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Fucoidan induces nitric oxide production via p38 mitogen-activated protein kinase and NF-κB-dependent signaling pathways through macrophage scavenger receptors

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Abstract

It has been reported that ligands of the macrophage scavenger receptor (MSR) induce a range of cellular responses including urokinase-type plasminogen activator and the production of inflammatory cytokines. Although nitric oxide (NO) is an important regulatory molecule in physiological functions such as vascular homeostasis, neurotransmission, and host defense, the effect of MSR ligands on NO production from macrophages was unknown. Here, we demonstrate that the MSR ligand, fucoidan, but neither oxidized low-density lipoprotein, acetylated LDL, maleylated bovine serum albumin nor dextran sulfate induces activation of inducible nitric oxide synthase (iNOS) promoter or NO production in RAW264.7 cells. Furthermore, we investigated the molecular mechanism by which fucoidan induces iNOS promoter activation. Using different inhibitors, we showed that the stimulation of fucoidan was mediated by both the p38 mitogen-activated protein kinase and the NF-κB-dependent pathways. Although these two pathways were independent, heat shock protein 90 (HSP90) played a significant role in both pathways. Our previous study showed that HSP90 directly interacts with the cytoplasmic domain of MSR. These results provide the evidence that HSP90 bound to the cytoplasmic domain of MSR is implicated in MSR-mediated signal transduction. Moreover, fucoidan-induced NO production by peritoneal macrophages from MSR-knockout ($MSR^{-/-}$) mice significantly decreases compared with those from wild-type mice. This is the first indication that MSR transduces the signal of fucoidan to iNOS gene expression.

Keywords: Macrophage scavenger receptor; Fucoidan; Nitric oxide; HSP90; p38 mitogen-activated protein kinase; NF-κB

The class A scavenger receptor (SR-A) is a multidomain trimeric molecule composed of three glycosylated protein chains [1]. SR-A mediates the high affinity binding and internalization of modified low-density lipoprotein (LDL), including the oxidized LDL (OxLDL) implicated in the development of atherosclerosis [2,3]. In addition to modified LDL, a diverse group of polyanionic compounds are listed as ligands for SR-A [4]. Broad ligand specificity in SR-A may explain the receptor multifaceted functions of macrophages such as the clearance of pathologic substances [5,6], host defenses [7,8], adhesion [7], and signal transduction [9–12]. It has recently been shown that macrophage interaction with SR-A ligands, such as maleylated bovine serum albumin (mBSA), the double-stranded (ds) DNA molecule (poly(dI) poly(dC)), the dsRNA molecule (poly(I) poly(C)), and lipoteichoic acid, induces distinct patterns of gene expression in the murine macrophage cell line RAW264.7, and this response is largely endocytosis dependent [13]. It has also been demonstrated that another ligand of SR-A, fucoidan, induces protein tyrosine phosphorylation of protein kinases, protein kinase C (PKC)

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activity, and specifically stimulates the activity of p21-activated kinase, the mitogen-activated kinase (MAPK) extracellular signal-regulated kinase (ERK), c-Jun N-terminal protein kinase (JNK), and p38 MAPK leading to inflammatory cytokine secretion and urokinase-type plasminogen activator expression [14,15]. Thus, although SR-A ligands initiate various signaling pathways, the influence of SR-A ligands on nitric oxide (NO) production from macrophages was unknown. NO, a free radical species, participates in the numerous physiological functions of macrophages such as vascular homeostasis, neurotransmission, and host defense [16–18]. NO is synthesized by nitric oxide synthase (NOS)-catalyzed conversion of L-arginine to L-citrulline. Whereas the activity of neuronal and endothelial NOS is mainly regulated post-translationally by cytoplasmic calcium levels or by phosphorylation by various protein kinases, the inducible isoform (iNOS) is primarily regulated at the transcriptional level [19].

This study investigates the effects of various SR-A ligands, including AcLDL, OxLDL, fucoidan, mBSA, and dextran sulfate, on NO production from macrophages. Only fucoidan was found to induce iNOS promoter activation and NO production in RAW264.7 cells. Fucoidan, a polyanionic, sulfated polymer of L-fucose obtained from brown marine algae, has been used as an effective competitor for OxLDL or modified LDL in studies on receptor binding, and it is reported to have anticoagulant activity [20,21] and antitumor activity [22,23]. However, the molecular mechanism of iNOS promoter activation and NO production by fucoidan is unknown. We demonstrate that the signal of fucoidan transduces via p38 MAPK- and nuclear factor-κB (NF-κB)-dependent pathways. Furthermore, although these two pathways are independent, both pathways need heat shock protein 90 (HSP90). HSP90 is an ATP-dependent molecular chaperone involved in the folding and activation of numerous substrate proteins including protein kinase and transcription factors [24]. Many of these substrates are critical elements in signal transduction. Since our previous study demonstrated that HSP90 binds to the cytoplasmic domain of SR-A [25], our current data strongly suggest that the HSP90 bound to the cytoplasmic domain of SR-A is implicated in SR-A-mediated signal transduction. Moreover, fucoidan-induced NO production significantly decreased in peritoneal macrophages from SR-A-knockout ($SR\text{-}A^{-/-}$) mice. These results are the first indication that SR-A transduces the stimulation of fucoidan.

Materials and methods

Materials. Fucoidan, SB203580, PD98059, calphostin C, wortmannin, MG-132, AG490, and geldanamycin were purchased from Sigma.

Cell culture. RAW264.7 cells and peritoneal macrophages were cultured in an RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum. Peritoneal macrophages were collected from SR-A knockout and wild-type mice (male, 9–12 weeks old).

Determination of nitric oxide. RAW264.7 cells (1×10^5) or peritoneal macrophages (2×10^5) were seeded on a 96-well plate and incubated for 24 h with AcLDL (25 μg/ml), OxLDL (25 μg/ml), fucoidan (50 μg/ml), mBSA (100 μg/ml), and dextran sulfate (100 μg/ml). NO production was determined by measuring the accumulation of nitrite in the culture medium using the Griess reaction [26]. Briefly, 100 μl of cultured supernatants was mixed with an equal volume of the Griess reagent [1% sulfanilamide, 0.1% *N*-(1-naphthyl)ethylenediamine in 5% phosphoric acid] for 10 min. The optical density of the reaction mixture was read at 550 nm with a microplate reader (Bio-Rad). The concentration of nitrite was determined using sodium nitrite as a standard. In the case of inhibitor experiments, cells were preincubated with or without SB203580 (20 μM), PD98059 (50 μM), calphostin C (100 nM), wortmannin (50 nM), MG-132 (5 μM), AG490 (50 μM) or geldanamycin (5 μM) for 1 h and then further incubated with fucoidan (50 μg/ml) for 24 h. NO production was determined as described above. The cytotoxicity of the inhibitors and fucoidan was evaluated using the 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyltetrazolium bromide (MTT) assay.

Plasmid constructs. The 1749-bp fragment (−1588 to +161) of the 5′-flanking region of mouse *iNOS* gene was cloned from a genomic library derived from RAW264.7 cells and inserted in front of the luciferase gene in PGV-B (TOYO INK) to form piNOS. The nucleotide sequences were confirmed by a DNA sequencer (ABIPRISM™310 Genetic Analyzer).

Transient transfection and luciferase assay. RAW264.7 cells (5×10^5) were plated on a 24-well plate and transfected with piNOS using LipofectAMINE 2000 reagent (invitrogen) according to the manufacturer's protocol. Briefly, cells were exposed to OPTI-MEM (invitrogen) containing 0.5 μg piNOS and 0.5 μg liposomes for 5 h. These cells were then washed and incubated with the medium alone or the medium containing either SB203580 (20 μM), PD98059 (50 μM), calphostin C (100 nM), wortmannin (50 nM), MG-132 (5 μM), AG490 (50 μM) or geldanamycin (5 μM) for 1 h and then further incubated with fucoidan (50 μg/ml) for 24 h. The cells were lysed with 100 μl lysis buffer [1% Triton X-100, 25 mM Tris-phosphate (pH 7.8), 2 mM dithiothreitol, 2 mM 1,2-diaminocyclohexane-*N*,*N*,*N*′,*N*′-tetraacetic acid (pH 7.8), and 10% glycerol]. Luciferase activity was assayed with 20 μl of lysate in the luciferase assay reagent [20 mM Tris–NaOH, 2.67 mM $MgSO_4$, 0.1 mM EDTA (pH 8.0), 33.3 mM dithiothreitol, 270 μM Co enzyme A, 530 μM ATP, and 470 μM luciferin] using a Lumat LB9501 luminometer (Berthold). The protein content in the cell lysates was measured using the Bradford assay, and each luciferase activity was normalized to each protein content.

RT-PCR. RAW264.7 cells (2.5×10^5) were plated on a 6-well plate and incubated for 24 h with or without fucoidan (50 μg/ml). Total RNA was extracted using RNeasy Mini Kit (Qiagen), and cDNA was reverse transcribed from 1μg total RNA using Thermoscript RT-PCR system (Invitrogen). PCR amplification of cDNA was carried out with specific primers for iNOS and β-actin, and the products were visualized by electrophoresis through 2% agarose gel containing ethidium bromide. PCR was performed in a Gene Amp PCR System 9700 (Applied Biosystems). The thermal cycle is as follows: 30 s of denaturation at 94 °C, 30 s annealing at 52 °C (iNOS) or 55 °C (β-actin), and 30 s of extension at 72 °C. For iNOS, the primers were sense, 5′-AAGTCAAATCCTACCAAAGTGA-3′; antisense, 5′-CAATAATACTGGTTGATGAACT-3′, and the PCR product was 410 bp. The primers for β-actin were sense, 5′-TCCTTCTTG GGTATGG-3′; antisense, 5′-ACGCAGCTCAGTAACAG-3′, and the PCR product was 355 bp.

Western blot analysis. RAW264.7 cells (2.5×10^5) were plated on a 6-well plate and incubated for 24 h with or without fucoidan (50 μg/ml). In the case of inhibitor experiments, cells were preincubated with or without geldanamycin (5 μM), SB203580 (20 μM) or MG-132 (5 μM) for 1 h and then further incubated with fucoidan (50 μg/ml) for 2 h. Cells were lysed with TNE buffer (10 mM Tris–HCl (pH 7.8), 1% (w/v) Nonidet P-40, 150 mM NaCl, and 50 mM EDTA) followed by centrifugation. The supernatants (50μg/lane) were subjected to 10% SDS–PAGE and subsequent immunoblotting, as described previously [26]. The proteins were detected using anti-iNOS (M-19, Santa Cruz Biotechnology), anti-β-actin (AC-15, sigma), anti-p38 MAP kinase (#9212, Cell Signaling), anti-phospho-p38 MAP kinase (#9211, Cell Signaling), and the ECL Western blot analysis system (Amersham Biosciences).

Preparation of nuclear extracts. RAW264.7 cells (1×10^7) were preincubated with SB203580, MG-132 or geldanamycin for 1 h and then further incubated with fucoidan (50 μg/ml) for 3 h. All extraction procedures were performed on ice with ice-cold reagents. RAW264.7 cells (1×10^7) treated with the inhibitor and fucoidan were washed with PBS twice and then harvested in 1 ml PBS using a cell scraper. The cells were collected by centrifugation (5000*g*, 5 min). The pellet was dispersed in 400 μl buffer A [10 mM Hepes–KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA (pH 8.0), 0.1% Nonidet P-40, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 2 μg/ml leupeptin]. The nuclei were pelleted by centrifugation (5000*g*, 5 min). The pelleted nuclei were dispersed in buffer B [50 mM Hepes–KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA (pH 8.0), 5 mM $MgCl_2$, 2% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 2 μg/ml leupeptin]. The suspended nuclei were gently shaken for 30 min and centrifuged (12,000*g*, 15 min). The clear supernatants containing nuclear proteins were stored at −80 °C before use. Protein concentrations were determined using the Bradford assay.

Electrophoretic mobility shift assay. The oligonucleotide sequences for electrophoretic mobility shift assay (EMSA) were as follows: 5′-CAGTT GAGGGGACTTTCCCAGGC-3′ (sense), 5′-GCCTGGGAAAGTCCC CTCAACT-3′ (antisense) for NF-κB and 5′-CCGCTTGATGACTCAG CCGGAA-3′ (sense), 5′-TTCCGGCTGAGTCATCAAGCG-3′ (antisense) for activator protein-1 (AP-1). The sense and complementary strand was annealed to the sense strand, and then the double-stranded fragment was labeled with [α-^{32}P]dGTP using the Klenow fragment (Takara). A total of 10 μg of nuclear extract was incubated with 7000 cpm of ^{32}P-labeled NF-κB or AP-1 probes in the binding buffer [10 mM Tris–HCl (pH 7.5), 5% glycerol, 1 mM dithiothreitol, and 1 mM EDTA] in the presence of 2 μg poly(dI–dC) at room temperature for 30 min, and then the mixture was further incubated at 4 °C for 30 min. The DNA–protein complexes were separated from the free probe on 5% native polyacrylamide gels in TAE buffer [6.7 mM Tris–HCl (pH 7.5), 3.3 mM sodium acetate, and 1 mM EDTA (pH 8.0)]. The gel was dried and analyzed using BAS1500 (Fuji Film). For the supershift experiments, specific antibodies against p50, p65, c-rel, RelB, p52, c-Jun or c-Fos (all of which were purchased from Santa Cruz Biotechnology) were added to the nuclear extract before the addition of the labeled probe.

Statistical analysis. Statistical comparisons were made by Student's *t* test, with a value of $P < 0.05$ considered to be significant.

Results

Effect of SR-A ligands on nitric oxide production from RAW264.7 cells

We investigated the effects of SR-A ligands on NO production from RAW264.7 cells. Cells were incubated with either 25 μg/ml AcLDL, 25 μg/ml OxLDL, 50 μg/ml fucoidan, 100 μg/ml mBSA or 100 μg/ml dextran sulfate for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture medium. As shown in Fig. 1A, the basal level of NO production from RAW264.7 cells was less than 3 μM. Significant NO production increase was detected only in the case of fucoidan treatment (6-fold). We also detected an increase in NO production of RAW264.7 cells in a dose-dependent manner with fucoidan treatment (Fig. 1B).

SR-A is responsible for the fucoidan-inducing nitric oxide production of macrophages

Fucoidan, a polyanionic polysaccharide, has been used as an effective competitor for OxLDL or AcLDL in studies on ligand–receptor binding [4]. Furthermore, it has been reported that fucoidan stimulates the production of cytokines in macrophages [4,11,15]. However, there is no direct evidence that the stimulation of fucoidan is mediated through SR-A. To clarify the role of SR-A in NO production in macrophages, we used peritoneal macrophages from SR-A-knockout (SR-A$^{-/-}$) mice [7]. Peritoneal macrophages from wild-type or SR-A$^{-/-}$ mice were incubated with 50 μg/ml fucoidan for 24 h. NO production was detected by measuring the accumulation of nitrite in the culture medium. As shown in Fig. 1C, the macrophage of SR-A$^{-/-}$ mice could not induce NO production by fucoidan. This indicates that almost all NO production of macrophages by fucoidan is mediated by SR-A. This is the first time in which it was shown directly that the stimulation of macrophages by fucoidan is certainly via the SR-A-dependent pathway.

Fucoidan induces iNOS promoter activation, iNOS mRNA expression, and iNOS protein in RAW264.7 cells

We next examined whether or not fucoidan-induced NO production from macrophages was catalyzed by iNOS. RAW264.7 cells were transformed by piNOS and then incubated with 0, 10, 25, 50 or 100 μg/ml fucoidan for 24 h. The cells were lysed, and luciferase activity was measured. The results show that fucoidan increased the expression of the luciferase from the iNOS promoter in a dose-dependent manner (Fig. 2A). These data strongly suggest that the increase in NO production in RAW264.7 cells by fucoidan is mediated by the induction of the iNOS expression. Furthermore, we checked both iNOS mRNA level and iNOS protein level when fucoidan was added to RAW264.7 cells. The results show that fucoidan induced iNOS mRNA expression (Fig. 2B), and hence increased iNOS protein level in RAW264.7 cells (Fig. 2C).

Effects of inhibitors on fucoidan-induced nitric oxide production and iNOS promoter activation in RAW264.7 cells

To further investigate the molecular mechanism of fucoidan-induced NO production from RAW264.7 cells, we examined the effect of various inhibitors that block signal transduction pathways. It has recently been reported that the fucoidan inducing interleukin-1β (IL-1β) expression is mediated by MAPKs [15]. It has also been demonstrated that the binding of fucoidan to SR-A induced protein tyrosine phosphorylation including phosphatidylinositol-3-OH kinase (PI 3-kinase) and increased PKC activity, which leads to an increase in the expression of the urokinase-type plasminogen activator [14]. In addition to the binding sites for AP-1 proteins in mouse iNOS promoter, it also contains multiple binding sites for NF-κB and Stat1, which are required for maximal responses to lipopolysaccharide (LPS) and interferon-γ (IFN-γ), respectively [27]. Our previous study showed that HSP90 directly interacts with the cytoplasmic domain of SR-A, and it suggested that HSP90

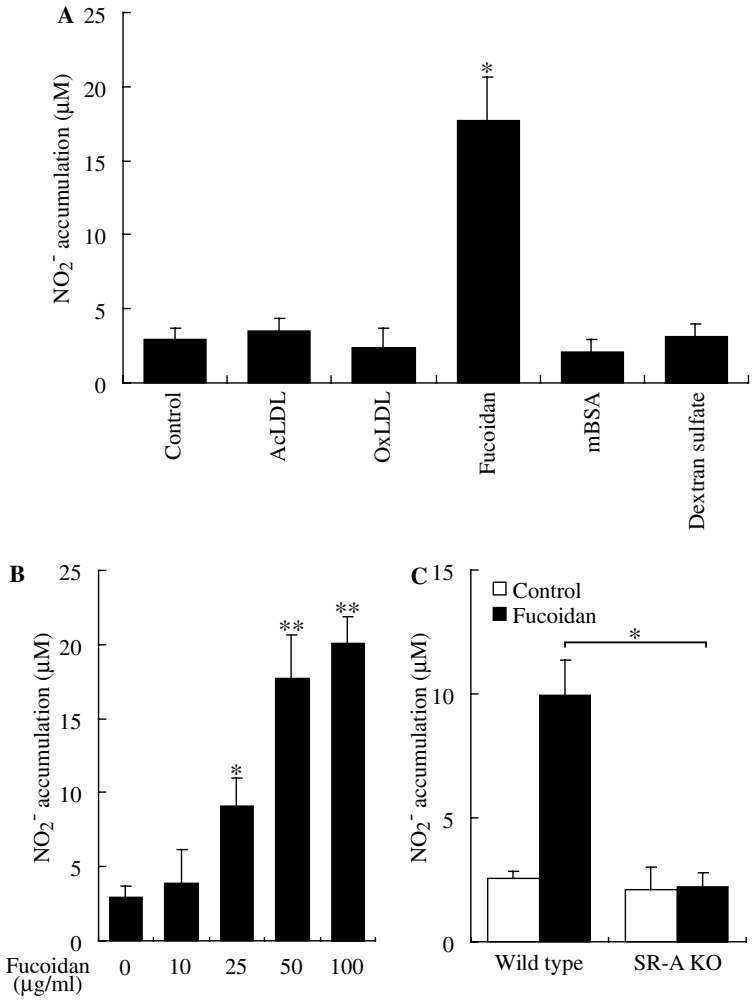

Fig. 1. Effects of SR-A ligands on NO production in RAW264.7 cells and peritoneal macrophages. (A) RAW264.7 cells (1×10^5) were incubated with AcLDL (25 μg/ml), OxLDL (25 μg/ml), fucoidan (50 μg/ml), mBSA (100 μg/ml) or dextran sulfate (100 μg/ml) for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture medium. Data are means ± SD of triplicate cultures from three independent experiments. $^*P < 0.001$ compared with control value. (B) RAW264.7 cells (1×10^5) were incubated with various concentrations of fucoidan (0, 10, 25, 50, and 100 μg/ml) for 24 h. NO production was determined as described above. Data are means ± SD of triplicate culture from three independent experiments. $^*P < 0.01$, $^{**}P < 0.001$ compared with control value. (C) Peritoneal macrophages from wild type or SR-$A^{-/-}$ mice were incubated with fucoidan (50 μg/ml) for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture medium. Data are means ± SD of triplicate cultures from three independent experiments. $^*P < 0.001$ compared with SR-A-KO macrophages incubated under the same conditions.

bound to SR-A may be implicated in signal transduction [25]. Based on these demonstrations, we investigated the effects of p38 MAPK inhibitor SB203580, mitogen-activated protein kinase kinase (MEK) inhibitor PD98059, PKC inhibitor calphostin C, PI 3-kinase inhibitor wortmannin, inhibitor of inhibitory κB-α (IκB-α) degradation MG-132, janus kinase 2 (JAK2) inhibitor AG490, and HSP90 inhibitor geldanamycin on NO production and iNOS promoter activation by fucoidan in RAW264.7 cells. Cells were preincubated with either SB203580, PD98059, calphostin C, wortmannin, MG-132, AG490 or geldanamycin for 1 h and then further incubated with fucoidan for 24 h. NO production and iNOS promoter activity were determined as described in Materials and methods. The results show that pretreatment of RAW264.7 cells with SB203580, MG-132 or geldanamycin blocked fucoidan-induced NO production by 40%, 60%, and 69%, respectively, and there were no obvious inhibitory effects with pretreatment with PD98059, calphostin C, wortmannin or AG490 (Fig. 3A). Similar inhibitory effects were observed on the activation of iNOS promoter. As shown in Fig. 3B, SB203580, MG-132, and geldanamycin blocked the fucoidan inducing iNOS promoter activation by 47%, 75%, and 79%, respectively; there were no obvious inhibitory effects with pretreatment with PD98059, calphostin C, wortmannin or AG490. These observations encouraged us to analyze p38 MAPK pathway of fucoidan-induced NO production. Upon fucoidan stimulation, the phosphorylation of p38 MAPK was detected around 30 min and was still apparent after 120 min (Fig. 4). However, fucoidan-induced phosphorylation of p38 MAPK was significantly inhibited by pretreatment with geldanamycin, suggesting that HSP90 acts upstream of p38 MAPK (Fig. 4). SB203580 can inhibit various kinases at doses only slightly higher than those that

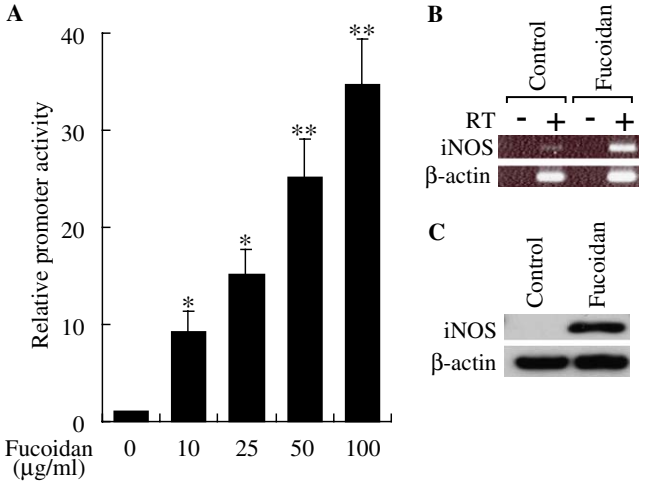

Fig. 2. Fucoidan induces iNOS promoter activation, iNOS mRNA expression, and iNOS protein expression in RAW264.7 cells. (A) RAW264.7 cells (5×10^5) were transfected with a mouse iNOS promoter construct (piNOS). Cells were treated with the indicated concentrations of fucoidan for 24 h and lysed for luciferase activity analysis. Data are means ± SD of triplicate cultures from three independent experiments. $^{*}P < 0.01$, $^{**}P < 0.001$ compared with control value. (B) RAW264.7 cells were cultured with or without fucoidan (50 μg/ml) for 24 h. iNOS mRNA expression was detected by RT-PCR. (C) RAW264.7 cells were cultured with or without fucoidan (50 μg/ml) for 24 h. iNOS protein was detected by Western blotting.

inhibit p38 MAPK. To rule out this possibility, we examined the effect of SB203580 on fucoidan-induced phosphorylation of p38 MAPK. As shown in Fig. 4, fucoidan-induced phosphorylation of p38 MAPK was not inhibited by SB203580, indicating that SB203580 did not inhibit its upstream kinases in this experiment. There were no obvious inhibitory effects with pretreatment of MG-132, either. Taken together, p38 MAPK, NF-κB, and HSP90 play an important role in fucoidan-induced NO production, and HSP90 acts upstream of p38 MAPK.

Effects of fucoidan on signal pathways through NF-κB and AP-1 activation in RAW264.7 cells

To clarify the mechanisms by which fucoidan activates the mouse iNOS promoter, we investigated the *cis*-elements of the iNOS promoter and transcription factors in RAW264.7 cells. As stated above, mouse iNOS promoter contains multiple binding sites for NF-κB, AP-1, and signal transducer and activator of transcription 1 (Stat1). Since JAK2 inhibitor AG490 had no effect on the fucoidan inducing NO production (Fig. 3A) and iNOS promoter activation (Fig. 3B), the signaling pathway through JAK/STAT is not involved in the fucoidan that induces NO production and iNOS promoter activation in RAW264.7 cells. For this reason, we then investigated the role of NF-κB and AP-1 in the induction of NO production by fucoidan. We checked the binding proteins to the iNOS promoter fragment including either the NF-κB or the AP-1 response sequence by EMSA. The results shown in Figs. 5A and B indicate that treatment with fucoidan induces the binding of NF-κB or AP-1 to each fragment. The NF-κB binding was inhibited by geldanamycin or MG-132 but not by SB203580, strongly supporting the indication that HSP90 and the degradation of IκB are necessary for the activation of the iNOS promoter by fucoidan. The compositions of NF-κB complexes were identified by supershift assays (Fig. 5A). The NF-κB–DNA complexes were supershifted when the nuclear extract was preincubated with antibodies against p50, p65, and c-rel. But supershifted bands were not observed with antibodies against RelB or p52 or rabbit IgG (control IgG). Thus, the binding complexes appear to be composed of p50/p65 and p50/c-rel heterodimers and p50 homodimers.

As shown in Fig. 4B, treatment with fucoidan also induced the binding of AP-1 proteins to the DNA element, and this binding was reduced by adding geldanamycin or SB203580 but not MG-132. This also supports the results in which HSP90 and p38 MAPK are related to the signal transduction from SR-A to the iNOS promoter activation by fucoidan. The compositions of the AP-1 complexes were also identified by supershift assays (Fig. 5B). Supershifted bands were observed with antibodies against c-Jun or c-Fos, but were not observed with rabbit IgG. Thus, AP-1–DNA complex appears to be composed of c-Jun/c-Fos heterodimers. Together, these results suggest that the fucoidan-induced NO production and the iNOS promoter activation are mediated by NF-κB and AP-1, and that HSP90 is important in both NF-κB and AP-1 activation by fucoidan.

Discussion

Among several SR-A ligands, such as AcLDL, OxLDL, fucoidan, mBSA, and dextran sulfate, only fucoidan mediated NO production in RAW264.7. Therefore, SR-A-mediated signaling pathways may be influenced not only by binding to the receptor but also by a certain property of the ligand. Similarly, it has been demonstrated that fucoidan but not OxLDL induces the production of IL-1β from macrophages [15]. Two possible reasons for this are as follows. First, after endocytosis, ligand or degradation products of the ligand activate a certain signal transduction pathway. For example, it has been shown that two of the major oxidized lipid components of OxLDL, 9-HODE, and 13-HODE, bind to peroxisome proliferator-activated receptor-γ (PPAR-γ) and inhibit the transcriptional responses mediated by the NF-κB, AP-1 and Stat1 transcription factors [28–30]. Second, each ligand binds to other molecules as a co-receptor, through which or with which each ligand transduces each signal.

Even though fucoidan has been used as an effective competitor for OxLDL and AcLDL in numerous studies on SR-A binding and SR-A-mediated signal transduction, it was unclear whether the stimulation of fucoidan uses the SR-A route. To ascertain whether NO production induced by fucoidan in macrophages is derived from SR-A, we

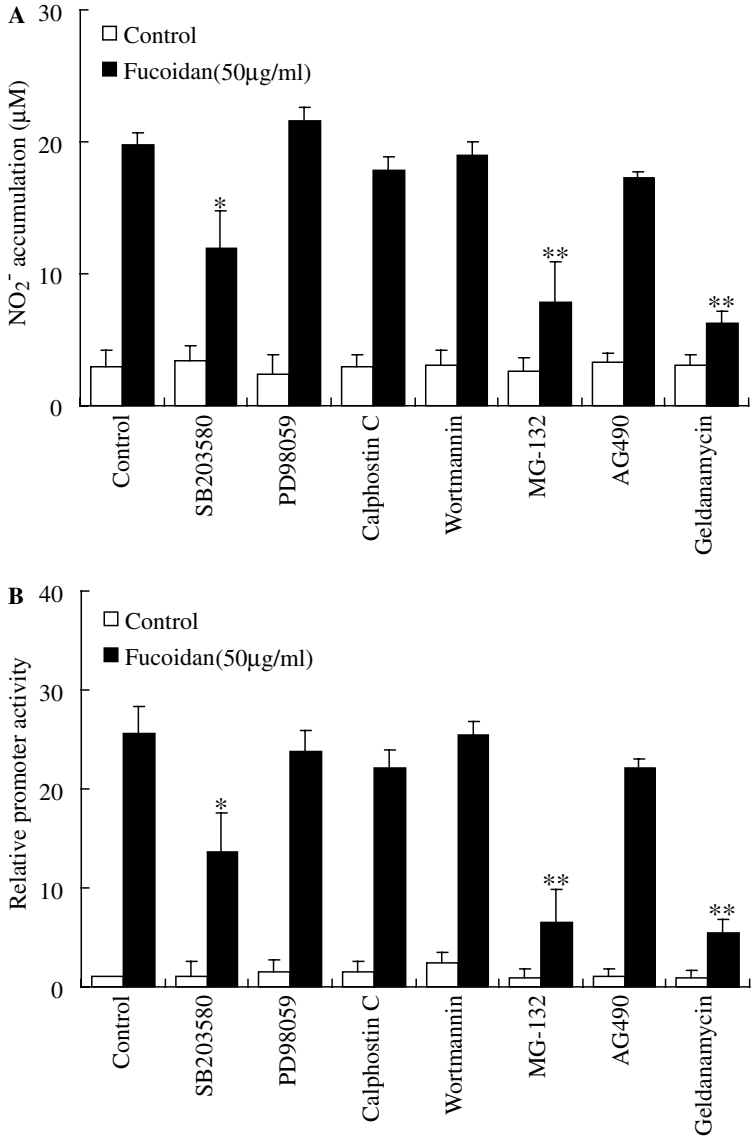

Fig. 3. Effects of downstream signaling inhibitors on fucoidan-induced NO production and mouse iNOS promoter activity. (A) RAW264.7 cells (1×10^5) were pretreated with SB203580 (20 μM), PD98059 (50 μM), calphostin C (100 nM), wortmannin (50 nM), MG-132 (5 μM), AG490 (50 μM) or geldanamycin (5 μM) for 1 h and then further incubated with fucoidan (50 μg/ml) for 24 h. NO production was determined by measuring the accumulation of nitrite in the culture medium. Data are means ± SD of triplicate cultures from three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with control value. (B) RAW264.7 cells (5×10^5) were transfected with piNOS. Cells were pretreated with SB203580 (20 μM), PD98059 (50 μM), calphostin C (100 nM), wortmannin (50 nM), MG-132 (5 μM), AG490 (50 μM) or geldanamycin (5 μM) for 1 h and then further incubated with or without fucoidan (50 μg/ml) for 24 h and analyzed for luciferase activity. Data are means ± SD of triplicate cultures from three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ compared with control value.

used peritoneal macrophages from SR-$A^{-/-}$ mice. We found that the NO production from macrophages from SR-$A^{-/-}$ mice significantly decreased compared with those from wild-type mice. This strongly suggests that the stimulation of fucoidan uses the SR-A route and that this is the major route for NO production by fucoidan. This is the first direct evidence that SR-A certainly transduces the stimulation of fucoidan.

We next then investigated the molecular mechanism of fucoidan-induced NO production. It has been demonstrated that SR-A ligands induce signal transduction pathways in macrophages [9–15]. For example, the binding of fucoidan to SR-A induced the PKC signaling pathway that mediates IL-1β production in macrophages [15], and also stimulates the phosphorylation of the tyrosine of phospholipase C-γ1, PI 3-kinase, and PKC leading to up-regulation of urokinase-type plasminogen activator expression [14]. It has also been suggested that tyrosine phosphorylation and activation of the MAPK are key components of the SR-A-mediated signal transduction cascades [12]. On the other hand, it has been reported that activation of the iNOS promoter is mediated by NF-κB, AP-1, and Stat1 [27]. Our previous study showed that HSP90 directly interacts with the cytoplasmic domain of SR-A, and suggests that

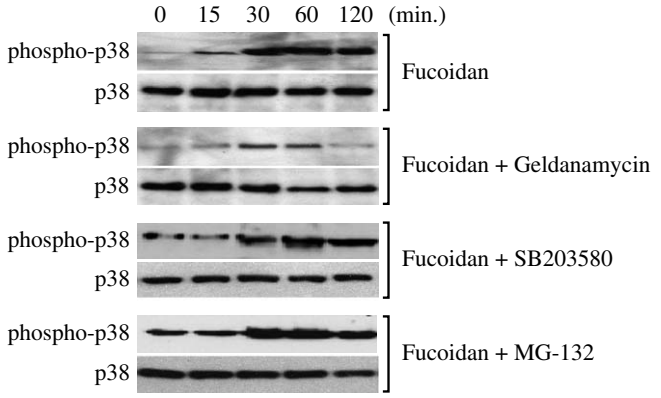

Fig. 4. Fucoidan induces phosphorylation of p38 MAPK. RAW264.7 cells were pretreated with or without geldanamycin (5 μM), SB203580 (20 μM) or MG-132 (5 μM) for 1 h and then further incubated with fucoidan (50 μg/ml) for 15, 30, 60, and 120 min. p38 MAPK and phosphorylated p38 MAPK protein levels were detected by Western blotting.

HSP90 bound to SR-A is implicated in signal transduction [25]. Based on these demonstrations, we used several different inhibitors such as SB203580, PD98059, calphostin C, wortmannin, MG-132, AG490, and geldanamycin, which inhibit p38 MAPK, MEK, PKC, PI 3-kinase, IκB-α degradation, JAK2, and HSP90, respectively. We found that p38 MAPK, NF-κB, and HSP90 but not MEK, PKC, PI 3-kinase, and JAK2 may play an important role in NO production. In addition, we investigated the relationship between HSP90 and p38 MAPK. The result demonstrated that HSP90 acts upstream of p38 MAPK. The study also revealed that the same combination of inhibitors affected the activation of the iNOS promoter in RAW264.7 cells, suggesting that the signal pathway to NO production is directly linked to activation of the iNOS promoter. Therefore, we then investigated the regulation of the iNOS promoter in fucoidan-stimulated RAW264.7 cells by using EMSA. In the case of NF-κB, treatment with fucoidan induced a significant increase in the binding activity of NF-κB, and this activation was inhibited by geldanamycin and MG-132 but not by SB203580. We also identified by the supershift assay that the binding NF-κB complexes are composed of p50/p65, p50/c-rel heterodimers, and p50 homodimers. On the other hand, the treatment with fucoidan induced the binding activity of AP-1 proteins as well, and this activation was inhibited by geldanamycin and SB203580 but not by MG-132. The compositions of AP-1 complexes identified by supershift assay are c-Jun/c-Fos heterodimers. Although earlier studies indicate that both p38 MAPK and NF-κB are involved in cytokine-stimulated NO production, the relation between these two signals is not clear. This study clarifies that fucoidan induces both the p38 MAPK and NF-κB pathways and that these two pathways lie in two distinct pathways that independently contribute to the regulation of iNOS promoter activity and NO production. p38 MAPK has been shown capable of activating several transcription factors, includ-

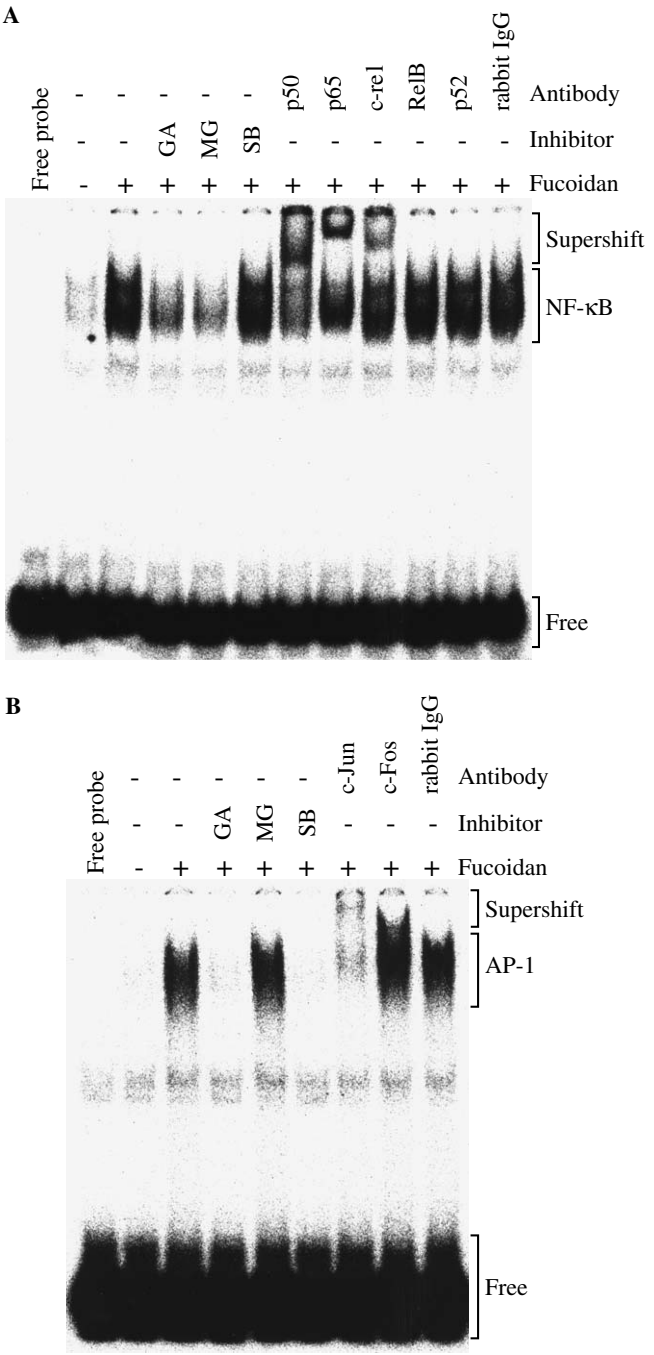

Fig. 5. Activation of NF-κB and AP-1 binding by fucoidan in RAW264.7 cells. (A) RAW264.7 cells were pretreated with geldanamycin (GA) (5 μM), MG-132 (MG) (5 μM) or SB203580 (SB) (20 μM) for 1 h and then further incubated with fucoidan (50 μg/ml) for 3 h. Nuclear extracts were prepared from these cells. EMSA was performed on 10 μg of nuclear extracts. A consensus ^{32}P-labeled oligonucleotide containing an NF-κB response sequence was used. The binding of NF-κB to the DNA element was determined by supershift experiments using antibodies against p50, p65, c-rel, RelB, p52 or rabbit IgG. (B) RAW264.7 cells were pretreated with geldanamycin (GA) (5 μM), MG-132 (MG) (5 μM) or SB203580 (SB) (20 μM) for 1 h and then further incubated with fucoidan (50 μg/ml) for 3 h. Nuclear extracts were prepared from these cells. EMSA was performed on 10 μg of nuclear extracts. A consensus ^{32}P-labeled oligonucleotide containing an AP-1 response sequence was used. The binding of AP-1 to the DNA element was determined by supershift experiments using antibodies against c-Jun, c-Fos or rabbit IgG.

ing ATF-1, CREB, MEF2C, and AP-1 [31–34], and is involved in the control of many processes for gene expression. p38 MAPK can also regulate gene expression at the post-transcriptional level [35,36]. Therefore, the activation of p38 MAPK in the fucoidan-stimulated macrophages results in a cooperative effect with NF-κB, possibly through affecting AP-1 serving as the transcriptional activation factor or at the post-transcriptional level.

We also demonstrated that although p38 MAPK and NF-κB lie in the independent signal pathway for fucoidan that induces NO production and iNOS promoter activation, HSP90 is essential in both the p38 MAPK and NF-κB pathways. According to recent reports, HSP90 is related to the functions of various signaling molecules, such as tyrosine kinase v-Src and LcK, and serine/threonine kinases Raf-1 and casein kinase II [24]. Hsu et al. [15] demonstrated that fucoidan induces the tyrosine phosphorylation of pp60Src, and the HSP90 and pp60Src complex plays an important role in this signal transduction. In this case, the association with HSP90 is important for signal transducer activity. There are several examples in which the membrane receptor itself interacts with HSP90, such as the insulin receptor [37] and CD14 [38,39]. In these cases, HSP90 is related to signal transduction. Previous studies show that HSP90 directly interacts with the cytoplasmic domain of SR-A and may be implicated in SR-A-mediated signal transduction [25]. As stated above, HSP90 interacts with various signaling molecules. It is suggested that HSP90 bound to SR-A functions in the signal transduction pathway of NO production by interacting with certain kinases. Considering the physiological conditions, the sequential biological effects need further investigation.

NO couples with superoxide anions (O_2^-) at a diffusion-controlled rate to produce peroxynitrite [40]. This reaction in macrophages contributes to their cytotoxic activity toward invading pathogens because peroxynitrite and its conjugated acid, peroxynitrous acid, are potent oxidizing agents [40]. The definitive role of SR-A in host defense in vivo has been clarified through the study on SR-A$^{-/-}$ mice. SR-A$^{-/-}$ mice show an increased susceptibility to infection with *Listeria monocytogenes* and the herpes simplex virus type-1, indicating that SR-A plays a part in host defense against pathogens [7]. However, the mechanisms by which SR-A protects the host have not been fully understood. Our findings here may explain in part the mechanism in which SR-A mediates in host defense.

In summary, these demonstrations show that fucoidan but not other SR-A ligands such as AcLDL, OxLDL, mBSA, and dextran sulfate induces iNOS promoter activation and NO production in macrophages via the p38 MAPK- and NF-κB-dependent pathways. Although these two pathways are independent, both pathways need HSP90. Furthermore, we showed for the first time that stimulation of fucoidan certainly transduces the signal through SR-A. Considering the biological role of NO in macrophages, SR-A-mediated NO production in macrophages may explain the physiological function of SR-A such as host defense.

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