Mol. Cells, Vol. 16, No. 1, pp. 97-105

Molecules and Cells

Astaxanthin Inhibits Nitric Oxide Production and Inflammatory Gene Expression by Suppressing IκB Kinase-dependent NF-κB Activation

Seon-Jin Lee, Se-Kyung Bai, Kwang-Soon Lee, Seung Namkoong, Hee-Jun Na, Kwon-Soo Ha, Jeong-A Han, Sung-Vin Yim[1], Kwang Chang, Young-Guen Kwon, Sung Ki Lee[2], and Young-Myeong Kim*

Vascular System Research Center and Department of Molecular and Cellular Biochemistry, Kangwon National University Biology, Chunchon 200-701, Korea;

[1] Department of Pharmacology, School of Medicine, Kangwon National University Biology, Chunchon 200-701, Korea;

[2] Department of Food Science in Animal resources, College of Animal Resource Science, Kangwon National University Biology, Chunchon 200-701, Korea.

(Received June 4, 2003; Accepted June 18, 2003)

Astaxanthin, a carotenoid without vitamin A activity, has shown anti-oxidant and anti-inflammatory activities; however, its molecular action and mechanism have not been elucidated. We examined *in vitro* and *in vivo* regulatory function of astaxanthin on production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) as well as expression of inducible NO synthase (iNOS), cyclooxygenase-2, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β). Astaxanthin inhibited the expression or formation production of these pro-inflammatory mediators and cytokines in both lipopolysaccharide (LPS)-stimulated RAW264.7 cells and primary macrophages. Astaxanthin also suppressed the serum levels of NO, PGE_2, TNF-α, and IL-1β in LPS-administrated mice, and inhibited NF-κB activation as well as iNOS promoter activity in RAW264.7 cells stimulated with LPS. This compound directly inhibited the intracellular accumulation of reactive oxygen species in LPS-stimulated RAW264.7 cells as well as H_2O_2-induced NF-κB activation and iNOS expression. Moreover, astaxanthin blocked nuclear translocation of NF-κB p65 subunit and IκBα degradation, which correlated with its inhibitory effect on IκB kinase (IKK) activity. These results suggest that astaxanthin, probably due to its antioxidant activity, inhibits the production of inflammatory mediators by blocking NF-κB activation and as a consequent suppression of IKK activity and IκB-α degradation.

* To whom correspondence should be addressed.
Tel: 82-33-250-8831; Fax: 82-33-244-3286
E-mail: ymkim@kangwon.ac.kr

Keywords: Cytokine; Inflammation; NF-κB; Nitric Oxide; Reactive Oxygen Species.

Introduction

Inflammation is characterized by the release of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), as well as inflammatory mediators, including nitric oxide (NO) and prostaglandin E_2 (PGE_2), that are synthesized by inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX). These inflammatory mediators and cytokines are involved in the causation of many human diseases including rheumatoid arthritis, asthma, atherosclerosis, and endotoxin-induced multiple organ injury (Guslandi, 1998; Ritchlin *et al.*, 2003; Simons *et al.*, 1996). Anti-inflammatory agents reduce the inflammatory response by suppressing the production of inflammatory cytokines and mediators (Leach *et al.*, 1998; Makarov, 2000).

Nuclear factor-κB (NF-κB) has a seminal role in immunity, because it activates pro-inflammatory genes encoding

Abbreviations: COX-2, cyclooxygenase-2; $DCFH_2$-DA, 2′, 7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; IFNγ, interferon-gamma; IKK, IκB kinase; IL-1β, interleukin-1beta; iNOS, inducible nitric oxide synthase; LPS, lipopolysaccharide; NF-κB, nuclear factor-kappa B; NO, nitric oxide; NOx, nitrite plus nitrate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; RT-PCR, reverse transcriptase-polymerase chain reaction; TNF-α, tumor necrosis factor-alpha.

iNOS, COX-2, TNF-α, IL-1β, and IL-6 (de Vera *et al.*, 1996; Makarov, 2000). It is activated by phosphorylation, ubiquitination, and subsequent proteolytic degradation of the IκB protein by activated IκB kinase (IKK) (de Martin *et al.*, 1993). The liberated NF-κB translocates to the nucleus and binds as a transcription factor to κB motifs in the promoters of target genes, leading to their transcription. Aberrant NF-κB activity is associated with various inflammatory diseases, and most anti-inflammatory drugs suppress inflammatory cytokine expression by inhibiting the NF-κB pathway (Castrillo *et al.*, 2001; Keifer *et al.*, 2001). Thus, an NF-κB inhibitor has clinical potential in inflammatory diseases.

Antioxidants such as α-tocopheryl succinate (Neuzil *et al.*, 2001) and probucol (Dichtl *et al.*, 1999) inhibit NF-κB activity and block the expression of pro-inflammatory genes as well as production of NO and PGE_2 (Pahan *et al.*, 1998). Astaxanthin is a non-pro-vitamin A carotenoid, abundant in vegetables and fruits; it is also present in marine animals. Astaxanthin and astaxanthin-like products are commonly indicated as antioxidants (Kurashige *et al.*, 1990) and immune modulators (Bennedsen *et al.*, 1999). One effect of astaxanthin is to scavenge reactive oxygen species (ROS) (Mortensen *et al.*, 1997). However, the mechanism of its anti-inflammatory action has not been elucidated.

We hypothesized that astaxanthin regulated the production of NO, PGE_2, and pro-inflammatory cytokines by inhibiting NF-κB activation. We showed in RAW264.7 cells stimulated with LPS that it inhibited *in vitro* and *in vivo* production of NO and PGE_2 as well as production of pro-inflammatory cytokines, such as TNF-α and IL-1β. Furthermore, it suppressed NF-κB activation and iNOS promoter activity by inhibiting IKK activity. These results support the idea that astaxanthin prevents inflammatory processes by blocking the expression of pro-inflammatory genes as a consequence of suppressing NF-κB activation.

Materials and Methods

Materials Dulbecco's modified Eagle's medium (DMEM), penicillin, and streptomycin were from Life Technology Inc. (Rockville, MD). LPS (*Escherichia coli* O111:B4) and astaxanthin were obtained from Sigma (USA). Poly (dI-dC) and NF-κB-specific oligonucleotide were from Promega (Madison, WI), and monoclonal iNOS antibody was from Transduction Laboratories (Lexington, KY). Polyclonal antibodies for IκB kinase (IKK), COX-2, TNF-α and IL-1β were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). 2′, 7′-dichlorofluorescin diacetate ($DCFH_2$-DA) was purchased from Molecular Probes (Eugene, OR). Other chemicals were from Sigma (USA) unless otherwise indicated.

Macrophage isolation and cell culture Cells of the murine macrophage cell line RAW264.7 were cultured in DMEM (2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin) containing 10% fetal bovine serum (HyClone Labs, Logan, UT). Peritoneal macrophages were collected from the peritoneal cavity of 6- to 8-week-old female BALB/c mice (Daihan-Biolink, Korea) and given an intraperitoneal (i.p.) injection of 1.5 ml of thioglycollate broth (4%) 7 days before harvest. Cells were cultured in 12-well plates (3×10^6 cells/well) or 6-well culture dishes (5×10^6 cells/well) at 37°C in 5% CO_2/95% air for 8 h. They were treated with LPS (10 μg/ml) in the presence or absence of various concentrations of astaxanthin.

Animal treatment Mice (6 to 8 week-old female BALB/c mice, Daihan-Biolink, Korea) were injected i.p. with LPS (4 mg/kg) and/or astaxanthin (40 mg/kg). After 12 h, blood was withdrawn by cardiac puncture. Serum was prepared by centrifugation at $12{,}000 \times g$ for 20 min at 4°C and kept at −20°C until use.

Measurement of nitric oxide metabolites, cytokines, and PGE_2 Nitrite, a stable oxidized product of NO, was measured in culture media using the Griess reagents (Kim *et al.*, 1997). Serum nitrite plus nitrate (NOx) concentration was determined with a nitrate reductase-based colorimetric assay kit (Alexis San Diego, USA). PGE_2 concentrations were determined by enzyme immunoassay (Amersham Pharmacia Biotech, Piscataway, NJ) and levels of TNF-α and IL-1β in culture media and sera were determined by ELISA (R&D Systems, Minneapolis, MN).

Western blot analysis Cells were incubated with or without LPS in the presence or absence of astaxanthin. They were harvested, washed twice with ice-cold phosphate-buffered saline (PBS), resuspended in PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed by three cycles of freezing and thawing (Koo *et al.*, 2002). Cytosolic fractions were obtained as supernatants after centrifugation at $12{,}000 \times g$ at 4°C for 20 min. Protein content was determined by the BCA method (Pierce, Rockford, IL), and samples (30 μg of protein) were separated on 8% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST) for 2 h, and incubated with monoclonal mouse-iNOS or polyclonal COX-2 antibodies in PBST containing 1% nonfat milk for 2 h. After washing three times with PBST, the membranes were hybridized with horseradish peroxidase-conjugated secondary antibodies for 1 h. Following five washes with PBST, they were incubated with chemiluminescent solution for 2 min, and protein bands visualized on X-ray film.

Reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was obtained from RAW264.7 using the Trizol reagent kit (Life Technology Inc., USA). Three μg of mRNA was converted to cDNA by treatment with 200 units 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 and three μl of the cDNA mixture was used for enzy-

matic 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 units of Taq DNA polymerase, and 0.1 μM each of primers for iNOS, COX-2, TNF-α, and IL-1β. Amplification conditions were: denaturation at 94°C for 5 min for the first cycle and for 45 s starting from the second cycle, annealing of iNOS at 47°C for 45 s, annealing of COX-2, TNF-α, and IL-1β at 51°C for 45 s, and extension at 72°C for 30 s for 35 cycles. Final extension was performed at 72°C for 10 min. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide. The primers used were 5′-TTTGGAGCAG-AAGTGCAAAGTCTC-3′ (sense) and 5′-GATCAGGAGGGA-TTTCAAAGACCT-3′ (antisense) for iNOS, 5′-CCGTGGTG-AATGTATGAGCA-3′ (sense) and 5′-CCTCGCTTCTGATCTG-TCTT-3′ (antisense) for the COX-2, 5′-ATGAGCACAGAAAG-CATG-3′ (sense) and 5′-TCACAGAGCAATGACTCC-3′ (antisense) for TNF-α, 5′-ATGGCAACTGTTCCTGAAC-3′ (sense) and 5′-TTAGGAAGACACGGATTC-3′ (antisense) for IL-1β, and 5′-TCCTTCGTTGCCGGTCCACA-3 (sense) and 5′-CGT-CTCCGGAGTCCATCACA-3′ (antisense) for β-actin.

Electrophoretic mobility shift assay RAW264.7 macrophages were treated with LPS in the presence or absence of astaxanthin for 2 h and nuclear extracts prepared as described previously (Kim *et al.*, 1997). A double stranded NF-κB-specific oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was labeled with [γ-^{32}P] ATP by 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 (Kim *et al.*, 1997), and samples separated on a 5% native polyacrylamide gel that was dried and subjected to autoradiography.

Assay of iNOS promoter activity Transient transfection and assay of iNOS promoter activity were carried out as described (Kim *et al.*, 2001). In brief, a murine iNOS promoter-luciferase construct was transfected into RAW264.7 cells by the liposome method. The cells were cultured overnight in DMEM supplemented with 10% fetal bovine serum and treated with LPS in the presence or absence of astaxanthin for 12 h. Cells were lysed with Reporter lysis buffer (Promega) or 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.

Immunoprecipitation and kinase assays Cells treated with LPS or astaxanthin were harvested and washed with PBS, and the pellets resuspended in 80 μl of immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 10% glycerol, 1% Nonidet P-40, 5 mM EDTA, 1 mM DTT, 100 mM NaF, 2 mM sodium pyrophosphate, 2 mM sodium orthovanadate, 1 mM PMSF, 10 μg aprotinin, and 10 μg leupeptin per milliliter) and stored on ice for 20 min before centrifugation (14,000 × *g*, 20 min, 4°C). The IKK complex was immunoprecipitated by incubation for 1 h at 4°C with polyclonal IKKα antibody bound to protein-A Sepharose. The immunoprecipitates were washed twice with immunoprecipitation buffer and twice with kinase buffer (20 mM HEPES, pH 7.4, 20 mM β-glycerophosphate, 20 mM $MgCl_2$, 2 mM DTT, 0.1 mM sodium orthovanadate). Kinase assays were initiated by the addition of 2 μM GST-IκBα fusion protein as substrate, and 0.5 μCi [γ-^{32}P]ATP. Reactions were incubated for 30 min at 30°C and stopped by the addition of 2× SDS-PAGE sample buffer. Phosphorylation of the IκBα proteins was measured by SDS-PAGE followed by autoradiography and densitometry.

Measurement of intracellular ROS RAW264.7 cells cultured in a 60 mm-dish at 5×10^5 cells/dish were treated with LPS for 20 min. $DCFH_2$-DA (5 μM) was added to the cultures and incubated for 30 min. The cells were harvested and washed three times with PBS, and intracellular ROS was quantified by flow cytometry with excitation at 488 nm and emission at 525 nm.

Statistical analysis Data are presented as the means ± SD of at least three separate experiments. Comparisons between groups were analyzed by Student's *t*-test. *P* values of 0.05 or less were considered statistically significant.

Results

Astaxathin inhibits the production of NO and PGE_2 by suppressing iNOS and COX-2 expression NO and PGE_2 are produced by immune activated macrophages, and at inflammatory sites (Guastadisegni *et al.*, 2002; Mino *et al.*, 1998). We determined if astaxanthin influences the production of NO and PGE_2 by RAW264.7 cells exposed to LPS. The cells accumulated nitrite, as a stable oxidized product of NO, in the culture medium when stimulated with LPS, and astaxanthin inhibited NO production with an IC_{50} of ~5 μM (Fig. 1A); it did not affect cell viability as measured by the crystal violet staining method (data not shown). To see whether astaxanthin inhibited NO production by suppressing iNOS gene expression, we examined its effect on levels of iNOS protein. Western blot analysis showed that stimulation of iNOS protein levels by LPS was completely inhibited by 50 μM astaxanthin (Fig. 1B). It also inhibited induction of iNOS mRNA (Fig. 1C), and blocked the increase in PGE_2 with an IC_{50} of ~30 μM (Fig. 1D). In addition, western blot and RT-PCR analyses showed that astaxanthin inhibited expression of COX-2 protein and mRNA, and blocked COX-2 expression at a concentration of 50 μM (Figs. 1E and 1F). These results show that astaxanthin inhibits NO and PGE_2 production by suppressing, respectively, iNOS and COX-2 expression in LPS-stimulated RAW264.7 macrophages.

Astaxanthin suppresses production of TNF-α and IL-

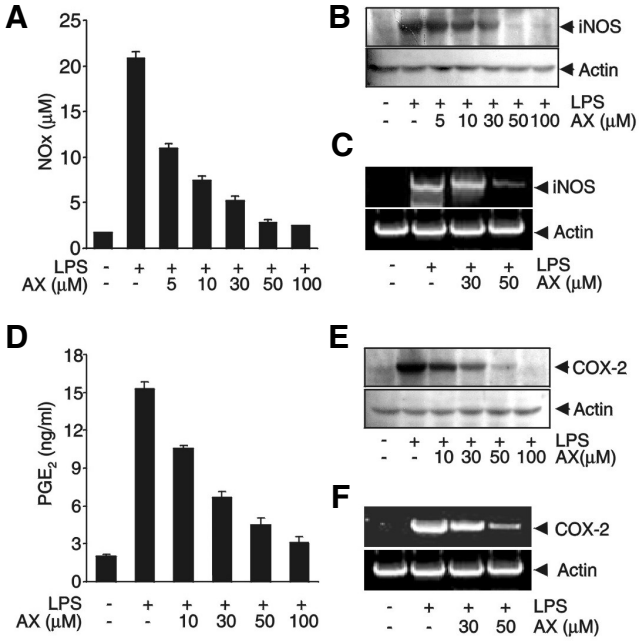

Fig. 1. Astaxanthin (AX) inhibits production NO and PGE_2 as well as expression of iNOS and COX-2. RAW264.7 cells were stimulated with LPS (10 μg/ml) in the presence or absence of different concentrations of astaxanthin. Nitrite (A) and PGE_2 levels (D) were measured after 16 h. Data shown are means ± SD ($n \geq 3$). (B) After 16 h, cells were harvested and levels of iNOS (B) and COX-2 (E) were measured by Western blotting. The blot was rehybridized with actin antibody to verify equal loading of protein. Levels of iNOS (C) and COX-2 mRNAs (F) were determined in LPS-stimulated cells after 8 h by RT-PCR. Actin was used as an internal control. Further details in **Materials and Methods**.

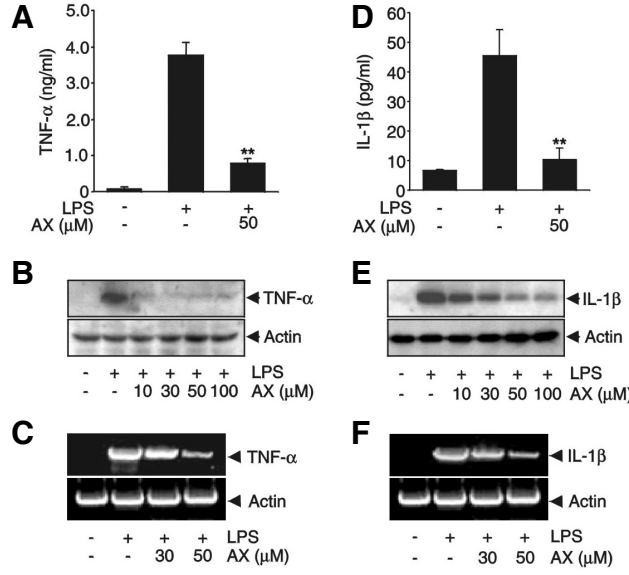

Fig. 2. Astaxanthin inhibits TNF-α and IL-1β expression in LPS-stimulated RAW264.7 cells. RAW264.7 cells were cultured with LPS (10 μg/ml) in the presence or absence of astaxanthin for 12 h. and TNF-α (A) and IL-1β levels (D) were determined in the culture media by ELISA. ** $p < 0.01$. Intracellular levels of TNF-α (B) and IL-1β (E) were measured by Western blot analyses and levels of TNF-α (C) and IL-1β mRNAs (F) were determined in LPS-stimulated cells after 8 h by RT-PCR. Further details in **Materials and Methods**.

1β To examine the effects of astaxanthin on inflammatory cytokine production, levels of TNF-α and IL-1β were measured in culture media by ELISA. Stimulation of RAW264.7 cells with LPS dramatically increased secreted TNF-α and IL-1β levels, and these increases were substantially inhibited by astaxanthin (50 μM) (Figs. 2A and 2D). 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 (Black *et al.*, 1997; Thornberry *et al.*, 1992). Western blot analyses showed that astaxanthin treatment reduced intracellular levels of these pro-forms (Figs. 2B and 2E), and also reduced levels of their mRNAs (Figs. 2C and 2F). Thus astaxanthin inhibits expression of TNF-α and IL-1β, probably at the transcriptional level.

Astaxanthin suppresses NO, TNF-α, and IL-1β production by peritoneal macrophages We also examined the effect of astaxanthin on NO, TNF-α, and IL-1β production by primary cultured peritoneal macrophages isolated from female BALB/c mice and cultured in 12-well plates for 8 h. NO production increased upon LPS-stimulation, and this increase was suppressed by 50 μM axtaxanthin (Fig. 3A) as was the increase in iNOS (Fig. 3B) and in TNF-α and IL-1β levels (Figs. 3C and 3D).

Astaxanthin inhibits NO, PGE_2, TNF-α, and IL-1β production *in vivo* NO, PGE_2, TNF-α, and IL-1β are critical mediators of the inflammatory process and of organ injury in endotoxemia and sepsis (Cunha *et al.*, 1994; Simons *et al.*, 1996). To examine the anti-inflammatory effects of astaxanthin in septic animals, we tested whether astaxanthin affects the production of NO, PGE_2, TNF-α, and IL-1β in LPS-treated mice. As expected astaxanthin inhibited the increases in plasma nitrite plus nitrate (NOx) and PGE_2 (Figs. 4A and 4B) as well as those of TNF-α and IL-1β (Figs. 4C and 4D). Clearly astaxanthin inhibits the production of inflammatory mediators under inflammatory conditions.

Astaxanthin suppresses NF-κB activation and iNOS promoter activity NF-κB activation plays a critical role in the expression of the inflammation-associated enzymes, iNOS and COX-2, as well as of the cytokines, TNF-α and IL-1β (Kiemer *et al.*, 2003) that are involved in the chronic inflammation and pathogenesis of human inflammatory diseases (Guslandi, 1998). We tested whether

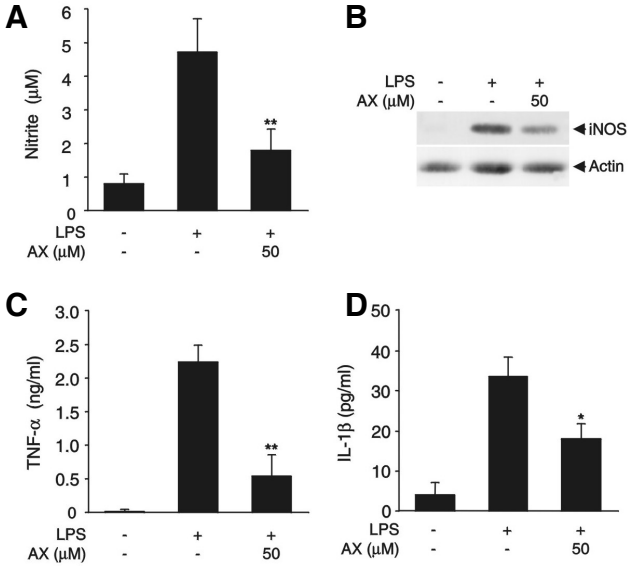

Fig. 3. Effects of astaxanthin on TNF-α and IL-1β production in mouse peritoneal macrophages. Peritoneal macrophages from female BALB/c mice (6- to 8-week old) were cultured in 6-well culture dishes (5×10^6 cells/well) for 8 h and treated with LPS (10 μg/ml) in the presence or absence of different concentration of astaxanthin for 24 h. (A) Nitrite levels in the culture medium and (B) intracellular iNOS levels. TNF-α (C) and IL-1β (D) were measured in the culture medium by ELISA. * $p < 0.05$, ** $p < 0.01$. Further details in **Materials and Methods**.

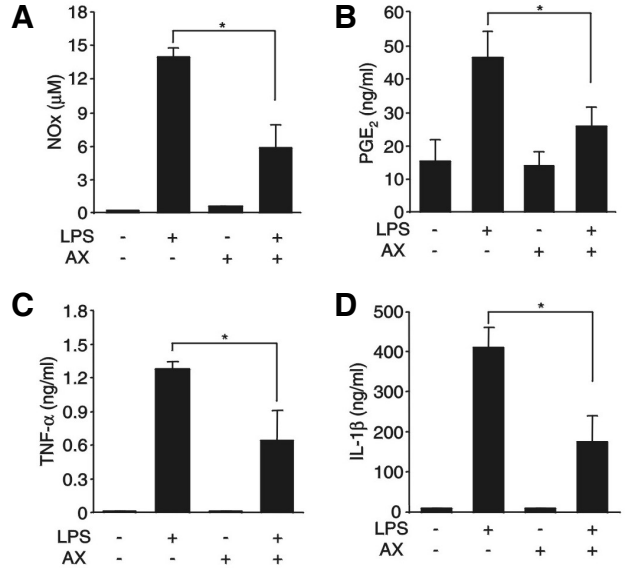

Fig. 4. Astaxanthin decreases plasma levels of NO, PGE_2, TNF-α, and IL-1β production. Mice were injected i.p. with LPS (4 mg/kg) following pretreatment with astaxanthin (40 mg/kg). After 12 h, whole blood samples were withdrawn by cardiac puncture. (A) Plasma levels of nitrite plus nitrate (NOx) measured with a nitrate assay kit, and plasma levels of PGE_2 (B), TNF-α (C), and IL-1β (D) measured by ELISA. Data are means ± SD (n = 6). * $p < 0.05$. Further details in **Materials and Methods**.

astaxanthin regulates NF-κB activation in LPS-stimulated RAW264.7 cells and found that the increase in NF-κB-DNA binding activity in nuclear extracts of LPS-stimulated macrophages was markedly inhibited (Fig. 5A) suggesting that astaxanthin inhibits the expression of iNOS, COX-2, TNF-α, and IL-1β by blocking NF-κB activation. Murine and human iNOS promoters contain functional NF-κB binding sites (de Vera *et al.*, 1996; Xie *et al.*, 1994) and NF-κB activation plays a key role in the transcriptional activation of the iNOS gene (Yokoseki *et al.*, 2001). We examined whether astaxanthin suppresses iNOS promoter activity by blocking NF-κB activation. LPS treatment of RAW264.7 cells resulted in a 3.7-fold increase in iNOS promoter activity that was suppressed by the NF-κB inhibitor pyrrolidine dithiocarbamate (PDTC) as well as by astaxanthin (Fig. 5B).

Astaxanthin inhibits LPS-dependent nuclear translocation of the NF-κB p65 subunit, degradation of IκBα, and IKK activation Translocation of NF-κB to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα (Baeuerle and Baichwal, 1997). We measured cytosolic and nuclear NF-κB p65 subunit levels following treatment with LPS in the presence or absence of astaxanthin by Western blot analysis. LPS treatment decreased cytosolic p65 subunit and increased nuclear p65 (Fig. 6A) and astaxanthin treatment inhibited this translocation. Since nuclear translocation of NF-κB is directly linked to IκB degradation (Butcher *et al.*, 2001), we next examined the effect of astaxanthin on this proteolytic event. Western blot analysis showed that levels of IκBα declined within 30 min of LPS treatment, and this decrease was blocked by astaxanthin (Fig. 6B). Degradation of IκBα is largely dependent on its phosphorylation by IKK activation (Pan *et al.*, 2000), and we found that a three-fold increase in IκBα phosphorylation and IKK activity in response to LPS was suppressed by astaxanthin (Figs. 6C and 6D).

Astaxanthin suppresses the LPS-induced increase in ROS and H_2O_2-induced NF-κB activation NF-κB activation by LPS is inhibited by antioxidants (Ma and Kinneer, 2002). We measured intracellular levels of ROS by FACS analysis following treatment of cells with PBS in the presence of $DCFH_2$-DA. LPS induced a marked increase in intracellular ROS, and this increase was strongly inhibited by astaxanthin (Fig. 7A). Astaxanthin pretreatment also suppressed the increase in intracellular ROS in RAW264.7 cells that were subsequently incubated with exogenous H_2O_2 (data not shown). We next examine whether astaxanthin regulates H_2O_2-mediated NF-κB activation. H_2O_2 treatment activated NF-κB, and activation

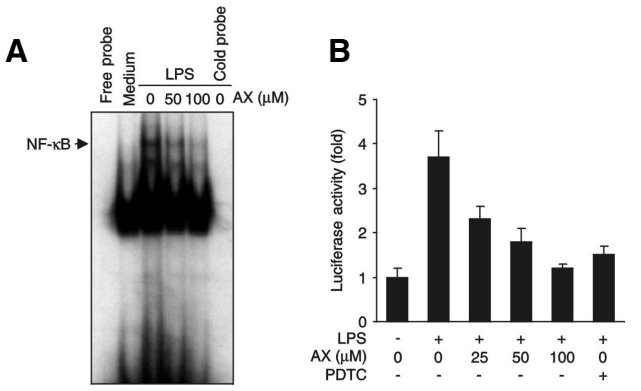

Fig. 5. Effects of astaxanthin on NF-κB activation and iNOS promoter activity. **A.** RAW264.7 cells were treated with LPS in the presence or absence of astaxanthin for 2 h. Nuclear NF-κB activity was analyzed by EMSA in the presence or absence of excess cold probe. **B.** RAW264.7 cells were transiently transfected by the lipofectamine method with a 1.6 kbp murine iNOS promoter fragment upstream of luciferase. Cells were treated with LPS in the presence or absence of astaxanthin for 12 h and luciferase activity in cell extracts was measured with a luminometer. Data shown are means ± SD (n = 3).

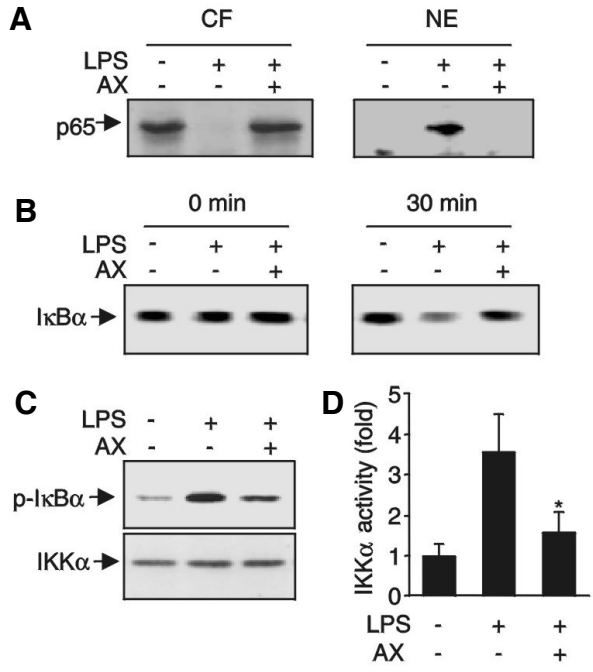

Fig. 6. Astaxanthin inhibits nuclear translocation of NF-κB p65 subunit, IκBα degradation, and IKK activation in LPS-stimulated RAW264.7 cells. Cells were incubated with LPS in the presence or absence of astaxanthin for 2 h and cytosolic (CF) and nuclear extracts (NE) prepared. **A.** Samples of 50 μg protein were separated on SDS-PAGE, and the NF-κB p65 subunit was visualized by Western blot analysis. **B.** Whole cell lysates from RAW264.7 cells treated with LPS in the presence or absence of astaxanthin for 0 and 30 min were separated on SDS-PAGE and IκBα protein was visualized by Western blot analysis. **C.** The IKK complex was immunoprecipitated with an anti-IKKα antibody and *in vitro* kinase assayed with 2 μM GST-IκBα fusion protein and 5 μCi [γ-^{32}P]ATP. After SDS-PAGE, phosphorylated IκBα protein was visualized by autoradiogaraphy. **D.** IKKα activity measured by densitometry of autoradiographic films. Data shown are means ± SD (n = 3). * $p < 0.05$. Further details in **Materials and Methods**.

was suppressed by astaxanthin (Fig. 7B). We further examined whether astaxanthin regulates H_2O_2-induced iNOS protein levels and NO production. Interferon-gamma (IFNγ) treatment induced iNOS protein expression in peritoneal macrophages, while H_2O_2 had no effect on its own (Fig. 7C). However, IFNγ-induced iNOS expression was significantly increased by simultaneous exposure to H_2O_2, and this synergistic effect was suppressed by astaxanthin. IFNγ-induced NO production was similarly elevated by treatment with H_2O_2, and again, this increase was suppressed by astaxanthin (Fig. 7D).

Discussion

The present study was undertaken to elucidate the effects of astaxanthin on the *in vitro* and *in vivo* production of inflammatory cytokines and mediators. We showed that it inhibited the expression of iNOS, COX-2, TNF-α, and IL-1β as well as production of NO and PGE_2 in LPS-stimulated macrophages and LPS-administrated septic animals. It also inhibited NF-κB activation and iNOS promoter activity, as well as LPS-induced nuclear translocation of cytosolic NF-κB p65, IκBα degradation, and IKK activity. Astaxanthin had a strong antioxidant action, inhibiting intracellular ROS accumulation and blocking H_2O_2-induced NF-κB activation; this may account for its anti-inflammatory action by suppressing NF-κB activation. Our results suggest that astaxanthin is a candidate treatment for chronic inflammatory diseases such as sepsis, rheumatoid arthritis, atherosclerosis, and inflammatory bowel disease.

Activated macrophages produce a large amount of NO and PGE_2 via COX-2 and iNOS, as well as the pro-inflammatory cytokines TNF-α and IL-1β (Chang *et al.*, 2001). These inflammatory mediators and cytokines activate other immune cells and can cause inflammatory diseases such as rheumatoid arthritis and endotoxemia-induced multiple organ injury (Guslandi, 1998; Ritchlin *et al.*, 2003; Simons *et al.*, 1996). 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 iNOS and COX-2 (Leach *et al.*, 1998; Makarov, 2000). Moreover inhibition of iNOS, COX-2, TNF-α, and IL-1β with neutralizing

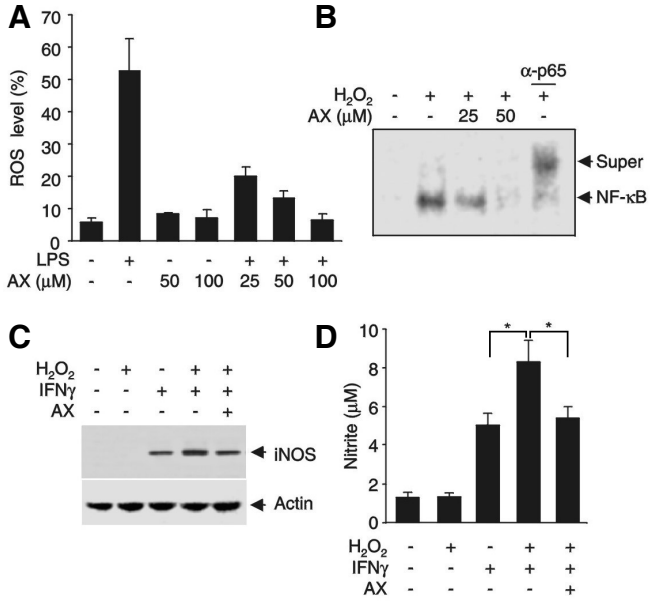

Fig. 7. Effect of astaxanthin on ROS generation, NF-κB activation, and iNOS expression. **A.** RAW264.7 cells were treated with LPS in the presence or absence of astaxanthin for 30 min, incubated with $DCFH_2$-DA (5 μM) for an additional 30 min and intracellular levels of ROS analyzed by FACS. **B.** RAW264.7 cells were treated with 1 μM H_2O_2 in the presence or absence of astaxanthin for 2 h. and nuclear NF-κB activity was analyzed by EMSA. Supershifting was performed with antibody against the NF-κB p65 subunit (α-p65). **C.** Peritoneal macrophages were treated with H_2O_2 and/or IFNγ (10 units/ml) in the presence or absence of astaxanthin (50 μM) for 14 h. and levels of iNOS measured by Western blotting. **D.** Nitrite measured in the culture medium after 24 h culture. Data shown are means ± SD (n = 3). * $p < 0.05$. Further details in **Materials and Methods**.

antibodies, inhibitors, or gene targeting, dramatically decreases local inflammation and the progression of rheumatoid arthritis (Dinarello, 2001; Feldmann *et al.*, 1995; Kagari *et al.*, 2002; Pinheiro and Calixto, 2002; Stefanovic-Racic *et al.*, 1994), and has beneficial effects on septic shock (Mathison *et al.*, 1988). We showed that astaxanthin inhibited expression of iNOS and COX-2, resulting in reduced NO, PGE_2, TNF-α and IL-1β production in both RAW264.7 and primary macrophage cells stimulated with LPS. In addition, it reduced plasma levels of NO, PGE_2, TNF-α, and IL-1β, in an animal model of sepsis. These results support our hypothesis that astaxanthin could have a therapeutic effect in chronic inflammatory diseases.

The transcription factor NF-κB regulates the expression of many inflammatory genes including iNOS, COX-2, TNF-α, and IL-1β (Li and Verma, 2002), and aberrant activation of NF-κB is associated with a number of chronic inflammatory diseases. Anti-inflammatory drugs probably suppress the NF-κB pathway (Berg *et al.*, 1999; Kiemer *et al.*, 2003) and suppression of NF-κB activation may be essential to prevent or treat inflammatory diseases. We showed that a relatively low concentration (50 μM) of astaxanthin blocked NF-κB activation and inhibited iNOS promoter activity and that it inhibited nuclear translocation of the NF-κB p65 subunit and IκBα protein degradation.

The key step in NF-κB activation is the rapid degradation of IκB, which requires IκB phosphorylation by elevated levels of IKK activity. The LPS receptor interacts with the small GTP-binding protein Rac1, and then associates with $p47^{phox}$ and $p67^{phox}$ (two subunits needed for NADPH oxidase $p91^{phox}$ function) at the cell membrane, leading to ROS production (Sanlioglu *et al.*, 2001). Increases in intracellular ROS augment NF-κB activation by enhancing IKK activity, and the antioxidant enzymes, superoxide dismutase and catalase, and pharmacologic antioxidants like N-acetyl cysteine and PDTC, inhibit NF-κB-dependent gene expression including expression of TNF-α (Sanlioglu *et al.*, 2001). Therefore, ROS-induced oxidative stress may play an important role in NF-κB activation and pro-inflammatory cytokine production in LPS-primed macrophages. Taken together the results reported here indicate that the ability of astaxanthin to modulate pro-inflammatory gene expression through suppression of NF-κB activation may be based on its antioxidant activity.

We showed previously that the antioxidant enzymes catalase, peroxidase, and myeloperoxidase inhibited NF-κB activation, iNOS expression, and NO production in macrophages stimulated by LPS (Han *et al.*, 2001) and suggested that ROS generated by macrophages is important for NF-κB-dependent gene expression. We found here that, like LPS, exogenous H_2O_2 activated NF-κB; however, unlike LPS, H_2O_2 alone was not enough to induce iNOS expression and NO production. This suggests that LPS stimulates other signal pathways activating iNOS gene expression, including other transcription factors such as Oct-1 (Xie, 1997). LPS induces NO production in macrophages and acts in a synergistic manner to increase IFNγ-induced NO production by activating NF-κB (Kim *et al.*, 2001), suggesting that activation of a single transcription factor may not be enough for maximal NOS production. This is consistent with our observation that exogenous H_2O_2, which activated NF-κB but did not induce iNOS expression, synergized with IFNγ in inducing iNOS expression and NO production.

In summary, our findings demonstrate that the natural product astaxanthin inhibited signaling cascade of pro-inflammatory gene expression in LPS-stimulated macrophages by suppressing NF-κB activation, probably as a result of scavenging intracellular ROS. This inhibitory effect has important implications for the development of anti-inflammatory drugs and strategies to limit pathological inflammation.

Acknowledgments This work was supported by a Vascular System Research Center grant (Y.-M.K) and a Basic Research Program grant R01-2000-000-00809-0 (S.-K.L) from Korea Science & Engineering Foundation.

References

Baeuerle, P. A. and Baichwal, V. R. (1997) NF-κB as a frequent target for immunosuppressive and anti-inflammatory molecules. *Adv. Immunol.* **65**, 111–137.

Bennedsen, M., Wang, X., Willen, R., Wadstrom, T., and Andersen, L. P. (1999) Treatment of H. pylori infected mice with antioxidant astaxanthin reduces gastric inflammation, bacterial load and modulates cytokine release by splenocytes. *Immunol. Lett.* **70**, 185–189.

Berg, J., Fellier, H., Christoph, T., Grarup, J., and Stimmeder, D. (1999) The analgesic NSAID lornoxicam inhibits cyclooxygenase (COX)-1/-2, inducible nitric oxide synthase (iNOS), and the formation of interleukin (IL)-6 *in vitro*. *Inflamm. Res.* **48**, 369–379.

Black, R. A., Rauch, C. T., Kozlosky, C. J., Peschon, J. J., Slack, J. L., Wolfson, M. F., Castner, B. J., Stocking, K. L., Reddy, P., Srinivasan, S., Nelson, N., Boiani, N., Schooley, K. A., Gerhart, M., Davis, R., Fitzner, J. N., Johnson, R. S., Paxton, R. J., March, C. J., and Cerretti, D. P. (1997) A metalloproteinase disintegrin that releases tumour necrosis factor-α from cells. *Nature* **385**, 729–733.

Butcher, B. A., Kim, L., Johnson, P. F., and Denkers, E. Y. (2001) Toxoplasma gondii tachyzoites inhibit proinflammatory cytokine induction in infected macrophages by preventing nuclear translocation of the transcription factor NF-κB. *J. Immunol.* **167**, 2193–2201.

Castrillo, A., de Las, H. B., Hortelano, S., Rodriguez, B., Villar, A., and Bosca, L. (2001) Inhibition of the nuclear factor kappa B (NF-κB) pathway by tetracyclic kaurene diterpenes in macrophages. Specific effects on NF-κB-inducing kinase activity and on the coordinate activation of ERK and p38 MAPK. *J. Biol. Chem.* **276**, 15854–15860.

Chang, Y. H., Lee, S. T., and Lin, W. W. (2001) Effects of cannabinoids on LPS-stimulated inflammatory mediator release from macrophages: involvement of eicosanoids. *J. Cell. Biochem.* **81**, 715–723.

Cunha, F. Q., Assreuy, J., Moss, D. W., Rees, D., Leal, L. M., Moncada, S., Carrier, M., O'Donnell, C. A., and Liew, F. Y. (1994) Differential induction of nitric oxide synthase in various organs of the mouse during endotoxaemia: role of TNF-α and IL-1β. *Immunology* **81**, 211–215.

de Martin, R., Vanhove, B., Cheng, Q., Hofer, E., Csizmadia, V., Winkler, H., and Bach, F. H. (1993) Cytokine-inducible expression in endothelial cells of an IκB-α-like gene is regulated by NF-κB. *EMBO J.* **12**, 2773–2779.

de Vera, M. E., Shapiro, R. A., Nussler, A. K., Mudgett, J. S., Simmons, R. L., Morris, S. M. Jr., Billiar, T. R., and Geller, D. A. (1996) Transcriptional regulation of human inducible nitric oxide synthase (NOS2) gene by cytokines: initial analysis of the human NOS2 promoter. *Proc. Natl. Acad. Sci. USA* **93**, 1054–1059.

Dichtl, W., Nilsson, L., Goncalves, I., Ares, M. P., Banfi, C., Calara, F., Hamsten, A., Eriksson, P., and Nilsson, J. (1999) Very low-density lipoprotein activates nuclear factor-κB in endothelial cells. *Circ. Res.* **84**, 1085–1094.

Dinarello, C. A. (2001) Anti-cytokine therapies in response to systemic infection. *J. Investig. Dermatol. Symp. Proc.* **6**, 244–250.

Feldmann, M., Brennan, F. M., Williams, R. O., Elliott, M. J., and Maini, R. N. (1995) Cytokine expression and networks in rheumatoid arthritis: rationale for anti-TNF-α antibody therapy and its mechanism of action. *J. Inflamm.* **47**, 90–96.

Guastadisegni, C., Nicolini, A., Balduzzi, M., Ajmone-Cat, M. A., and Minghetti, L. (2002) Modulation of PGE_2 and TNF-α by nitric oxide and LPS-activated RAW 264.7 cells. *Cytokine* **19**, 175–180.

Guslandi, M. (1998) Nitric oxide and inflammatory bowel diseases. *Eur. J. Clin. Invest.* **28**, 904–907.

Han, Y. J., Kwon, Y. G., Chung, H. T., Lee, S. K., Simmons, R. L., Billiar, T. R., and Kim, Y. M. (2001) Antioxidant enzymes suppress nitric oxide production through the inhibition of NF-κB activation: role of H_2O_2 and nitric oxide in inducible nitric oxide synthase expression in macrophages. *Nitric Oxide* **5**, 504–513.

Kagari, T., Doi, H., and Shimozato, T. (2002) The importance of IL-1β and TNF-α, and the noninvolvement of IL-6, in the development of monoclonal antibody-induced arthritis. *J. Immunol.* **169**, 1459–1466.

Keifer, J. A., Guttridge, D. C., Ashburner, B. P., and Baldwin, A. S. Jr (2001) Inhibition of NF-κB activity by thalidomide through suppression of IκB kinase activity. *J. Biol. Chem.* **276**, 22382–22387.

Kiemer, A. K., Hartung, T., Huber, C., and Vollmar, A. M. (2003) Phyllanthus amarus has anti-inflammatory potential by inhibition of iNOS, COX-2, and cytokines via the NF-κB pathway. *J. Hepatol.* **38**, 289–297.

Kim, Y. M., de Vera, M. E., Watkins, S. C., and Billiar, T. R. (1997) Nitric oxide protects cultured rat hepatocytes from tumor necrosis factor-α-induced apoptosis by inducing heat shock protein 70 expression. *J. Biol. Chem.* **272**, 1402–1411.

Kim, K. M., Chun, S. B., Koo, M. S., Choi, W. J., Kim, T. W., Kwon, Y. G., Chung, H. T., Billiar, T. R., and Kim, Y. M. (2001) Differential regulation of NO availability from macrophages and endothelial cells by the garlic component S-allyl cysteine. *Free Radical Biol. Med.* **30**, 747–756.

Koo, M. S., Kwo, Y. G., Park, J. H., Choi, W. J., Billiar, T. R., and Kim, Y. M. (2002) Signaling and function of caspase and c-jun N-terminal kinase in cisplatin-induced apoptosis. *Mol. Cells* **13**, 194–201.

Kurashige, M., Okimasu, E., Inoue, M., and Utsumi, K. (1990) Inhibition of oxidative injury of biological membranes by astaxanthin. *Physiol. Chem. Phys. Med. NMR* **22**, 27–38.

Leach, M., Hamilton, L. C., Olbrich, A., Wray, G. M., and Thiemermann, C. (1998) Effects of inhibitors of the activity of cyclooxygenase-2 on the hypotension and multiple organ dysfunction caused by endotoxin: a comparison with dexamethasone. *Br. J. Pharmacol.* **124**, 586–592.

Li, Q. and Verma, I. M. (2002) NF-κB regulation in the immune system. *Nat. Rev. Immunol.* **2,** 725–734.

Makarov, S. S. (2000) NF-κB as a therapeutic target in chronic inflammation: recent advances. *Mol. Med. Today* **6**, 441–448.

Ma, Q. and Kinneer, K. (2002) Chemoprotection by phenolic

antioxidants. Inhibition of tumor necrosis factor-α induction in macrophages. *J. Biol. Chem.* **277**, 2477–2484.

Mathison, J. C., Wolfson, E., and Ulevitch, R. J. (1988) Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. *J. Clin. Invest.* **81**, 1925–1937.

Mino, T., Sugiyama, E., Taki, H., Kuroda, A., Yamashita, N., Maruyama, M., and Kobayashi, M. (1998) Interleukin-1α and tumor necrosis factor-α synergistically stimulate prostaglandin E_2-dependent production of interleukin-11 in rheumatoid synovial fibroblasts. *Arthritis. Rheum.* **11**, 2004–2013.

Mortensen, A., Skibsted, L. H., Sampson, J., Rice-Evans, C., and Everett, S. A. (1997) Comparative mechanisms and rates of free radical scavenging by carotenoid antioxidants. *FEBS Lett.* **418**, 91–97.

Neuzil, J., Schroder, A., von Hundelshausen, P., Zernecke, A., Weber, T., Gellert, N., and Weber, C. (2001) Inhibition of inflammatory endothelial responses by a pathway involving caspase activation and p65 cleavage. *Biochemistry* **40**, 4686–4692.

Pahan, K., Sheikh, F. G., Namboodiri, A. M., and Singh, I. (1998) N-acetyl cysteine inhibits induction of NO production by endotoxin or cytokine stimulated rat peritoneal macrophages, C6 glial cells and astrocytes. *Free Radical Biol. Med.* **24**, 39–48.

Pan, M. H., Lin-Shiau, S. Y., Ho, C. T., Lin, J. H., and Lin, J. K. (2000) Suppression of lipopolysaccharide-induced nuclear factor-κB activity by theaflavin-3,3′-digallate from black tea and other polyphenols through down-regulation of IκB kinase activity in macrophages. *Biochem. Pharmacol.* **59**, 357–367.

Piaggio, E., Sanceau, J., Revelli, S., Bottasso, O., Wietzerbin, J., and Serra, E. (2001) Trypanocidal drug benznidazole impairs lipopolysaccharide induction of macrophage nitric oxide synthase gene transcription through inhibition of NF-κB activation. *J. Immunol.* **167**, 3422–3426.

Pinheiro, R. M. and Calixto, J. B. (2002) Effect of the selective COX-2 inhibitors, celecoxib and rofecoxib in rat acute models of inflammation. *Inflamm Res.* **51**, 603–610.

Ritchlin, C. T., Haas-Smith, S. A., Li, P., Hicks, D. G., and Schwarz, E. M. (2003) Mechanisms of TNF-α- and RANKL-mediated osteoclastogenesis and bone resorption in psoriatic arthritis. *J. Clin. Invest.* **111**, 821–831.

Sanlioglu, S., Williams, C. M., Samavati, L., Butler, N. S., Wang, G., McCray, P. B., Jr Ritchie, T. C., Hunninghake, G. W., Zandi, E., and Engelhardt, J. F. (2001) Lipopolysaccharide induces Rac1-dependent reactive oxygen species formation and coordinates tumor necrosis factor-α secretion through IKK regulation of NF-κB. *J. Biol. Chem.* **276**, 30188–30198.

Simons, R. K., Junger, W. G., Loomis, W. H., and Hoyt, D. B. (1996) Acute lung injury in endotoxemic rats is associated with sustained circulating IL-6 levels and intrapulmonary CINC activity and neutrophil recruitment--role of circulating TNF-α and IL-β? *Shock* **6**, 39–45.

Stefanovic-Racic, M., Meyers, K., Meschter, C., Coffey, J. W., Hoffman, R. A., and Evans, C. H. (1994) N-monomethyl arginine, an inhibitor of nitric oxide synthase, suppresses the development of adjuvant arthritis in rats. *Arthritis. Rheum.* **37**, 1062–1069.

Thornberry, N. A., Bull, H. G., Calaycay, J. R., Chapman, K. T., Howard, A. D., Kostura, M. J., Miller, D. K., Molineaux, S. M., Weidner, J. R., and Aunins, J., *et al.* (1992) A novel heterodimeric cysteine protease is required for interleukin-1β processing in monocytes. *Nature* **356**, 768–774.

Xie, Q. (1997) A novel lipopolysaccharide-response element contributes to induction of nitric oxide synthase. *J. Biol. Chem.* **272**, 14867–14872.

Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994) Role of transcription factor NF-κB/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**, 4705–4708.

Yokoseki, O., Suzuki, J., Kitabayashi, H., Watanabe, N., Wada, Y., Aoki, M., Morishita, R., Kaneda, Y., Ogihara, T., Futamatsu, H., Kobayashi, Y., and Isobe, M. (2001) cis Element decoy against nuclear factor-κB attenuates development of experimental autoimmune myocarditis in rats. *Circ. Res.* **89**, 899–906.