Inhibitory Effects of Lutein on Endotoxin-Induced Uveitis in Lewis Rats

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PURPOSE. Lutein deposits in the macula and lens of human eyes with high concentration and is well known as an eye-protective nutrient for its beneficial effects on eye disease such as age-related macular degeneration and cataract. The purpose of the present study was to investigate the effects of lutein on endotoxin-induced uveitis (EIU) in rats.

METHODS. EIU was induced in male Lewis rats by subcutaneous injection of 200 μg lipopolysaccharide. Lutein or dexamethasone was administered intravenously at 30 minutes before, at the same time as, and at 30 minutes after LPS treatment. The aqueous humor was collected at 24 hours after LPS injection, the number of infiltrating cells, the protein concentration, and the levels of nitric oxide (NO), tumor necrosis factor (TNF)-α, interleukin (IL)-6, prostaglandin (PG)-E2, monocyte chemoattractant protein ( MCP)-1, and macrophage inflammatory protein (MIP)-2 in the aqueous humor were determined. Immunochemical staining with a monoclonal antibody against activated nuclear factor (NF)-κB was performed to evaluate the effect of lutein on NF-κB activation in the iris-ciliary body (ICB) of rats. A mouse macrophage cell line (RAW264.7 cells) was stimulated with LPS in the presence or absence of lutein. Expression of inducible NO synthase (iNOS), cyclooxygenase-2 (COX-2), and degradation of inhibitor (I)κB (IkB) were analyzed by Western blot analysis.

RESULTS. Lutein suppressed the development of EIU in a dose-dependent fashion. The anti-inflammatory effect of 100 mg/kg lutein was as strong as that of 1 mg/kg dexamethasone. Treatment with lutein reduced the concentrations of NO, TNF-α, IL-6, PG-E2, MCP-1, and MIP-2 in aqueous humor. Lutein also suppressed the activation of NF-κB in the ICB as well as iNOS and COX-2 expression and IkB degradation in RAW cells.

CONCLUSIONS. These findings indicate that lutein has anti-inflammatory effects on EIU by inhibiting the NF-κB dependent signaling pathway and the subsequent production of proinflammatory mediators. (Invest Ophthalmol Vis Sci. 2006;47:2562-2568) DOI:10.1167/iovs.05-1429

Lutein is one of the most widely found carotenoids in frequently consumed fruits and vegetables. Chemically, lutein and its structural isomer zeaxanthin differ from other carotenoids in that they each have two hydroxyl groups, one on each side of the molecule (Fig. 1) and thus are referred to as xanthophylls.1,2 Lutein is abundantly present in dark, leafy green vegetables, such as spinach and kale.3 Humans are not capable of synthesizing carotenoids de novo and thus, their presence in human blood and tissues is entirely due to the ingestion of food or supplement sources. Of all the carotenoids present in the human diet and in serum, only lutein and zeaxanthin are present in the macula and lens—two eye tissues critical to vision.4,5 The macular pigment is made up entirely of lutein and zeaxanthin. Many observational and intervention studies showed that lutein consumption reduces the risk of age-related macular degeneration (AMD), cataracts, and other eye diseases.6 Lutein is believed to act in two ways to protect ocular tissue against photooxidative damage: first as a filter for damaging blue light and second as an antioxidant that quenches and scavenges photo-induced reactive oxygen species.3,6 Lutein may also serve to protect skin from ultraviolet-induced damage and may help reduce the risk of cardiovascular disease and several types of cancer.7–10 Although lutein is known to many as the eye-protective nutrient, so far there has been no report on its effect on ocular inflammation.

Endotoxin-induced uveitis (EIU) is an animal model of acute anterior segment intraocular inflammation that is induced by injection of endotoxin, the lipopolysaccharide (LPS) component of the Gram-negative bacterial cell wall.11 Cellular infiltration and protein leakage into the anterior chamber of the eye reaches a maximum at 24 hours after LPS injection.12 Although the exact mechanism of EIU remains poorly understood, numerous data indicate that cytokines play an essential role in the development of EIU. Elevated expression of cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, and macrophage inflammatory protein (MIP)-2 were observed concomitant with maximum EIU.12,13 Other inflammatory mediators such as nitric oxide (NO)14 and prostaglandin (PG)-E215 are also involved in the pathogenesis of EIU.

Production and release of inflammatory cytokines by LPS depends on inducible gene expression mediated by the activation of transcription factors. Nuclear factor (NF)-κB, which is one of the most ubiquitous transcription factors, has been suggested to play a key role in these reactions.16 Under quiescent conditions, NF-κB is sequestered in the cytosol bound to the inhibitor (I)κB. Exposure of cells to LPS triggers phosphorylation cascades that ultimately lead to phosphorylation and degradation of IκB. Once IκB dissociates from the complex, NF-κB translocates into the nucleus where binding to specific DNA motifs in the promoter region occurs, leading to increased gene transcription.17 The purpose of the present study was to investigate the effects of lutein on EIU in rats. We investigated the effects of lutein on cellular infiltration, protein leakage, and levels of TNF-α, IL-6, MCP-1, MIP-2, PG-E2, and NO in the aqueous humor.
humor. To elucidate the mechanism underlying the anti-inflammation effects of lutein, we investigated the activation of NF-κB in the iris-ciliary body (ICB) of the rats treated with lutein. In addition, we investigated the effects of lutein on the LPS-induced expression of inducible NO synthase (iNOS) and cyclooxygenase (COX)-2 and the degradation of IκB in RAW 264.7 cells.

**MATERIALS AND METHODS**

**Animal Groups and EIU**

Eight-week-old male Lewis rats (180 –220 g) were used. EIU was induced by subcutaneous injection with 200 μg LPS from *Salmonella typhimurium* (Sigma-Aldrich, St. Louis, MO) that had been diluted in 0.2 mL phosphate-buffered saline (PBS, pH 7.4). Rats were injected intravenously with 1, 10, or 100 mg/kg lutein (Kemin Industries, Des Moines, IA) or 1 mg/kg dexamethasone (Sigma-Aldrich) diluted in 0.1 mL PBS containing 0.1% dimethyl sulfoxide (DMSO; Sigma-Aldrich). Both lutein and dexamethasone were administrated three times: simultaneously and 30 minutes before and after the LPS injection. In the LPS group, 0.1% DMSO in PBS was administered intravenously on the same schedule as in the lutein group. In the control group, neither LPS nor lutein was injected into the rats. Experimental animals were treated in accordance with the ARVO Statement for Use of Animals in Ophthalmic and Vision Research.

**Number of Infiltrating Cells and Protein Concentration in Aqueous Humor**

At 24 hours after LPS injection, the rats were euthanatized, and the aqueous humor was collected immediately from both eyes by an anterior chamber puncture (15–20 μL/rat) using a 30-gauge needle under the surgical microscope. The aqueous humor was then accurately diluted 10-fold with PBS (pH 7.4).

For cell counting, the aqueous humor sample was suspended in an equal amount of Türk stain solution, and the cells were counted with a hemocytometer under a light microscope. The number of cells per field (an equivalent of 0.1 μL) 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 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 instructions. The absorbance values were measured at 540 nm in the microtiter plate reader.
Levels of TNF-α, IL-6, PGE2, MCP-1, and MIP-2 in Aqueous Humor

The levels of TNF-α, IL-6, PGE2, MCP-1, and MIP-2 in the aqueous humor were assessed with a commercially available ELISA kit (PGE2; R&D Systems, Minneapolis, MN; the others, BioSource International, Camarillo, CA) according to the manufacturer’s instructions. The ELISA assay was performed in duplicate.

Histopathologic Evaluation

At 24 hours after LPS injection, rats were euthanatized. The eyes were enucleated immediately and stored in a mixture of 10% formalin and 2.5% glutaraldehyde for 24 hours, and then the eyes were embedded in paraffin. Sagittal sections (5 μm thick) were cut near the optic nerve head and stained with hematoxylin and eosin.

Immunohistochemical Studies for NF-κB

At 3 hours after LPS injection, rats were anesthetized, and the eyes were fixed by an intracardiac perfusion of 4% paraformaldehyde in 0.1 M PBS. The eyes were enucleated and immersed in the same fixative for 12 hours and embedded in paraffin. Next, 5-μm sagittal sections were cut near the optic nerve head. Sections were dewaxed with xylene and rehydrated with ethanol. Antigen retrieval was performed by heating sections in a microwave oven. The sections were rinsed in PBS twice and incubated with normal goat serum and then with the primary antisera. The primary antisera were conjugated goat anti-rabbit IgG (dilution, 1:200; Jackson ImmunoResearch Laboratories, Inc, West Grove, PA). Nuclei were then stained with PBS containing YO-PRO-1 (Invitrogen, Eugene, OR) for 5 minutes. The sections were examined by laser scanning confocal microscopy (MRC-1024; Bio-Rad, Richmond, CA; and LSM 510: Carl Zeiss Meditec, Inc., Oberkochen, Germany). Within each ICB sample, two areas were randomly photographed, and the number of activated NF-κB–positive cells was counted by a masked researcher. The results of two areas were averaged for each sample and in each group. This analysis was performed in the eight eyes of four rats in each group.

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 (Sigma-Aldrich) supplemented with 2 mM glutamine, antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) and 10% heat-inactivated fetal bovine serum (Invitrogen-Gibco, Grand Island, NY) and maintained at 37°C in a humidified incubator containing 5% CO2. These cells were pretreated with 1, 10, 100 μg/mL lutein for 24 hours and subsequently stimulated with 1 μg/mL LPS from Salmonella typhimurium (Sigma-Aldrich) for 24 hours, unless otherwise stated.

Western Blot Analysis

RAW cells were washed with ice-cold PBS and then lysed in cold NP-40 lysis buffer (50 mM Tris-Cl [pH 7.6], 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/mL each of leupeptin, aprotinin, and pepstatin) for 15 minutes at 4°C. Plates were then scraped, and crude lysates were cleared by centrifugation at 14,000g for 10 minutes at 4°C. Aliquots of the lysates were diluted with 2× SDS sample buffer and boiled for 2 minutes. Lysates were separated on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). The membranes were then incubated in blocking solution (3% wt/vol dried low-fat milk and 0.1% vol/vol Tween 20 in PBS). Subsequently, the membranes were incubated with anti-COX-2 antibody (Alexis Biochemicals, Carlsbad, CA), anti-iNOS antibody (Upstate Biotechnology, Lake Placid, NY), anti-IκB antibody (Santa Cruz Biotechnology), anti-α-tubulin antibody (Laboratory Vision, Westinghouse Drive Fremont, CA). The membranes were then probed with horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Piscataway, NJ) and visualized by chemiluminescence (GE Healthcare).

Cell Viability

Cell viability was determined by MTT assay after a 48-hour exposure to various concentrations of lutein. 3(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5 mg/mL; Chemicon, Temecula, CA) was added to 0.1 mL of cell suspension for 4 hours, and the formazan formed was then dissolved in isopropanol. Optical density was measured with a plate reader at 570 nm.

Statistical Analysis

Data are expressed as the mean ± SD. Data were analyzed by analysis of variance (ANOVA). The Tukey-Kramer test was used as a post hoc comparison to compare the two treatment groups; *P < 0.05 was considered to be statistically significant.

RESULTS

Effect of Lutein on Cellular Infiltration and Protein Concentration in Aqueous Humor

Severe inflammation was found in the anterior segment of rats with EIU at 24 hours after LPS administration (Fig. 2B). Significant reductions of inflammation were observed in eyes treated with 100 mg/kg of lutein as well as 1 mg/kg of dexamethasone (Figs. 2C, 2D). In the LPS group, the number of inflammatory cells in aqueous humor 24 hours after LPS administration was 20.57 ± 4.74 × 105 cells/mL (n = 8). Treatment with 10 and 100 mg/kg lutein significantly reduced the number of inflammatory cells (10 mg/kg: *P < 0.05; 100 mg/kg: *P < 0.01), whereas 1 mg/kg of lutein did not result in a reduction in the cell count. No infiltrating cell was detected in the aqueous humor of rats that were treated with 1, 10, or 100 μg/mL lutein for 24 hours.

![FIGURE 3. Effect of lutein on cellular infiltration and protein concentration in the aqueous humor collected 24 hours after LPS treatment. Data are the mean ± SD of results in eight rats. The dose of dexamethasone was 1 mg/kg. *P < 0.05, **P < 0.01, significantly different from the LPS group.](image-url)
humor of the control group. The effect of 100 mg/kg lutein on the number of cells in the aqueous humor was almost the same as that in the 1-mg/kg dexamethasone group (Fig. 3A). The protein concentration in the groups treated with lutein was significantly lower than that in the LPS group (*P < 0.01), and the decrease occurred in a dose-dependent fashion. There was no significant difference between the levels of protein in 100-mg/kg lutein-treated group and the 1-mg/kg dexamethasone-treated group (Fig. 3B).

**Effect of Lutein on NO, TNF-α, IL-6, PGE2, MCP-1, and MIP-2 in Aqueous Humor**

Considerable production of NO, TNF-α, IL-6, PGE2, MCP-1, and MIP-2 in the aqueous humor was seen in LPS group. Lutein treatment significantly reduced their concentrations in aqueous humor with a dose-dependent fashion. The reduction of these inflammatory mediators in the 100 mg/kg of lutein group was almost the same as that in the 1-mg/kg dexamethasone group (Fig. 4).

**Immunohistochemistry of NF-κB p65 in the ICB after LPS Injection**

Three hours after injection of LPS, activated NF-κB p65 immunoreactivity was strongly expressed in the ICB. In contrast, the number of activated NF-κB-positive cells were lower in the ICB of rats treated with lutein. Normal control experiments showed only background levels (Fig. 5A).

To obtain a quantitative measure of NF-κB activity in the ICB, the active NF-κB-positive cells were counted. In the control group, no active NF-κB-positive cells were detected in the ICB. LPS injection resulted in a marked increase in the percentages of active NF-κB-positive cells in the ICB at 3 hours (31.79% ± 3.62%). In the lutein groups, the percentages of active NF-κB-positive cells decreased significantly in a dose-dependent fashion (*P < 0.01; Fig. 5B).

**Expression of iNOS Protein and COX-2 Protein**

No iNOS or COX-2 signals were detected in unstimulated RAW cells (Fig. 6, lane 1). LPS stimulation induced considerable
expression of iNOS and COX-2 proteins (Fig. 6, lane 2). Lutein inhibited the expression of both iNOS and COX-2 proteins in a dose-dependent fashion (Fig. 6, lanes 3–5). It did not decrease the cell viability of RAW264.7 cells when these cells were incubated with 100 μg/mL lutein for 48 hours (data not shown).

**Effects of Lutein on the Degradation of I-κBα**

To examine whether the inhibitory action of lutein is attributable to the degradation of I-κB, we examined the cytoplasmic levels of I-κBα protein in lutein-treated RAW cells by Western blot.

A large amount of I-κBα protein was detected in the cytoplasmic extract of nontreated RAW cells (control group; Fig. 7, lane 1). After stimulation with LPS for 15 minutes, the cytosolic I-κBα protein decreased significantly (Fig. 7, lane 2). However, when RAW cells were incubated with lutein for 24 hours before the stimulation of LPS, the degradation of I-κBα was inhibited (Fig. 7, lanes 3–5).

**DISCUSSION**

Lutein has been used in the treatment of eye diseases and to protect visual function since the 1950s. It is well known for its beneficial effects on AMD, which is a chronic, progressive, degenerative disease of the macula and is the leading cause of central vision loss among elderly people in the Western world. Although the exact mechanisms of AMD remain unclear, inflammation may be involved in its pathogenesis and has led to the consideration of anti-inflammatory therapy as treatment for the early stages of the disease. In this study, we investigated the effect of lutein on EIU, an animal model for acute ocular inflammation in humans. We found for the first time that lutein has an anti-inflammatory effect on intraocular inflammation and the anti-inflammatory activity of 100 mg/kg of lutein was comparable to that of 1 mg/kg dexamethasone. We administered lutein intravenously in a single dose at first. The number of infiltrating cells in aqueous humor was not always suppressed. To get a stable result, three consecutive doses of lutein were administered. Actually, a total dose of 300 mg/kg of lutein was received by rats in 100 mg/kg of the lutein-treated group. In our other study of lutein, we found the half-life of plasma lutein obtained by intravenous injection is less than 15 minutes; thus, three consecutive doses of 100 mg/kg of lutein seems can maintain high level of plasma lutein longer time than single dose of 300 mg/kg of lutein. In keeping with the route of administration of lutein, 1 mg/kg of dexamethasone was injected three times, as well.

To elucidate the anti-inflammatory mechanism of lutein, we measured several proinflammatory mediators including NO, PGE2; cytokines TNF-α and IL-6; and chemokines MCP-1 and MIP-2 all of which contribute to the pathophysiology of EIU. NO is a highly reactive radical that is regarded as a key mediator in uveitis. NO can be produced in large amounts by iNOS in response to endotoxin and cytokines. Excessive production of NO by iNOS mediates increased protein leakage as well as hemodynamic and vascular permeability changes associated with EIU. A large increase in iNOS mRNA was detected in the ICB and retina during EIU. Many reports have shown that inhibition of iNOS protects against the development of EIU which suggests that activation of iNOS and large amounts of NO contribute to the pathogenesis of EIU. In our study, we demonstrated that lutein reduced NO production in aqueous humor significantly. The expression of iNOS in LPS-stimulated RAW cells was also inhibited by lutein. Thus, it appears that lutein suppresses NO production by blocking iNOS protein expression. PGE2 is another inflammatory mediator involved in the pathogenesis of EIU and is also thought to contribute to breakdown of the blood-aqueous barrier during EIU. Cyclooxygenase (COX) is an enzyme that converts arachidonic acid to PGs. COX-2 is an inducible form responsible for the production of large amounts of proinflammatory PGs at the inflammatory sites in animals as well as patients with inflammatory diseases. Our data showed that lutein could suppress LPS-induced COX-2 expression in RAW264.7 cells.
and PGE2 production in the aqueous humor. Therefore, our results suggest that lutein suppresses PGE2 through inhibition of the COX-2 enzyme. The inhibition of the iNOS-NO pathway and the COX-2-PGE2 pathway would improve the therapeutic management of uveitis.15

The contribution of cytokines TNF-α and IL-6 to EIU is pleiotropic. Some data suggest these two cytokines have a protective role in ocular inflammation.26 On the contrary, both cytokines appear to be proinflammatory. Injection of TNF-α or IL-6 into the vitreous of rats produced severe intraocular inflammation.29-30 TNF-α and IL-6 are produced principally by activated macrophages and monocytes, whose recruitment depends on chemokine MIP-2 and MCP-1, respectively. Both chemokines are considered to be essential in the pathogenesis of EIU.31-32 Data from this study have shown that lutein can decrease the cytokines and chemokines concentrations in aqueous humor in a dose-dependent manner. These findings suggest that part of the mechanism of lutein suppression of EIU must include the prevention of the cytokines and chemokines in the anterior chamber.

Considering that NF-κB response elements are present on the promoters for iNOS, COX-2, as well as TNF-α, IL-6, MCP-1, and MIP-2 genes,33-34 and lutein significantly inhibits the expression of iNOS and COX-2 and the levels of these inflammatory cytokines, it is reasonable to postulate that lutein regulates the expression of these proteins by inhibiting NF-κB activation. Our results indicate that lutein suppressed LPS-induced activation of NF-κB in the ICB. Thus, the inhibition of NF-κB activation and subsequent synthesis of inflammatory mediators can help explain the beneficial effect of lutein in EIU treatment.

Although the precise primary targets involved in the inhibition of NF-κB activation by lutein have not yet been identified, our data have shown that lutein can block the degradation of IκBα from the cytosolic fraction in activated macrophages. Thus, IκB still binds to NF-κB and prevents NF-κB translocation to the nucleus where the NF-κB positively regulates the expression of genes involved in the immune and inflammatory responses. Although a possibility that lutein may also inhibit other transcription factors induced by LPS and suppress the gene transcription of inflammatory mediators remains, our results demonstrate that lutein-induced inhibition the expression of inflammatory mediators is at least partially due to the inhibition of IκB degradation. Whether there are other path-

![Figure 6](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932938/)

**Figure 6.** Inhibition of LPS-induced iNOS protein and COX-2 protein by lutein in RAW macrophages. RAW cells were pretreated with 1, 10, or 100 μg/mL lutein for 24 hours and subsequently incubated with 1 μg/mL of LPS for another 24 hours. Expression of iNOS protein and COX-2 protein were detected by Western blot. In graphs, the signal intensity of iNOS and COX-2 are expressed in relation to α-tubulin signal intensity to account for minor difference in loading between lanes. Lane 1: control; lane 2: LPS; lane 3: 1 μg/mL lutein; lane 4: 10 μg/mL lutein; lane 5: 100 μg/mL lutein.

![Figure 7](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932938/)

**Figure 7.** The effect of lutein on LPS-induced IκBα degradation in RAW macrophages. IκBα protein was assessed by Western blot. RAW cells were pretreated with 1, 10, or 100 μg/mL lutein for 24 hours and subsequently incubated with 1 μg/mL of LPS for 15 minutes. In graphs, the signal intensity of IκBα is expressed in relation to α-tubulin signal intensity to account for minor difference in loading between lanes. Lane 1: control; lane 2: LPS; lane 3: 1 μg/mL lutein; lane 4: 10 μg/mL lutein; lane 5: 100 μg/mL lutein.

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ways involved in the anti-inflammatory mechanism of lutein needs further investigation.

We have measured the plasma lutein concentration of the rat at different times after three injections of lutein by the method of HPLC (data not shown). The plasma lutein concentration reaches the peak immediately after lutein injection, then decreases rapidly. Park et al. reported plasma lutein concentration in mice fed a diet containing lutein rapidly increased on day 3 and no further increase or decrease was observed during lutein uptake. These results suggest that the acute effect of lutein injections may not be the same as the effect of chronic accumulation of lutein obtained from the diet. The anti-inflammatory effects of lutein obtained from the diet should be investigated further. Human plasma lutein levels can be increased by taking supplement or dietary modification, but the effects of lutein on human uveitis still remain unclear.

In summary, this study indicates that lutein shows a dose-dependent anti-inflammatory effect on EIU. In particular, the ocular anti-inflammatory effect of 100 mg/kg lutein was as strong as that of 1 mg/kg dexamethasone. A possible mechanism for this effect of lutein may depend on its ability to inhibit the activation of NF-κB and the subsequent production of proinflammatory mediators.

Acknowledgments

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References

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