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Inulin stimulates NO synthesis via activation of PKC-α and protein tyrosine kinase, resulting in the activation of NF-κB by IFN-γ-primed RAW 264.7 cells

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Abstract

Inulin, an active component of Chicorium intybus root, has been shown to stimulate the growth of bifidobacteria, and inhibit colon carcinogenesis. NO mediates a number of the host-defense functions of activated macrophages, including antimicrobial and tumoricidal activity. We examined the effect of inulin on the synthesis of NO in RAW 264.7 cells. Inulin alone had no effect, whereas inulin with IFN-γ synergistically increased the NO production and inducible NO synthase (iNOS) expression in RAW 264.7 cells. Synergy between IFN-γ and inulin was mainly dependent on inulin-induced TNF-α secretion. Also, protein kinase C (PKC)-α was involved in the inulin-induced NO production. Inulin-mediated NO production was inhibited by the protein tyrosine kinase (PTK) inhibitor, tyrphostin AG126. Since iNOS gene transcriptions have been shown to be under the control of the NF-κB/Rel family of transcription factors, we assessed the effect of inulin on NF-κB/Rel using an EMSA. Inulin produced strong induction of NF-κB/Rel binding, whereas AP-1 binding was slightly induced in RAW 264.7 cells. Inulin stimulated phosphorylation and degradation of IκB-α. These results suggest that in IFN-γ-primed RAW 264.7 cells inulin might stimulate NO synthesis via activation of PKC-α and PTK, resulting in the activation of NF-κB.

Keywords: Inulin; Nitric Oxide (NO); NF-kappa B (Nuclear factor-kappa B); PKC (protein kinase C)-alpha; Protein tyrosine kinase; RAW 264.7 cells

1. Introduction

Inulin (~5.5 kDa) is a polysaccharide purified from the root of Chicorium intybus L [1]. Inulin has been shown to stimulate the growth of bifidobacteria, which are regarded as beneficial strains in the colon, and inhibit colon carcinogenesis in laboratory animal models [2–4]. Inulin has also been reported to influence serum and liver lipid concentrations, cecal short-chain fatty acid concentrations, and fecal lipid excretion in rats [5]. Pure nonhydrolyzed inulin was directly converted to ethanol in a simultaneous saccharification and fermentation process [6].

Macrophages play a significant role in host defense mechanisms. When activated, they inhibit the growth of a wide variety of tumor cells and microorganisms [7–10]. NO was reported to participate in the cytolytic function of macrophages [11]. Treatment of tumor cells with S-nitroso-N-acetylpenicillamine, a NO-generating compound, showed a similar tumoricidal effect. Stimulation of murine macrophages by LPS and IFN-γ results in the expression of an inducible NO synthase (iNOS), which catalyzes the production of large amounts of NO from $_{L}$-arginine and molecular oxygen [12]. The iNOS enzyme can be specifically and

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stoichiometrically inhibited by the structural analogues of $_L$-arginine, such as N^G-monomethyl-$_L$-arginine (N^GMMA).

Previous studies have shown that LPS binds to LPS-binding protein and then binds to membrane CD14 and activates phosphatidylinositol-phospholipase C via tyrosine phosphorylation to induce protein kinase C (PKC) activation; this results in the stimulation of NF-κB-specific DNA-protein binding, initiating the expression of iNOS and finally, the release of NO [13]. Macrophages and monocytic cells express the Ca^{2+}-dependent isoenzymes α, áI and áII, the Ca^{2+}-independent isoenzymes δ and ϵ, and the atypical isoenzyme ζ [14–17]. In addition, there is evidence that both the production of TNF-α and its activities are mediated by protein tyrosine kinases (PTKs) [18, 19], and therefore these events can be blocked by PTK inhibitors such as tyrphostins [20]. Although both the actions of LPS and TNF-α are mediated by tyrosine phosphorylation reactions, the precise identities of the PTKs involved have not been elucidated.

NF-κB appears to play a primary role in the transcriptional regulation of iNOS and TNF-α gene expression [21]. In unstimulated cells, NF-κB is an inactive heterodimer of p50/p65 subunits bound to the NF-κB inhibitor protein IκB. Upon stimulation, IκB is phosphorylated on specific serine residues which target IκB for degradation in a ubiquitine-dependent manner [22]. The antioxidants inhibitors of NF-κB activation, pyrrolidinedithiocarbamate (PDTC) and diethyldithiocarbamic acid, prevent LPS plus IFN-γ-induced iNOS expression and NO production by RAW 264.7 cells [23], indicating that NF-κB participates in LPS plus IFN-γ-induced iNOS expression.

In the present study, we investigated whether inulin induces NO synthesis and activates NF-κB in RAW 264.7 cells. We showed that inulin synergistically induced NO synthesis through activation of NF-κB in IFN-γ-primed RAW 264.7 cells.

2. Materials and Methods

2.1. Materials

Murine IFN-γ (1×10^6 U/mL) was purchased from Genzyme (München, Germany). Inulin, N-(1-naphtyl)-ethylenediamine dihydrochloride, LPS, sodium nitrite, and sulfanilamide were purchased from Sigma (St. Louis, MO). Recombinant TNF-α and TNF-α Ab were obtained from R & D system Inc. (Minneapolis, MN). Anti-iNOS, p50, p65, p-IκB-α, IκB-α and PKC-α Ab was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). N^GMMA and tyrphostin AG126 were purchased from Calbiochem (San Diego, CA). All reagents and media for culture experiments were tested for their LPS content with use of a colorimetric Limulus amoebocyte lysate assay (detection limit, 10 pg/mL; Whittaker Bioproducts, Walkersville, MD). None of these reagents contained endotoxins.

2.2. Cell cultures

RAW 264.7 cells, a macrophage-like cell line, were obtained from the Korean Cell Line Bank (Seoul, ROK). The cells were grown in RPMI 1640 medium (Gibco BRL, Grand Island, NY) with 10% heat-inactivated FBS, 1% penicillin-streptomycin at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air.

2.3. Measurement of nitrite concentration

NO synthesis in cell cultures was measured by a modified microplate assay method, as previously described [14]. To measure nitrite, 100 μL aliquots were removed from a conditioned medium and incubated with an equal volume of Griess reagent (1% sulfanilamide/0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) at room temperature (RT) for 10 min. The absorbance at 540 nm was determined in a Titertek Multiskan (Flow Laboratories, North Ryde, Australia). NO_2^- was determined by using sodium nitrite as a standard. The cell-free medium alone contained 5 to 8 μmol of NO_2^-; this value was determined in each experiment and subtracted from the value obtained from the medium with cells.

2.4. Western blot analysis

Whole cell lysates were made by boiling cells in a sample buffer (62.5 mmol Tris-Cl, pH 6.8, 2% SDS, 20% glycerol, 10% 2-mercaptoethanol). Proteins in the cell lysates were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 10% skim milk in PBS-tween-20 for 1 h at RT and incubated with each Ab. After washing in PBS-tween-20 three times, the blot was incubated with secondary Ab for 30 min and the Ab-specific proteins were visualized by an enhanced chemiluminescence detection system according to the recommended procedure (Amersham Corp, Piscataway, NJ).

2.5. Assay of TNF-α secretion

TNF-α concentration derived culture supernatants were measured by a modified ELISA, as described elsewhere [24]. The ELISA was devised by coating 96-well plates of mouse mAb with specificity for mouse TNF-α. Before use and between subsequent steps in the assay, coated plates were washed with PBS containing 0.05% tween-20. All reagents used in this assay were incubated for 2 h at 37°C. Recombinant mouse TNF-α were diluted and used as a standard. Serial dilutions starting from 1 pg/mL were used to establish the standard curve. Assay plates were exposed sequentially to mouse anti-TNF-α Ab, and phosphatase conjugated goat anti-rabbit IgG Ab and avidine peroxidase, and ρ-nitropheny1 phosphate and ABTS substrate solution containing 30% H_2O_2. The plates were read at 405 nm.

2.6. Preparation of nuclear extract and EMSA

Cells were washed with ice-cold PBS, scraped with a rubber policeman, and the subsequently formed pellet rapidly was frozen at −80°C. After thawing the pellet, it was suspended in buffer A (10 mmol Tris-HCl pH 7.9, 10 mmol KCl, 1.5 mmol $MgCl_2$, 0.5 mmol DTT). The cells were then resuspended in modified buffer A (10 mmol Tris-HCl pH 7.9, 10 mmol KCl, 1.5 mmol $MgCl_2$, 0.5 mmol DTT, 0.1% NP-40). The nuclei were gently resuspended in buffer C (20 mmol Tris-HCl pH7.9, 0.2 mmol EDTA, 1.5 mmol $MgCl_2$, 0.5 mmol DTT, 0.5 mmol PMSF, 25% glycerol, 420 mmol NaCl) and incubated for 15 min at 4°C. An established EMSA method, with slight modifications, was used [25]. Nuclear protein (10 μg) was incubated for 20 min at RT with 20 μg of BSA, 2 μg of poly (dI-dC), 2 μL of buffer C (20 mmol HEPES/KOH, 20% glycerol, 100 mmol KCl, 0.5 mmol PMSF, pH 7.9), 4 μL of buffer F (20% ficoll-400, 100 mmol HEPES/KOH, 300 mmol KCl, 10 mmol DTT, 0.5 mmol PMSF, pH 7.9), and 20,000 cpm of a ^{32}P-labeled probe encoding the NF-κB consensus sequence (5′-AGTTGAGGGGACTTTCCCAGGC-3′) or AP-1 consensus sequence (5′-CGCTTGATGAGTCAGCCGGAA-3′) in a final volume of 20 μL. DNA-protein complexes were resolved at 30 mA for 1 h in native 4% polyacrylamide gel. After electrophoresis, the gel was dried and autoradiographed.

2.7. Statistical analysis

Collected data were expressed as means ± S.E. Statistical analysis was performed by a Student's *t*-test to express the difference between two groups.

3. Results

3.1. Inulin-induced NO production

Initially, we investigated whether RAW 264.7 cells could be stimulated by inulin, either alone or in combination with IFN-γ, to induce NO production. RAW 264.7 cells were cultured with IFN-γ (5 U/mL) and then stimulated with various concentrations (0.01, 0.1, 1 mg/mL) of inulin. NO release was measured using the Griess method. As shown in Fig. 1A, Inulin alone had no effect, whereas inulin with IFN-γ significantly increased the NO production in RAW 264.7 cells. The increased production of NO was observed upon 24 h and thereafter was decreased by addition of (?) inulin (Fig. 1B). The dose-dependent effects of inulin on NO production are shown in Fig. 1C. The synergistic effect was always maximal at 1 mg/mL of inulin. These results suggest that inulin with IFN-γ synergistically increases NO production.

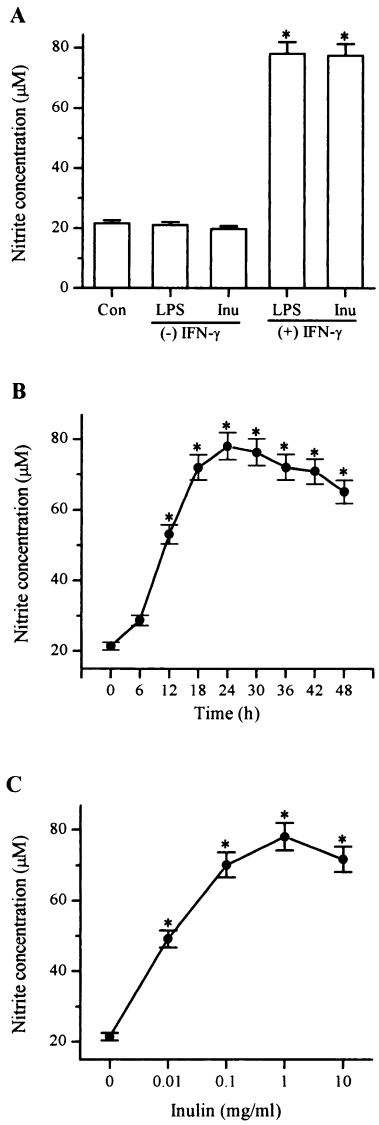

Fig. 1. Effects of inulin on NO production in RAW 264.7 cells. (A) The cells (1×10^6 cells/mL) were cultured with IFN-γ (5 U/mL) and then stimulated with LPS (100 ng/mL) or inulin (1 mg/mL) 6 h after incubation. (B) Time-dependent effect of inulin on NO production in IFN-γ-treated RAW 264.7 cells. (C) Dose-dependent effect of inulin on NO production in IFN-γ-treated RAW 264.7 cells. After 24 h of culture, NO release was measured by the Griess method. Values are means ± S. E. of duplicate determinations from three separate experiments (* $P < 0.01$).

3.2. Inulin-induced iNOS expression

Fig. 2A shows the effects of inulin plus IFN-γ treatments on the expression of iNOS in RAW 264.7 cells. We performed a Western blot analysis using an anti-iNOS Ab. Inulin alone did not induce the expression of iNOS, whereas inulin in combination with IFN-γ synergistically increased the expression of iNOS in RAW 264.7 cells. The iNOS expression of Fig. 2A is normalized to the control value using a personal densitometer (Fig. 2B).

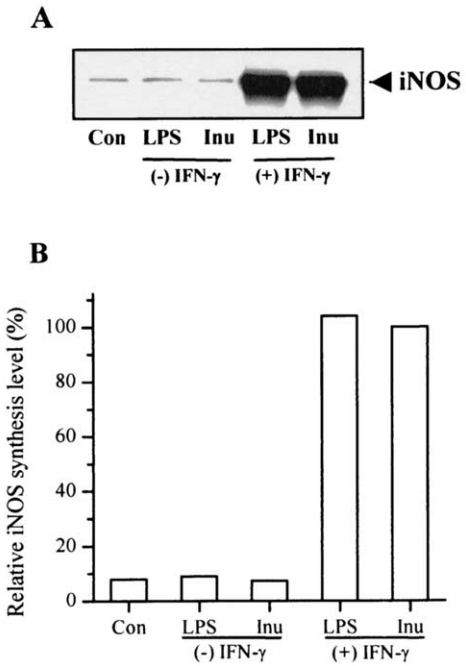

Fig. 2. Effect of inulin on the expression of iNOS in RAW 264.7 cells. (A) The cells (1×10^6 cells/mL) were cultured for 24 h, either in medium containing IFN-γ (5 U/mL), LPS (100 ng/mL) or inulin (1 mg/mL), and analyzed by Western blot. Lane 1, control; lane 2, LPS alone; lane 3, inulin alone; lane 4, IFN-γ plus LPS and lane 5, IFN-γ plus inulin. (B) The iNOS expression in panel A is normalized to the control value using a personal densitometer.

3.3. Inhibition of inulin-induced NO production by N^GMMA

To determine if the signal mechanism in inulin-induced NO production is involved in the $_L$-arginine-dependent pathway in RAW 264.7 cells, the cells were incubated for 6 h in the presence of IFN-γ plus N^GMMA. Inulin-induced NO production was progressively inhibited with increasing amounts of N^GMMA (data not shown). Analogues of $_L$-arginine, N^GMMA (1 mmol), significantly blocked the inulin-induced NO production (Fig. 3).

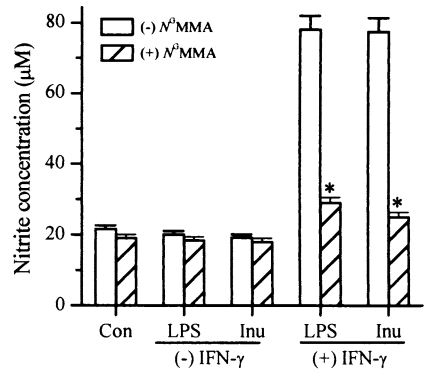

Fig. 3. Effect of N^GMMA on inulin-induced NO production in RAW 264.7 cells. The cells (1×10^6 cells/mL) were cultured with IFN-γ (5 U/mL) plus N^GMMA (1 mmol) and then stimulated with inulin (1 mg/mL) for 24 h. NO release was measured by the Griess method. Values are means ±S.E. of duplicate determinations from three separate experiments (* $P < 0.01$).

Table 1
Effect of inulin on TNF-α secretion by RAW 264.7 cells[1]

Treatment			TNF-α secretion (pg/mL)
IFN-α	LPS	Inulin	
−	−	−	310 ± 1.15
+	−	−	405 ± 3.20
−	+	−	350 ± 2.65
−	−	+	339 ± 1.73
+	+	−	770 ± 4.10*
+	−	+	688 ± 4.25*

[1] The cells (1×10^6 cells/mL) were incubated for 24 h in medium alone or in medium containing IFN-γ (5 U/mL), LPS (100 ng/mL), or inulin (1 mg/mL). The supernatants were collected and frozen at −80°C until being assayed for TNF-γ concentration. Values are means ± S.E. of duplicate determinations from three separated experiments. * $P < 0.05$ versus IFN-γ-treated control.

3.4. Inulin-induced TNF-α secretion and PKC-α activation

RAW 264.7 cells secreted very low levels of biologically active TNF-α after 24 h incubation with medium alone, 5 U/mL IFN-γ, or 1 mg/mL inulin (Table 1). However, RAW 264.7 cells secreted high levels of TNF-α after incubation with IFN-γ plus inulin. Inulin-induced NO production was progressively inhibited by the use of anti-murine TNF-α neutralizing Ab (data not shown). Furthermore, to analyze whether PKC-α is involved in inulin-induced NO production, we performed a Western blot analysis using an anti-PKC-α Ab. PKC-α is known to be involved in LPS-induced NO production in macrophages [13]. Fig. 4 shows the effects of inulin plus IFN-γ on the synthesis of PKC-α in RAW 264.7 cells. Inulin used in combination with IFN-γ strongly increased the expression of PKC-α in RAW 264.7 cells.

3.5. Inhibition of inulin-induced NO production by tyrphostin AG126

To determine whether PTK activity mediated NO production by inulin, the PTK inhibitor tyrphostin AG126 was simultaneously applied with inulin to RAW 264.7 cells. The

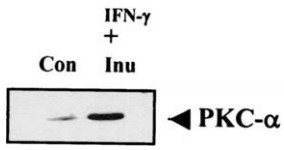

Fig. 4. Effect of inulin on the expression of PKC-α in RAW 264.7 cells. Fifty μg of total protein was resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane and analyzed by Western blotting using an anti-PKC-α Ab. The cells (1×10^6 cells/mL) were cultured with IFN-γ (5 U/mL) and then stimulated with inulin (1 mg/mL) 6 h after incubation.

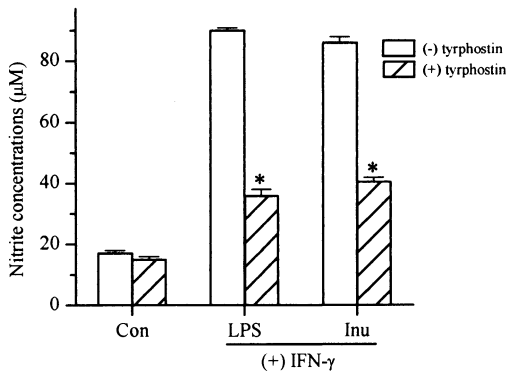

Fig. 5. Effect of PTK inhibitors of inulin-induced NO production in RAW 264.7 cells. The cells were treated for 5 min with tyrphostin AG126 (100 μmol) prior to the addition of IFN-γ (5 U/mL) and then stimulated with LPS (100 ng/mL) or inulin (1 mg/mL) for 24 h. NO release was measured by the Griess method. Values are means ± S.E. of duplicate determinations from three separate experiments (* $P < 0.01$).

cells were treated for 5 min with tyrphostin AG126 (100 μM) prior to the addition of IFN-γ (5 U/mL) and then stimulated with LPS (100 ng/mL) or inulin (1 mg/mL) for 24 h. As shown in Fig. 5, tyrphostin AG126 clearly inhibited inulin-induced NO production.

3.6. Inulin-induced activation of NF-κB

To further investigate the molecular mechanism of inulin-mediated activation of RAW 264.7 cells, we focused on the transcription factors whose binding sites are in the promoter of the iNOS gene. We assessed the effect of inulin on NF-κB activation using an EMSA (Fig. 6A). LPS or inulin treatment of RAW 264.7 cells induced a marked increase in NF-κB binding. We also assessed the effect of inulin on the AP-1 whose binding motifs are in the promoter of the iNOS gene (Fig. 6B). Treatment of RAW 264.7 cells with LPS produced strong induction of AP-1 binding. However, inulin slightly induced AP-1 binding activity. These results indicate that inulin differs from LPS in the mechanism of AP-1 activation.

As shown in Fig. 7A, stimulation of RAW 264.7 cells with LPS after the treatment of IFN-γ increased the synthesis of NF-κB subunits (p50 and p65). Inulin alone partially increased the NF-κB activation. When inulin was used in combination with IFN-γ, there was a marked cooperative activation of NF-κB. Activation of NF-κB required phosphorylation and proteolytic degradation of the inhibitory protein IκB-α. We assessed the kinetics of IκB-α phosphorylation and degradation by Western blotting. Partial IκB-α degradation could be noted within 30 min and considerable degradation of IκB-α occurred by 120 min after treatment of LPS or inulin. These results show that inulin increased NF-κB activation through stimulation of IκB-α phosphorylation and degradation. RAW 264.7 cells were pre-treated with PDTC (100 μM) for 30 min and stimulated with inulin (1 mg/mL) for 24 h. The inulin-induced NO production was significantly blocked by PDTC (Fig. 7B).

4. Discussion

In this study, we demonstrated that NO synthesis in RAW 264.7 cells by inulin, can be highly stimulated in combination with IFN-γ, and in a time- and dose-dependent manner. The synergistic effect was always maximal at 1 mg/mL of inulin. Concentrations of less than 0.01 mg/mL were considerably less effective and, in some cases, ineffective. NO, the initial product of oxidation of $_{L}$-arginine, exhibits a multitude of biological actions [26, 27]. The strong inhibition of NO production by N^{G}MMA indicates

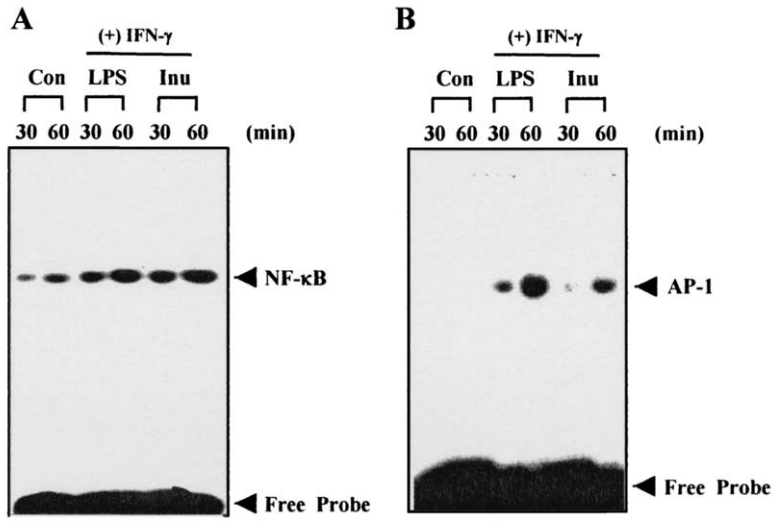

Fig. 6. Effects of inulin on the DNA binding activities of NF-κB and AP-1 in RAW 264.7 cells. Nuclear extracts from inulin-stimulated RAW 264.7 cells were incubated with ^{32}P-labeled oligonucleotides corresponding to NF-κB (A) and AP-1 (B). Reaction products were electrophoresed, and then the gel was dried and autoradiographed.

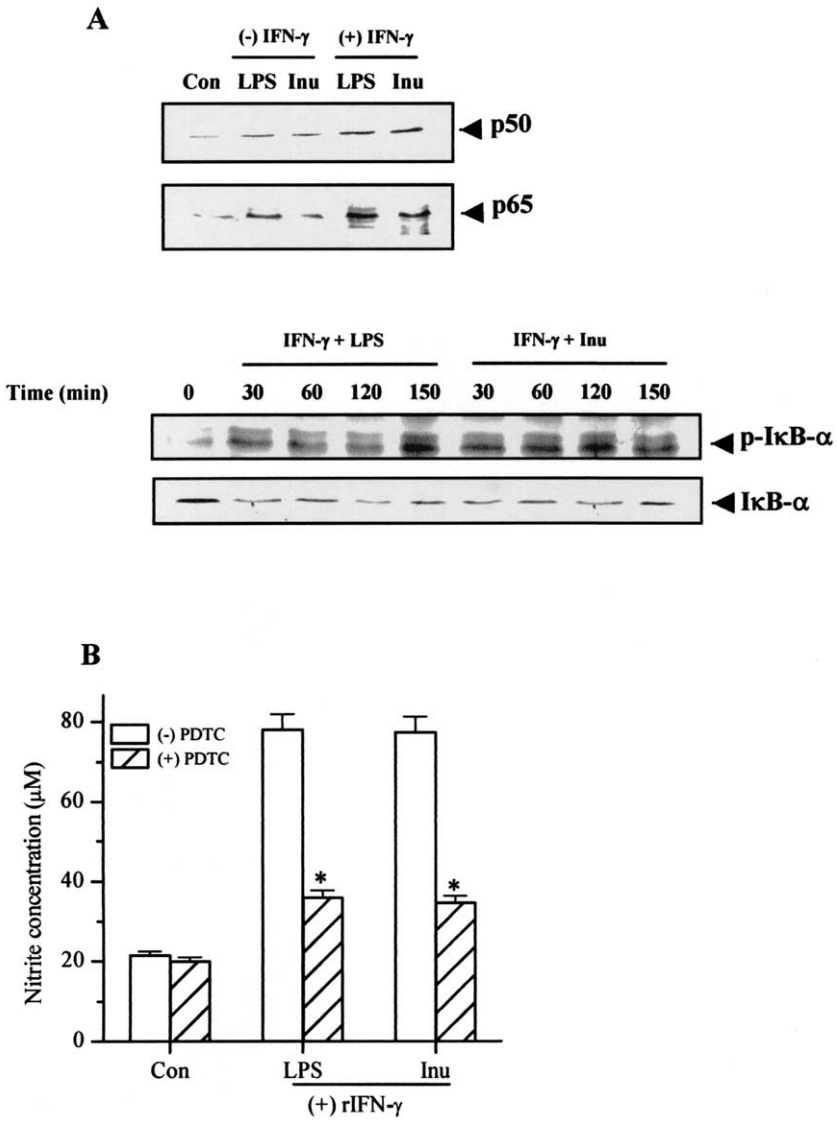

Fig. 7. Effects of inulin on NF-κB activation in RAW 264.7 cells. (A) Fifty μg of total protein was resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by Western blotting using an anti-p50, p65, p-IκB-α and IκB-α Ab. (B) The cells (1×10^6 cells/mL) were pre-incubated with PDTC (100 μmol) for 30 min and then stimulated with LPS (100 ng/mL) or inulin (1 mg/mL) in the presence of IFN-γ (5 U/mL) for 24 h. Values are means ± S.E. of duplicate determinations from three separate experiments (* $P < 0.01$).

that the signal mechanism in inulin-induced NO production is dependent on the $_L$-arginine pathway in RAW 264.7 cells. The addition of anti-murine TNF-α neutralizing Ab inhibits the synergistic effect of inulin with IFN-γ on NO production (data not shown). These results suggest that inulin-induced TNF-α secretion is crucial for synergistic induction of NO synthesis in RAW 264. 7 cells. PKC-α is known to be involved in the LPS-induced NO production in macrophages [13]. In this study, we demonstrated that inulin significantly increased the expression of PKC-α in RAW 264.7 cells. These results suggest that not only LPS but also inulin can activate PKC-α in NO production by RAW 264.7cells.

The induction of iNOS by LPS and cytokines in macrophages is initiated by the activation of PTKs [28]. We demonstrated that inulin-induced NO production was significantly blocked by tyrphostin AG126 (Fig. 5). This finding indicates that inulin may stimulate NO production through PTK activation.

NF-κB is thought to be a unique molecule that is activated by LPS and TNF-α [29–32]. It has been demonstrated that putative NF-κB binding sites (κB site) are present in mouse, rat, and human iNOS promoter regions, and that two positions on the murine macrophage iNOS gene promoter region containing a κB site and IFN-γ-activated site are necessary for iNOS induction in response to LPS/ IFN-γ [33]. The activation of NF-κB appears to require the phosphorylation and degradation of the IκB-α proteins, which thereby allows the rapid translocation of NF-κB from the cytoplasm to the nucleus [34]. The data presented in this

paper indicate that inulin treatment of RAW 264.7 cells results in degradation of IκB-α and activation of NF-κB, which may serve as a critical regulator for the inducible expression of many genes. However, inulin differs from LPS in the levels of AP-1-specific DNA-protein complex (Fig. 6). The NF-κB blocker PDTC inhibited both LPS- and inulin-induced NO production. These results support the involvement of NF-κB activation in inulin-induced iNOS induction.

Inulin could be a new class of activators to stimulate the induction of iNOS. NO production by iNOS is beneficial in fighting against bacteria, fungi, viruses, and parasites [35]. NO generation also influences the cytotoxicity of macrophages and tumor-induced immunosuppression. Chicory inulin is officially recognized as a natural food ingredient. It has been proposed to classify chicory inulin as a "colonic food," i.e., a "food entering the colon and serving as substrate for the endogenous bacteria" [36]. Inulin stimulates the growth of bifidobacteria and inhibits colon carcinogenesis [2–4]. At present, the precise physiological significance of NO synthesis by inulin is unknown.

In conclusion, our results demonstrate that inulin acts as an activator of RAW 264.7 cells pretreated by IFN-γ via a process involving $_{L}$-arginine-dependent NO production. Inulin stimulates NO production through the activation of PKC-α and PTK, resulting in the activation of NF-κB. Inulin may show tumoricidal effects. The synergistic mechanism of inulin on NO production in IFN-γ-primed RAW 264.7 cells remains to be further elucidated.

Acknowledgments

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