γ-induced NO production in glial cells and macrophages is mediated by opposite regulation of p38 activation and TNF-α production. The IFN-γ-induced increases in NO/iNOS production are selectively inhibited in glial cells by fucoidan. This inhibition may be mediated by blocking of p38 and JAK/STAT activation. In macrophages, IFN-γ-mediated NO/iNOS expression and p38 activation are more increased by fucoidan. Moreover, fucoidan increased TNF-α production which is positively regulated by p38 activation and resulted in an increased of NO production via synergistic effect between TNF-α and IFN-γ. In macrophages, JAK/STAT is a positive regulator whereas activated p38 is a negative regulator of IFN-γ-induced NO/iNOS production.

effect of fucoidan on IFN-γ-induced NO/iNOS production in RAW264.7 cells. On the one hand, p38 directly downregulates IFN-γ-induced NO/iNOS production; but on the other hand, it maintains the level of fucoidan-induced TNF-α release, which strongly synergizes with IFN-γ to induce iNOS expression in macrophage cell type. While we found that fucoidan alone increased p-p38 in RAW264.7 cells (Fig. 5), there was no increase in NO production, implying that p38 activation alone is not enough to induce iNOS expression in RAW264.7 cells.

It is believed that fucoidan is a ligand of class A scavenger receptors (SR-A) [Baba et al., 1998; Patel et al., 2002; O'Leary et al., 2004]. It has been shown that SR-A is directly involved in cell signaling and cytokine production [Pollaud-Chérion et al., 1998; Coller and Paulnock, 2001; Hsu et al., 2001; Nakamura et al., 2006]. There has been also reported that the possibility of involvement with other receptors besides SR-A for fucoidan to induce biological effects in cells [Ordija and Freeman, 2003; Oomizu et al., 2006]. In this study, SR-A1 blocking antibody did not change the effect of fucoidan on IFN-γ-induced iNOS in RAW264.7 cells (data not shown). Therefore, other receptors may responsible for the effects of fucoidan on IFN-γ-induced iNOS in RAW264.7 cells.

NO over-production is thought to play a crucial role in the induction of tissue injury in neuron-inflammatory diseases. Therefore, prevention of iNOS expression represents an important therapeutic goal [Shinoda and Whittle, 2001]. In this study, we found that fucoidan inhibited NO production and iNOS expression induced by IFN-γ in glial cells. Recent research indicated that fucoidan shows protective effect on neuron death in vivo system. The longer duration of pretreatment of fucoidan showed the better protective effect on neuron perhaps despite of its relatively poor ability to pass blood–brain barrier [Luo et al., 2009]. These data suggest fucoidan as a potential therapeutic agent for reducing inflammatory-related neuronal injury such as Alzheimer's disease.

As summarized in Figure 6, our data suggest that fucoidan suppresses IFN-γ-induced NO/iNOS production in glial cells via inhibition of JAK/STAT/IRF-1 and p-p38. In contrast, fucoidan increased IFN-γ-induced iNOS in RAW264.7 cells via stimulation of TNF-α production and p-p38. We also found that p-p38 activation has a dual role which increases the IFN-γ-induced NO/iNOS production as a positive regulator in glial cells and decreases NO/iNOS as a negative regulator in macrophages. It is supposed that this dual direction led by activated p38 might depend on cell states and cell types. These different effects of fucoidan on iNOS expression through IFN-γ-mediated signaling between two cell types can suggest the possibility not only as a promising candidate for treating inflammatory-related neuronal injuries but also as a immune modulating nutrient for altering sensitivity of cells to the p38 activation.

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