

Effects of Astaxanthin on Lipopolysaccharide-Induced Inflammation In Vitro and In Vivo

Kazuhiko Ohgami,¹ Kenji Shiratori,¹ Satoshi Kotake,¹ Tomomi Nishida,² Nobuhisa Mizuki,² Kazunaga Yazawa,³ and Shigeaki Ohno¹

PURPOSE. Astaxanthin (AST) is a carotenoid that is found in marine animals and vegetables. Several previous studies have demonstrated that AST exhibits a wide variety of biological activities including antioxidant, antitumor, and anti-*Helicobacter pylori* effects. In this study, attention was focused on the antioxidant effect of AST. The object of the present study was to investigate the efficacy of AST in endotoxin-induced uveitis (EIU) in rats. In addition, the effect of AST on endotoxin-induced nitric oxide (NO), prostaglandin E2 (PGE2), and tumor necrosis factor (TNF)- α production in a mouse macrophage cell line (RAW 264.7) was studied in vitro.

METHODS. EIU was induced in male Lewis rats by a footpad injection of lipopolysaccharide (LPS). AST or prednisolone was administered intravenously at 30 minutes before, at the same time as, or at 30 minutes after LPS treatment. The number of infiltrating cells and protein concentration in the aqueous humor collected at 24 hours after LPS treatment was determined. RAW 264.7 cells were pretreated with various concentrations of AST for 24 hours and subsequently stimulated with 10 μ g/mL of LPS for 24 hours. The levels of PGE2, TNF- α , and NO production were determined in vivo and in vitro.

RESULTS. AST suppressed the development of EIU in a dose-dependent fashion. The anti-inflammatory effect of 100 mg/kg AST was as strong as that of 10 mg/kg prednisolone. AST also decreased production of NO, activity of inducible nitric oxide synthase (NOS), and production of PGE2 and TNF- α in RAW264.7 cells in vitro in a dose-dependent manner.

CONCLUSIONS. This study suggests that AST has a dose-dependent ocular anti-inflammatory effect, by the suppression of NO, PGE2, and TNF- α production, through directly blocking NOS enzyme activity. (*Invest Ophthalmol Vis Sci.* 2003;44:2694-2701) DOI:10.1167/iov.02-0822

From the ¹Department of Ophthalmology and Visual Sciences, Hokkaido University Graduate School of Medicine, Sapporo, Japan; the ²Department of Ophthalmology, Yokohama City University School of Medicine, Yokohama, Japan; and the ³Laboratory of Nutraceuticals and Functional Foods Science, Graduate School of Fisheries Science, Tokyo University of Fisheries, Tokyo, Japan.

Supported in part by a grant for Researches on Sensory and Communicative Disorders, Ministry of Health, Labour and Welfare, Japan, and in part by grants-in-aid for Scientific Research, Ministry of Education, Culture and Science, Japan.

Submitted for publication August 14, 2002; revised December 16, 2002; accepted January 6, 2003.

Disclosure: K. Ohgami, None; K. Shiratori, None; S. Kotake, None; T. Nishida, None; N. Mizuki, None; K. Yazawa, None; S. Ohno, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Kazuhiko Ohgami, Department of Ophthalmology and Visual Sciences, Hokkaido University School of Medicine, N17 W5, Kita-ku, Sapporo, 060-8638 Japan; kohgami@med.hokudai.ac.jp.

Endotoxin-induced uveitis (EIU) is an animal model of acute anterior segment intraocular inflammation that is induced by an injection of lipopolysaccharide (LPS) or lipoteichoic acid.¹⁻⁵ In this model, LPS may directly activate the vascular endothelium, macrophages, and other cells. Cellular infiltration and protein extravasation in the anterior part of the eye reaches a maximum at 20 to 24 hours after LPS treatment.⁶ In the vitreous and retina, cellular infiltration reaches a maximum at 48 hours after LPS treatment.⁷ Exposure to outer bacterial toxins such as LPS stimulates cellular inflammatory responses and releases factors, such as nitric oxide (NO),^{8,9} prostaglandin E2 (PGE2),¹⁰⁻¹² cytokines including tumor necrosis factor (TNF)- α ,¹³ and eicosanoid mediators, that promote inflammatory responses. In particular, increased plasma TNF- α levels during endotoxemia and Gram-negative sepsis contributes to lethality as suggested by the protective effects afforded by TNF- α neutralizing antibodies.¹⁴

Three types of nitric oxide synthase (NOS) isoforms have been identified in cells. Endothelium NOS and neural NOS are both constitutive NOS. The NO produced by constitutive NOS acts to maintain normal vasoactivity in an active state of vasodilation through a Ca⁺²-dependent pathway and acts as a neurotransmitter in neuron signal transmission. NOS in macrophages and hepatocytes is inducible (iNOS) and its activation is Ca⁺² independent. After exposure to endogenous and exogenous stimulators, iNOS is induced quantitatively in various cells, such as macrophages, smooth muscle cells, and hepatocytes to trigger several disadvantageous cellular responses and cause inflammation.¹⁵ Therefore, NO production induced by iNOS may reflect the degree of inflammation. Thus, we can evaluate the effect of an anti-inflammatory drug by measuring NO levels. *N*-nitro-L-arginine methyl ester (L-NAME) showed effective inhibitory activity in LPS-induced NO production by directly blocking the NOS enzyme activities.¹⁶⁻¹⁸

Carotenoids are a family of more than 700 natural lipid-soluble pigments that are only produced by phytoplankton, algae, plants, and a limited number of fungi and bacteria. Astaxanthin (AST) is one of the most common carotenoids and is found in the red pigment of crustacean shells (crabs, shrimps, for example), salmon, and the asteroidean.¹⁹ The chemical structure of AST is shown in Figure 1. The AST of the xanthophylls group possesses no provitamin A activity in contrast to α -carotene. The AST content of salmon is 1.7 to 2.6 mg/100 g. Several previous studies have demonstrated that AST exhibits a wide variety of biological activities, including antioxidant,²⁰ antitumor,²¹ and anti-*Helicobacter pylori* effects.²² The antioxidant activities of carotenoids are related to the stability of formed free radicals after they react with active free radicals. As a result, AST could suppress the production of NO. Several in vitro and in vivo studies have indicated that L-NAME may also function as an anti-inflammatory mediator.^{16,18} There has been no report on the effects of AST on LPS-induced inflammation in vivo or in vitro.

In the present study, we investigated the influence of AST on LPS-induced uveitis in rats. In addition, we also investigated the NO production in RAW 264.7 cells treated with AST in vitro to clarify the anti-inflammatory effect. Furthermore, in

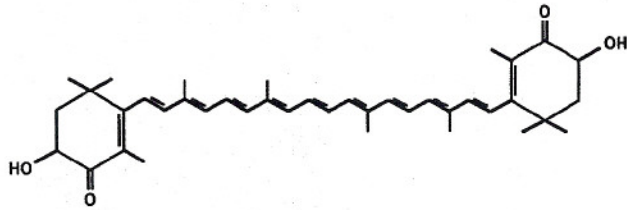


FIGURE 1. Chemical structure of astaxanthin.

vivo, the anti-inflammatory potency of AST was compared with that of prednisolone. Also, in vitro, the inhibitory effect of AST on NO production was compared with that of L-NAME, a known inhibitor of NO production.

MATERIALS AND METHODS

Animals Groups and EIU Induction

Eight-week-old male Lewis rats, weighing 180 to 220 g, were used. EIU was induced by injection into one footpad of 200 μ g of LPS from *Salmonella typhimurium* (Sigma, St. Louis, MO) that had been diluted in 0.2 mL of sterile water.

The rats were injected intravenously with 1, 10, or 100 mg/kg AST (Sigma) or 10 mg/kg prednisolone (Sigma) in 1 mL/kg 60% polyethylene glycol (Wako, Osaka, Japan). Each compound was administered a three time points: simultaneously and 30 minutes before and after the LPS injection. For the LPS group, 60% polyethylene glycol was administered intravenously on the same schedule as the AST group.

Animals were handled and cared for according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Number of Infiltrating Cells and Protein Concentration in Aqueous Humor

Suzuma reported that cellular infiltration in the aqueous humor reached a maximum level at 24 hours after LPS treatment in this model.¹ The number of cells infiltrating the aqueous humor and the aqueous humor protein concentration were used as indicators of the degree of anterior inflammation.

At 24 hours after LPS injection, rats were killed and the aqueous humor was collected immediately. Briefly, the aqueous humor was collected from both eyes by an anterior chamber puncture (15–20 μ L/rat) with a 30-gauge needle under a surgical microscope. For cell counting, the aqueous humor sample was suspended in an equal amount of Türk stain solution, and the cells were counted, using a hemocytometer under a light microscope. The number of cells per field (an equivalent of 0.1 mL) was manually counted, and the number of cells per microliter was obtained by averaging the results of four fields from each sample. The total protein concentration in the aqueous

humor samples was measured with a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). The aqueous humor samples were stored in ice water until testing, and cell counts, and total protein concentrations were measured on the day of sample collection.

Determination of NO Levels in Aqueous Humor

The total level of nitrate plus nitrite in the aqueous humor was measured by using a total nitrite colorimetric assay kit (Oxis International, Portland, OR), according to the manufacturer's instruction. The aqueous humor from both eyes of a rat was diluted up to 50 μ L and used for one assay. The NO assay was repeated once or twice.

Levels of TNF- α and PGE2 in Aqueous Humor

The levels of TNF- α and PGE2 in the aqueous humor obtained from rats with EIU were assessed with a commercially available ELISA kit (R&D Systems, Minneapolis, MN), according to the manufacturer's instructions. The ELISA assay was performed in duplicate. The data represent the mean of eight determinations \pm SD.

Cell Culture and LPS Stimulation

RAW 264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in RPMI-1640 medium supplemented with 2 mM glutamine, antibiotics (100 U/mL each of penicillin and streptomycin), and 10% heat-inactivated fetal bovine serum (Gibco-BRL, Grand Island, NY) and maintained at 37°C in a humidified incubator containing 5% CO₂. RAW 264.7 cells were seeded onto a 24-well plate (5 \times 10⁴ cells/well) for experiments. The cells were pretreated with 2.5, 5, 12.5, and 25 μ M AST for 24 hours and subsequently stimulated with 10 μ g/mL of LPS from *S. typhimurium* for 24 hours, unless otherwise stated.

AST was dissolved in 0.01% dimethyl sulfoxide (DMSO). For the control group, RAW cells were cultured with 0.01% DMSO alone. The effects of AST were compared with those of L-NAME, a known inhibitor of NO production.

Determination of Nitrite Concentration in Medium

NO was measured as its end product, nitrite, by using Griess reagent (Sigma), as described elsewhere.²³ The culture supernatant (100 μ L) was mixed with 100 μ L of Griess reagent for 10 minutes, and the absorbance at 550 nm was measured in a microplate reader. The concentration of nitrite in the samples was determined with reference to a sodium nitrite standard curve. The data represent the mean of eight determinations \pm SD.

Determination of iNOS Enzyme Activity

A NO synthase assay kit (Calbiochem-Novabiochem, San Diego, CA) was used to determine iNOS enzyme activity. AST- or L-NAME-pre-

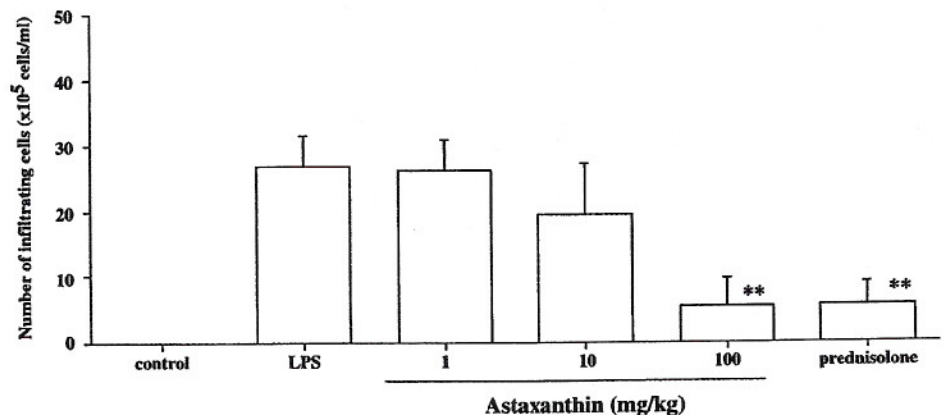


FIGURE 2. Effect of astaxanthin on cellular infiltration in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Cell numbers are expressed as the mean \pm SD ($n = 8$). No infiltrating cells were detected in the aqueous humor from rats without LPS (control group). The dose of prednisolone was 10 mg/kg. ** $P < 0.01$, compared with the control group.

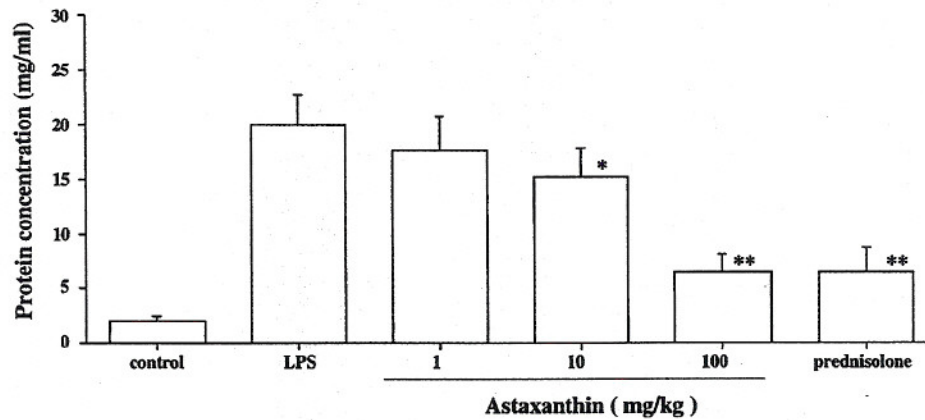


FIGURE 3. Effect of astaxanthin on protein concentration in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each value represents the mean \pm SD ($n = 8$). The dose of prednisolone was 10 mg/kg. * $P < 0.05$ and ** $P < 0.01$, compared with the LPS group.

treated cells were incubated with LPS (10 μ g/mL) for 24 hours. The cells were washed three times with PBS, scraped into cold PBS, and centrifuged at 500g for 10 minutes at 4°C. The cell pellet was resuspended in 0.4 mL of hypotonic buffer that contained 10 mM HEPES, 10 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM EDTA (pH 7.4). The total protein concentrations in solution samples were measured using a BCA protein assay kit (Pierce).

Levels of TNF- α and PGE2

The levels of TNF- α and PGE2 in the medium were measured by ELISA (R&D Systems) according to the manufacturer's instruction. The ELISA assay was performed in duplicate.

Cell Viability

For determination of cell viability, 50 mg/mL of methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (Sigma) was added to 1 mL of cell suspension (5.3×10^4 cells/mL in 24-well plates) for 24 hours, and the MTT formazan formed was dissolved in acidic-2-propanol. Optical density was measured with a plate reader used at 590 nm. The optical density of the Formosan formed by untreated cells was taken as 100%.

Statistical Analysis

The values were expressed as mean \pm SD. A Student's unpaired *t*-test was used to assess the statistical significance of differences. $P < 0.05$ was regarded as significant.

RESULTS

Number of Inflammatory Cells in Aqueous Humor

In the LPS group, the number of inflammatory cells that infiltrated the aqueous humor 24 hours after LPS treatment was $26.9 \pm 4.7 \times 10^5$ cells/mL (mean \pm SD, $n = 7$). The group treated with 100 mg/kg of AST showed a significantly reduced number of inflammatory cells ($5.4 \pm 4.3 \times 10^5$ cells/mL) compared with the control group ($P < 0.01$, Fig. 2). The effect of 100 mg/kg AST on the number of cells in the aqueous humor was almost the same as that for 10 mg/kg prednisolone ($5.7 \pm 4.3 \times 10^5$ cells/mL, Fig. 2). Treatment with 10 mg/kg of AST showed a mild reduction in number of cells ($19.6 \pm 7.7 \times 10^5$ cells/mL), and there was no significant difference from the LPS group. No infiltrating cells were detected in the aqueous humor from rats without LPS (control group).

Aqueous Humor Protein Concentration

The protein concentration in the aqueous humor of rats without LPS (control group) was 2.0 ± 0.5 mg/mL and in that of rats with LPS was 20.1 ± 2.8 mg/mL. The protein concentrations in the groups treated with 10 mg/kg and 100 mg/kg AST were significantly lower than that in the LPS group (10 mg/kg: 15.3 ± 2.6 mg/mL, $P < 0.05$; 100 mg/kg: 6.2 ± 1.7 mg/mL, $P < 0.01$, Fig. 3). The reduction of protein concentration in the AST 100-mg/kg group was almost the same as that in the pred-

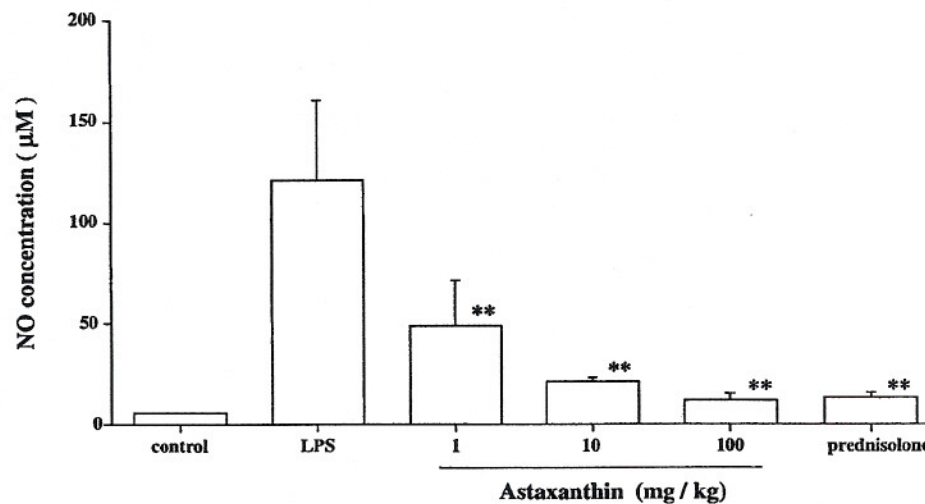


FIGURE 4. Effect of astaxanthin on NO levels in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each value represents mean \pm SD ($n = 8$). The dose of prednisolone was 10 mg/kg. ** $P < 0.01$, compared with the LPS group.

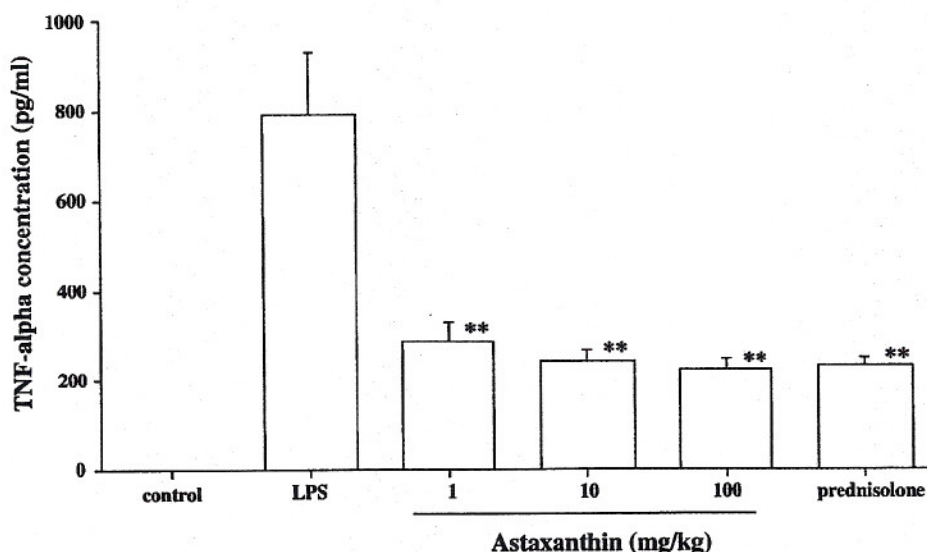


FIGURE 5. Effect of astaxanthin on TNF- α concentrations in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each value represents the mean \pm SD ($n = 8$). The dose of prednisolone was 10 mg/kg. ** $P < 0.01$, compared with the LPS group.

nisolone group (6.5 ± 2.3 mg/mL, Fig. 3). Treatment with 1 mg/kg of AST produced only a mild reduction in protein concentration (17.7 ± 3.2 mg/mL), and there was no significant difference from level in the LPS group.

Levels of NO in EIU

The NO production in the LPS group was 122.0 ± 39.1 μ M ($n = 8$). The NO production in the groups treated with AST differed significantly from that in the LPS control group, in a dose-dependent manner. (1 mg/kg: 49.1 ± 23.2 μ M, $P < 0.01$; 10 mg/kg: 21.1 ± 2.3 mg/mL, $P < 0.01$; 100 mg/kg: 12.1 ± 2.9 μ M, $P < 0.01$; Fig. 4). The reduction in NO production in the AST 100-mg/kg group was almost the same as that in the prednisolone group (13.3 ± 3.2 μ M, Fig. 4). The NO concentration in the aqueous humor of rats without LPS (control group) was 5.7 ± 0.4 μ M.

Levels of TNF- α in Aqueous Humor

In the LPS group, the TNF- α concentration in the aqueous humor was 792.3 ± 140.1 pg/mL. The TNF- α concentration in

the AST group showed a tendency to decrease in a dose-dependent fashion. Treatment with AST significantly reduced the TNF- α concentration compared with that of the LPS group (1 mg/kg: 287.6 ± 42.8 pg/mL, $P < 0.01$; 10 mg/kg: 241.0 ± 27.5 pg/mL, $P < 0.01$; 100 mg/kg: 223.1 ± 24.3 pg/mL, $P < 0.01$, Fig. 5). The reduction in TNF- α concentration in the AST 100-mg/kg group was almost the same as that in the prednisolone group (233.0 ± 2.3 pg/mL, Fig. 5). However, TNF- α was not detected in the aqueous humor of the control rats.

PGE2 Concentration in EIU

The PGE2 concentration in the LPS control group was 10.2 ± 1.7 ng/mL. Treatment with AST significantly reduced PGE2 concentration, compared with that in the LPS group (1 mg/kg: 2.6 ± 0.9 ng/mL, $P < 0.01$; 10 mg/kg: 1.2 ± 0.4 ng/mL, $P < 0.01$; 100 mg/kg: 0.8 ± 0.3 ng/mL, $P < 0.01$; Fig. 6). The reduction in PGE2 concentration in the AST 100-mg/kg group was almost the same as that in the prednisolone group (1.0 ± 0.3 ng/mL, Fig. 6). PGE2 was not detected in the aqueous humor of the control rats.

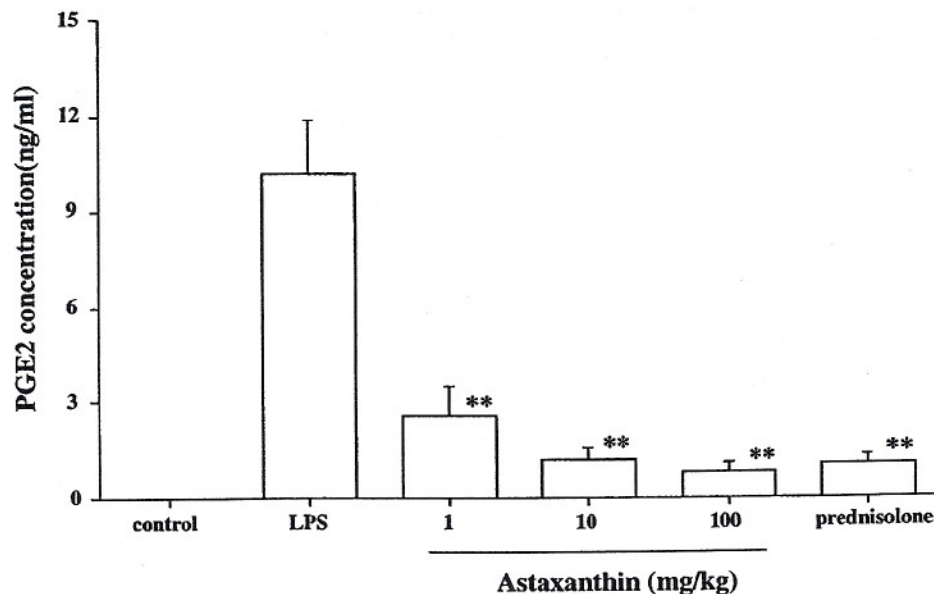


FIGURE 6. Effect of astaxanthin on PGE2 concentrations in the aqueous humor. The aqueous humor was collected 24 hours after LPS treatment. Each value represents the mean \pm SD ($n = 8$). The dose of prednisolone was 10 mg/kg. ** $P < 0.01$, compared with the LPS group.

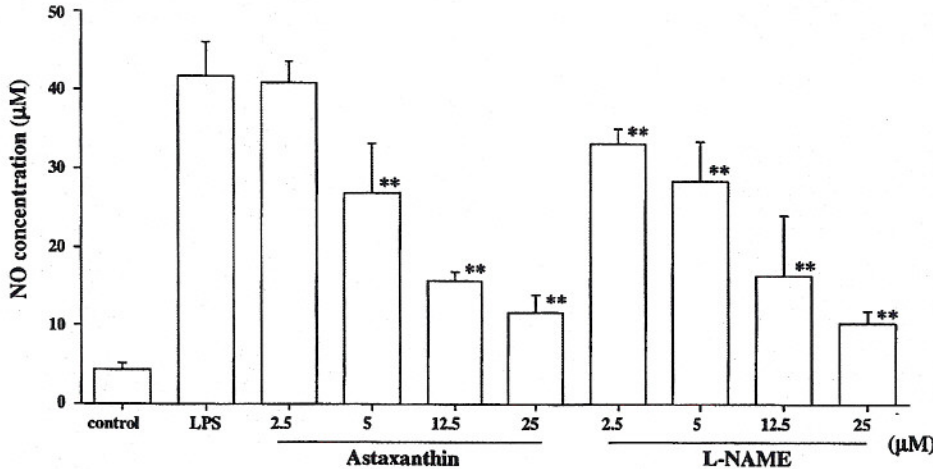


FIGURE 7. Effect of astaxanthin on NO production in LPS-induced RAW 264.7 cells. RAW cells were pretreated with various concentrations of astaxanthin and L-NAME for 24 hours. Astaxanthin- or L-NAME-pretreated RAW cells were incubated with LPS (10 µg/mL) for 24 hours. The nitrite concentration in the culture supernatants was determined for NO production. RAW cells in the normal group were cultured with 0.01% DMSO. Data are expressed as the mean ± SD ($n = 8$). ** $P < 0.01$, compared with the LPS group.

NO Production in RAW 264.7 Cells

The content of nitrite without LPS in RAW 264.7 cells (control group) was 4.2 ± 0.9 µM ($n = 8$). After the treatment with 10 µg/mL of LPS for 24 hours (LPS group), nitrite concentration in the medium increased substantially, by approximately 10-fold (41.7 ± 4.4 µM). Treatment with 2.5, 12.5, or 25 µM AST significantly reduced NO production compared with that in the LPS group (2.5 µM: 28.7 ± 6.5 µM, $P < 0.01$; 12.5 µM: 15.6 ± 1.4 µM, $P < 0.01$; 25 µM: 11.5 ± 2.5 µM, $P < 0.01$; Fig. 7). The effect of 25 µM AST on LPS-induced NO production in RAW 264.7 cells was almost the same as that of 25 µM L-NAME (10.3 ± 1.6 µM, Fig. 7). AST did not decrease cell viability in RAW 264.7 cells when these cells were incubated with 25 µM AST alone for 24 hours (data not shown).

iNOS Enzyme Activity

NOS enzyme activity in RAW 264.7 cells without stimulation was 0.54 ± 0.20 nmol/min per milligram protein ($n = 8$). After LPS stimulation, the NOS enzyme activity was approximately two times higher than in the normal group (1.23 ± 0.16 nmol/min per milligram protein). Enzyme activity showed a tendency to decrease in the AST-treated group in a dose-dependent manner. Treatment with AST significantly reduced iNOS activity compared with that in the LPS group (5 µM: 0.97 ± 0.28 nmol/min per milligram protein; $P < 0.05$, 12.5 µM: 0.88 ± 0.28 nmol/min per milligram protein, $P < 0.01$;

and 25 µM: 0.66 ± 0.22 nmol/min per milligram protein, $P < 0.01$, Fig. 8). The effect of 25 µM AST on LPS-induced NO production in RAW 264.7 cells was almost the same as that of 25 µM L-NAME (10.3 ± 1.6 µM).

TNF-α Concentration

The TNF-α concentration induced by LPS was approximately 10 times higher than in the normal group. The TNF-α concentration in the AST group showed a tendency to decrease in a dose-dependent manner. Treatment with AST significantly reduced TNF-α concentration compared with that in the LPS group (5 µM: 893.0 ± 95.0 pg/mL, $P < 0.05$; 12.5 µM: 501.8 ± 86.7 pg/mL, $P < 0.01$; and 25 µM: 375.3 ± 35.9 pg/mL, $P < 0.01$; Fig. 9). Treatment with L-NAME significantly reduced TNF-α levels compared with those of the LPS group. However, the TNF-α levels of the 25-µM AST group was significantly lower than those of the 25-µM L-NAME group (AST; 375.3 ± 35.9 pg/mL versus L-NAME; 787.0 ± 117.0 pg/mL, $P < 0.01$, Fig. 9).

PGE2 Concentration

The PGE2 concentration induced by LPS was approximately four times higher than in the normal group. The PGE2 concentration in the AST group showed a tendency to decrease in conjunction with decreases in AST concentrations. Treatment with AST significantly reduced PGE2 concentration compared with that in the LPS group (12.5 µM: 4529.4 ± 598.2 pg/mL,

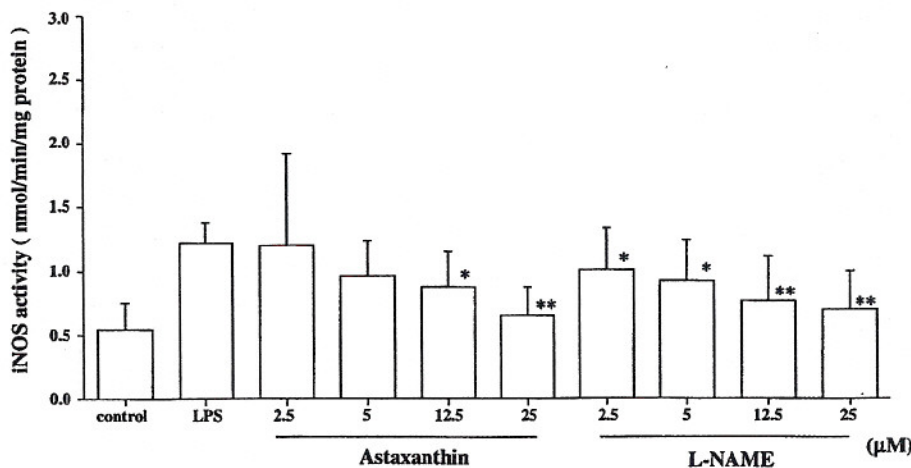


FIGURE 8. Effect of astaxanthin on NOS activity in LPS-induced RAW 264.7 cells. RAW cells were pretreated with various concentrations of astaxanthin and L-NAME for 24 hours. Astaxanthin- or L-NAME-pretreated RAW cells were incubated with LPS (10 µg/mL) for 24 hours. RAW cells in the normal group were cultured with 0.01% DMSO. Data are expressed as the mean ± SD ($n = 8$). * $P < 0.05$ and ** $P < 0.01$, compared with the LPS group.

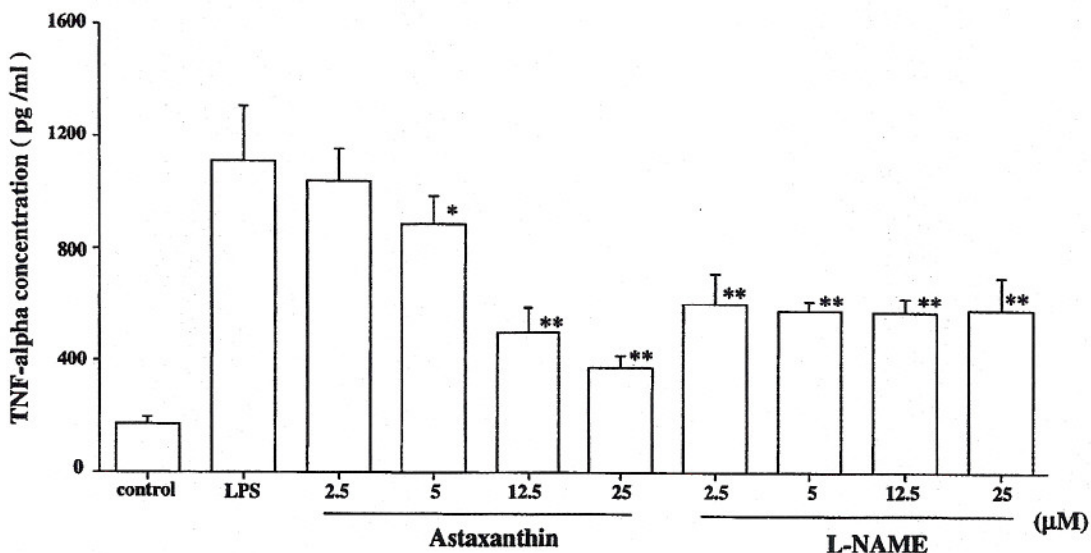


FIGURE 9. Effect of astaxanthin on TNF- α levels in LPS-infected RAW 264.7 cells. RAW cells were pretreated with various concentrations of astaxanthin and L-NAME for 24 hours. Astaxanthin- or L-NAME-pretreated RAW cells were incubated with LPS (10 μ g/mL) for 24 hours. RAW cells of normal group were cultured with 0.01% DMSO. Data are expressed as the mean \pm SD ($n = 8$). * $P < 0.05$ and ** $P < 0.01$, compared with the LPS group.

$P < 0.01$; and 25 μ M: 3797.7 ± 401.7 pg/mL, $P < 0.01$; Fig. 10). Treatment with L-NAME showed mild PGE2 concentration reduction and there were no significant differences from the LPS group.

DISCUSSION

The results of this study indicate that AST suppresses the development of EIU in a dose-dependent fashion. In particular, the ocular anti-inflammatory effect of 100 mg/kg of AST was as strong as that of a 10-mg/kg dose of prednisolone.

Carotenoids are known to take part in protecting marine animals against damage from free radicals and singlet oxygen reactive species.²² AST has a strong quenching capability

against damage from singlet oxygen in vitro²³—80 times stronger than α -tocopherol and twice as strong as β -carotene.^{22,23} It is understandable why AST has a strong singlet oxygen-quenching capability, considering its molecular structure. The reactivity to other molecular oxygen decreases, because singlet-oxygen-associated, carbon-centered radicals of AST can form more stable resonance structures by the attachment of the carbonyl group and the hydroxyl group to the β -ionone ring of AST.²³ AST can remove the chain carrying lipid peroxy radicals in the liposomal suspension more efficiently than β -carotene but less efficiently than α -tocopherol, because the hydrogen bonds by the carbonyl group in the β -ionone ring of AST and hydrophobic association by the polyene chain allows AST to fit in the membrane phospholipid structure well.²⁴⁻²⁷

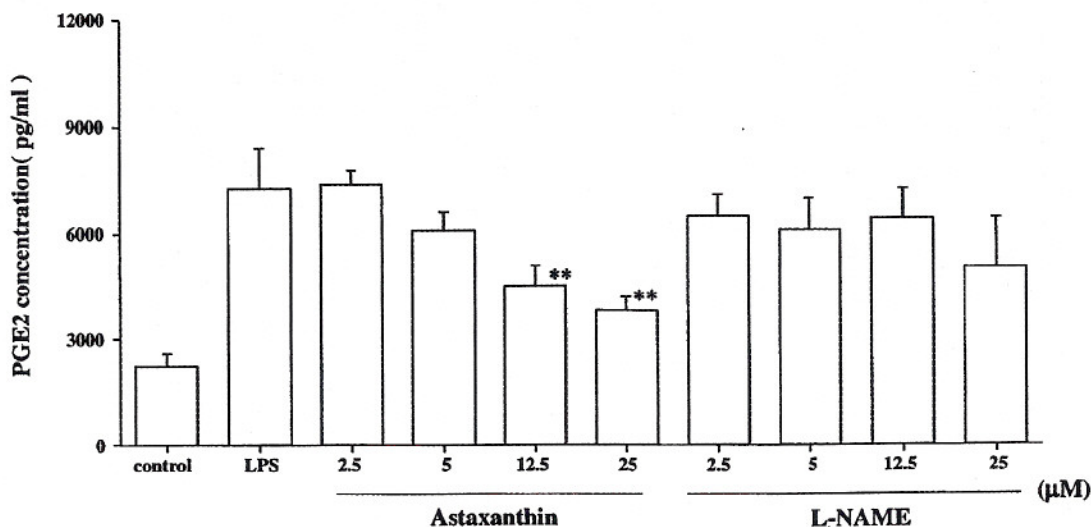


FIGURE 10. Effect of astaxanthin on PGE2 concentrations in LPS-infected RAW 264.7 cells. RAW cells were pretreated with various concentrations of astaxanthin and L-NAME for 24 hours. Astaxanthin- or L-NAME-pretreated RAW 264.7 cells were incubated with LPS (10 μ g/mL) for 24 hours. RAW 264.7 cells of the normal group were cultured with 0.01% DMSO. Data are expressed as the mean \pm SD ($n = 8$). ** $P < 0.01$, compared with the LPS group.

To elucidate the anti-inflammatory mechanism of AST, we focused our attention on the antioxidant effect of AST and measured the concentration of NO in the aqueous humor in vivo. AST suppressed the NO production in the aqueous humor in a dose-dependent manner. Also, we investigated the effect of AST on LPS-induced NO production and PGE2 and TNF- α levels in the RAW 264.7 macrophage cell, with L-NAME as a positive control. AST decreased NO production and iNOS enzyme activity in a dose-dependent manner, thus agreeing with the results of the in vivo experiment. These results demonstrate that AST suppresses NO production by directly inhibiting the NOS enzyme activity similar to the NOS inhibitor L-NAME. Large amounts of NO production induced by bacterial lipopolysaccharide or cytokines play an important role in endotoxemia and inflammatory conditions.¹⁰ Therefore, we propose that AST, which inhibits NO production through inhibiting iNOS enzyme activity, has beneficial therapeutic effects in the treatment of inflammation. Our results indicate that the compound, even at the concentration of 25 μ M, did not change cell viability. Therefore, inhibition of LPS-induced NO production by AST was not the result of its cytotoxicity on the cells.

TNF- α is a pleiotropic cytokine produced principally by activated macrophages and monocytes and also has an major role in the nonspecific resistance against various infectious agents.^{28,29} The results of the present study indicate that AST decreased TNF- α concentration in a dose-dependent manner, both in vivo and in vitro. The results of TNF- α inhibition by AST correspond to the results of inhibited NOS activity and decreased NO production, with the application of AST.

The mechanism of the NO-induced suppression of TNF synthesis is not known. A potential link is PGE2. It has been reported recently that NO activates cyclooxygenase enzymes and thereby leads to a marked increase in PGE2 production.^{30,31} A suppressive effect of PGE2 on TNF synthesis through elevated cAMP levels has been convincingly demonstrated.^{28,31-33} In the present study, AST suppressed the levels of LPS-induced PGE2 and TNF- α in a dose-dependent manner in vivo and in vitro. Our results support the argument for a regulatory role of NO on TNF production in the pathophysiology of EIU.

In summary, in the current study AST had a dose-dependent anti-inflammatory effect on EIU. In particular, the ocular anti-inflammatory effect of 100 mg/kg of AST was as strong as that of 10 mg/kg prednisolone. A possible mechanism for the ocular anti-inflammatory effect of AST is the suppression of production of NO, PGE2, and TNF- α by directly blocking NOS enzyme activity. These results suggest that AST may be a promising agent for the treatment of ocular inflammation.

References

- Suzuma I, Mandai M, Suzuma K, Ishida K, Tojo SJ, Honda Y. Contribution of E-selectin to cellular infiltration during endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 1998;39:1620-1630.
- Hikita N, Chan CC, Whitcup SM, Nussenblatt RB, Mochizuki M. Effects of topical FK506 on endotoxin-induced uveitis (EIU) in the Lewis rat. *Curr Eye Res*. 1995;14:209-214.
- Bhattacharjee P, Williams RN, Eakins KE. An evaluation of ocular inflammation following the injection of bacterial endotoxin into the rat foot pad. *Invest Ophthalmol Vis Sci*. 1983;24:196-202.
- Baatz H, Tonessen B, Prada J, Pleyer U. Thalidomide inhibits leukocyte-endothelium interaction in endotoxin-induced uveitis. *Ophthalmic Res*. 2001;33:256-263.
- Ohta K, Nakayama K, Kurokawa T, Kikuchi T, Yoshimura N. Inhibitory effects of pyrrolidine dithiocarbamate on endotoxin-induced uveitis in Lewis rats. *Invest Ophthalmol Vis Sci*. 2002;43:744-750.
- Rosenbaum JT, McDevitt HO, Guss RB, Egbert PR. Endotoxin-induced uveitis in rats as a model for human disease. *Nature*. 1980;286:611-613.
- Miyamoto K, Ogura Y, Hamada M, Nishiwaki H, Hiroshiba N, Honda Y. In vivo quantification of leukocyte behavior in the retina during endotoxin-induced uveitis. *Invest Ophthalmol Vis Sci*. 1996;37:2708-2715.
- Chen YC, Shen SC, Lee WR, Hou WC, Yang LL, Lee TJ. Inhibition of nitric oxide synthase inhibitors and lipopolysaccharide induced inducible NOS and cyclooxygenase-2 gene expressions by rutin, quercetin, and quercetin pentaacetate in RAW 264.7 macrophages. *J Cell Biochem*. 2001;82:537-548.
- Boujedaini N, Liu J, Thuillez C, Cazin L, Mensah-Nyagan AG. In vivo regulation of vasomotoricity by nitric oxide and prostanoids during gestation. *Eur J Pharmacol*. 2001;427:143-149.
- Bellot JL, Palmero M, Garcia-Cabanes C, Espi R, Hariton C, Orts A. Additive effect of nitric oxide and prostaglandin-E2 synthesis inhibitors in endotoxin-induced uveitis in the rabbit. *Inflamm Res*. 1996;45:203-208.
- Murakami A, Nakamura Y, Tanaka T, et al. Suppression by citrus auraptene of phorbol ester- and endotoxin-induced inflammatory responses: role of attenuation of leukocyte activation. *Carcinogenesis*. 2000;21:1843-1850.
- Hoekzema R, Verhagen C, van Haren M, Kijlstra A. Endotoxin-induced uveitis in the rat: the significance of intraocular interleukin-6. *Invest Ophthalmol Vis Sci*. 1992;33:532-539.
- Tracey KJ, Cerami A. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu Rev Med*. 1994;45:491-503.
- Tracey KJ, Fong Y, Hesse DG, et al. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. *Nature*. 1987;330:662-664.
- Wadsworth TL, Koop DR. Effects of Ginkgo biloba extract (EGb 761) and quercetin on lipopolysaccharide-induced release of nitric oxide. *Chem Biol Interact*. 2001;137:43-58.
- Rahman MM, Bhoola KD, Elson CJ, Lemon M, Dieppe PA. Identification and functional importance of plasma kallikrein in the synovial fluids of patients with rheumatoid, psoriatic, and osteoarthritis. *Ann Rheum Dis*. 1995;54:345-350.
- Kluft C. Determination of prekallikrein in human plasma: optimal conditions for activating prekallikrein. *J Lab Clin Med*. 1978;91:83-95.
- Pastor C, Teisseire B, Vicaut E, Payen D. Effects of L-arginine and L-nitro-arginine treatment on blood pressure and cardiac output in a rabbit endotoxin shock model. *Crit Care Med*. 1994;22:465-469.
- Miki W, Yamaguchi K, Konosu S. Comparison of carotenoids in the ovaries of marine fish and shellfish. *Comp Biochem Physiol B*. 1982;71:7-11.
- Esterbauer H, Jurgens G, Quehenberger O, Koller E. Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. *J Lipid Res*. 1987;28:495-509.
- Tanaka T, Makita H, Ohnishi M, Mori H, Satoh K, Hara A. Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin. *Cancer Res*. 1995;55:4059-4064.
- Wang X, Willen R, Wadstrom T. Astaxanthin-rich algal meal and vitamin C inhibit Helicobacter pylori infection in BALB/cA mice. *Antimicrob Agents Chemother*. 2000;44:2452-2457.
- Sewer MB, Barclay TB, Morgan ET. Down-regulation of cytochrome P450 mRNAs and proteins in mice lacking a functional NOS2 gene. *Mol Pharmacol*. 1998;54:273-279.
- Palozza P, Krinsky NI. Antioxidant effects of carotenoids in vivo and in vitro: an overview. *Methods Enzymol*. 1992;213:403-420.
- Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys*. 1989;274:532-538.
- Lim BP, Nagao A, Terao J, Tanaka K, Suzuki T, Takama K. Antioxidant activity of xanthophylls on peroxyl radical-mediated phospholipid peroxidation. *Biochim Biophys Acta*. 1992;1126:178-184.
- Shibata A, Kiba Y, Akati N, Fukuzawa K, Terada H. Molecular characteristics of astaxanthin and beta-carotene in the phospholipid monolayer and their distributions in the phospholipid bilayer. *Chem Phys Lipids*. 2001;113:11-22.
- Eigler A, Moeller J, Endres S. Exogenous and endogenous nitric oxide attenuates tumor necrosis factor synthesis in the murine macrophage cell line RAW 264.7. *J Immunol*. 1995;154:4048-4054.

29. Friden BE, Runesson E, Hahlin M, Brannstrom M. Evidence for nitric oxide acting as a luteolytic factor in the human corpus luteum. *Mol Hum Reprod*. 2000;6:397-403.
30. Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P. Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci USA*. 1993;90:7240-7244.
31. Endres S, Fulle HJ, Sinha B, et al. Cyclic nucleotides differentially regulate the synthesis of tumour necrosis factor-alpha and interleukin-1 beta by human mononuclear cells. *Immunology*. 1991;72:56-60.
32. Eisenhut T, Sinha B, Grottrup-Wolfers E, Semmler J, Siess W, Endres S. Prostacyclin analogs suppress the synthesis of tumor necrosis factor-alpha in LPS-stimulated human peripheral blood mononuclear cells. *Immunopharmacology*. 1993;26:259-264.
33. Spengler RN, Spengler ML, Lincoln P, Remick DG, Strieter RM, Kunkel SL. Dynamics of dibutyryl cyclic AMP- and prostaglandin E2-mediated suppression of lipopolysaccharide-induced tumor necrosis factor alpha gene expression. *Infect Immun*. 1989;57:2837-2841.