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Effects of Astaxanthin on the Production of NO and the Expression of COX-2 and iNOS in LPS-Stimulated BV2 Microglial Cells

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Astaxanthin has shown antioxidant, antitumor, and anti-inflammatory activities; however, its molecular action and mechanism in the nervous system have yet to be elucidated. We examined the *in vitro* effects of astaxanthin on the production of nitric oxide (NO), as well as the expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) in lipopolysaccharide (LPS)-stimulated BV2 microglial cells. Astaxanthin inhibited the expression or formation of nitric oxide (NO), iNOS and COX-2 in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. Astaxanthin also suppressed the protein levels of iNOS and COX-2 in LPS-stimulated BV2 microglial cells. These results suggest that astaxanthin, probably due to its antioxidant activity, inhibits the production of inflammatory mediators by blocking iNOS and COX-2 activation or by the suppression of iNOS and COX-2 degradation.

Keywords: Astaxanthin, anti-inflammatory activity, BV2 microglial cell, Infalmmatory mediator

Astaxanthin is one of the naturally occurring carotenoids responsible for the pink-red pigmentation in a variety of living organisms. Astaxanthin is known to be a very strong natural antioxidant, and exhibits strong singlet oxygen/free-radical scavenging activity and protects cells against lipid peroxidation. Several studies have compared the antioxidant activity of astaxanthin with that of other antioxidants. Its ability to quench singlet oxygen is 500 times stronger than that of vitamin E and 40 times stronger than that of beta-carotene. Furthermore, astaxanthin prevents lipid peroxidation more effectively than beta-carotene, lutene, and zeaxanthin. The very strong antioxidant activity of astaxanthin is attributed to its many conjugated double bonds.

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Inflammation, a self-defensive reaction against various pathogenic stimuli, may become a harmful self-damaging process. Increasing evidence has linked chronic inflammation to a number of neurodegenerative disorders, including Alzheimer's disease (AD), Parkinson's disease (PD) [15], and multiple sclerosis (MS) [1, 3], as well as acquired immune deficiency syndrome (AIDS) dementia [8].

Astaxanthin reduced the inflammation caused by carrageenan injection in rats. Foot edema was induced by carrageenan injection, but the oral administration of astaxanthin reduced the degree of carrageenan-induced foot edema. Astaxanthin also reduced the inflammation induced by *Helicobacter pylori*. Astaxanthin inhibited the thymic weight reduction caused by restriction stress, and also inhibited lipid peroxidation in the liver and bowels in mice. After exposure to endogenous and exogenous stimulators, iNOS is quantitatively induced in various cell types, such as macrophages, smooth muscle cells, and hepatocytes, to trigger several disadvantageous cellular responses and cause inflammation [4, 18].

Microglia are the resident macrophages and immune surveillance cells of the CNS, and they play an important role in brain inflammatory immune and degenerative processes [14]. Although they form the first line of defense for the neural parenchyma, the uncontrolled activation of microglia may be directly toxic to neurons owing to the release of various substances, such as inflammatory cytokines (IL-1β, TNF-α, IL-6), NO, and PGE2, which mediate the inflammatory processes in the central nervous system [6, 16, 23].

NO and proinflammatory cytokines, such as IL-1β and TNF-α, have been implicated as important mediators in the process of inflammation. The activation of microglia induced by CNS injury or infection is associated with neurodegenerative disorders and the release of NO and subsequent releases of proinflammatory cytokines [2, 9]. Specifically, the high level of NO produced by iNOS has been defined as an indicator of cytotoxicity in inflammation and endotoxemia [19]. It has also been reported that

cytokines, such as IL-1β and TNF-α, are proinflammatory both *in vitro* and *in vivo* [7, 11]. These proinflammatory cytokines are known to activate the transcription of the COX-2 and iNOS genes [21, 22].

There has been no report on the effects of astaxanthin in LPS-stimulated BV2 microglial cells *in vitro* [10, 12]. The aim of the present study was to investigate the effects of astaxanthin on the expression of iNOS and COX-2 in LPS-stimulated BV2 microglial cells. We also investigated the production of NO in BV2 microglial cells treated with astaxanthin *in vitro* to clarify its anti-inflammatory effect.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) containing L-arginine (200 mg/l), fetal bovine serum (FBS), and other tissue culture reagents were purchased from Gibco (Grand Island, NY, U.S.A.). Lipopolysaccharide (LPS), Tween-20, bovine serum albumin (BSA), *p*-nitrophenyl phosphate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Poly (dI-dC) and RNase-free DNase were obtained from Promega (Madison, WI, U.S.A.), and monoclonal iNOS antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.). Polyclonal antibody for COX-2 was obtained from Santa Cruz Biotechnology (Santa Cruz, U.S.A.). The antibody against β-actin, peroxidase-labeled anti-rabbit immunoglobulin, was purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise indicated.

Cell Culture

The murine BV2 microglial cells were obtained from Choi (Konkuk University, Korea). The cells were maintained in DMEM supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C under 5% CO_2 [10, 12]. Cells were prepared in a 96-well plate at a concentration of 8×10^4. Cells were treated with various concentrations of astaxanthin (1 μM, 10 μM, and 100 μM) and LPS (100 ng/ml), either alone or with the indicated combinations. Astaxanthin was dissolved in 0.01% (v/v) dimethyl sulfoxide (DMSO). For the control group, BV2 microglial cells were cultured with 0.01% DMSO alone.

MTT Assay

An MTT assay was performed to measure the cytotoxicity of astaxanthin. BV2 microglial cells were treated with astaxanthin at concentrations of 1 μM, 10 μM, and 100 μM for 1 h [13] and then LPS was (100 ng/ml) added for 24 h. After the addition of 100 μl of MTT solution (5 mg/ml), the cells were incubated at 37°C for 4 h and formed formazan crystals resolved with 100 μl of DMSO. The absorbance was then determined at 540 nm using an ELISA reader. The optical density of the formazan formed by the untreated cells was taken as 100%.

Measurement of Nitric Oxide

In order to determine the effects of astaxanthin on NO synthesis, the NO level in cell cultures was measured using a Griess reaction, as described previously [17, 20]. Nitrite was taken as a measure of NO production. Cells were prepared in a 96-well plate at a concentration of 8×10^4. Astaxanthin, with or without LPS (100 ng/ml), was added to the culture medium of BV2 microglial cells for 24 h [18]. One hundred-μl aliquots of cell supernatants from each well were mixed with 100 μl of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4) in a 96-well microtiter plate reader and incubated for 10 min at 24°C. The absorbance at 540 nm was determined using a microplate reader (Bio-Tek Instruments). Fresh culture medium was used as a blank in all experiments. The amount of nitrite in the samples was calculated using a sodium nitrite (Sigma) standard curve freshly prepared in culture medium.

RT-PCR

BV2 microglial cells were treated with 100 μM astaxanthin in a 6-well plate and incubated for 1 h. The cells were then treated with LPS alone or with the indicated combinations [5, 11]. After 4 h, the test medium was removed from the culture dishes, and the cells were washed with ice-cold PBS and scraped. The cells were harvested in a lysis buffer, and the harvested cells were added to 1 ml of Trizol reagent (BRL, MD, U.S.A.), homogenized, and incubated at 24°C for 2~3 min. Contaminating DNA was removed using RNase-free DNase (Promega, WI, U.S.A.). The cells were then centrifuged at 12,000 rpm for 15 min at 4°C, and the supernatant was removed. The remaining RNA pellet was added to 1 ml of 75% ethanol treated with diethyl pyrocarbonate (DEPC). For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was reverse-transcribed for 1 h at 37°C in a reaction mixture containing RNA, polyd(T) primer, random primer, and reverse transcriptase. Three μg of mRNA were 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 37°C for 1 h. The reaction was terminated by heating at 70°C for 15 min, and 3 μl of the cDNA mixture was then 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 units of *Taq* DNA polymerase, and 0.1 μM each of primer for iNOS and COX-2. The 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 at 51°C for 45 s, and extension at 72°C for 30 s for 35 cycles. The final extension was performed at 72°C for 10 min. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide. The following primers were used for PCR:

iNOS (F-5'-GTG TTC CAC CAG GAG ATG TTG); iNOS-R (5'-CTC CTG CCC ACT GAG TTC GTC), COX-2-F (5'-TGC ATG TGG CTG TGG ATG TCA TCA A); COX-2-R (5'-CAC TAA GAC AGA CCC GTC ATC TCC A), β-actin-F (5'-CTT TGA TGT CAC GCA CGA TTT C); β-actin-R (5'-GGG CCG CTC TAG GCA CCA A).

Western Blot Analysis

BV2 microglial cells were treated with astaxanthin for 1 h and then treated with LPS for 6 h. The cells were incubated with or without LPS in the presence or absence of astaxanthin. They were harvested, washed twice with ice-cold PBS, resuspended in PBS containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and lysed by three cycles of freezing and thawing. Cytosolic fractions were obtained as

supernatants after centrifugation at 12,000 ×*g* at 4°C for 20 min. The total protein concentrations in the solution samples were measured using a BCA protein assay (Pierce, Rockford, IL, U.S.A.). Protein samples containing 20 μg of protein were separated on 8% SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gels) and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in PBS containing 0.1% Tween 20 (PBST) for 1 h, and then incubated with monoclonal iNOS (1:10,000) and polyclonal COX-2 antibodies (1:10,000) in PBS-T containing 1% nonfat milk for 2 h. The membranes were then washed three times with PBS-T and incubated with secondary antibody HRP-conjugated anti-rabbit antibody for 1 h. Following five washes with PBS-T, the membranes were incubated with chemiluminescent solution for 2 min. The iNOS and COX-2 protein levels were detected using an ECL kit.

Statistical Analysis

Data are reported as the means±SD of three independent determinations. All experiments were performed at least three times, each time with three or more independent observations. Statistical analysis was performed using the Student's *t* test. *P* values of 0.05 or less were considered statistically significant.

Results

Cytotoxic Effect of Astaxanthin, Evaluated by MTT Assay in LPS-Stimulated BV2 Microglial Cells

The cytotoxicity of astaxanthin was evaluated in the presence or absence of LPS by MTT assay, a method used to measure viable cells. The viabilities of the cells incubated with astaxanthin at 0 μM, 1 μM, 10 μM, and 100 μM were 100±0.02%, 97.44±0.03%, 94.75±0.10%, and 93.30±0.11%, respectively. The viabilities of the cells incubated with astaxanthin at 0 μM, 1 μM, 10 μM, and 100 μM in 100 ng/ml of LPS were 93.57±0.10%, 91.92±0.08%, 90.11±0.05%, and 92.09±0.03%, respectively. Astaxanthin did not decrease the viability of the BV2 microglial cells when they were incubated with or without LPS (100 ng/ml) in the presence or absence of astaxanthin (0, 1, 10, or 100 μM). The MTT assay revealed that astaxanthin exerted no significant cytotoxicity on BV2 microglial cells (Fig. 1).

Astaxanthin Inhibits NO Production in LPS-Stimulated BV2 Microglial Cells.

The effect of astaxanthin on NO production in LPS-stimulated BV2 microglial cells was investigated. NO is an important intracellular and intercellular signaling molecule involved in the regulation of diverse physiological and pathophysiological mechanisms in the cardiovascular, nervous, and immunological systems. Therefore, we investigated the anti-inflammatory activity of astaxanthin by testing the effects of astaxanthin on NO production in LPS-stimulated BV2 microglial cells.

NO is produced by immune-activated macrophages at various sites of inflammation. The cells accumulated nitrite, a stable oxidized product of NO, in culture medium when stimulated with LPS. Nontreated cells and astaxanthin-treated cells without LPS produced 14.06±1.77 μM and 9.43±0.90~11.08±0.35 μM, respectively (Fig. 2). When the LPS-stimulated cells were treated with 0 μM, 1 μM, 10 μM, or 100 μM astaxanthin, they produced 33.79±

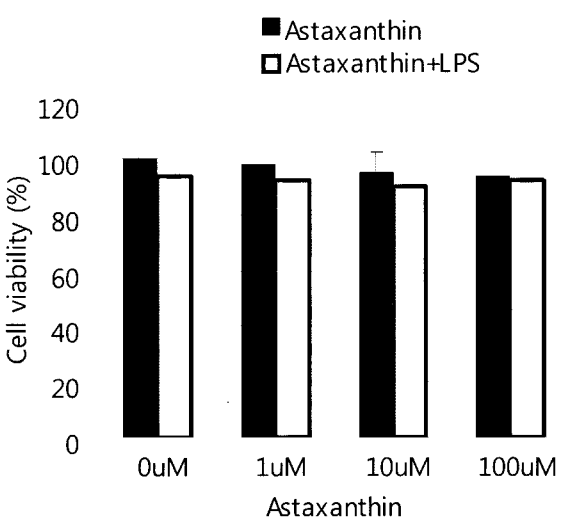

Fig. 1. Effects of astaxanthin on cell viability in BV-2 microglial cells.

Cells were pretreated with 1 μM, 10 μM, or 100 μM astaxanthin for 1 h followed by 100 μg/ml of LPS treatment for 24 h. The cells were added to 100 μl of MTT reagent and incubated at 37°C for 4 h. Cell viability was determined *via* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

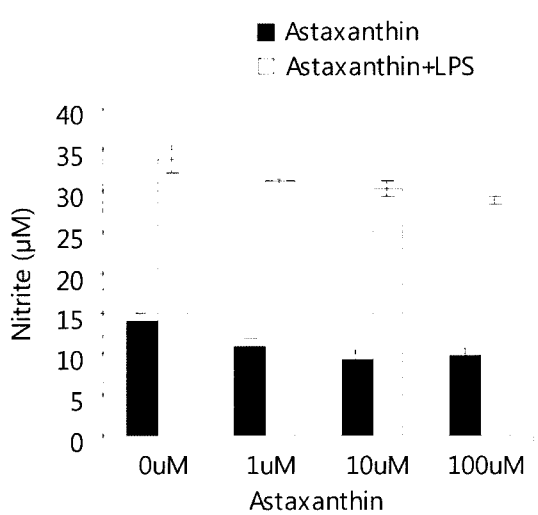

Fig. 2. Measurement of nitrate oxide (NO) in BV2 microglial cells.

Cells were pretreated with 1 μM, 10 μM, or 100 μM astaxanthin for 1 h followed by 100 μg/ml LPS treatments for 24 h. One hundred μl aliquots of cell supernatant from each well were mixed with 100 μl of Griess reagent in a 96-well microtiter plate reader and incubated for 10 min at 24°C.

0.61 μM, 31.14±0.40 μM, 30.22±0.80 μM, and 28.69± 0.50 μM, respectively (Fig. 2). In this study, the NO production in the groups treated with LPS differed significantly from that in the nontreated groups. NO production in the astaxanthin-treated groups not receiving LPS stimulation differed from that in the control cells (0 μM astaxanthin without LPS) ($P<0.05$). Specifically, the production of NO decreased significantly in the groups treated with LPS as compared with that in the control cells (0 μM astaxanthin with LPS) ($P<0.01$). These results suggest that astaxanthin inhibited the release of NO in LPS-stimulated BV2 microglial cells in a dose-dependent manner (Fig. 2).

Effects of Astaxanthin on the mRNA Expression Levels of iNOS and COX-2

NO and COX-2 are critical mediators of the inflammatory process and of organ injury in endotoxemia and sepsis. The production of NO in the body is catalyzed by a family of enzymes called nitric oxide synthases. To examine the anti-inflammatory effects of astaxanthin in septic animals, we tested whether astaxanthin affects the production of iNOS and COX-2 mRNA in LPS-treated BV2 microglial cells. The BV2 microglial cells were incubated with or without astaxanthin (100 μM) for 1 h, respectively. The cells were then stimulated with LPS (100 ng/ml) for 4 h. In this study, the mRNA levels of COX-2 and iNOS in the control cells were used as a control value (100.00). The level of iNOS mRNA in nontreated cells stimulated with 100 ng/ml LPS was markedly increased to a density of 182.64±4.32 in comparison with the control cells. When the cells were treated with 100 μM astaxanthin, the cells with and without LPS decreased to densities of 94.03±6.34 and 143.86±5.27 in comparison with the nontreated cells stimulated with LPS, respectively. We also investigated the effect of astaxanthin treatment on COX-2 mRNA level.

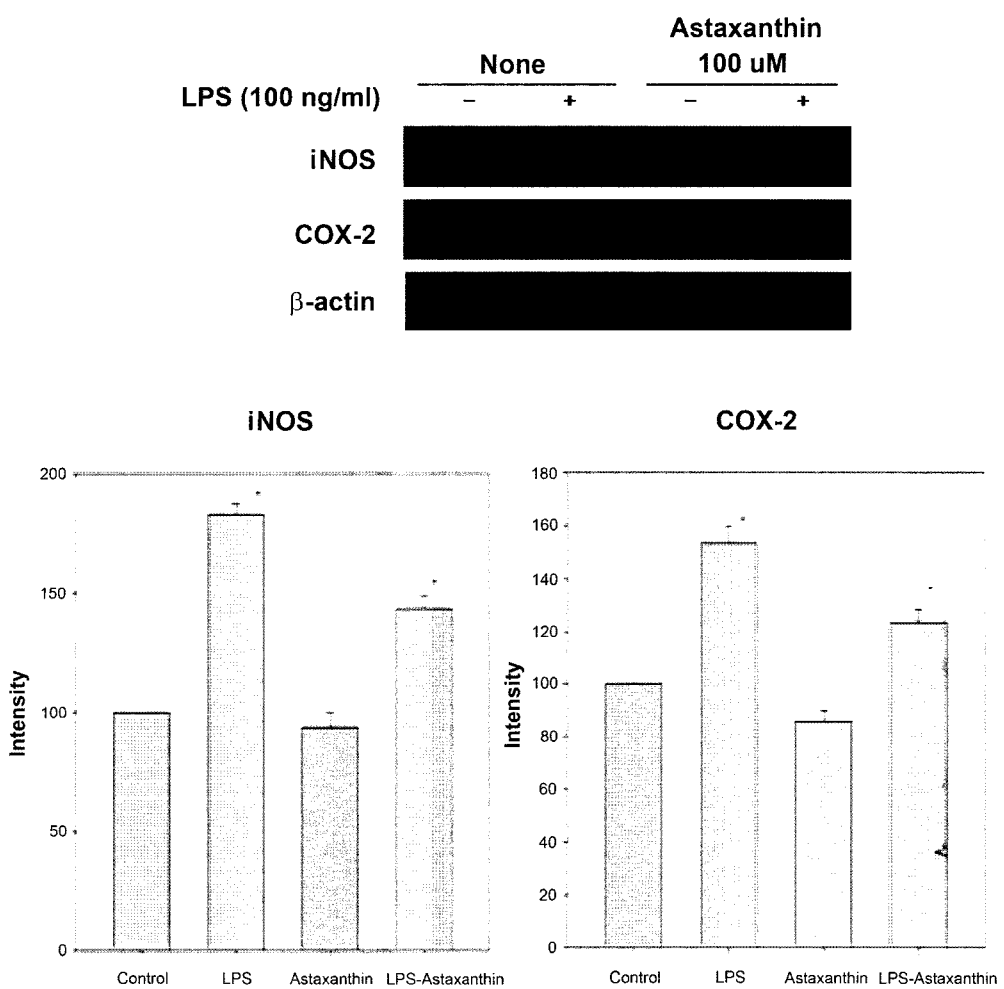

Fig. 3. RT-PCR analysis of the mRNA expression levels of COX-2 and iNOS.
Cells were pretreated with nothing 100 μM astaxanthin for 1 h followed by no or 100 ng/ml LPS treatment for 4 h. β-Actin was used as the internal control (to verify equal loading of protein).

Nontreated cells stimulated with LPS and astaxanthin-treated cells not receiving LPS stimulation expressed densities of 153.52±6.32 and 85.78±4.39 in comparison with the control cells. The astaxanthin-treated cells stimulated with LPS were decreased to a density of 123.2±5.23 in comparison with the nontreated cells stimulated with LPS. As shown in Fig. 3, this analysis indicated that the LPS-stimulated expression levels of COX-2 and iNOS mRNA were decreased by astaxanthin. RT-PCR analysis indicated that the LPS-stimulated expression of COX-2 mRNA was significantly decreased by astaxanthin treatment.

Effects of Astaxanthin on iNOS Synthesis

It remains unclear whether the reduction in NO production by astaxanthin is a result of the inhibition of iNOS expression or the inhibition of its enzymatic activity. To determine whether the inhibitory effect of astaxanthin on NO production was due to a decrease in the cytosolic iNOS protein level, BV2 microglial cells were treated with LPS alone or with astaxanthin, and then Western blot analysis was used to analyze the amount of iNOS. The BV2 microglial cells were incubated with 100 μM astaxanthin for 1 h, and the cells were then stimulated with LPS (100 ng/ml) for 6 h. In this study, the protein levels of iNOS in the control cells were used as a control value (100.00). The protein level of iNOS in the nontreated cells stimulated with LPS was markedly increased to a density of 167.21 in comparison with the control cells, whereas the cells treated with and without LPS stimulation decreased to densities of 109.51 and 73.37 in comparison with the non-treated cells stimulated with LPS, respectively. As shown in Fig. 4, pretreatment with astaxanthin significantly decreased the iNOS protein levels in BV2 microglial cells. The result of Western blot analysis indicated that astaxanthin directly modulates the iNOS expression level in LPS-stimulated BV2 microglial cells.

Effects of Astaxanthin on COX-2 Synthesis

Several reports have previously shown that LPS strongly induces the expression of the COX-2 gene leading to the

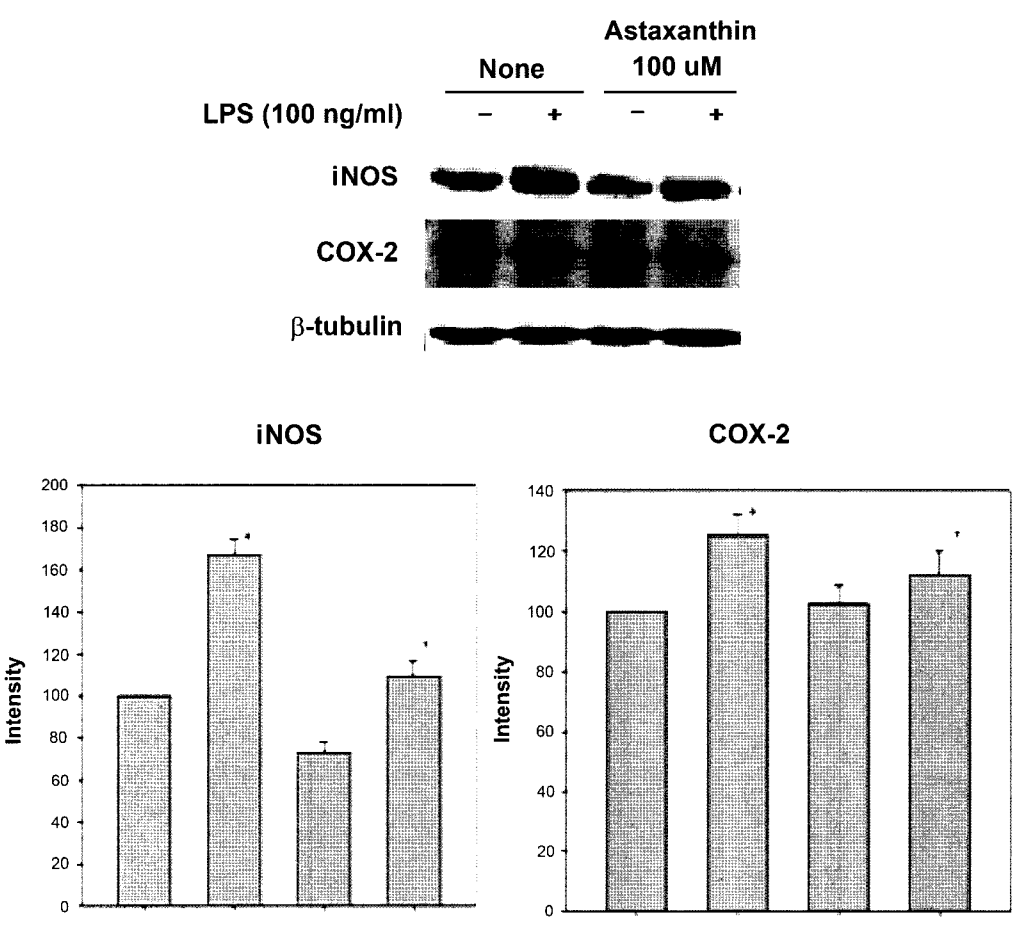

Fig. 4. Modulation of LPS- induced iNOS and COX-2 expression by astaxanthin in BV-2 microglial cells.
BV-2 cells were treated with astaxanthin for 1 h, and then treated with LPS for 6 h. B-tubulin was used as the internal control.

synthsis of PGE2. As shown in Fig. 3, we determined whether astaxanthin inhibits the expression of COX-2 mRNA. We investigated whether astaxanthin could affect COX-2 protein expression in LPS-stimulated BV2 microglial cells. BV2 microglial cells were incubated with 100 μM astaxanthin for 1 h, and the cells were then stimulated with LPS (100 ng/ml) for 6 h. Western blot analysis was used to analyze the amount of COX-2. In this study, the protein levels of COX-2 in the control cells were used as a control value (100.00). The level of the COX-2 protein in nontreated cells stimulated with LPS was increased to a density of 125.23 in comparison with the control cells, whereas cells treated with and without LPS stimulation were slightly decreased to densities of 112.01 and 102.58 in comparison with nontreated cells stimulated with LPS, respectively. As shown in Fig. 4, the expression of the COX-2 protein in LPS-stimulated BV2 microglial cells was also inhibited by astaxanthin treatment. The result of Western blot analysis indicated that astaxanthin directly modulates the COX-2 expression level in LPS-stimulated BV2 microglial cells.

Discussion

After exposure to endogenous and exogenous stimulators, iNOS is quantitatively induced in various cells, such as macrophages, smooth muscle cells, and hepatocytes, in order to trigger several disadvantageous cellular responses and cause inflammation. Inflammation and oxidative stress are major components of chronic neurodegenerative diseases. Microglia are the resident macrophages and immune surveillance cells of the central nervous system, and they play an important role in brain inflammatory immune and degenerative processes. The present study was undertaken in an effort to elucidate the effects of astaxanthin on the production of inflammatory cytokines and mediators *in vitro*. We showed that astaxanthin inhibited the expression of iNOS and COX-2 as well as the production of NO in LPS-stimulated BV2 microglial cells. NO is produced by immune-activated macrophages at various sites of inflammation. 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 the levels of NO. We investigated whether astaxanthin influences the production of NO by BV2 microglial 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 the production of NO (Fig. 2). Astaxanthin did not affect cell viability as measured by the MTT assay (Fig. 1). It is not known whether the reduction in NO production by astaxanthin is a result of the inhibition of iNOS expression or the inhibition of its enzymatic activity. Therefore, this study was undertaken in order to investigate whether astaxanthin could affect iNOS protein expression in LPS-stimulated BV2 microglial cells. Western blot analysis showed that stimulation of iNOS protein levels by LPS was markedly inhibited by treatment with 100 μM astaxanthin, and that the astaxanthin treatment also inhibited the induction of iNOS mRNA (Figs. 3 and 4). In addition, Western blot and RT-PCR analyses showed that astaxanthin inhibited the expression of the COX-2 protein and mRNA expression at a concentration of 100 μM (Figs. 3 and 4). The results suggest that astaxanthin could exert its anti-inflammatory actions by suppressing the expression and syntheses of NO, COX-2, and iNOS.

In summary, astaxanthin inhibited LPS-stimulated NO, COX-2, and iNOS. As the effects of astaxanthin are mediated *via* the inhibition of iNOS, astaxanthin could potentially be used in pathological processes involving the induction of NO. It is very possible that astaxanthin can offer a valuable mode of therapy for the treatment of brain inflammatory diseases.

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