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Corn silk induces nitric oxide synthase in murine macrophages

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Abbreviations: HRP, horseradish peroxidase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor kappa B; NO, nitric oxide; PDTC, pyrolidine dithiocarbamate

Abstract

Corn silk has been purified as an anticoagulant previously and the active component is a polysaccharide with a molecular mass of 135 kDa. It activates murine macrophages to induce nitric oxide synthase (NOS) and generate substantial amounts of NO in time and dose-dependent manners. It was detectable first at 15 h after stimulation by corn silk, peaked at 24 h, and undetectable by 48 h. Induction of NOS is inhibited by pyrolidine dithiocarbamate (PDTC) and genistein, an inhibitor of nuclear factor kappa B (NF-κB) and tyrosine kinase, respectively, indicating that iNOS stimulated by corn silk is associated with tyrosine kinase and NF-κB signaling pathways. IκB-α degradation was detectible at 10 min, and the level was restored at 120 min after treatment of corn silk. Corn silk induced nuclear translocation of NF-κB by phosphorylation and degradation of IκB-α.

Keywords: macrophages; nitric oxide; nitric oxide synthase; NF-κB

Introduction

Corn silk is an outer thread-like part of corn. It has been used to treat a pathological swelling and asthma, and used for diuretics in oriental medicine. Although recently more interests have been focused on biological activities of medicinal plants, most researches are still in the preliminary stage. Methanol extracts of corn silk showed an antioxidative activity on the level of lipid peroxidation (Maksimovic and Kovacevic, 2003). Volatiles from corn silk inhibited the growth of *Aspergillus flavus*, indicating that it has an antifungal activity (Ziringue, 2000). In addition, extract of corn silk inhibited TNF and LPS-induced cell adhesion, but not cytotoxic activity or TNF production (Habtemariam, 1998).

Macrophages play key roles during immune response after activation (Caleda and Nathan, 1994). A variety of mechanisms can activate macrophages including exposure to cytokines such as IFN-γ and interleukin-2. Macrophage activation can be also stimulated by infection of bacteria, bacterial products, and their particulates. They can destroy tumor cells after treatment with both IFN-γ and LPS, indicating that two stimuli are necessary for complete activation (Drysdale *et al*., 1988). Each molecule interacts with specific receptor on the surface of macrophages (Lorsbach *et al*., 1993). Macrophages can also kill bacteria, virus, or parasites directly, secrete proinflamatory cytokines, act as an antigen presenting cells, and finally participate in tissue remodeling. Macrophage activation was accompanied by increased cell size, cytoplasmic spreading, increased NO production, increased cytokines and adhesion molecules, and Fc receptors.

NO has been identified as an important signaling molecule involved in regulating a variety of biological activities. Macrophages release NO from guanidino moiety of L-arginine *via* reaction catalyzed by the inducible form of nitric oxide synthase (iNOS) by a variety of agents such as endotoxin, IL-1, TNF-α, and IFN-γ (Chen *et al*., 1999; Tracey, 2002). A major part of inducible nitric oxide synthase (iNOS) regulation occurs at the transcriptional levels and changes in iNOS mRNA lead to different concentration of NO production in iNOS-expressing cells (Lowenstein *et al*., 1993; Yang *et al*., 2002).

Although the signal transduction pathway for iNOS is not yet clear, many researches have been studied for iNOS expression induced by proinflammatroy cytokines. The induction of iNOS is triggered and regulated by a series of signaling pathways including nuclear factor kappa B (NF-κB) transcription factor. The promoter region of the iNOS gene contains several transcription factor binding sites including that for NF-κB (Lowenstein *et al*., 1993). The transcription factor, NF-κB has been implicated as an essential part of pathogen- and stress-related responses of host organisms. Direct or indirect signals from pathogens or stress potently activated NF-κB, which can

induce many defense-related genes transcriptionally. The family of NF-κB protein was required for the enhanced iNOS gene expression when macrophages were exposed to LPS and other signals such as serum deprivation (Xie *et al*., 1994; Liu *et al*., 2001). In naive cells, NF-κB is associated with an inhibitory protein, IκB-α in the cytoplasm, binding to NF-κB dimers and keeping its inactive state, but activation by appropriate signals leads to the phosphorylation of IκB-α by IκB-α kinase. Phosphorylated IκB-α is released from NF-κB complex, allowing the translocation of NF-κB into the nucleus, binding to DNA, and leading to activate the responsive genes (Lin *et al*., 1995).

In this study, we report that stimulation of macrophages with corn silk induced significant amounts of nitric oxide synthase (NOS) with production of NO. NOS induction was mediated by nuclear translocation of NF-κB by degradation of IκB-α.

Materials and Methods

Cells and reagents

RAW264.7 cells, a murine macrophage cell line, were obtained from the ATCC and cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin in 6 well plates or in 10 cm dishes. DMEM, FBS, penicillin, streptomycin, polymyxin B, LPS (from *Escherichia coli* serotype 055: B5), pyrolidine dithiocarbamate (PDTC), SB203580, PD98059, and genistein were obtained from Sigma Chemical Co. (St. Louis, MO). mAb for iNOS, p50 subunit of NF-κB, p65 subunit of NF-κB, and polyclonal Ab for IκB-α were from Santa Cruz Biotechnology (Santa Cruz, CA).

Purification of corn silk active fraction

Active fraction of corn silk was prepared by the methods of Choi *et al*. (2004). Corn silk was obtained from local market, washed with distilled water, and disrupted for 5 min with pulse in an ice-chilled bead-beater (Biospec Products, Bartleville, OK). The homogenate was filtered by several layers of cheese cloths, and the filtrates were centrifuged at 10,000 *g* for 40 min in a refrigerated centrifuge, T-324 using A-8.24 rotor (Kontron Instruments, Zurich, Sweden). The supernatant was fractionated with ethyl acetate, and the aqueous fraction was applied on a Sephadex G-100 column, a phenyl Sepharose column, and finally a Sephadex G-150 column to get a purified fraction. All corn silk preparations were pre-treated with polymyxin B for 2 h at room temperature to prevent corn silk from endotoxin contamination.

Immunoblot analysis

Ten μg of cell lysate were separated by SDS-PAGE and transferred onto nitrocellulose membranes as described before (Shin *et al*., 2002). The blots were then washed in Tris-Tween buffered saline [TTBS, 20 mM Tris-HCl, pH 7.6 containing 137 mM NaCl and 0.05% (v/v) Tween 20], blocked overnight with 5% (w/v) BSA, and probed with mAb for iNOS, p50 or p65 NF-κB and polyclonal Ab for IκB-α in 5% (w/v) BSA dissolved in TTBS. Using HRP-conjugated secondary anti-mouse Abs (iNOS, p50 or p65 NF-κB), or anti-rabbit Ab (IκB-α), blots were detected by enhanced chemiluminescence.

Determination of NO concentration

NO production in culture supernatants was assayed by measuring nitrite, its stable degradation product, using Griess reagent. After stimulation, the supernatants were centrifuged and its aliquots were mixed with 0.25 ml of Griess reagent to make final volume of 0.5 ml and then incubated for 10 min at room temperature before measuring the absorbance at 540 nm. $NaNO_2$ was used as a standard.

Results

The anticoagulant was purified and characterized from corn silk (Choi *et al*., 2004). Since inflammation and coagulation are closely related, we have tested whether it activates macrophages. Macrophages incubated with corn silk expressed high level of iNOS and produced significant amount of NO in a dose-dependent manner (measured as NO_2^-)(Figure 1A). Production of NO was detectable at 2.5 μg/ml of corn silk after 24 h stimulation. Above 25 μg/ml of corn silk activation, the level of NO decreased. The expression of iNOS was also time-dependent. It is detectable first at 15 h after corn silk activation, remained up to 32 h, and undetectable by 48 h (Figure 1B). However, released NO was detectable after 8 h stimulation with corn silk and the level of NO increased up to 48 h. To clarify that NO production and expression of iNOS were due to carbohydrate part of corn silk, corn silk was treated with nonspecific protease, subtilisin B, or was oxidized by periodic acid. As shown in Figure 1C, protease-treated corn silk did not change the level of iNOS expression, whereas oxidized one did not induce NOS at all, indicating that carbohydrate of corn silk is responsible for the stimulation of macrophages. In case of buffered control treated corn silk, the level of iNOS was reduced probably due to loss during oxidation processes.

To determine signaling molecules contributing to

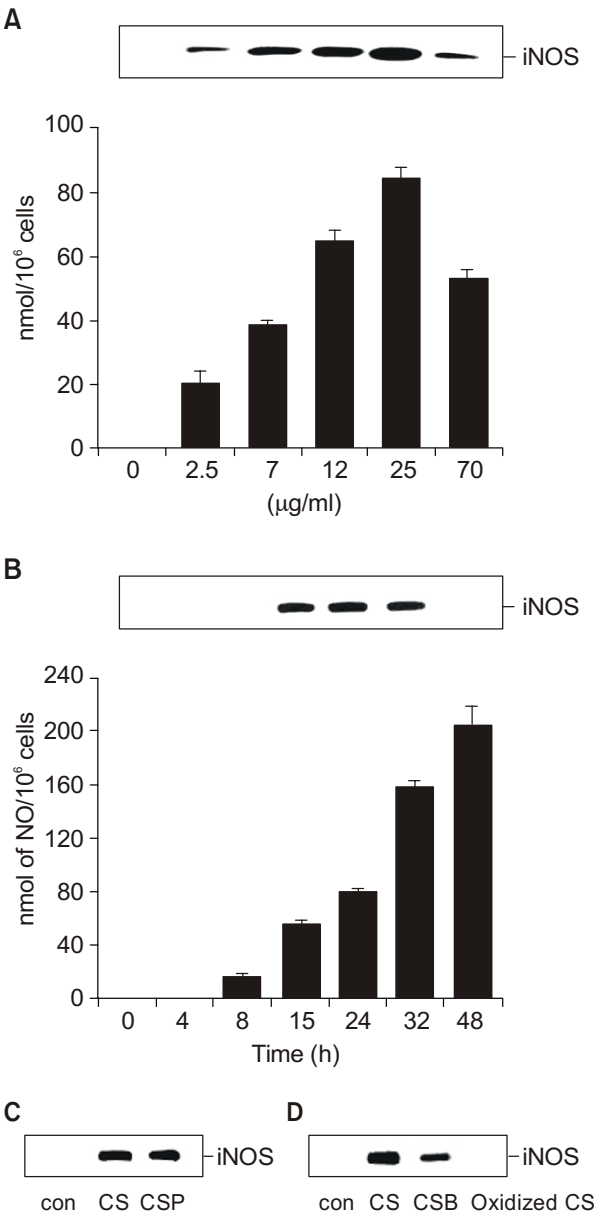

Figure 1. Production of NO by corn silk in RAW 264.7 cells. (A) Cells were treated with corn silk at various concentrations for 24 h, followed by the Griess reagent assay for NO. (B) Cells were treated with 25 μg/ml of corn silk for indicated incubation times. (C) Cells were treated with 25 μg/ml of corn silk preincubated without (CS) or with subtilisin B (CSP) for 18 h. (D) Corn silk was oxidized with periodate (CSP) for 24 h. CSB was buffer treated instead of periodic acid. Then, cells were incubated with corn silk for 18 h. The data shown represent the mean ± SD of three independent assays. The cells used in the NO assay were subjected to electrophoresis and Western blot analysis using iNOS-specific Ab as described in Materials and Methods. Results were representative of three independent experiments.

corn silk-induced iNOS production, we tested the effects of inhibitors of MEK (PD98059), p38 (SB20 3580), tyrosine kinase (genistein), tyrosine phosphatase (sodium orthovanadate), and NF-κB (PDTC) on NOS induction by corn silk. Corn silk-induced NOS production was not inhibited by the addition of PD98059 and SB203580, indicating that MEK and p38 MAPK are not involved in signal transduction pathway stimulated by corn silk (Figure 2). However, PDTC and genistein inhibited NOS induction significantly, while sodium orthovanadate potentiated NOS expression (Figure 2). These results suggested that NOS induction stimulated by corn silk is via activation of NF-κB and inhibition of tyrosine phosphatase.

Next, we have determined degradation of IκB-α to investigate the potential role of activation of NF-κB in the expression of iNOS by corn silk in mouse macrophages. The cells were stimulated with corn silk for 0, 5, 10, 15, 30, 60, and 120 min. Cells were lysed, fractionated, and analyzed by Western blotting. As shown in Figure 3A, corn silk induced the degradation of Iκ B-α. Degradation of Iκ B-α began at 10 min after corn silk stimulation, and the level of Iκ B-α was restored after 120 min. As a positive control, LPS also showed degradation of Iκ B-α, but the time course was different. Next, to examine the effect of corn silk on nuclear translocation of NF-κB, cells were treated 0, 60, and 120 min with corn silk, and cytoplasmic and nuclear extracts were examined for p50 and p65 NF-κB by immunoblots. Figure 3B showed that corn silk caused nuclear translocation of p50 and p65 subunits after 60 min of corn silk stimulation, and decreased the cytoplasmic levels of these two proteins. Consistent with corn silk-induced degradation of Iκ B-α, it induced nuclear translocation of p50 and p65 NF-κB, and caused these proteins to stimulate the responsive gene.

Discussions

We demonstrated that corn silk extract activates macrophages to express high level of iNOS and generates large amounts of NO in time and dose-dependent manners. The corn silk extract was purified and characterized to have an activity of anticoagulation both in human plasma and in thrombin-induced purified fibrinogen system (Choi *et al*., 2004). Since processes of coagulation and inflammation are closely related and share mediators, a possible effect of the anticoagulant from corn silk on macrophages was tested. The anticoagulant from corn silk delayed thrombin time twice at 8.1 μg/ml using human plasma, and analysis of the inhibition of thrombin- catalyzed fibrin formation gave an apparent K_i value of 3.1 μg/ml using purified fibrinogen (Choi *et al*., 2004). Our data showed that it also activated macrophage and generated NO at the concentration range of 2.5-70 μg/ml. Its anticoagulating activity occurred at the

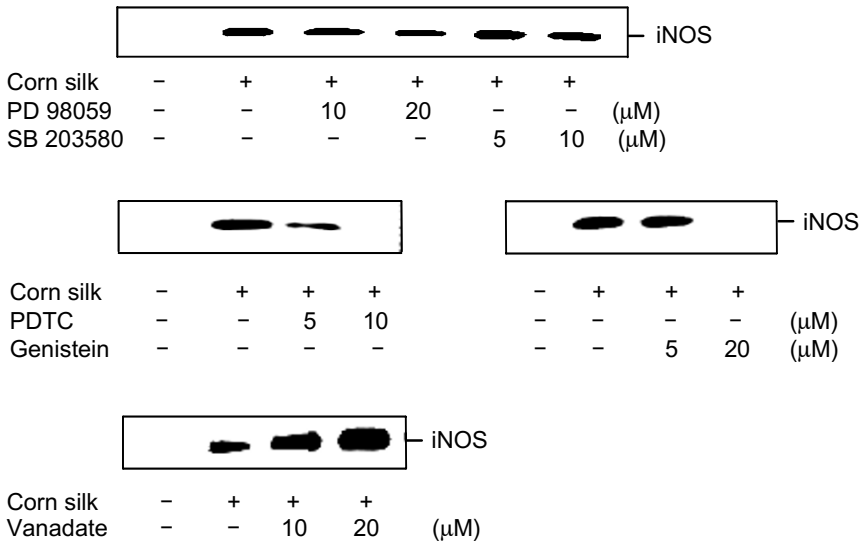

Figure 2. Effects of inhibitors involved in signal transduction pathways on expression of iNOS by corn silk in RAW 264.7 cells. Cells were pretreated with each inhibitor for 1 h, and stimulated with 25 μg/ml of corn silk for 24 h. The cell lysates were subjected to electrophoresis and Western blot analysis using iNOS-specific Ab as described in Materials and Methods. Results were representatives of three independent experiments.

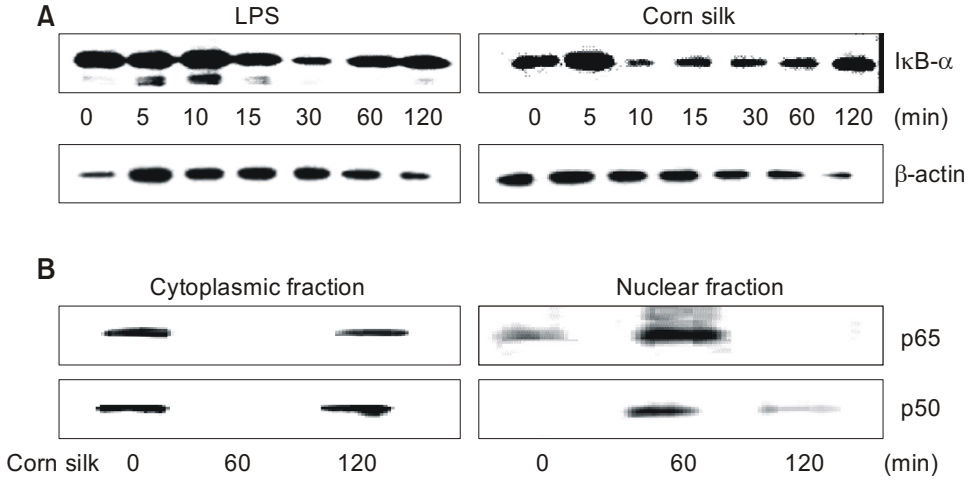

Figure 3. Effects of corn silk on NF-κB signaling pathways in RAW 264.7 cells. (A) Cells were stimulated with 25 μg/ml of corn silk for 0, 5, 10, 15, 30, 60, and 120 min. The cytoplasmic fractions of cell lysates were subjected to electrophoresed and Western blot analysis using IkB-α specific Ab as described in Materials and Methods. LPS (1 μg/ml) was treated as a positive control. (B) Cells were stimulated with 25 μg/ml of corn silk for 0, 60, and 120 min. Cells were lysed, and cytoplasmic and nuclear fractions were separated. Cytosols and nuclear extracts were analyzed for p50 and p65 NF-κB by immunoblots. Results were representative of three independent experiments.

similar concentration range to its inflammatory activity such as NO production. As bacterial outer shell component, LPS which activates macrophages has a strong stimulatory effect on factor X-activating activities (Pejler, 1999). An activator of coagulation, tissue factor can directly augment macrophage activation by factor VII1-dependent induction of proinflammatory changes in macrophages (Cunningham *et al*., 1999). Tissue factor also induces arthritis in mice by strong inflammatory properties mediated by monocytes (Bokarewa *et al*., 2002).

Production of NO was detectable at 2.5 μg/ml of corn silk and reached at the maximum at 25 μg/ml after 24 h stimulation. Latter condition was used for following NO production assays. Above 25 μg/ml of corn silk reduced the iNOS level. We have not determined whether corn silk induces other molecules. However, the decrease could be explained by induction of other proteins such as heme oxygenase-1 (Otterbein *et al*., 2003). Inhibition of NO accumulation by peroxysome proliferator-activated receptor agonists was associated with a fall in inducible NOS protein

and an induction of heme oxygenase-1 (Colville-Nash *et al*., 1998).

Activations of NF-κB and inhibition of tyrosine phosphatase were involved in the induction process of NOS in murine macrophages stimulated by corn silk. Through a series of signaling pathways, the activation of the multisubunit inhibitor of IκB (IκB) kinase (IKK) complex induces IκBα and IκBβ phosphorylation. Phosphorylation of these inhibitory proteins leads to degradation and the release of Rel/ NF-κB proteins from cytosol to the nucleus. iNOS is among the genes that are induced by NF-κB transcription factors and contain key NF-κB regulatory sites in its promoter. Although the membrane receptor of corn silk has not been determined yet, certain membrane proteins are assumed to act as a receptor in macrophages. A possible candidate could be CR3 which is identified as the leukocyte membrane receptor for β-glucan (Thornton *et al*., 1996). Angelan, a polysaccharide from Angelica gigas, also stimulated macrophages and induced NF-κB in murine macrophages (Jeon and Kim, 2001). Since PDTC, a relatively specific inhibitor of the activation of NF-κB in macrophages, blocked the NOS induction, it appeared that NF-κB is involved in the induction of iNOS gene itself in corn silk-stimulated macrophages. However, we have not tested the effects of PDTC on binding of NF-κB proteins to the iNOS promoter. However, we demonstrated that corn silk caused degradation of IκB-α in murine macrophages. Corn silk also induced nuclear translocation of p50 and p65 subunits of NF-κB after degradation of IκB-α. Our results strongly suggest that corn silk induced NOS by NF-κB pathway. A role for tyrosine phosphatase in control of iNOS expression was reported in IFN-γ-treated peritoneal macrophages (Diaz-Guerra *et al*., 1999). Treatment with sodium orthovanadate of macrophages stimulated with IFN-γ potentiated the activation of NF-κB and the expression of iNOS. Analysis of iNOS promoter activity showed that inhibition of tyrosine phophatase increased the cooperation between IFN-γ-activated transcription factors and NF-κB, indicating a negative role for tyrosine phosphatase. Importance of tyrosine phosphatase in the regulation of iNOS was also reported in *Leishmania*-infected macrophages (Nandan *et al*., 1999). Infection with *Leishmania* attenuated iNOS expression with reduced MAPK signaling and it was recovered by treatment of sodium orthovanadate.

In summary, we have shown that corn silk activates macrophages to express NOS *via* NF-κB and tyrosine kinase activation. Corn silk induced nuclear translocation of p50 and p65 subunits of NF-κB after degradation of IκBα.

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