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Inhibition of acrolein-stimulated MUC5AC production by fucoidan in human bronchial epithelial cells

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Fucoidan, a marine sulfated polysaccharide has both antithrombotic and anti-inflammatory effects. We determined the effect of fucoidan on *MUC5AC* expression in a human bronchial epithelial cell line, NCI-H292. Reverse transcription-polymerase chain reaction (RT-PCR) analysis showed that 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-κB (NF-κB) 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.

1. Introduction

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), smoking 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).

Fucoidan is a marine sulfated polysaccharide that has antiviral, anti-angiogenic, antitumor, a contraceptive, antithrombotic, anticoagulant, and anti-inflammatory properties (Berteau et al. 2003). The brown seaweed, *Laminaria japonica* Aresch. (Laminariales), is consumed as a marine vegetable in East Asia. Fucoidan, one of its main components, is also available as a food supplement in Japan and the United States. Fucoidan inhibits inflammation by inhibiting complement (Blondin et al. 1996) and leukocyte migration (Linnemann et al. 2000). We have recently reported that high concentrations of fucoidan (10–100 μg/ml) inhibit iNOS expression and subsequent nitric oxide production in activated macrophages (Yang et al. 2006). 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.

2. Investigations, results and discussion

Mucin synthesis in epithelial layers is controlled via transcriptional (Li et al. 1998; Manna et al. 1995) and post-transcriptional regulation (Velcich and Augenlicht 1993). We first determined whether acrolein stimulates *MUC5AC* expression in NCI-H292 cells. Acrolein 0.3 and 1 ng/ml significantly increased *MUC5AC* mRNA (Fig. 1A). 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).

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 (0.3 and 1 ng/ml) significantly increased luciferase activity in cells transfected with the *MUC5AC*-Luc reporter plasmid (Fig. 1B), suggesting that the induction of *MUC5AC* is mediated through transcriptional activation.

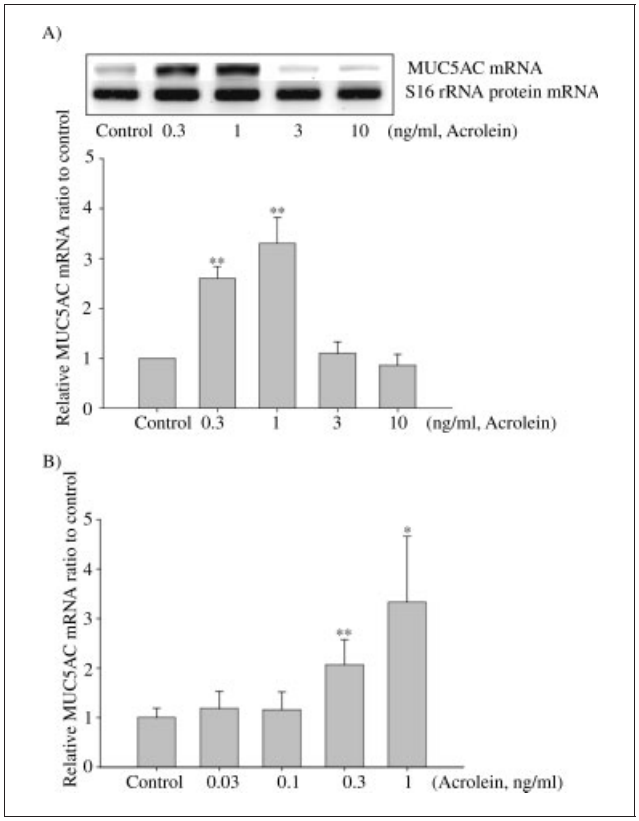

Fig. 1: (A) Effect of acrolein on *MUC5AC* mRNA expression in NIC-H292 cells. NCI-H292 cells were incubated in a medium containing acrolein (0.3–10 ng/ml) for 4 h and *MUC5AC* mRNA levels were monitored by RT-PCR. Relative *MUC5AC* mRNA levels were determined by measuring band intensities by scanning densitometry. Data represent the means ± SD of three separate experiments (significant compared to the untreated control, $^{**}p < 0.01$). (B) Effect of acrolein on *MUC5AC*-Luc reporter activity. Induction of luciferase activity by acrolein in the NIC-H292 cells 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, $^{*}p < 0.05$, $^{**}p < 0.01$)

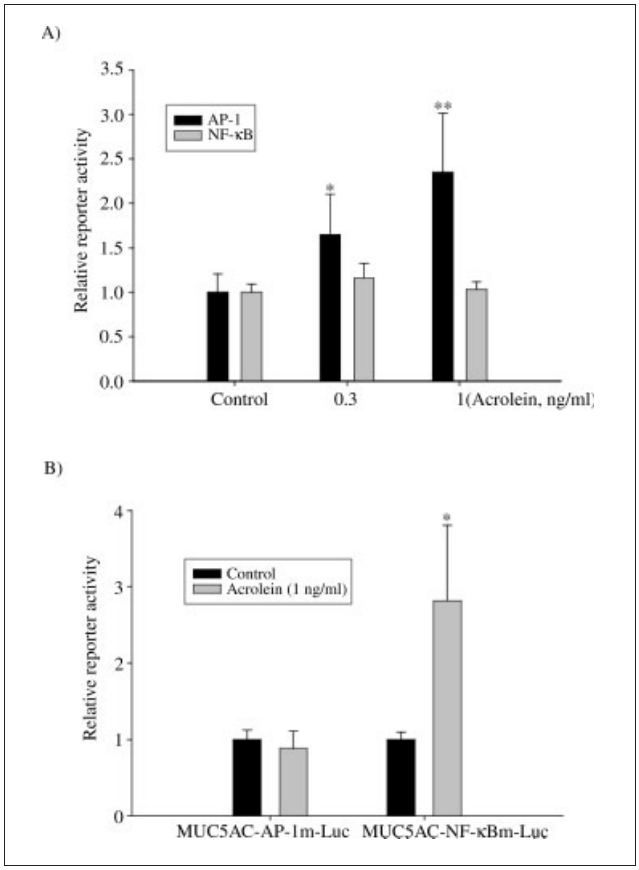

Fig. 2: (A) Effect of acrolein on NF-κB and AP-1 reporter activities. NCI-H292 cells were transfected with pAP-1-Luciferase or pNF-κB-Luciferase minimal reporter plasmid, and reporter gene analysis was performed as described in the legend of Fig. 1, panel (B). Data represent the means ± SD of 4 different samples (significant compared to the untreated control, $^{*}p < 0.05$, $^{**}p < 0.01$). (B) 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-κBm-Luc (NF-κB site mutation) and luciferase activities were measured 18 h after 1 ng/ml acrolein treatment as described in the legend of Fig. 1, panel (B). Data represent the means ± SD of 3 different samples (significant compared to the untreated control, $^{*}p < 0.05$)

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-κB (NF-κB) binding site (Kato et al. 2006; Young et al. 2007). Two adjacent transcription factor binding sites for NF-κB 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-κB via generation of reactive oxygen species (Korkmaz et al. 2007). Therefore, we first assessed whether acrolein activated AP-1 and NF-κB using pNF-κB-Luciferase and pAP-1-Luciferase minimal reporter plasmids. Acrolein significantly increased AP-1- but not NF-κB-driven reporter activity (Fig. 2A), 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-κB 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-κB site did not (Fig. 2B). These results demonstrate that AP-1 activation is critical for *MUC5AC* induction by acrolein.

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 a 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. 3A). Fucoidan concentration-dependently inhibited this up regulation, with 30 μg/ml fucoidan completely blocking the induction of *MUC5AC* (Fig. 3A).

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 μM) 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).

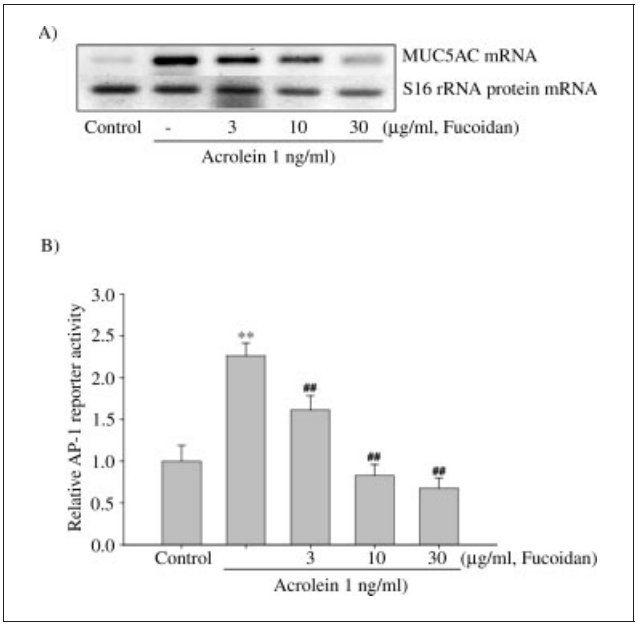

Fig. 3: (A) Effect of fucoidan on *MUC5AC* mRNA increase by acrolein. NCI-H292 cells were pre-incubated with fucoidan (3–30 μg/ml) or vehicle for 30 min and then the cells were exposed to acrolein (1 ng/ml) for 4 h. *MUC5AC* mRNA levels were monitored by RT-PCR. (B) Effect of fucoidan on AP-1 reporter activity. NCI-H292 cells were transfected with pAP-1-Luciferase plasmid, and incubated with fucoidan (3–30 μg/ml) and acrolein (1 ng/ml) for 18 h. Reporter gene analysis was performed as described in the legend of Fig. 1, 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, $^{\#\#}p < 0.01$)

In summary, 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.

3. Experimental

All reagents, including fucoidan, were supplied by Sigma (St. Louis, MO). 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% CO_2/95% air atmosphere in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin.

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.

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