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Inhibitory effects of fucoidan on the iNOS-mediated nitric oxide production and MUC5AC release

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ABSTRACT

후코이단의 iNOS 매개성 nitric oxide 생성과 MUC5AC 분비 억제 효과

Inhibitory effects of fucoidan on the iNOS-mediated nitric oxide production and MUC5AC release

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Fucoidan, a marine sulfated polysaccharide has both antithrombotic and anti-inflammatory effects. . This study evaluated the effect of fucoidan on the expression of inducible nitric oxide synthase (iNOS) in a macrophage cell line, RAW264.7 and the effect of fucoidan on *MUC5AC* expression in a human bronchial epithelial cell line, NCI-H292.

Low concentration range of fucoidan (10 mg/ml) increased the basal expression level of iNOS in quiescent macrophages. However, we found for the first time that fucoidan inhibited the release of nitric oxide (NO) in RAW264.7 cells stimulated with lipopolysaccharide (LPS). Western blot analysis revealed that fucoidan suppressed the LPS-induced expression of the inducible nitric oxide synthase (*iNOS*) gene. Moreover, the activation of both nuclear factor-kB (NF-kB) and activator protein 1 (AP-1) are key steps in the transcriptional activation of the *iNOS* gene.

Here, it was revealed that fucoidan selectively suppressed AP-1 activation, and that the activation of AP-1 appears to be essential for

the induction of iNOS in activated macrophages. This inhibitory effect on AP-1 activation by fucoidanmight be associated with its NO blocking and anti-inflammatory effects.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis showedthat fucoidan inhibited *MUC5AC* expression and protein secretion in cells stimulated with acrolein, a toxic aldehyde present in tobacco smoke. The activation of both nuclear factor-kB (NF-kB) and activator protein 1 (AP-1) are key steps in the transcriptional activation of *MUC5AC*. We found that the acrolein-mediated transactivation of *MUC5AC* was selectively dependent on AP-1 activation and was suppressed by fucoidan. Fucoidan-induced AP-1 inhibition and *MUC5AC* repression might be associated with fucoidan's protective effects against respiratory diseases.

Key Word:

acrolein, AP-1, fucoidan, MUC5AC-1,iNOS, macrophages,
nitric oxide

ABSTRACT

후코이단의 iNOS 매개성 nitric oxide 생성과 MUC5AC 분비 억제 효과

Inhibitory effects of fucoidan on the iNOS-mediated nitric oxide production and MUC5AC release

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해조류에서 유래된 황화다당류인 후코이단은 항혈전 작용과 항염 작용을 가지고 있다. 이 연구에서는 마크로파지세포인 RAW264.7 셀라인에서 inducible nitric oxide synthase (iNOS)의 발현과 인간기관지 상피세포로부터 유래한 NCI-H292 셀 라인에서의 *MUC5AC*의 발현을 평가하였다.

낮은 농도 범위((10 μg/ml)에서의 후코이단은 활동이 없는 마크로파지에서의 iNOS의 발현을 증가 시킨다. 그러나 후코이단은 lipopolysaccharide (LPS)에 의하여 자극 되어진 RAW264.7 셀에서의 nitric oxide (NO)의 분비 억제를 나타낸다. 또한 후코이단은 Western blot analysis 상에서 LPS에 발현된 iNOS를 억제 하는 것으로 밝혀졌다. 더욱이 nuclear factor-kB (NF-kB)와 activator protein 1 (AP-1) 활성이 iNOS 유전자 전사 활성화에 중요한 단계임을 알아냈다.

여기서, 후코이단은 선택적으로 AP-1 활성화를 억제하며 또한 AP-1의 활성화된 마크로파지의 iNOS 유도에 필수적임을 알 수 있다. 그리하여 후코이단이 AP-1 활성에 대한 방해작용이 항염작염, NO blocking과 연관되어 있을 것으

로 사료된다.

Reverse transcription-polymerase chain reaction (RT-PCR) 결과에 따르면 후코이단은 담배연기에 존재하는 독성 알데하이드인 acrolein에 의한 *MUC5AC*의 발현을 억제 하는 것으로 나타났다. NF-kB와 AP-1의 활성화는 *MUC5AC*의 전사 활성에 중용한 단계이다. acrolein을 매개로 한 *MUC5AC*의 전사는 AP-1의 활성화와 AP-1을 억제하는 후코이단에 의하여 선택적으로 발현된다.

후코이단에 의한 AP-1의 억제와 *MUC5AC*의 억제는 후코이단의 호흡기 질환에 대한 보호작용과 연관되어져 있다고 생각한다.

Key Word:

acrolein, AP-1, fucoidan, MUC5AC-1, iNOS, macrophages, nitric oxide

Introduction

Algal fucoidan, which is extracted from brown algae (e.g., Ascophyllumnodosum and Fucus vesiculosus), is a marine sulfated polysaccharide that is endowed with important biological activities including antiviral, anti-angiogenic, antitumoral, contraceptive, antithrombotic, anticoagulant and anti-inflammatory effects [1]. The brown seaweed, *Laminaria japonica*Aresch. (Laminariales), is distributed widely in East Asia and is consumed as a marine vegetable.Fucoidan, one of its main constituents, is also available as food supplement in Japan and United States.

Fucoidan contains a substantial percentage of L-fucose and sulfate ester groups, and the structural characteristics of fucoidan are very similar to those of heparin. Therefore, it exhibits potent anti-thrombotic activity[1]. Another outstanding physiological function of fucoidan is anti-inflammatory properties through anti-complementary [2], anti-leukocyte migration [3] and anti-proliferation effects on smooth muscle cell [4]. In addition, fucoidan as a ligand for the macrophage scavenging receptor 1 (MSR1) increased the levelof tumor necrosis factor-a (TNF-a) and interleukin-1 secretion [5]. It was recently reported that fucoidan increases the level of nitric oxide (NO) production in quiescent macrophages, which was related with p38 kinase-dependent NF-kB activation [6].

Although several studies on the biological activities of fucoidans have been performed, with particular focus on its immunomodulatory actions, it is unclear if fucoidan affects the formation of NO in

lipopolysaccharide (LPS; endotoxin, a representative Toll-like receptor 4 agonist)-stimulated macrophages. This study we unexpectedly found that fucoidan inhibits the LPS-mediated expression of inducible nitric oxide synthase (iNOS) byblocking AP-1 activation in macrophages.

Excess mucus production is a hallmark in the pathogenesis of several airway diseases, including chronic bronchitis, asthma, and cystic fibrosis (Lundgren and Shelhamer, 1990). Mucins, a class of mucus glycoproteins, provide airway secretions with characteristic adhesiveness, elasticity, and viscosity. Several mucin genes (e.g., *MUC1-MUC4*, *MUC5AC*, *MUC5B*, and *MUC6-MUC8*) are present in the respiratory, gastrointestinal, and reproductive tracts (Gendler and Spicer, 1995; Borchers et al., 1999). MUC5AC and MUC5B proteins are major constituents of the mucous layer in humans (Reid et al., 1997; Thornton et al., 1997).

Acrolein is a highly electrophilic and volatile liquid with an irritating odor (Witz, 1989) that is produced by a wide variety of natural and synthetic processes, including incomplete combustion of organic materials such as fuels and tobacco. In addition, patients treated with the anti-cancer agent, cyclophosphamide, are frequently exposed to acrolein as a metabolite (Sladek, 1988). Because acrolein production is relatively high (50-70 ppm) in tobacco smoke (Ayer and Yeager, 1982), its production is associated with various respiratory diseases, including chronic obstructive pulmonary disease (COPD) (Borchers et al., 1999 Leikauf, 2002). Moreover, both acrolein and tobacco smoke extract stimulate the secretion of mucins from bronchial epithelial cells (Gensch et al., 2004).

However, the effect of fucoidan on bronchial mucin secretion has not been elucidated. Here, we show that fucoidan inhibits acrolein-mediated expression of *MUC5AC* by blocking AP-1 activation in NCI-H292 cells, a human bronchial epithelial cell line.

Matrials and methods

Materials: The 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium solutions were purchased from Promega (Madison, WI); the anti-murine iNOS polyclonal antibody from Transduction Laboratories (Lexington, KY); the anti-phospho-I-kB ?antibody from Cell Signaling Technology (Beverly, MA), and the anti-c-Rel (p65) and I-kBa ?antibodies from Santa Cruz Biotechnology (Santa Cruz, CA). The horseradish peroxidase-conjugated donkey anti-rabbit and alkaline phosphatase-conjugated donkey anti-mouse IgGs were obtained from Jackson Immunoresearch Laboratories (West Grove, PA). All the reagents including fucoidan were supplied by Sigma (St. Louis, MO).

Cell culture: The RAW264.7 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and incubated at 37C in a 5% CO2/95% air atmosphere in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), 50 units/ml penicillin, and 50 mg/ml streptomycin. For all experiments, the cells were grown until they reached 80% to 90% confluence and were subjected to no more than 20 cell passages. NCI-H292 cells (human lung mucoepidermoid carcinoma) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and incubated at 37 C in a 5% CO2/95% air atmosphere in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin.

Measurement of NO: The RAW264.7 cells (5x105 cells) were preincubated in serum-free medium at 37C for 12 h and the level of NO production was monitored by measuring the nitrite levels in the culture media using the Griess reagent [7]. The absorbance was measured at 540 nm after incubating the culture mediumwith the Griess reagent for 10 min.

Preparation of nuclear extract: The cells were preincubated for 10 min in the culture medium in the presence or absence of sumaflavone or robustaflavone, and then exposed to LPS (1 mg/ml). The cells were then removed using a cell scraper, centrifuged at 2,500*g* at 4C for 5 min, and swollen by adding 100 ml of a lysis buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet-P40, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonylfluoride]. The cells were vortexed in order to disrupt the cell membranes, and the samples were incubated for 10 min on ice followed by centrifugation for 5 min at 4C. Pellets containing the crude nuclei were resuspended in 60ml of the extraction buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonylfluoride, and incubated for 30 min on ice. The samples were then centrifuged at 15,800*g* for 10 min to obtain a supernatant containing nuclear extracts, which were stored at -80C until needed.

Western blot analysis: Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblot analyses were performed, as described previously [7]. The cells were lysed in a buffer containing 20 mM TrisCl (pH 7.5), 1% Triton X-100, 137 mM sodium chloride, 10% glycerol, 2 mM

EDTA, 1 mM sodium orthovanadate, 25 mM -glycerophosphate, 2 mM sodium pyrophosphate, 1 mM phenylmethylsulfonylfluoride, and 1 mg/ml leupeptin. The lysates were centrifuged at 12,000*g*for 10 min to the remove debris, fractionated by 10 % gel electrophoresis, electrophoretically transferred to nitrocellulose paper, incubated with the primary antibodies and then with the alkaline phosphatase- or horseradish peroxidase-conjugated secondary antibodies. Finally, the papers were developed using either 5-bromo-4-chloro-3-indoylphosphate and nitroblue tetrazolium or an ECL chemiluminescence detection kit [8].

Construction of an iNOS Promoter-luciferase Construct and NF-kB reporter gene assays: The pGL-miNOS-1588 luciferase reporter assay system was used to determine the transcriptional activity of the *iNOS* gene. In order to generate the miNOS promoter-luciferase construct (pGL-miNOS-1588), the miNOS promoter region from -1588 bp to +165 bp was amplified by a polymerase chain reaction (PCR) and ligated into the pGEM-T easy vector (Promega, Madison, WI). The amplified product was subcloned into the KpnI/BglII site of the pGL3-basic plasmid [9].

The cells were plated at a density of 3105 cells/well in a 12-well plate and transfected the following day. A dual-luciferase reporter assay system (Promega, Madison, WI) was used to determine the promoter activity. Briefly, the cells were transiently transfected with 1 mg of pGL-miNOS1588, pNF-kB-Luciferase, or pAP-1-Luciferase plasmid and 4 ng of the pRL-SV plasmid (Promega, Madison, WI) using the GenejuiceReagent (Novagen, Madison, WI) and then exposed to LPS for 18 h. The firefly and *Renilla* luciferase activities in the cell lysates were measured using a

luminometer (Turner Designs; TD-20, CA). The relative luciferase activities were calculated by normalizing the iNOS, NF-kB, or AP-1 promoter-driven firefly luciferase activities versus that of *Renilla* luciferase.

Gel shift assay: The double stranded DNA probe for the consensus sequence of AP-1 (1.75 *p*mol/ml, 5'-CGCTTGATGAGTCAGCCGGAA-3') was purchased from Promega (Madison, WI) and used for the gel shift analysis after end-labeling the probe with [-32P]ATP and T4 polynucleotide kinase. The reaction mixture contained 2 ml of 5 a binding buffer containing 20% glycerol, 5 mM MgCl2, 250 mM NaCl, 2.5 mM EDTA, 2.5 mM dithiothreitol, 0.25 mg/ml poly dI-dC, 50 mM Tris-Cl (pH 7.5), 10 mg of the nuclear extracts, and sterile water to a total volume of 10 l. After a 10 min preincubation period, a 1 l probe (106 cpm)was added and the mixture was incubated at room temperature for 20 min. The specificity of DNA/protein binding was determined by a competition reaction using a 20-fold molar excess of the unlabeled AP-1 oligonucleotide. The samples were loaded onto 5% polyacrylamide gels at 100 V. The gels were then removed, dried, and autoradiographed.

Scanning densitometry and statistics: Scanning densitometry was carried out using FLA-7000 Image Scan & Analysis System (Fujifilm, Japan), and a paired Student's *t*-test was used to examine the significant inter-group differences. Statistical significance was accepted at either $p<0.05$ or $p<0.01$.

RT-PCR assay of MUC5AC : Reverse transcription-polymerase chain reaction (RT-PCR), and reporter gene assays were performed as described (Choi et al., 2005). PCR was performed using selective primers for human *MUC5AC* (sense primer: 5'-TCC GGC CTC ATC TTC TCC-3'; antisense primer: 5'-ACT TGG GCA CTG GTG CTG-3')(Borchers et al., 1999) and *S16 ribosomal protein* (*S16r*) (sense: 5'-TCCAAGGGTCCGCTGCAGTC-3', antisense: 5'-CGTTCACCTTGATGAGCCCATT-3'). Paired Student's *t*-tests were used to examine inter-group differences. Statistical significance was set at either the $p<0.05$ or <0.01 level as indicated.

Results

Induction of iNOS by low concentration range of fucoidan in quiescent macrophages: Fucoidan was reported to upregulate iNOS expression in quiescent macrophages [6]. The present studyalso confirmed that 10 mg/ml fucoidan slightly, but significantly increased the levels of the iNOS protein in theun-stimulated RAW264.7 cells (Fig. 1). However, the enhanced expression of iNOS disappeared in cell lysates obtained from macrophages treated with 30 or 100 mg/ml fucoidan. Hence, 10 mg/ml of fucoidan may act as a weak inducer of the iNOS gene in quiescent macrophages as a ligand of MSR but fucoidan at concentration above 30 mg/ml may have another function to repress the induction of iNOS.

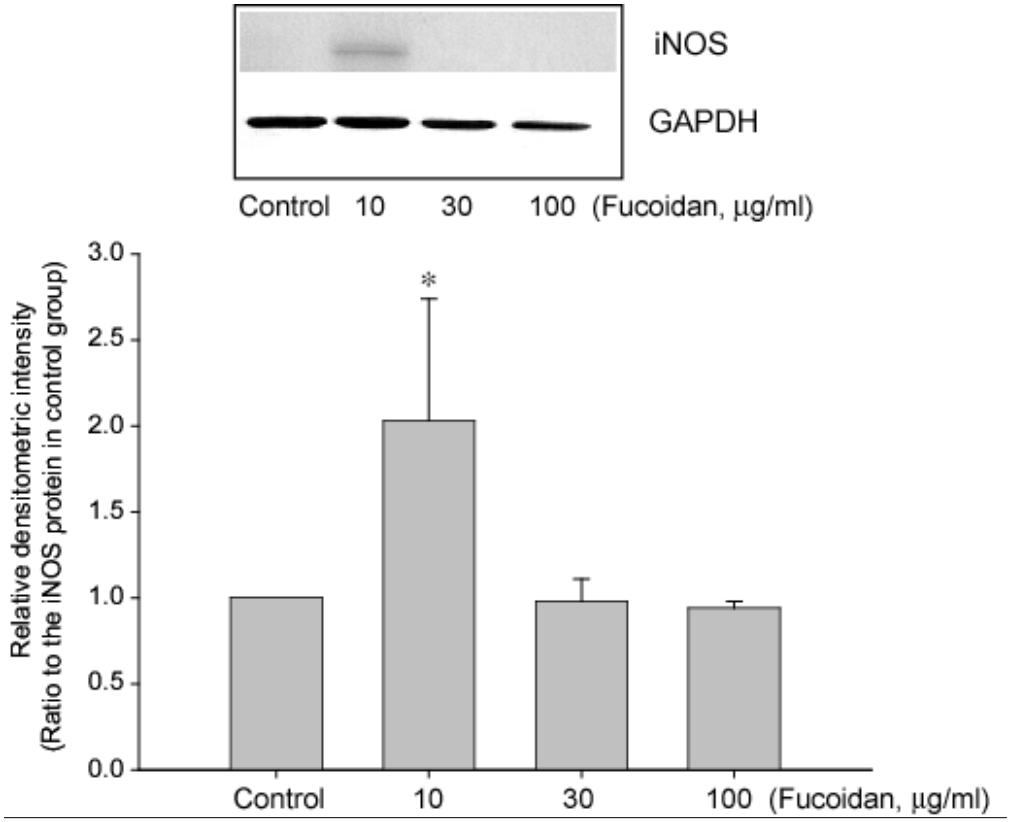

Fig. 1. Effect of fucoidan on iNOS expression in quiescent macrophages. RAW264.7 cells were incubated in a medium containing fucoidan (10-100 mg/ml) for 18 h and iNOS protein levels were monitored in the cell lysates. Relative iNOS protein levels were determined by measuring immunoblot band intensities by scanning densitometry. Data represent the means SD of three separate experiments (significant compared to the untreated control, *p<0.05).

Inhibitory effects of fucoidan on the induction of iNOS in activated macrophages: Quiescent macrophages can be activated by exposure to proinflammatory cytokines or LPS. When LPS (1 mg/ml) was added to the RAW264.7 cells, the level of NO production increased from 12 h (8.5 fold) and peaked at 48 h (13.2 fold) (Fig. 2A). Fucoidan significantly inhibited this increase in NO production in a concentration dependent manner, with 100 mM fucoidan completely blocking the LPS-inducible NO production at 24 h (Fig. 2A).

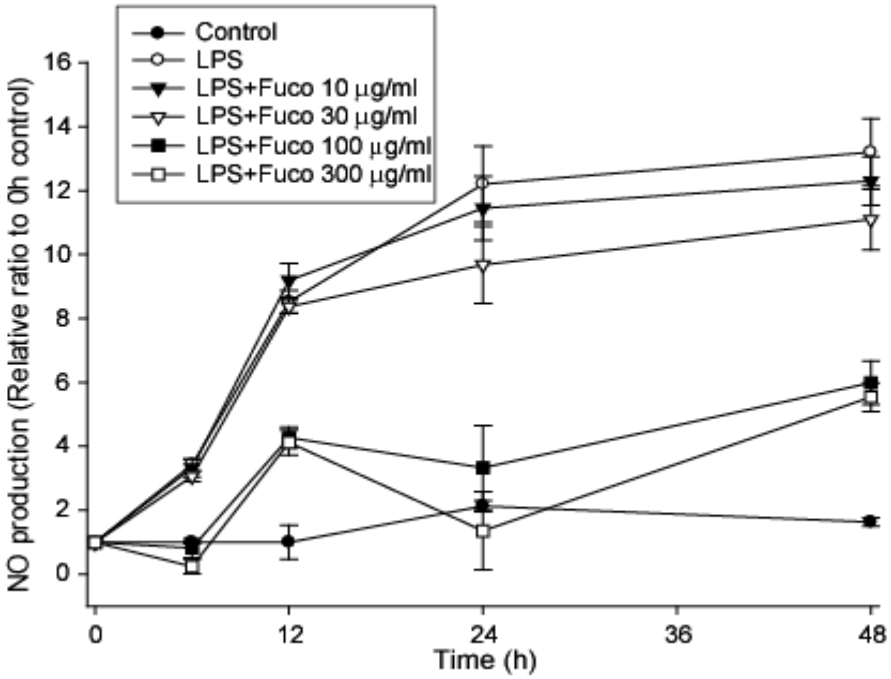

Fig 2A.)Effect of fucoidan on NO productionin LPS-stimulated macrophages. RAW264.7 cells were incubated in a medium containing fucoidan (10-300 mg/ml) for 10 min and then treated with LPS at 1 mg/ml. The amount of nitrite generated in medium was monitored for 48 h. Data represent the means SD of 4 different samples.

It is unclear if the inhibition of NO formation by fucoidan is the result of the inhibition of iNOS gene expression. Therefore, the inhibitory effects of the different fucoidan concentrations on iNOS protein expression induced by LPS (1 mg/ml) were assessed. As shown in Fig. 1B, fucoidanconcentration-dependently inhibited iNOS protein expression at 30-300 mM, and iNOS induction by LPS was completely blocked by fucoidan concentrations > 100 mM (Fig. 2B). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) levels were comparable among samples. These results show that fucoidan suppresses the de novo synthesis of iNOS in LPS-activated macrophages.

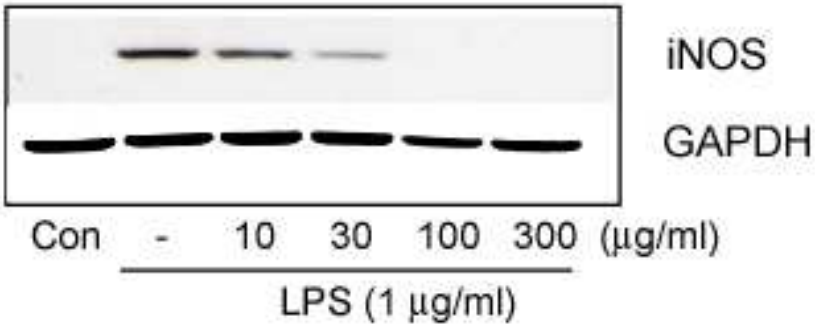

Fig 2B) Effect of fucoidan on iNOS expression in LPS-stimulated macrophages. iNOS protein levels were monitored 18 h after treating cells with LPS (1 mg/ml).

iNOS expression is mainly regulated at the transcriptional level [10]. In order to determine if the process of *iNOS* gene transcription is targeted by fucoidan, reporter gene analysis was carried out using macrophages transfected with the mammalian cell expression vector, pGL-miNOS1588, which contained luciferase cDNA and a 1.59 kb miNOS promoter [9]. LPS (1 mg/ml) increased the luciferase activity by approximately 4.2 fold, which was reversed by 30 or 100 mM fucoidan (Fig. 2C).

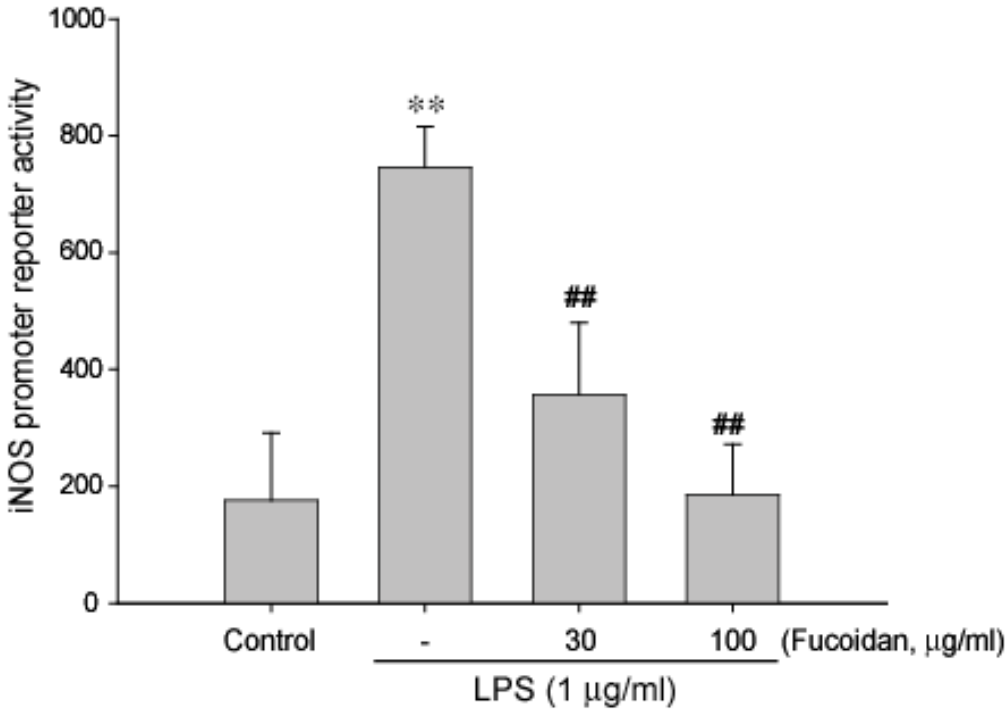

Fig 2C) Effect of fucoidan on the transactivation of *iNOS* gene. Induction of luciferase activity by LPS in the RAW264.7 cells transiently transfected with pGL-miNOS1588 construct, which contained -1588 bp iNOS promoter sequences, was confirmed using a luminometer. A dual luciferase reporter gene assay was performed on the lysed cells co-transfected with pGL-miNOS1588 (firefly luciferase) and pRL-SV (*Renilla* luciferase)(in a ratio of 250:1) after exposure to LPS (1 mg/ml) and fucoidan(30 and 100 mg/ml) for 18 h. The activation of the reporter gene was calculated as a relative change in the *Renilla* luciferase activity. Data represents the means SD of 4 separate samples (significant versus the control, $**p<0.01$; significant versus the LPS-treated group, $##p<0.01$).

No effect of fucoidan on LPS-inducible NF-kB activation: NF-kB is an essential transcription factor for the induction of several inflammatory mediators including, tumor necrosis factor-a, cyclooxygenase-2, and iNOS [12, 13]. Therefore, a reporter gene assay was carried out using a luciferase plasmid containing the NF-kB minimal promoter to determine if the inhibition of iNOS induction by fucoidan is due to the suppression of NF-B activation. The LPS treatment (18h) caused a 2.9 fold increase in NF-kB reporter activity (Fig. 3A). However, a pretreatment with 30 or100 mM fucoidan had no effect on the LPS-induced luciferase activity (Fig. 3A).

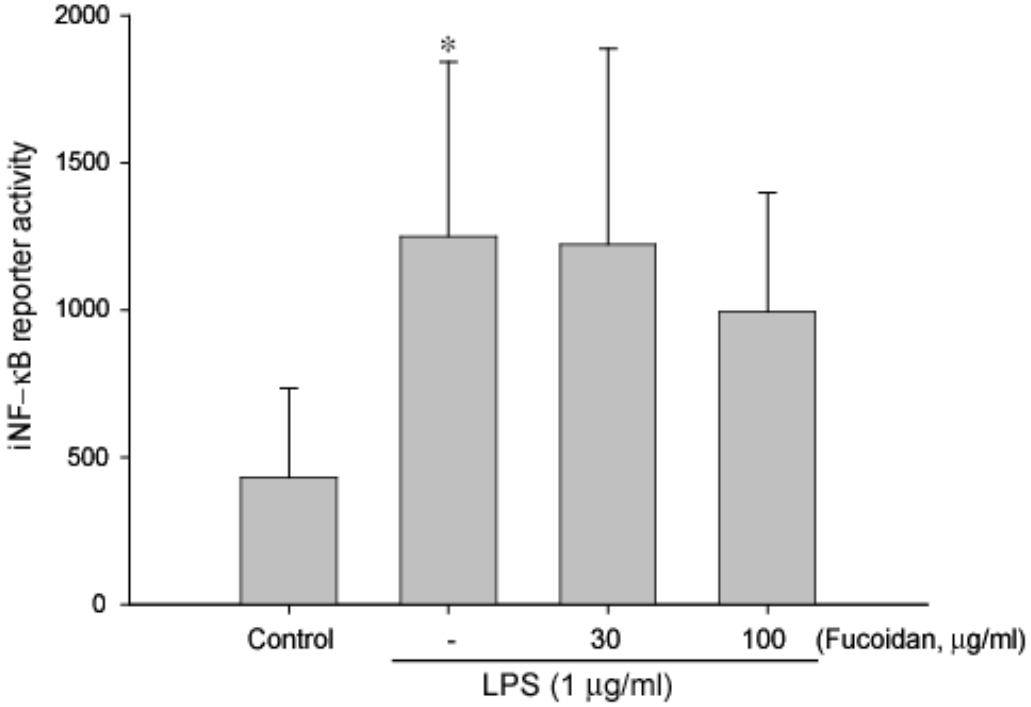

Fig 3A) NF-kB reporter gene analysis. Cells were transfected with pNF-kB-Luc plasmid, and reporter gene analysis was performed as described in the legend of Fig. 2, panel (C). Data represent the means SD of 5 or 7 separate samples (significant versus the control, $*p<0.05$).

The inhibitory protein I-Ba , sequesters the activated NF-kB (a p65/p50 heterodimer) in the cytoplasm as an inactive complex. Upon inflammatory stimulation, its inhibitory subunit, I-Ba,is phosphorylated and degraded, liberating an active p65, which is then translocated into the nucleus [14]. Therefore, the nuclear p65 levels were measured to confirm the result of the NF-kB reporter gene assay. The p65 protein levels in the nuclear fractions were increased 15 or 30 min after the LPS (1 mg/ml) treatment, and were not lower in the cells pretreated with 100 mM fucoidan (Fig. 3B).

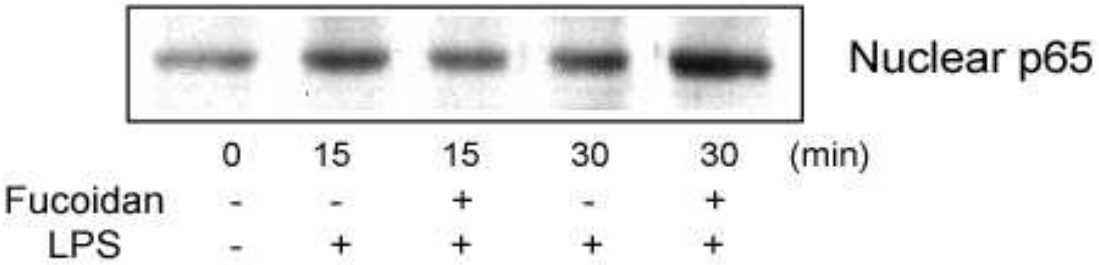

Fig 3B) Effects of fucoidan on the LPS-induced nuclear translocation of p65. RAW264.7 cells were treated with 1 mg/ml LPS for 15 min or 30 min in the presence or absence of 100 mg/ml fucoidan, and nuclear p65 protein was detected immunochemically using anti-p65 antibody. Equal loadings of nuclear protein were verified by Ponceau-S staining.

The protein levels of I-kBa and phosphorylated I-kBa in macrophages were also assessed. Immunoblot analysis using the specific antibodiesrevealed that 100 mM fucoidandid not reverse the phosphorylation and subsequent degradation of I-kBa by LPS (1 mg/ml) (Fig. 3C). These results show that NF-kB activation by LPS was unaffected by the fucoidan treatment.

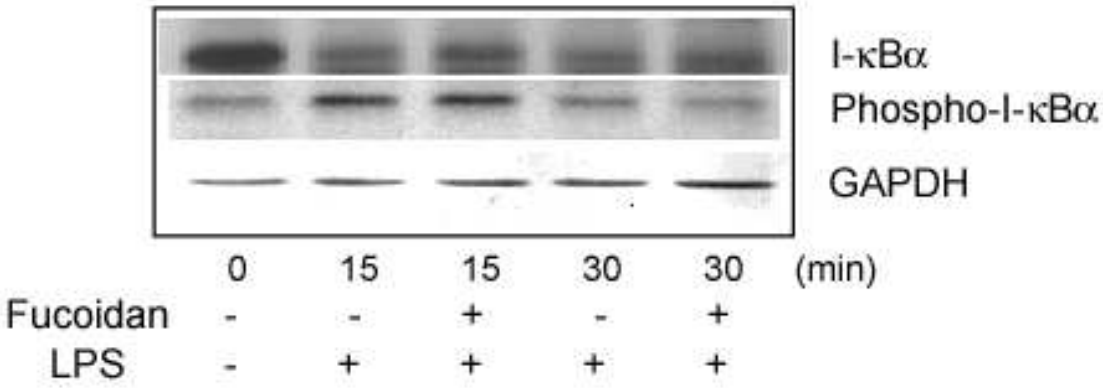

Fig 3C) Effect of fucoidan on LPS-induced I-kBa phosphorylation and degradation. The phosphorylation and degradation of I-kBa were immunochemically assessed by treating cells preincubated with 100 mg/ml fucoidanfor 10 min with LPS.

Inhibitory effect of fucoidan on LPS-inducible AP-1 activation: The expression of the *iNOS* gene is also controlled by the transcription factor, AP-1 [15-17]. The level of AP-1 activation was assessed by a reporter gene assay using the luciferase plasmid containing the AP-1 consensus sequences. Fucoidan (30 or 100 mM) significantly inhibited the LPS-induced increases in AP-1 reporter activity (Fig. 4A). This suggests that blocking AP-1 activation may be the mechanistic basis for the inhibition of iNOS expression by fucoidan.

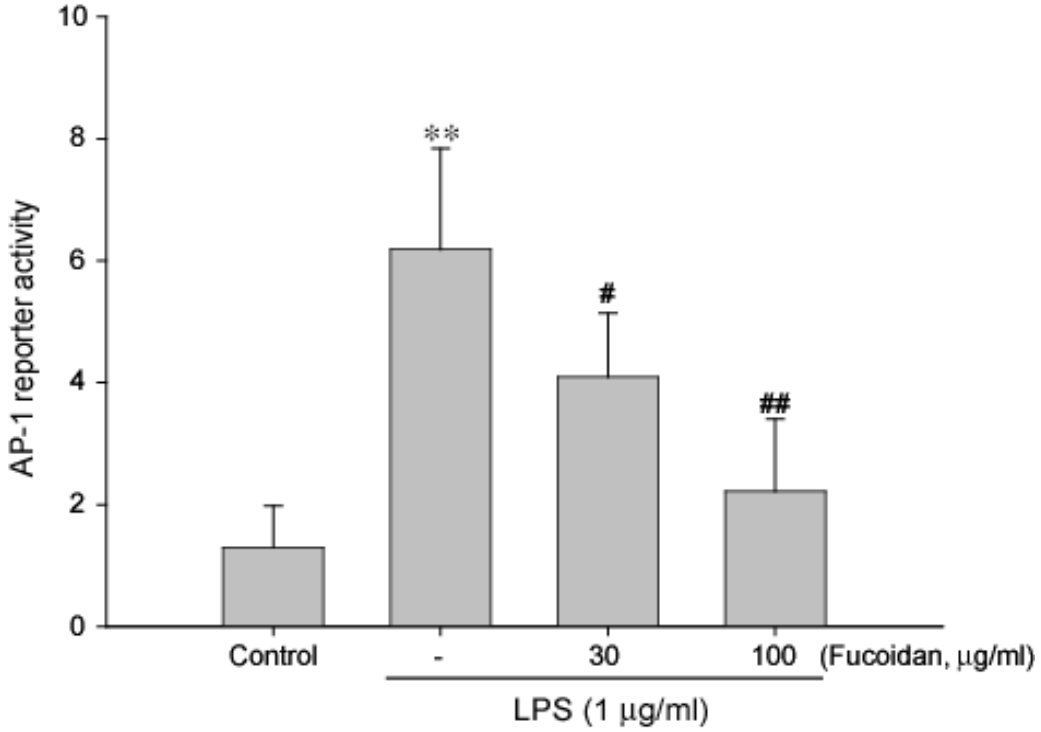

Fig 4A) (A) AP-1 reporter gene assay. Cells were transfected with pAP-1-Luc plasmid, and reporter gene analysis was performed as described in the legend of Fig. 2, panel (C). Data represent the means SD of 4 separate samples (significant versus the control, **p<0.01; significant versus the LPS-treated group, #p<0.05; ##p<0.01). (A) AP-1 reporter gene assay. Cells were transfected with pAP-1-Luc plasmid, and reporter gene analysis was performed as described in the legend of Fig. 2, panel (C). Data represent the means SD of 4 separate samples (significant versus the control, **p<0.01; significant versus the LPS-treated group, #p<0.05; ##p<0.01).

In order to confirm fucoidan's inhibitory effect on AP-1 activation, gel shift analysis was performed using a radiolabeled AP-1 consensus sequence. The band intensity of the slow migrating complex was increased 1 h after the LPS treatment, which confirmed that AP-1 was activated by 1 mg/ml LPS (Fig. 4B). The addition of a 20-fold excess of an unlabeled AP-1 to the nuclear extract obtained from the LPS-stimulated cells abolished the AP-1 binding (Fig. 4B). Fucoidan (30 or 100 mM) inhibited the LPS-inducible AP-1 DNA binding, with complete inhibition being observed at 100 mM of fucoidan (Fig. 4B).

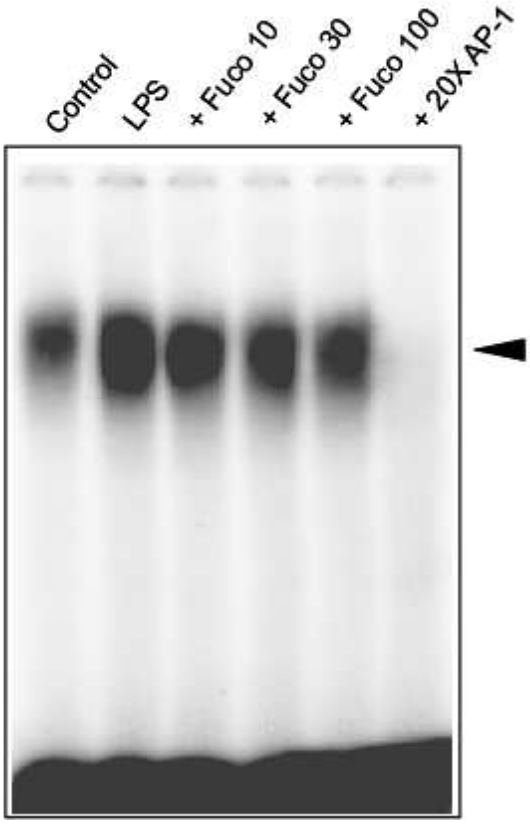

Fig 4B) Gel shift analyses of AP-1. Gel shift assays were performed with nuclear extracts prepared from RAW264.7 cells cultured with or without LPS (1 mg/ml) and fucoidan (10-100 mg/ml) for 1 h. All lanes were loaded with 10 mg of nuclear extracts and labeled AP-1 DNA consensus sequence. The arrowhead indicates the AP-1 binding complex.

Stimulatory effect of acrolein on MUC5AC expression in NCI-H292 cells: Mucin synthesis in epithelial layers is controlled via transcriptional (Li et al., 1998; Manna et al., 1995) and posttranscriptional regulation (Velcich and Augenlicht, 1993). We first determined whether acrolein stimulates MUC5AC expression in NCI-H292cells. 0.3 and 1 ng/ml of acrolein significantly increased MUC5AC mRNA (Fig. 5A). However, MUC5AC was not induced at higher concentrations of acrolein (>3 ng/ml), probably because of acrolein's cytotoxicity (Minsonou et al., 2006; Nardini et al., 2002).

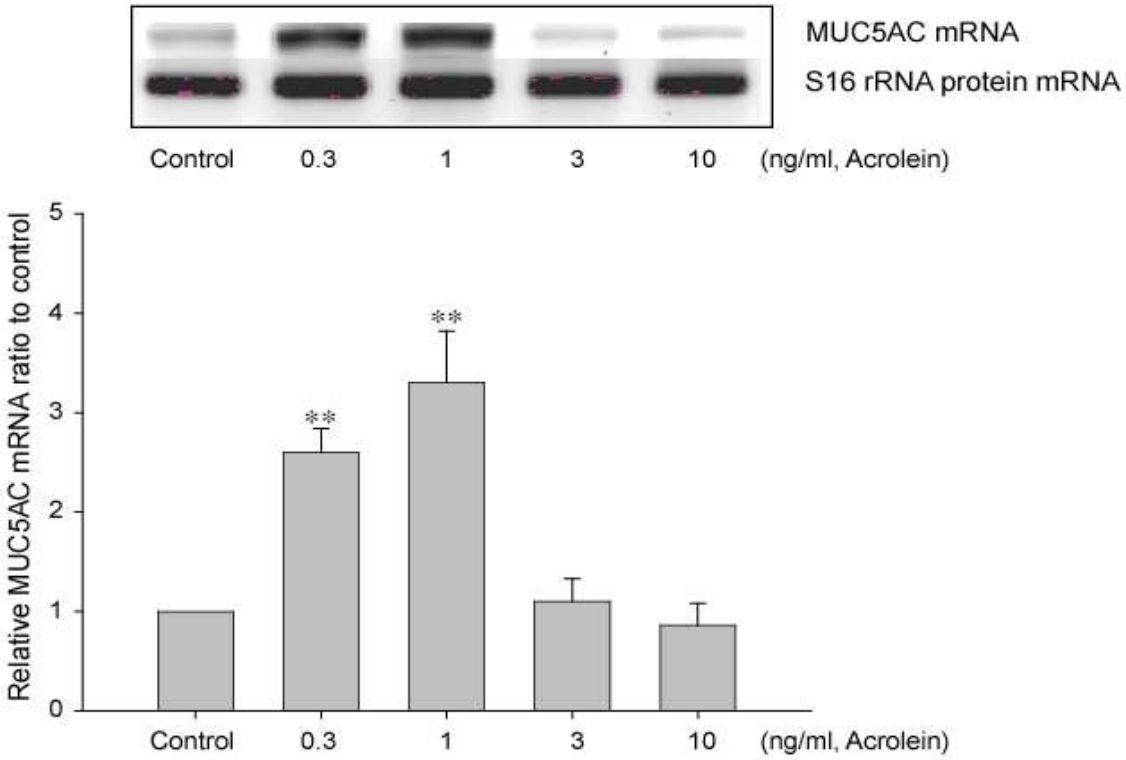

Fig. 5. (A) Effect of acrolein on MUC5AC mRNA expression in NIC-H292 cells. NCI-H292cells were incubated in a medium containing acrolein (0.3-10 ng/ml) for 4 h and MUC5AC mRNA levels were monitored by RT-PCR. Relative MUC5AC mRNAlevels were determined by measuring band intensities by scanning densitometry. Data represent the means SD of three separate experiments (significant compared to the untreated control, $^{**}p<0.01$).

The transcriptional mechanism of MUC5AC expression: We then evaluated transcriptional regulation of MUC5AC using a MUC5AC-Luc reporter plasmid that contained the luciferase gene and 3.7 kb of the human *MUC5AC* promoter (Wang et al., 2002). Acrolein (0.1 and 0.3 ng/ml) significantly increased luciferase activity in cells transfected with the MUC5AC-Luc reporter plasmid (Fig. 5B), suggesting that the induction of *MUC5AC* is mediated through transcriptional activation.

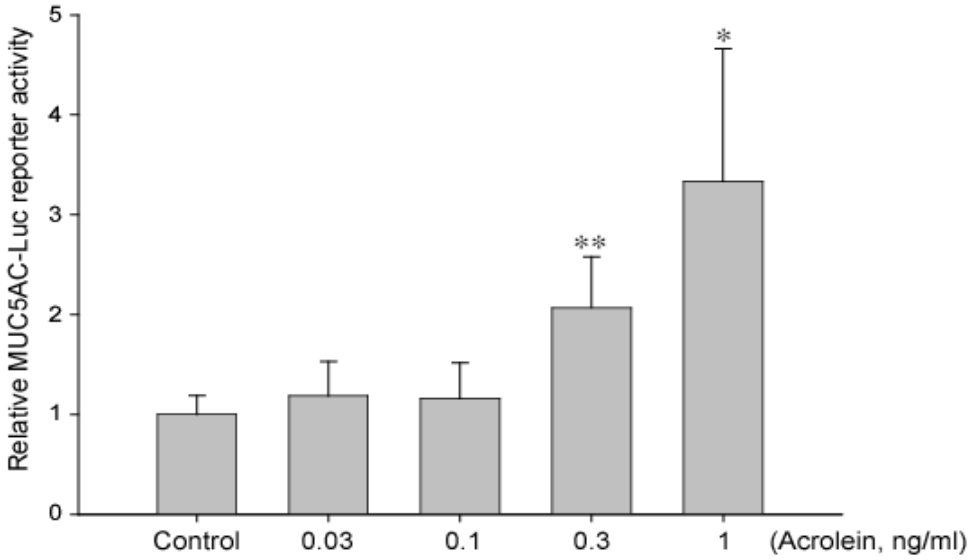

Fig 5B Effect of acrolein on MUC5AC-Luc reporter activity. Induction of luciferase activity by acrolein in the NIC-H292cells transiently transfected with MUC5AC-Luc reporter construct was confirmed using a luminometer. A dual luciferase reporter gene assay was performed on the lysed cells co-transfected with MUC5AC-Luc (firefly luciferase) and phRL-SV (h*Renilla* luciferase) after exposure to acrolein (0.03-1 ng/ml) for 18 h. Data represent the means SD of 4 different samples (significant compared to the untreated control, *p<0.05, **p<0.01).

Several *cis*-acting elements are located in the promoter region of *MUC5AC*, including a SMAD4 binding site, hypoxia responsive element and specific protein-1 binding sites, an activator protein-1 (AP-1) binding site, and a nuclear factor-kB (NF-kB) binding site (Kato et al., 2006; Young et al., 2007). Two adjacent transcription factor binding sites for NF-kB and AP-1 regulate *MUC5AC* expression in response to *Haemophilus influenzae* lipoprotein P6 (Chen et al., 2004). One proposed mechanism of acrolein cytotoxicity is the activation of AP-1 and NF-kB via generation of reactive oxygen species (Korkmaz et al., 2007). Therefore, we first assessed whether acrolein activated AP-1 and NF-kB using pNF-kB-Luciferase and pAP-1-Luciferase minimal reporter plasmids. Acrolein significantly increased AP-1- but not NF-B-driven reporter activity (Fig. 6A), indicating that acrolein selectively activated AP-1 in NCI-H292 cells. We next confirmed that acrolein-mediated AP-1 activation was required for acrolein-inducible *MUC5AC* transcription by performing selective mutagenesis of the AP-1 and NF-B binding sites in MUC5AC-Luc reporter plasmid. A mutation in the AP-1 site (Chen et al., 2004) reduced the acrolein-mediated MUC5AC-Luc reporter activity, but mutating the NF-B site did not. (Fig. 6B). These results demonstrate that AP-1 activation is critical for *MUC5AC*induction by acrolein.

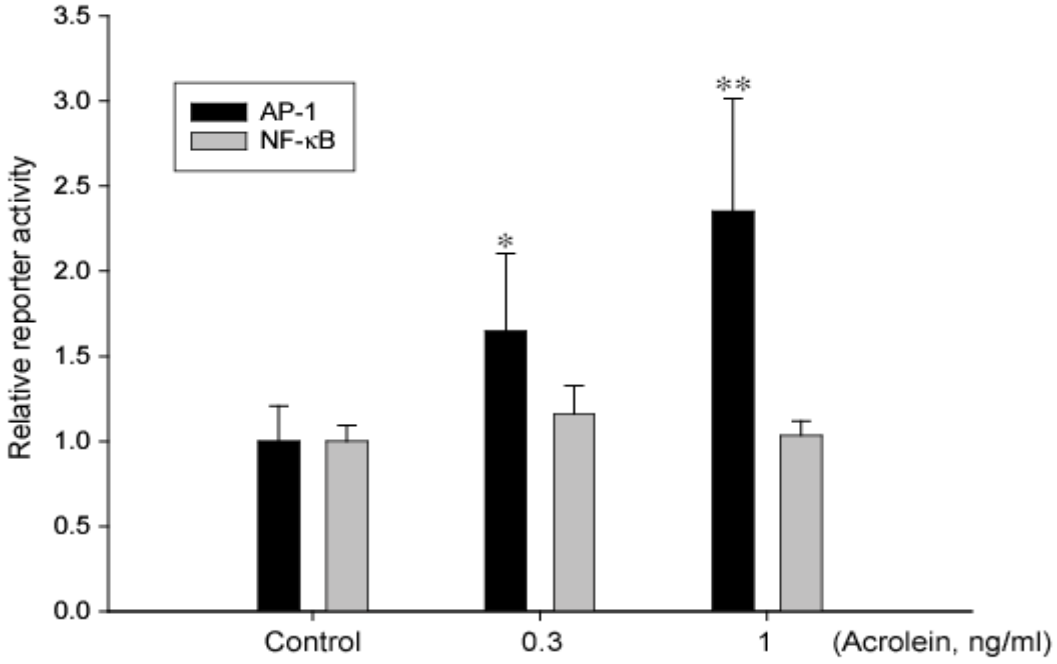

Fig 6A) Effect of acrolein on NF-kB and AP-1 reporter activities. NCI-H292 cells were transfected with pAP-1-Luciferase or pNF-kB-Luciferase minimal reporter plasmid, and reporter gene analysis was performed as described in the legend of Fig. 5, panel. Data represent the means SD of 4 different samples (significant compared to the untreated control, *p<0.05, **p<0.01).

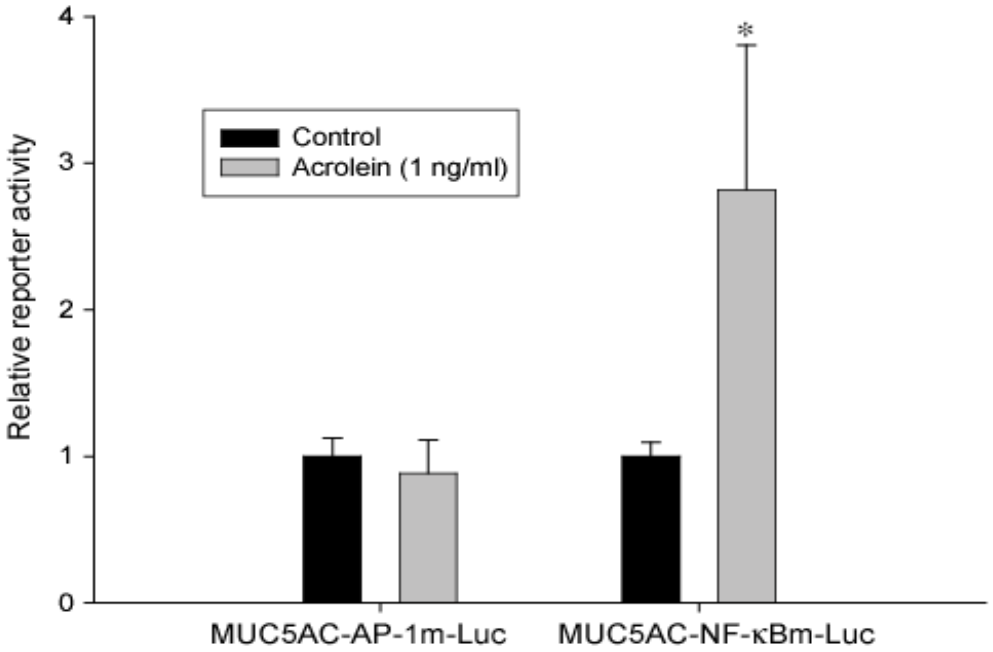

Fig 6B) Role of AP-1 in acrolein-inducible MUC5AC expression. NCI-H292 cells were transfected with MUC5AC-AP-1m-Luc (AP-1 site mutation) or MUC5AC-NF-kBm-Luc (NF-kB site mutation) and luciferase activities were measured 18 h after 1 ng/ml acrolein treatment as described in the legend of Fig. 5, panel (B). Data represent the means SD of 3 different samples (significant compared to the untreated control, $*p<0.05$).

Maruyama *et al*. (2005) showed that fucoidan inhibited the production of Th2 cytokines in bronchoalveolar lavage fluid in mice. We also showed that fucoidan potently inhibited the production of nitric oxide, a marker of pro-inflammatory mediator release from activated macrophages (Yang et al., 2006). Hence, fucoidan could have potential in treating respiratory disease. We therefore determined the effect of fucoidan on the acrolein-induced mRNA expression of *MUC5AC*. Acrolein (1 ng/ml) increased *MUC5AC* mRNA levels within 4 h (Fig. 7A).

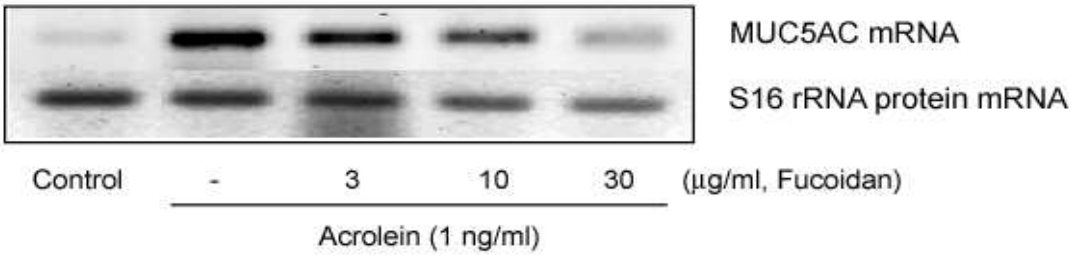

Fig 7A) Effect of fucoidan on MUC5AC mRNA increase by acrolein. NCI-H292 cells were pre-incubated with fucoidan (3-30 mg/ml) or vehicle for 30 min and then the cells were exposed to acrolein (1 ng/ml) for 4 h. MUC5AC mRNA levels were monitored by RT-PCR.

Fucoidan concentration-dependently inhibited this up regulation, with 30 mg/ml fucoidan completely blocking the induction of *MUC5AC* (Fig. 7B).

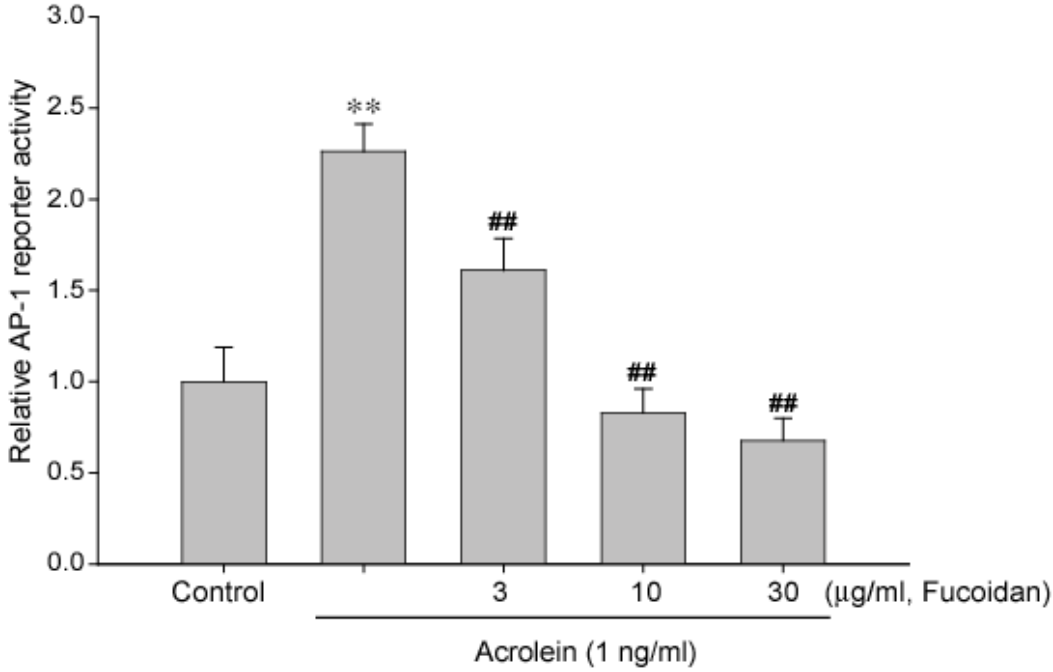

Fig 7B) Effect of fucoidan on AP-1 reporter activity. NCI-H292 cells were transfected with pAP-1-Luciferase plasmid, and incubated with fucoidan (3-30 ?g/ml) and acrolein (1 ng/ml) for 18 h. Reporter gene analysis was performed as described in the legend of Fig. 5, panel (B). Data represent the means SD of 8 different samples (significant compared to the untreated control, **p<0.01; significant compared to the acrolein-treated sample, ##p<0.01).

Discussion

The production of excess NO by iNOS induction in activated macrophages plays a key role in severe inflammatory diseases such as sepsis and arthritis [18, 19]. In particular, NO induces the collapse of the vascular reactivity and causes pathological alterations [20]. Therefore, the selective inhibition of iNOS expression in inflammatory cells, such as macrophages, may offer a new therapeutic strategy against inflammation.

MSR1 or the class A scavenger receptor on macrophages or transformed foam cells in atherosclerotic lesions is involved in internalizing the modified lipoproteins, and is believed to be one of the pathological mediators during atherosclerosis progression [21, 22]. Fucoidan is recognized as a ligand for MSR1, and can be taken up by macrophages via an endocytosis dependent mechanism [6]. It was recently reported that an interaction between macrophages and fucoidan causes the production of NO via a p38 kinase and NF-kB-dependent mechanism [6]. In contrast, we found for the first time that fucoidan, a main constituent of brown algae, inhibits the expression of iNOS in LPS-activated macrophages via the selective blocking of AP-1 activation. Fucoidan has several biological activities. Recently, its anti-inflammatory and anti-complement actions have attracted considerable attention [1]. Theunique suppressing effects on iNOS induction in activated macrophages might be one of explanations for its anti-inflammatory actions.

Inflammatory cytokines such as TNF-a exert their biological effects through members of the cytokine receptor superfamily, whereas, LPS

triggers the initial signals via TLR4 [11]. Hence, the mechanisms for regulating the induction of iNOS by LPS appear to be different from those caused by inflammatory cytokines. We also found that fucoidan blocked iNOS expression in TNF-a-stimulated macrophages (data not shown). This suggests that fucoidan may not act on the extracellular receptors but on the intracellular signaling machinery such as the process of transcription factor activation.

The *iNOS* gene promoter contains several homologous consensus sequences for binding the transcription factors such as NF-kB, AP-1, and C/EBP [23, 24], and NF-kB and AP-1 are believed to be essential for *iNOS* transcription [25, 26]. In this study, reporter gene analysis using the NF-kB minimal promoter and Western blot analysis using p65, I-kBa and phospho-I-kBa antibodies, revealed that fucoidan minimally affected the activation of NF-kB by LPS. However, the AP-1 reporter gene and gel shift analyses (Fig. 4A and 4B) revealed that LPS-induced AP-1 activation was suppressed by fucoidan. These results suggest that the inhibitory effect of fucoidan on the induction of iNOS in activated macrophages is closely related to the blocking of inflammatory signal(s)-inducible AP-1 activation. The most representative physiological effect of fucoidan is the anti-coagulation effect, which is due to its structure similarity to heparin [27]. Heparin can migrate into the nucleus and suppress AP-1 mediated transcription in smooth muscle cells and hepatoma cells [28, 29]. Hence, fucoidan may freely translocate to the nucleus and affect the activity of some transcription factors.

The activation status of a variety of transcription factors is

controlled by the generation of ROS [30], and the activation of AP-1 is dependent on the cellular redox status [31, 32]. Recently, it was suggested that fucoidan acts as a potential antioxidant [33] and it was also found that the sulfated polysaccharide efficiently scavenges hydroxyl and peroxyl radicals as well as peroxynitrite ions in the Total Oxidant Scavenging Capacity (TOSC) assay system (Kim SK et al., unpublished data). Hence, the blocking of AP-1 activation by fucoidan in activated macrophages may be associated with its antioxidant effect.

In conclusion, fucoidan inhibits nitric oxide production in macrophages activated by LPS or TNF-a through the selective inhibition ofAP-1 activation. This inhibition can explain some of the anti-inflammatory effects of fucoidan.

Since acrolein-mediated *MUC5AC* transcription is dependent on AP-1 activation, we further determined the inhibitory effect of fucoidan on AP-1 activity. Fucoidan (30 or 100 mM) significantly inhibited the acrolein-induced increases in AP-1 reporter activity (Fig. 3B). These results support the notion that fucoidan may block AP-1 activation and inhibit *MUC5AC*expression, a similar mechanism by which it blocks induction of nitric oxide synthase in activated macrophages (Yang et al., 2006).

In conclusion, fucoidaninhibits nitric oxide production in macrophages activated by LPS or TNF-a through the selective inhibition of AP-1 activation. This inhibition can explain some of the anti-inflammatory effects of fucoidan. fucoidan suppressed acrolein-induced *MUC5AC* expression in human bronchial epithelial cells through inhibition of AP-1 activation. Since the main pathological markers of asthma and COPD

are chronic inflammation and mucin production, fucoidan might have therapeutic potential for chronic respiratory diseases.

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감사의 글

이 자리를 빌어서 학위 과정 동안 많은 도움을 주셨던 분들에게 작을 글로나마 감사의 마음을 전하고자 합니다.

학문의 견지를 넓혀주시고 훌륭한 가르침을 주신 강건욱 교수님께 깊은 감사를 드립니다. 이 논문이 완성되기 까지 지도와 격려를 아끼지 않으셨던 최후균, 정혜광 교수님께도 감사드립니다.

논문이 나오기까지 많은 도움을 준 같은 연구실의 대학원생들에게도 감사드립니다.

아울러 언제나 큰 커다란 힘이 되어주신 부모님과 아내 그리고 예원, 예섭에게 고마움을 전합니다.

저작물 이용 허락서

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논문제목	한글:후코이단의 iNOS 매개의 nitric oxide와 MUC5AC release의 억제 효과				
	영문:Inhibitory effects of fucoidan on the iNOS-mediated nitric oxide production and MUC5AC release				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건 아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함.
2. 위의 목적을 위하여 필요한 범위 내에서의 편집과 형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포・전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사 표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물 이용의 허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리침해에 대하여 일체의 법적 책임을 지지 않음.
7. 소속 대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송・출력을 허락함.

동의여부 : 동의(0) 반대( )

2009년 2월

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