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β-Carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated macrophages by suppressing redox-based NF-κB activation

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Abbreviations: COX-2, cyclooxygenase-2; $DCFH_2$-DA, 2',7'-dichlorofluorescin diacetate; DPI, diphenylene iodonium; IKK, IκB kinase; iNOS, inducible nitric oxide synthase; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NOx, nitrite plus nitrate; PDTC, pyrrolidine dithiocarbamate; RT-PCR, reverse transcriptase-PCR

Abstract

β-Carotene has shown antioxidant and anti-inflammatory activities; however, its molecular mechanism has not been clearly defined. We examined *in vitro* and *in vivo* regulatory function of β-carotene on the production of nitric oxide (NO) and PGE_2 as well as expression of inducible NO synthase (iNOS), cyclooxygenase-2, TNF-α, and IL-1β. β-Carotene inhibited the expression and production of these inflammatory mediators in both LPS-stimulated RAW264.7 cells and primary macrophages in a dose-dependent fashion as well as in LPS-administrated mice. Furthermore, this compound suppressed NF-κB activation and iNOS promoter activity in RAW264.7 cells stimulated with LPS. β-Carotene blocked nuclear translocation of NF-κB p65 subunit, which correlated with its inhibitory effect on IκBα phosphorylation and degradation. This compound directly blocked the intracellular accumulation of reactive oxygen species in RAW264.7 cells stimulated with LPS as both the NADPH oxidase inhibitor diphenylene iodonium and antioxidant pyrrolidine dithiocarbamate did. The inhibition of NADPH oxidase also inhibited NO production, iNOS expression, and iNOS promoter activity. These results suggest that β-carotene possesses anti-inflammatory activity by functioning as a potential inhibitor for redox-based NF-κB activation, probably due to its antioxidant activity.

Keywords: beta-carotene; cytokines; macrophages; nitric oxide; NF-κB; reactive oxygen species.

Introduction

Macrophages play an important role in host defense against noxious substances and are involved in a variety of disease processes, including autoimmune diseases, infections, and inflammatory disorders (Pierce, 1990). Inflammatory stimuli such as LPS and IFN-γ activate macrophages to produce a variety of pro-inflammatory cytokines such as TNF-α and IL-1β as well as other inflammatory mediators including PGE_2 and nitric oxide (NO), which are synthesized by cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS), respectively. These inflammatory mediators are involved in the pathogenesis of many inflammation-associated human diseases (Simons *et al.*, 1996; Guslandi, 1998; Ritchlin *et al.*, 2003). Anti-inflammatory drugs and agents reduce the inflammatory response by suppressing the production of the inflammatory mediators and in turn block the initiation and progression of inflammation-associated diseases (Leach *et al.*, 1998; Ritchlin *et al.*, 2003).

Expression of these cytokine and enzyme genes can be regulated by the activation of the transcription factor nuclear factor-kappa B (NF-κB), which is critically involved in several aspects of the pathogenesis of rheumatoid arthritis and other chronic inflammatory diseases (Makarov, 2000; Tak *et al.*, 2001). NF-κB is activated as a consequence of phosphorylation, ubiquitination, and subsequent proteolytic degradation of the IκB protein through activation of IκB kinase (IKK) (de Martin *et al.*, 1993). The

liberated NF-κB translocates into nuclei and binds to (B motifs in the promoters of pro-inflammatory genes such as iNOS, COX-2, TNF-α, and IL-1β, leading to the induction of their mRNA expression. Most of the anti-inflammatory drugs have been shown to suppress the expression of these genes by inhibiting the NF-κB activation pathway (Gilroy *et al.*, 2004). Thus, an NF-κB inhibitor may be useful as a potential therapeutic drug in clinical applications for regulating the inflammation associated human diseases.

It has been also demonstrated that antioxidants such as α-tocopheryl succinate (Neuzil *et al.*, 2001), probucol (Dichtl *et al.*, 1999), and astaxanthin (Lee *et al.*, 2003a) can inhibit NF-κB activity and block the expression of pro-inflammatory genes as well as production of NO and PGE_2 (Heiss *et al.*, 2001), probably by the inhibition of NF-κB activation. These observations indicate that reactive oxygen species (ROS) plays an important role in NF-κB activation and inflammatory gene expression. β-carotene acts as a powerful antioxidant in oxidative stress and chain breaking antioxidant in lipid peroxidation (Smith, 1998) and prevents the development of inflammatory diseases such as atherosclerosis (Vivekananthan *et al.*, 2003) and rheumatoid arthritis (Heliovaara *et al.*, 1994).

We here hypothesized that β-carotene may inhibit the expression of pro-inflammatory gene expression and the production of inflammatory mediators such as NO and PGE_2 through the inhibition of NF-κB activation. We found that β-carotene suppressed the expression of iNOS, TNF-α, IL-1β, and COX-2 as well as the production of NO and PGE_2 in LPS-stimulated macrophages and LPS-administrated mice. This compound also blocked iNOS promoter activity and NF-κB activation and reduced the accumulation of intracellular ROS level in LPS-activated macrophages. Thus, these results indicate that β-carotene possesses anti-inflammatory potential by suppressing inflammatory cytokines and modulators through the suppression of redox-based NF-κB activation.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium, penicillin, and streptomycin were purchased from Life Technology Inc. (Rockville, MD). LPS (*Escherichia coli* O111:B4) and β-carotene were obtained from Sigma (St. Louis, MO). Poly (dI-dC) and NF-κB-specific oligonucleotide were obtained from Promega (Madison, WI). Monoclonal iNOS antibody was purchased from Transduction Laboratories (Lexington, KY). Antibodies for IκBα, NF-κB p65, poly (ADP-ribose) polymerase (PARP), COX-2, TNF-α, and IL-1β were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). 2',7'-dichlorofluorescin diacetate ($DCFH_2$-DA) was purchased from Molecular probe (Eugene, OR). Other chemicals were obtained from Sigma (St. Louis, MO) unless indicated otherwise.

Macrophage isolation and cell culture

Murine macrophage cell line RAW264.7 was cultured in Dulbecco's modified Eagle's medium (2 mM L-glutamine; 100 U/ml penicillin; 100 μg/ml streptomycin) plus 10% fetal bovine serum (HyClone Labs, Logan, UT). Peritoneal macrophages were collected from the peritoneal cavity of 6-8 week old female BALB/c mice (Daihan-Biolink, Korea) given an intraperitoneal injection of 1.5 ml of thioglycollate broth (4%) 7 days before harvest. Cells were cultured in 6-well plates (3×10^6 cells/well) for 12 h. Cells were stimulated with LPS (1 μg/ml) in the presence or absence of β-carotene.

Animal treatment

Mice (6 to 8 week old female BALB/c mice, Daihan-Biolink, Korea) were intraperitoneally injected with LPS (4 mg/kg) and/or β-carotene (10 mg/kg). After 12 h, whole blood samples were withdrawn by cardiac puncture. Serum was prepared by centrifugation at 12,000 *g* for 20 min at 4°C and kept at -20°C until use.

Measurement of NO metabolites, cytokines, and PGE_2: nitrite, a stable oxidized product of NO, was determined in cell culture medium by Griess reagents (Kim *et al.*, 1997). Serum nitrite plus nitrate (NOx) concentration was determined by using a nitrate reductase-based colorimetric assay kit (Alexis San Diego, CA). PGE_2 concentration was determined using an enzyme immunoassay kit (Amersham Pharmacia Biotech, Piscataway, NJ). The levels of TNF-α and IL-1β in the culture medium and serum were determined using ELISA kits (R&D Systems, Minneapolis, MN).

Western blot analysis

Cells were incubated with or without LPS in the absence or presence of β-carotene. Cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), and resuspended in PBS containing 0.1 mM phenylmethylsulfonyl fluoride. The suspension was lysed by three cycles of freezing and thawing. The cytosolic fractions were obtained from the supernatant after centrifugation at 12,000 *g* at 4°C for 20 min. The protein content was determined by the BCA method (Pierce, Rockford, IL). Samples (40 μg of protein) were separated on SDS-PAGE and transferred onto nitrocellulose membranes. The mem-

branes were blocked with 5% nonfat-dried milk in PBS containing 0.1% Tween 20 (PBST) for 2 h, and then incubated with monoclonal mouse iNOS antibody in PBST containing 1% nonfat milk for 2 h. After washing three times with PBST, membrane was hybridized with horseradish peroxidase-conjugated secondary antibody for 1 h. Following five washes, membranes were incubated with chemiluminescent solution for 2 min, and the protein bands were visualized on X-ray film.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Three μg of mRNA was converted to cDNA by treatment with 200 U of reverse transcriptase and 500 ng of oligo-dT primer in 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, and 1 mM dNTPs at 42°C for 1 h. The reaction was stopped by heating at 70°C for 15 min. Three μl of the cDNA mixture was used for enzymatic amplification. PCR was performed in 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 2.5 U of *Taq* DNA polymerase, and 0.1 μM each of primers for iNOS, COX-2, TNF-α, and IL-1β. The amplification conditions and primers were the same as previously described (Lee *et al.*, 2003a).

Electrophoretic mobility shift assay

Nuclear extracts from RAW264.7 cells were prepared as described previously (Kim *et al.*, 2001). A double stranded NF-κB-specific oligonucleotide (5'-AGTTGAGGGGACTTTCCCAGGC-3') was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase and purified on a G-50 Sephadex column. The nuclear extracts (10 μg of protein) were incubated with ~40,000 cpm (~0.5 ng) of ^{32}P-labeled oligonucleotide for 20 min at room temperature as described previously (Kim *et al.*, 2001). Samples were separated on a 5% native polyacrylamide gel. The gel was dried and subjected to autoradiography.

iNOS promoter activity assay

Transient transfection and iNOS promoter activity assay were carried out as previously described (Kim *et al.*, 2001). A murine iNOS promoter-luciferase construct was transfected into RAW264.7 cells by the liposome method. The cells were cultured overnight in media supplemented with 10% fetal bovine serum and treated with LPS in the presence or absence of β-carotene for 12 h. Cells were lysed with buffer containing 1% Triton X-100, 5 mM dithiothreitol, 50% glycerol, 10 mM EDTA, and 125 mM Tris-phosphate (pH 7.8). Luciferase activity was measured by luminometer.

Measurement of intracellular ROS

The amount of intracellular ROS was measured by previous method (Lee *et al.*, 2003b). RAW264.7 cells cultured on round coverslips in phenol red-free medium were treated with or without 50 μM β-carotene for 30 min. For the last 10 min of stimulation, cells were incubated with 10 μM $DCFH_2$-DA in the presence or absence of 1 μg/ml LPS. Then, the cells were immediately observed by confocal laser-scanning microscopy. DCF fluorescence intensities were determined from the same numbers of cells in a randomly selected area.

Statistical analysis

Data are presented as the mean ± SD of at least three separate experiments. Comparisons between two groups were analyzed using Student's *t*-test. *P* values less than 0.05 were considered to be statistically significant.

Results

β-carotene inhibits inflammatory enzyme expression

Immune-activated macrophages up-regulate the expression of the inflammatory enzymes, such as iNOS and COX-2, which are involved in the pathogenesis of many human diseases. iNOS and COX-2 synthesize NO and PGE_2 from L-arginine and arachidonic acid, respectively. We first performed the experiments to determine whether β-carotene regulates the production and NO and PGE_2 in the murine macrophage cell line RAW264.7. Cells were stimulated with LPS in the presence or absence of β-carotene for 16 h, and the levels of NO and PGE_2 were measured in the culture medium by Griess reagents and ELISA kit, respectively. LPS-stimulated cells increased the accumulation of nitrite, a stable oxidized product of NO, and PGE_2 in the culture medium while control cells did not (Figures 1A and 1B). These increases were significantly reduced in a dose dependent manner by co-treatment with β-carotene with an IC_{50} value of ~30 μM. No cytotoxic effect of β-carotene was observed under the same experimental condition as measured by lactate dehydrogenase release and crystal violet staining (data not shown). We next examined the effects of β-carotene on the levels of iNOS and COX-2 proteins and mRNAs in LPS-stimulated cells. Western blot analyses showed that LPS-mediated increases in the protein and mRNA levels of these enzymes were suppressed in a dose-dependent manner by β-carotene treatment and that 50 μM β-carotene almost completely inhibited their

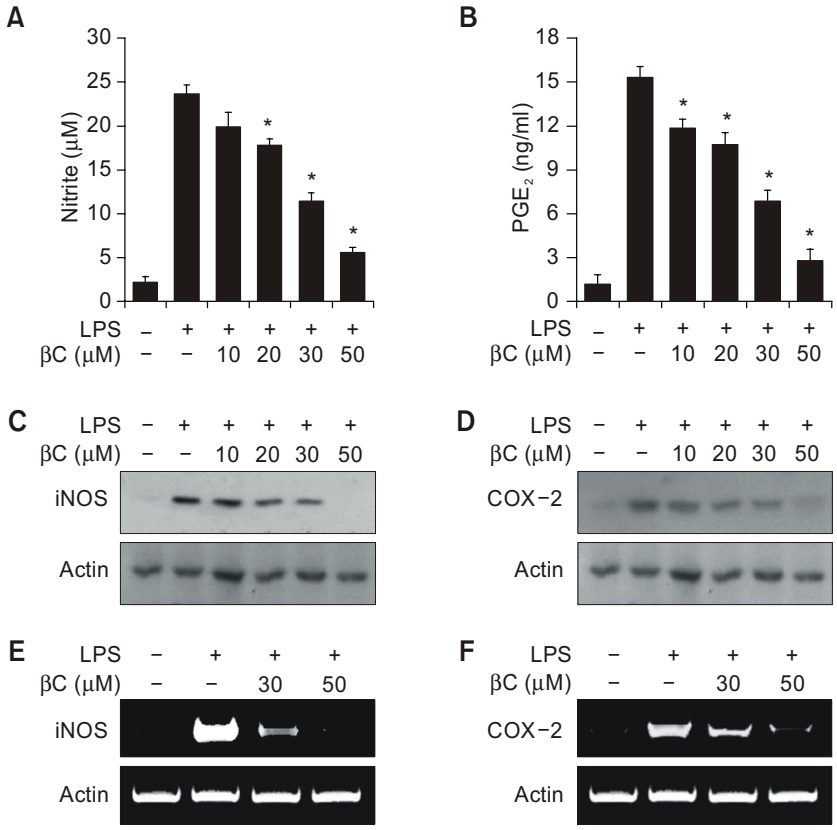

Figure 1. β-Carotene inhibits the production of NO and PGE_2 and the expression iNOS and COX-2 in LPS-stimulated RAW264.7 cells. (A and B) RAW264.7 cells were stimulated with LPS (1 μg/ml) in the presence or absence of different concentrations of β-carotene (βC). After 16 h, the levels of nitrite and PGE_2 were measured in the culture medium by Griess reagents and ELISA kit. Data shown are the mean ± SD ($n \geq 3$). *, $P < 0.01$ *versus* LPS alone. (C and D) After 16 h, cells were harvested, washed twice with ice-cold PBS, and lysed by three cycles of freezing and thawing. Cytosolic extracts were prepared by centrifugation at 12,000 *g* for 20 min. The protein levels of iNOS and COX-2 were measured by Western blot analyses using antibodies against mouse iNOS and COX-2, respectively. The membranes were rehybridized with actin antibody to verify equal loading of protein in each lane. (E and F) After 8 h stimulation, total RNAs were obtained from RAW264.7 cells using a Trizol reagent kit. The mRNA levels of iNOS and COX-2 were determined by RT-PCR analysis. Actin was used as an internal control.

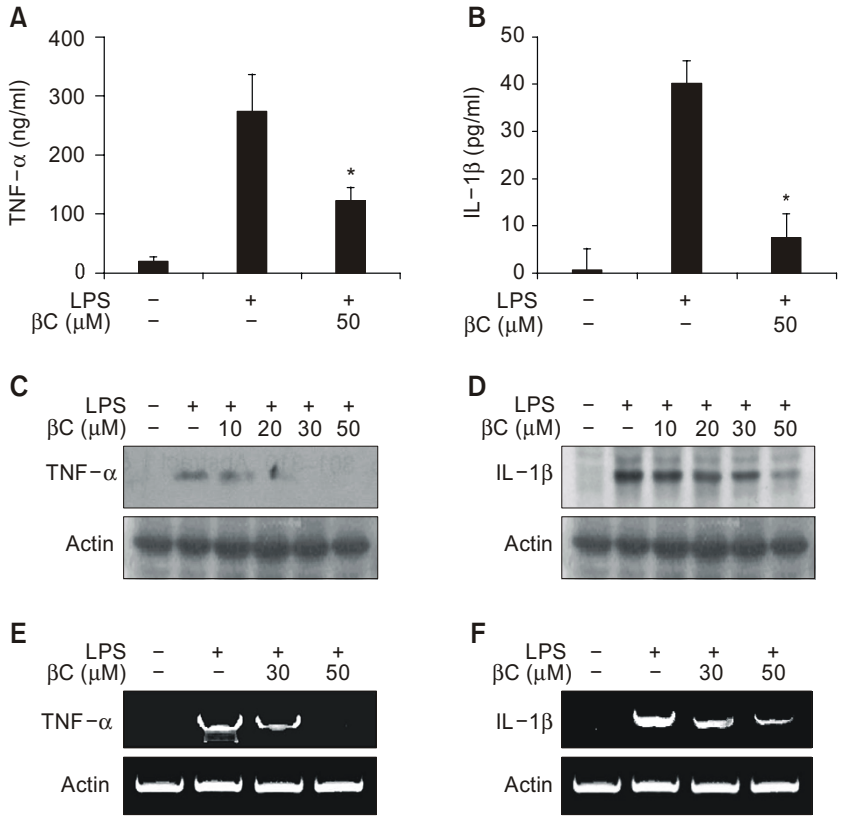

Figure 2. β-Carotene inhibits TNF-α and IL-1β production and expression in LPS-stimulated RAW264.7 cells. (A and B) RAW264.7 cells were cultured with LPS (1 μg/ml) in the presence or absence of indicated concentrations of β-carotene (βC) for 12 h. The levels of TNF-α and IL-1β were determined in the culture medium using ELISA kits. Data shown are the mean ± SD ($n \geq 3$). *, $P < 0.01$ *versus* LPS alone. (C and D) After 8 h, cells were harvested, washed twice with ice-cold PBS, and lysed by three cycles of freezing and thawing. Cytosolic extracts were prepared by centrifugation at 12,000 *g* for 20 min. The intracellular levels of TNF-α and IL-1β were measured by Western blot analyses using polyclonal antibodies for TNF-α and IL-1β. (E and F) After 6 h stimulation, total RNAs were obtained from RAW264.7 cells using a Trizol reagent kit. The levels of TNF-α and IL-1β mRNAs were determined by RT-PCR analysis. Actin was used as an internal control.

protein and mRNA levels (Figures 1C to 1F). These results indicates that β-carotene inhibits NO and PGE_2 production through the suppression of iNOS and COX-2 expression at the transcriptional step in LPS-stimulated RAW264.7 macrophages, respectively.

β-carotene inhibits pro-inflammatory cytokine expression in LPS-stimulated RAW264.7 cells

We next examined whether β-carotene regulates inflammatory cytokine production. RAW264.7 cells were stimulated with LPS in the presence or absence of β-carotene and the protein levels of TNF-β and IL-1β were measured in the culture medium by ELISA. Stimulation of RAW264.7 cells with LPS significantly increased the secreted TNF-α and IL-1β levels in the culture medium, and these increases were effectively inhibited by co-treatment with 50 μM β-carotene (Figures 2A and 2B). It has been shown that TNF-α and IL-1β are expressed as inactive pro-forms, cleaved into the active forms by TNF-α-converting enzyme and interleukin-1β-converting enzyme, and secreted into the culture medium or extracellular fluids (Thornberry *et al.*, 1992; Black *et al.*, 1997). We next examined whether β-carotene inhibits the intracellular protein levels of pro-TNF-α and pro-IL-1β. Western blot analyses showed that β-carotene treatment suppressed the intracellular levels of these pro-forms in a dose-dependent manner (Figures 2C and 2D). Furthermore, β-carotene inhibited the mRNA levels of these cytokines in RAW264.7 cells stimulated with LPS (Figures 2C and 2F). These results indicate that β-carotene inhibits the expression of pro-inflammatory TNF-β and IL-1β.

β-carotene inhibits NO production and inflammation-associated gene expression in LPS-stimulated peritoneal macrophages

We further examined the effects of β-carotene on iNOS expression in primary cultured murine peri-

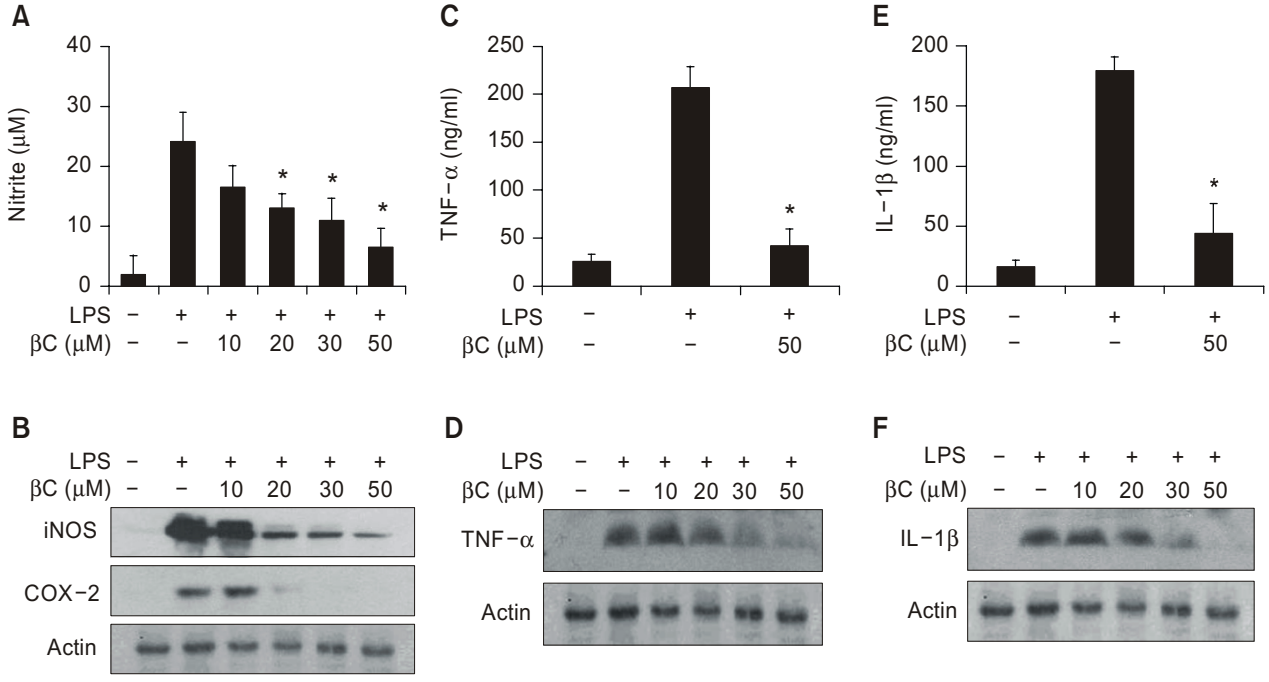

Figure 3. β-Carotene inhibits inflammatory gene expression in primary peritoneal macrophages. Peritoneal macrophages cultured in 6-well culture dishes were treated with LPS in the presence or absence of β-carotene (βC) for 16 h. (A) The levels of nitrite were measured in the culture media by Griess reagents. Data shown are the mean ± SD (n = 3). *, $P < 0.01$ *versus* LPS alone. (B) Cytosolic extracts were prepared by three cycles of freezing and thawing and centrifugation at 12,000 *g* for 20 min. The levels of iNOS and COX-2 were measured by Western blot analyses using antibodies against mouse iNOS and COX-2. The membranes were rehybridized with actin antibody to verify equal loading of protein in each lane. (C and E) The levels of TNF-α and IL-1β were determined in culture medium using ELISA kits. Data shown are the mean ± SD ($n \geq 3$). *, $P < 0.01$ *versus* LPS alone. (D and F) Cytosolic extracts were prepared by three cycles of freezing and thawing and centrifugation at 12,000 *g* for 20 min. The intracellular levels of TNF-α and IL-1β were measured by Western blot analyses using antibodies against mouse TNF-α and IL-1β. The membranes were rehybridized with actin antibody to verify equal loading of protein in each lane.

toneal macrophages. For this experiment, peritoneal macrophages isolated from female BALB/c mice were cultured in 6-well plates overnight and stimulated with LPS in the presence or absence of β-carotene, and NO production and expression of inflammation-associated genes were determined. LPS-stimulated peritoneal macrophages increased NO production and the expression of iNOS and COX-2 proteins, and these increases were significantly inhibited in a dose-dependent manner by co-treatment with β-carotene (Figures 3A and 3B). Moreover, treatment with β-carotene inhibited the increases in both the secreted and pro-forms of TNF-α and IL-1β in LPS-stimulated primary macrophages (Figures 3C to 3F).

β-carotene decreases the production of NO, PGE_2, TNF-α, and IL-1β *in vivo*

The data shown above provide evidence for a distinct anti-inflammatory action of β-carotene in RAW264.7 cells and peritoneal macrophages. To verify the *in vivo* relevance of β-carotene's *in vitro* anti-inflammatory role, we examined its effect on the production of the inflammation-associated mediators and gene products, such as NO, PGE_2, TNF-α, and IL-1β, in LPS-administrated mice as a well-established septic animal model. Serum levels of NOx and PGE_2 were significantly elevated by LPS administration compared with those of control mice injected with saline or β-carotene alone, and these increases were reduced by treatment with β-carotene (Figures 4A and 4B). We next examined the regulatory effects of β-carotene on the serum levels of TNF-α and IL-1β in LPS-administrated mice. LPS administration increased the serum levels of these cytokines, while control mice treated with saline or β-carotene alone did not (Figures 4C and D). LPS-induced increases in the serum levels of TNF-α and IL-1β were significantly suppressed by co-administration with β-carotene. These results indicate that β-carotene can inhibit the production of inflammatory mediators in *in vivo* pathologic inflammatory conditions.

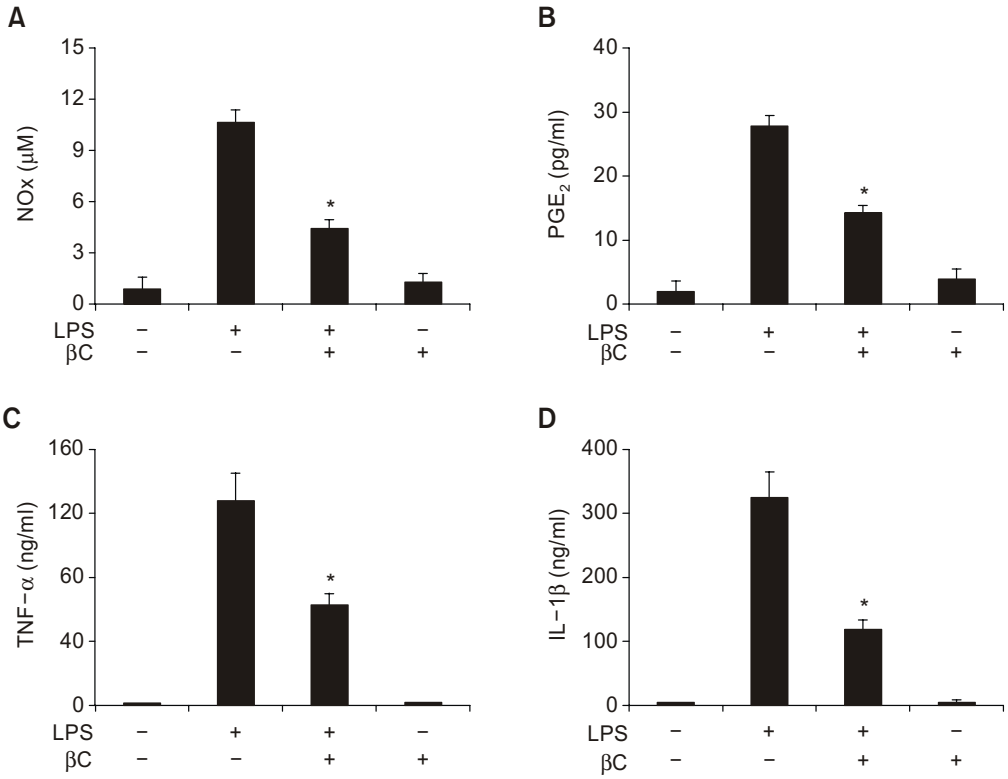

Figure 4. β-Carotene decreases the production of inflammatory mediators *in vivo*. Mice were injected i.p. with LPS (4 mg/Kg) following pretreatment with or without β-carotene (βC, 10 mg/Kg). After 12 h of LPS treatment, whole blood was collected by cardiac puncture and serum was obtained by centrifugation at 12,000 *g* for 20 min. (A) The serum levels of nitrite plus nitrate (NOx) were measured using a nitrate assay kit. The levels of PGE_2 (B), TNF-α (C), and IL-1β (D) were determined by ELISA kits. All data shown are the mean ± SD (n = 6). *, $P < 0.01$ *versus* LPS alone.

β-carotene suppresses NF-κB activation and iNOS promoter activity by inhibiting IκB-α degradation and nuclear translocation of NF-κB

It is well known that NF-κB is an important transcription factor for the inducibility of various inflammatory genes including iNOS, COX-2, TNF-α, and IL-1β in macrophages treated with LPS (Xie *et al.*, 1994; Rhodus *et al.*, 2005). To investigate whether NF-κB is an important target for the anti-inflammatory action of β-carotene in macrophages, we examined the effect of β-carotene on NF-κB activation by performing an electrophoretic mobility shift assay. The nuclear extract from LPS-stimulated macrophages showed an increase in NF-κB-DNA binding activity, while this activity was not seen in unstimulated cells (Figure 5A). The binding activity was suppressed in a dose-dependent manner by treatment with β-carotene, and specific interaction between DNA and NF-κB was demonstrated by a competitive assay with a 100-fold excess of unlabelled oligonucleotide (cold probe), suggesting that β-carotene inhibits the expression of the inflammatory genes through the suppression of NF-κB activation. An NF-κB element at -85 bp upstream in the murine iNOS promoter has been shown to be the major regulatory factor for murine iNOS gene expression in response to LPS (Xie *et al.*, 1994). We examined whether β-carotene suppresses iNOS promoter activity through the inhibition of NF-κB activation. Transient transfection in RAW264.7 cells was performed with a murine

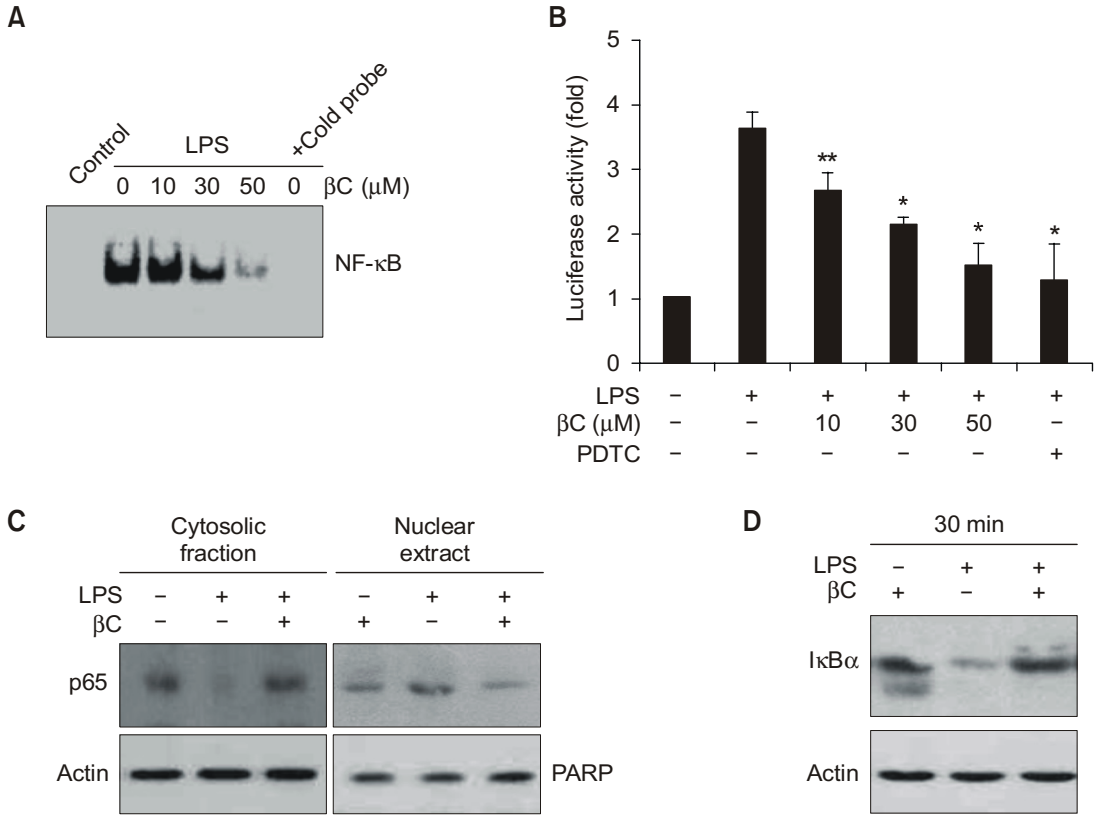

Figure 5. β-Carotene suppresses NF-κB activation and iNOS promoter activity. (A) RAW264.7 cells were treated with LPS in the presence or absence of β-carotene (βC) for 2 h. Cells were harvested, and nuclear extracts were prepared. Nuclear NF-κB activity was analyzed by electrophoretic mobility shift assay in the presence or absence of an excess amount of cold probe. (B) RAW264.7 cells were transiently transfected by a lipofectamine method with murine iNOS promoter. Cells were treated with LPS in the presence or absence of indicated concentrations of β-carotene or PDTC (10 μM) for 12 h. Luciferase activity in the cell extracts was measured by luminometer. Data shown are the mean ± SD (n = 3). **, $P < 0.05$; *, $P < 0.01$ *versus* LPS alone. (C) Cells were incubated with LPS in the presence or absence of β-carotene (50 μM) for 2 h. Cells were harvested, washed with ice-cold PBS, and resuspended in nuclear extraction buffer. Cytosolic fractions and nuclear extracts were prepared as described in the Method section. Both samples were separated on SDS-PAGE, and the NF-κB p65 subunit was visualized by Western blot analysis. The blot was rehybridized with antibodies for actin and PARP to verify equal loading of protein in each lane. (D) Whole cell lysates from RAW264.7 cells treated with LPS in the presence or absence of β-carotene (50 μM) for 30 min were separated in SDS-PAGE. IκBα protein was visualized by Western blot analysis using an antibody against IκBα.

iNOS promoter construct (piNOS-Luc) by lipofection. Treatment of the transfected cells with LPS resulted in about a 3.6-fold increase in luciferase activity (Figure 5B). This increase was suppressed in a dose-dependent manner by increasing concentrations of β-carotene and the addition of the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC). The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IκB-α (Traenckner *et al.*, 1995; Butcher *et al.*, 2001). To determine whether the inhibitory effect of β-carotene on LPS-induced NF-κB activation was due to inhibition of IκBα degradation and NF-κB translocation, we determined the cytosolic and nuclear NF-κB p65 subunit levels following treatment of LPS in the presence or absence of β-carotene. LPS treatment decreased the cytosolic p65 subunit level, resulting in an increase in the nuclear p65 level, while this subunit was mostly present in the cytosol from control cells (Figure 5C). β-carotene treatment inhibited LPS-induced translocation of the cytosolic p65 subunit to the nuclei. We next examined the effect of β-carotene on the proteolytic degradation of IκBα. LPS treatment significantly decreased the IκBα protein level within 30 min compared with control, and this effect was significantly blocked by β-carotene treatment (Figure

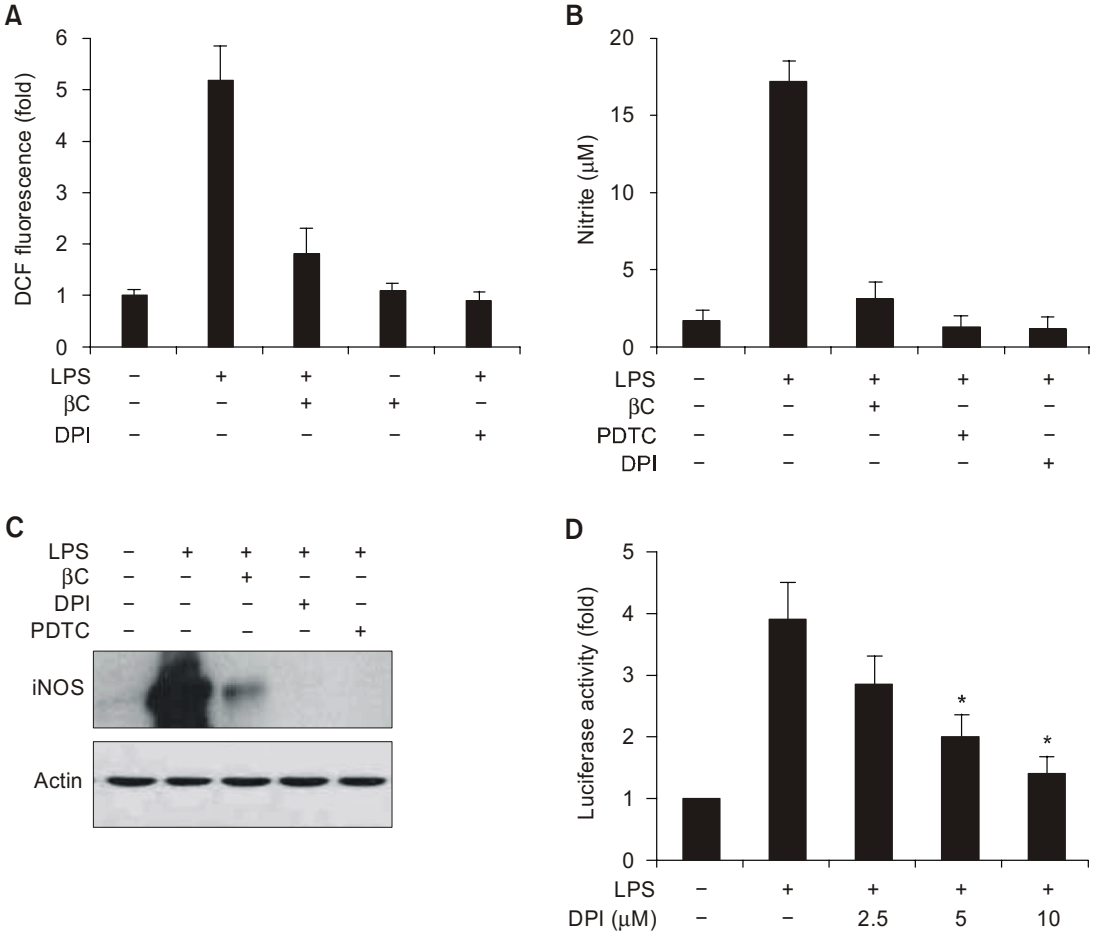

Figure 6. β-Carotene, NADPH oxidase inhibitor, and PDTC suppress the intracellular ROS level, iNOS expression, and iNOS promoter activity in RAW264.7 cells. RAW264.7 cells were treated with LPS in the presence or absence of β-Carotene (βC, 50 μM), DPI (10 μM), and PDTC (15 μM). (A) After 30 min incubation, cells were incubated with $DCFH_2$-DA (5 μM) for an additional 30 min. Cells were washed twice with PBS, and the intracellular levels of ROS were analyzed by confocal microscope. DCF fluorescence intensities were determined from the same numbers of cells in randomly selected area. Data shown are the mean ± SD ($n \geq 3$). (B) After 16 h incubation, the levels of nitrite were measured in culture medium by Griess reagents. Data shown are the mean ± SD ($n \geq 3$). (C) The iNOS level was determined by Western blot analysis as described in Figure 1. (D) RAW264.7 cells were transiently transfected by a lipofectamine method with murine iNOS promoter. Cells were treated with LPS in the presence or absence of indicated concentrations of DPI for 12 h. Luciferase activity in the cell extracts was measured by luminometer. Data shown are the mean ± SD ($n \geq 3$). *, $P < 0.01$ *versus* LPS alone.

5D). The results suggest that β-carotene inhibits both the LPS-induced translocation of the p65 subunit to the nucleus and the degradation of IκBα, indicating that the major target of β-carotene suppression of NF-κB activation may be the inhibition of IκBα degradation.

β-Carotene and NADPH oxidase inhibitor suppress LPS-induced increase in ROS level, iNOS expression, and iNOS promoter activity

Macrophages stimulated with LPS generate ROS via the activation of a membrane-bound NADPH oxidase (Bokoch and Knaus, 2003), and ROS plays an important role in NF-κB activation (Flohe *et al.*, 1997). These observations indicate that antioxidant chemicals and enzymes inhibit NF-κB activation and subsequently suppress NF-κB-dependent gene expression (Han *et al.*, 2001). We examined whether β-carotene inhibits ROS generation and NO production in RAW264.7 cells stimulated with LPS. Treatment with β-carotene as well as the NADPH oxidase inhibitor diphenylene iodonium (DPI) significantly reduced the intracellular levels of ROS in RAW264.7 cells stimulated with LPS (Figure 6A). Moreover, treatment with β-carotene, DPI, and PDTC suppressed the LPS-induced increase in NO production and iNOS expression compared with the control cells (Figures 6B and 6C). Since β-carotene inhibited NF-κB activation and iNOS promoter activity (Figure 5), we next examined whether DPI regulates iNOS promoter activity. Figure 6D showed that treatment with DPI suppressed iNOS promoter activity in a concentration-dependent manner as both β-carotene and PDTC did, as shown in Figure 5B. This result suggests that the antioxidant activity of β-carotene contributes to the suppression of NF-κB-dependent iNOS expression.

Discussion

β-Carotene is a major antioxidant present in a range of fresh fruits and vegetables. This compound is known to take part in protecting animals against damage from free radicals and singlet oxygen reactive species. The antioxidant properties of β-carotene have been implicated in the molecular basis for preventing several diseases, primarily owing to the putative role of oxidative stress in disease initiation and progression. It has been demonstrated that β-carotene may suppress *in vivo* oxidative stress-dependent lipid peroxidation (Everett *et al.*, 1996), carcinogenesis (Witschi, 2005), and inflammation (Curran *et al.*, 2000). Inflammation is a complex process and involves production of various inflammatory mediators, such as NO, cytokines, ROS, and bioactive lipid metabolites, which are involved in the development of inflammatory diseases. LPS, a potent immune activator, induces septic shock syndrome through the production of many inflammatory mediators, such as NO, TNF-α, IL-1β, prostanoids, and leukotrienes (Everett *et al.,* 1996; Kim *et al.,* 2001) by activating immune cells including macrophages. Since activated macrophages result in the increases in NF-κB-dependent inflammatory mediators, both NF-κB activation and the inflammatory gene expression are well-known biological markers for inflammatory responses and its level is highly correlated with the pathogenesis of many acute and chronic human inflammatory diseases, including asthma, rheumatoid arthritis, inflammatory bowel disease, and endotoxemia-induced multiple organ injury (Barnes and Karin, 1997; Guzik *et al.*, 2003). Thus, the suppression of NF-κB-dependent inflammatory gene expression may be an effective therapeutic strategy for preventing inflammatory processes and diseases.

The present study was undertaken to elucidate the biological and pharmacological effect of β-carotene on *in vitro* and *in vivo* production of inflammatory cytokines and mediators. We also investigated the mechanism for the molecular action of β-carotene, which is associated with the suppression of NF-κB activation. Although treatment of human leukemic cells (HL-60) with β-carotene alone can induce NF-κB activation by a significant increase in ROS production (Palozza *et al.*, 2003), we here showed that β-carotene inhibited the NF-κB-dependent expression of inflammatory genes, such as TNF-α, IL-1β, iNOS, and COX-2, and production of NO and PGE_2 in LPS-stimulated macrophages and LPS-administrated animals. These observations suggest that β-carotene may act as a dual functional molecule: the activation of NF-κB in tumor cells without any stimulation and the inhibition of NF-κB activation in immune cells stimulated with immune stimulants including LPS. In this study, we demonstrated that the molecular mechanism by which β-carotene inhibits the expression of these inflammatory mediators appeared to involve the inhibition of NF-κB activation by blocking LPS-induced IκBα degradation and nuclear translocation of the cytosolic NF-κB p65 subunit. Moreover, β-carotene showed to act as a potent antioxidant activity by inhibiting intracellular ROS accumulation in LPS-stimulated macrophages. This antioxidant activity of β-carotene is likely to suppress NF-κB activation and NF-κB-dependent inflammatory gene expression. These results evidently suggest that β-carotene may prevent organ or tissue injury during acute endotoxemia or sepsis by suppressing NF-κB-mediated inflammatory gene expression.

Activated macrophages produce a large amount of

the pro-inflammatory cytokines TNF-α and IL-1β and induce the expression of COX-2 and iNOS, which catalyze the biosynthesis of PGE_2 and NO, respectively. These inflammatory cytokines and mediators play an important role in the pathogenesis of acute and chronic inflammatory diseases and organ injury (Simons *et al.*, 1996; Guslandi, 1998). It has been shown that anti-inflammatory drugs such as dexamethasone, prednisone, sulfasalazine, and aspirin prevent the development of human inflammatory diseases by suppressing the production of pro-inflammatory cytokines and expression of COX-2 and iNOS in immune-activated macrophages (Leach *et al.*, 1998; Makarov, 2000; Tak *et al.*, 2001). Previous studies have demonstrated that the suppression of biological activities of TNF-α, IL-1β, COX-2, and iNOS by neutralizing antibodies, selective inhibitors or gene targeting have led to a dramatic improvement in the local inflammation and progression of rheumatoid arthritis (Tak *et al.*, 2001) and atherosclerosis (Napoli and Ignarro, 2001) and the beneficial effects for septic shock (Szabo *et al.*, 1994). It indicates that a potent drug with anti-inflammatory activity provides the beneficial advantage for treatment of inflammatory disorders. Our study showed that β-carotene effectively inhibited the production of the inflammatory cytokines and enzymes in a dose dependent manner with an IC_{50} value of ~30 μM in LPS-stimulated macrophages as well as in a septic mouse model. These results suggest that β-carotene has therapeutic potential in inflammatory diseases.

Activation of the transcription factor NF-κB by a wide variety of agents, such as ROS, TNF-β, and LPS, plays a crucial role in the expression of the inflammatory genes, including iNOS, COX-2, TNF-β, and IL-1β, which contain NF-κB-binding motifs within their respective promoters (Xie *et al.*, 1994; Rhodus *et al.*, 2005). Thus, the abnormal, constitutive activation of NF-κB has known to be associated with a number of chronic inflammatory diseases, such as asthma, rheumatoid arthritis, atherosclerosis, and inflammatory bowl disease. It has been shown that anti-inflammatory drugs including dexamethasone suppress the expression of inflammation-associated genes by the inhibition of the NF-κB signaling pathway (van der Saag *et al.*, 1996; Makarov, 2000). This fact indicates that the inhibition of the NF-κB pathway is an extremely attractive target for the intervention of inflammatory processes. In addition a role of NF-κB activation has been implicated in the pathogenesis of human diseases characterized by increases in the host immune and inflammatory response. Constitutive activation of the NF-κB pathway has also been implicated in the pathogenesis of some human cancers (Chiao *et al.*, 2002). Furthermore, activation of the NF-κB pathway may be involved in the initiation or potentiation of neuritic plaques and neuronal apoptosis during the early phases of Alzheimer's disease (Kaltschmidt *et al.*, 1999). Thus, it suggests that potential inhibitors of NF-κB pathway can be used for prevention of inflammation-associated human diseases, cancer, and Alzheimer's disease. Our previous study has been shown that the antioxidant astaxanthin acts as an inhibitor of the NF-κB pathway (Lee *et al.*, 2003a). In fact, the present study demonstrated that relatively low concentrations of β-carotene (30 to 50 μM) blocked NF-κB activation and inhibited iNOS promoter activity. It has been shown that the activation and nuclear translocation of NF-κB is regulated by the proteolytic degradation IκB after its phosphorylation and ubiquitination (de Martin *et al.*, 1993), indicating that IκB degradation is a critical step for NF-κB activation. Our results showed that β-carotene inhibited IκB degradation and subsequently blocked nuclear translocation of the NF-κB p65 subunit, resulting in the suppression of iNOS promoter activity. Thus, the molecular mechanism of β-carotene's anti-inflammatory effect is likely associated with the inhibition of NF-κB activation through the suppression of IκBα degradation.

LPS stimulates ROS via the activation of NADPH oxidase in macrophages. ROS is thought to be involved in inflammatory gene expression through the redox-based activation of NF-κB signaling pathway (Kabe *et al.*, 2005). It has been shown that antioxidants, such as PDTC, N-acetyl-L-cysteine, and astaxanthin, inhibit inflammatory gene expression and NO production by suppressing NF-κB activation through the removal of ROS (Meyer *et al.*, 1992; Schreck *et al.*, 1992; Lee *et al.*, 2003a). Therefore, ROS may play an important role in NF-κB activation and pro-inflammatory cytokine production in LPS-stimulated macrophages, probably by increasing tyrosine phosphorylation of IKK via the activation of protein kinase D (Storz and Toker, 2003). It also suggests that intracellular oxidative stress is an activating signal of NF-κB-dependent inflammatory gene expression. Our data demonstrated that β-carotene significantly suppressed LPS-induced increases in intracellular ROS accumulation, iNOS expression, NO production, and iNOS promoter activity in macrophages as the NADPH oxidase inhibitor DPI and PDTC did, indicating that ROS participated in NF-κB-dependent iNOS expression and NO production. Therefore, the molecular mechanism by which β-carotene inhibits NF-κB activation is likely to block IκB phosphorylation and degradation, probably by suppressing redox-based IKK activation.

Taken together, β-carotene inhibited the production of inflammation-associated gene expression and mediators via the suppression of IκB degradation and subsequent NF-κB activation by scavenging intra-

cellular ROS. It suggests that β-carotene may be beneficial in the prevention or treatment of human inflammatory diseases, such as sepsis, rheumatoid arthritis, and atherosclerosis.

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