Neuroprotective Effect of an Antioxidant, Lutein, during Retinal Inflammation

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PURPOSE. Lutein has been the focus of recent study as a possible therapeutic approach for retinal diseases, but the molecular mechanism of its neuroprotective effect remains to be elucidated. The aim of this study was to investigate, with the use of a mouse endotoxin-induced uveitis (EIU) model, the neuroprotective effects of lutein against retinal neural damage caused by inflammation.

METHODS. EIU was induced by intraperitoneal injection of lipopolysaccharide (LPS). Each animal was given a subcutaneous injection of lutein or vehicle three times: concurrently with and 5 hours before and after the LPS injection. Analysis was carried out 24 hours after EIU induction. Levels of rhodopsin protein and STAT3 activation were analyzed by immunoblotting. Lengths of the outer segments of the photoreceptor cells were measured. Dark-adapted full-field electroretinograms were recorded. Oxidative stress in the retina was analyzed by dihydroethidium and fluorescent probe. Expression of glial fibrillary acidic protein (GFAP) was shown immunohistochemically.

RESULTS. The EIU-induced decrease in rhodopsin expression followed by shortening of the outer segments and reduction in a-wave amplitude were prevented by lutein treatment. Levels of STAT3 activation, downstream of inflammatory cytokine signals, and reactive oxygen species (ROS), which are both upregulated during EIU, were reduced by lutein. Pathologic change of Müller glial cells, represented by GFAP expression, was also prevented by lutein.

CONCLUSIONS. The present data revealed that the antioxidant lutein was neuroprotective during EIU, suggesting a potential approach for suppressing retinal neural damage during inflammation. (Invest Ophthalmol Vis Sci. 2009;50:1433–1439) DOI:10.1167/iovs.08-2493

Recent studies have shown that the retinal inflammatory reaction is a pathogenic factor in most retinal diseases; therefore, regulating inflammation is an important therapeutic approach. Several inflammatory cytokines, such as vascular endothelial growth factor (VEGF), interleukin (IL)-6, and angiotensin II,3–5 are simultaneously upregulated and cause severe retinal damage during inflammatory diseases, including diabetic retinopathy6–8 and age-related macular degeneration (AMD).9,10 The entrapment of inflammatory extracellular cytokine signals is reported as an important therapeutic strategy for retinal inflammatory diseases.4 However, other simpler and more general therapeutic approaches might be achieved by targeting a more common intracellular pathway downstream of these cytokine signals.

Recent attention has focused on the link between inflammatory diseases and intracellular oxidative stress.10–13 Reactive oxygen species (ROS) generated through inflammatory reactions may attack DNA, protein, or lipid cellular components.14 Moreover, ROS may act as a secondary messenger to activate various signaling pathways by inducing stress-response genes or proteins.14 Thus, the regulation of ROS might inhibit the tissue damage caused by inflammatory reactions. In support of this idea, several reports using animal models suggest that the administration of antioxidants reduces ROS and is effective for preventing or treating inflammatory diseases such as rheumatoid arthritis,15 arteriosclerosis,16 vascular changes in diabetes,17 and inflammatory bowel disease.18 The involvement of oxidative stress in ocular inflammatory diseases is also reported. In experimental animal models, diabetic retinas show high levels of ROS,12 and an oxidized protein causes AMD-like lesions in the retina.10

Here we focus on lutein, one of the antioxidants that quench and scavenge ROS. It is classified as a xanthophyll carotenoid, along with its optical isomer, zeaxanthin. Lutein is distributed in the lens and macula and protects the ocular tissue by filtering blue light.19–21 In addition, lutein is reported to have an anti-inflammatory effect in ocular tissue.22 Moreover, we have reported that lutein administration suppresses the activation of NF-κB, one of the downstream effectors of inflammatory cytokine signaling in vascular endothelial cells, and inhibits the generation of laser-induced choroidal neovascularization.23 Lutein is anticipated to show a preventive effect on the progress of human AMD in the Age-Related Eye Disease Study 2. However, whether the administration of lutein reduces the level of ROS in retinal neural cells and has a protective effect on visual function remain to be elucidated.

We previously reported that various inflammatory cytokines, such as IL-6 and angiotensin II, are upregulated in retinas with endotoxin-induced uveitis (EIU).3,5 Activated STAT3, a downstream intracellular effector of these cytokines, is one of the causes of EIU-induced retinal dysfunction; it reduces the level of rhodopsin, an indispensable visual substance, by inducing excessive degradation through the ubiquitin-proteasome system (UPS)24 and causes visual function impairment that can be recorded by electroretinogram (ERG).3 In addition, glial fibrillary acidic protein (GFAP) expression in pathologically reactivated Müller glial cells is induced by activated STAT3 during EIU.3 Although excessive ROS may be also...
generated in the inflamed retina, whether ROS plays a role in retinal dysfunction during inflammation is still unclear. In this study, we investigated the ability of lutein to prevent retinal damage and to provide a neuroprotective effect during retinal inflammation, using the EIU model in mouse.

METHODS

Animals

C57BL/6 mice (8 weeks old) were purchased (Clea Japan, Tokyo, Japan). Each mouse received a single intraperitoneal injection of 6.0 mg/kg body weight lipopolysaccharide (LPS) from Escherichia coli (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS). The mice were killed and evaluated 24 hours after LPS injection. This time was chosen for analysis because most of the pathologic changes in the retina were obvious by then. Each mouse was given three concomitant injections of lutein (20% lutein in sunflower oil, 100 mg/kg body weight; provided by Wakasa Seikatsu Co., Ltd., Kyoto, Japan) or vehicle (sunflower oil) concurrently with the LPS injection, 3 hours before and 3 hours after the injection.

All animal experiments were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Immunoblot Analysis

Twenty-four hours after LPS injection, the eyes were immediately enucleated, and the retina was carefully isolated and placed in lysis buffer. The lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane (Millipore Corp., Bedford, MA). After they were blocked with 4% skim milk, the membranes were incubated overnight with a rabbit anti-rhodopsin antibody (1:10,000; DSL, Osaka, Japan) or rabbit anti-phospho-STAT3 antibody (1:1000; Cell Signaling Technology, Beverly, MA), and mouse anti-$\alpha$-tubulin (1:2000; Sigma), to assess the amount of each protein in each sample. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibody or biotin-conjugated secondary antibody followed by avidin-biotin complex ( Vectastain ABC Elite Kit; Vector Laboratories, Burlingame, CA). Finally, the signals were detected through the enhanced chemiluminescence (ECL Blotting Analysis System; Amersham, Arlington Heights, IL) and measured using the ImageJ program.

Immunohistochemistry

Samples were fixed with 4% paraformaldehyde and prepared for paraffin sections and cryosections. Sections were stained with hematoxylin and eosin, and outer segment (OS) lengths were measured in three sections of the midperipheral part of the retina and averaged. Apoptotic cells were detected with the TdT-DUTP terminal nick-end labeling (TUNEL) kit (Chemicon-Millipore, Billerica, MA). The sections were also incubated with rabbit anti-GFAP antibody (1:1000; DAKO, Carpinteria, CA) antibody followed by Alexa 568-conjugated goat anti-rabbit IgG, and nuclei were counterstained with the nuclear dye bisbenzamide (1:1000 from a stock solution of 10 mg/mL. (Hoechst 33258; Sigma). All the sections were examined under a microscope equipped with a digital camera (Carl Zeiss), and the intensity of the staining was measured using the ImageJ program.

Real-Time RT-PCR

Total RNA was extracted from the retina, and cDNA was synthesized after RNase-free DNase (Invitrogen) treatment. Real-time PCR was performed using an Mx3000p, with SYBR green (Takara Bio, Shiga, Japan). Primers for rhodopsin detection were described by Peng et al.60 Results are presented as the ratio of the mRNA of interest to the mRNA of an internal control gene, gpdh. The gpdh primer sequences were acccacagtcgtgcatcac (forward) and tccaccacgtggtgctgta (reverse).

Statistical Analysis

Data were expressed as mean $\pm$ SD and standard error. Statistical significance was tested with the unpaired two-tailed Student’s t-test, and differences were considered statistically significant at $P < 0.05$.

RESULTS

Preservation of Rhodopsin by Lutein in Retinas with EIU

We first measured the rhodopsin level in inflamed retinas of mice with EIU by immunoblot analysis. We found that the decrease in rhodopsin in the retinas with EIU was mostly prevented by the administration of lutein (Figs. 1A, 1B) mean $\pm$ SD (SE; 95% confidence interval [CI]); control, 1.00 $\pm$ 0.28 (0.094; 0.784–1.216); EIU, 0.74 $\pm$ 0.18 (0.061; 0.596–0.878); EIU with lutein, 1.00 $\pm$ 0.27 (0.074; 0.839–1.161); $n = 9$, $n = 9$, $n = 13$, respectively ($P < 0.05$).

We further compared OS lengths in the photoreceptor cells. Rhodopsin was most concentrated in the OS. OS length represented the level of rhodopsin in the individual photoreceptor cells, as shown in the heterozygote rhodopsin knockout mice.61 It was also shortened during EIU, correlating with the downregulation of rhodopsin induced by inflammatory cytokines, as we have previously reported.62 Shortening of OS caused by cytokine-induced rhodopsin reduction can be re-
versible when the rhodopsin level recovers,28 suggesting that OS length reflects the rhodopsin level. In this study, the shortening of OS length during EIU was avoided by lutein (Figs. 1C, 1D) control, 1.00 ± 0.05 (0.027); EIU, 0.74 ± 0.05 (0.017); EIU with lutein 0.98 ± 0.01 (0.005); n = 3 (P < 0.01), suggesting that the level of rhodopsin expression in each photoreceptor cell was downregulated during EIU and that this influence was avoided by lutein. Another photoreceptor protein, transducin, was not reduced during EIU, which supported the idea that the number of the photoreceptor cells might have been unchanged (data not shown). We further confirmed that TUNEL-positive cells were hardly observed and that photoreceptor cell death was not induced during EIU (data not shown). Thus, lutein preserved the expression level of rhodopsin protein in the photoreceptor cells during retinal inflammation.

Preservation of the Electroretinogram Response by Lutein during EIU

We next asked whether lutein was effective in protecting visual function. Importantly, the amplitude of the a-wave in full-field ERG, which represents photoreceptor cell function and decreases during EIU, was significantly preserved by the administration of lutein (Figs. 2A, 2B) control, 0.575 ± 0.127 (0.063); EIU, 0.393 ± 0.067 (0.033); EIU with lutein, 0.50 ± 0.075 (0.037) mV, n = 4 (P < 0.05). This was consistent with the rhodopsin level (Figs. 1A, 1B). For the other parameters of ERG, we did not observe significant changes with the administration of lutein (Figs. 2A, 2C–E). Therefore, functional analysis also showed that the visual dysfunction caused by photoreceptor cell damage during inflammation was successfully prevented by treatment with lutein.

Inhibitory Effect of Lutein on STAT3 Activation in the Retina with EIU

Given that reduction in rhodopsin is correlated with activated STAT3,3–23 we also checked the level of phosphorylated STAT3 by immunoblot analysis. As we expected, STAT3 activation during EIU was clearly suppressed by lutein (Figs. 3A, 3B); control, 1.00 ± 0.300 (0.113; 0.723–1.277); EIU, 1.98 ± 0.41 (0.154; 1.605–2.356); EIU with lutein, 1.44 ± 0.37 (0.124; 1.153–1.723); n = 9, n = 9, n = 13, respectively (P < 0.05). Although the STAT3 activation was not fully prevented by lutein, the reduction in rhodopsin was almost completely inhibited (Figs. 1A, 1B), suggesting that lutein sufficiently suppressed STAT3 activation to a level lower than a certain threshold for rhodopsin reduction23 and preserved rhodopsin protein. Thus, lutein succeeded in inhibiting inflammatory cytokine signaling in the retinal neural cells.

Reduction of Oxidative Stress by Lutein in the Retina with EIU

We tested whether ROS are induced during EIU and suppressed by treatment with lutein. For this purpose, we used DHE staining. DHE specifically reacts with intracellular O₂⁻, a ROS, and is converted to the red fluorescent compound ethidium in nuclei. In the retinas of mice with EIU, DHE fluorescence was clearly upregulated in all retinal neural cells, and this upregulation was efficiently suppressed by lutein (Figs. 4A–D); control, 1.00 ± 0.32 (0.162); EIU, 1.58 ± 0.30 (0.150); EIU with lutein, 1.00 ± 0.27 (0.135); n = 4 (P < 0.05).

We also checked the influence of another ROS, OH⁻, in the retina. Accumulation of OH⁻ may induce lipid peroxidation, which destabilizes the cell membrane.29 Levels of lipid peroxidation were analyzed by measuring the light emission of oxidized BODIPY-C11. Light emission from BODIPY-C11 converts irreversibly from red to green when oxidized. We found a substantial increase in the intensity of oxidized BODIPY-C11 in the OS during EIU, but this influence was inhibited, and the light emission remained red in the OS after lutein treatment (Figs. 4E–K) control, 1.00 ± 0.15 (0.08); EIU, 1.68 ± 0.38 (0.16); EIU with lutein, 0.96 ± 0.25 (0.13); n = 4, n = 6, n = 3, respectively (P < 0.05). Lipid peroxidation was predominantly observed in OS, consistent with the fact that the long-chain fatty acids primarily affected during the lipid peroxidation process are composed of polyunsaturated fatty acids (PUFAs), which are abundant in OS.29 Thus, treatment with lutein clearly inhibited the accumulation of ROS in the retinal neural cells during EIU.

Inhibitory Effect of Lutein on GFAP Expression in the Müller Glial Cells

In the retina, Müller glial cells maintain the microenvironment and support photoreceptor cell function. Although they ex-
press GFAP only in their endfeet under control conditions, they alter their characteristics to become reactive glial cells and express GFAP in their cell bodies when pathologic events occur. The induction of GFAP during EIU was inhibited after treatment with lutein (Figs. 5A–D). Thus, lutein was also effective on retinal glial cells to retain their normal condition during EIU.

DISCUSSION

We demonstrated that treatment with an antioxidant, lutein, prevented the EIU-induced decrease in rhodopsin protein (Fig. 1) and preserved the a-wave amplitude of ERG (Fig. 2), thus protecting the photoreceptor cell function in the inflamed retinas. STAT3 activation (Fig. 3) and oxidative stress (Fig. 4), downstream effects of the signaling induced by inflammatory cytokines, were effectively suppressed by lutein in the retinal neural cells. Induction of GFAP in the Müller glial cells during EIU was also prevented by lutein (Fig. 5).

Consistent with our previous report, the rhodopsin protein level was preserved when STAT3 activation was suppressed by lutein (Figs. 1, 3). STAT3 activation was clearly inhibited by treatment with lutein, which also reduced oxidative stress in the retina (Figs. 3, 4), suggesting that oxidative stress during inflammation promoted STAT3 activation and reduced the rhodopsin protein level. STAT3 is activated not only by extracellular signals through transmembrane receptors that recruit and activate Janus kinase (JAK) but also by high levels of intracellular ROS. One of these ROS-activated pathways is mediated by JAK, which is activated by ROS, most likely in a cell-autonomous fashion. For example, 200 μM H₂O₂, a physiological concentration of this representative ROS, induces the rapid activation of JAK in vascular smooth muscle cells within 5 minutes, suggesting that this activation is independent of newly induced cytokine expression. Because JAK is expressed in most of the retinal neural cells, ROS may rapidly accelerate STAT3 activation throughout the retina. Thus, the reduction of ROS by lutein may suppress the rapid activation of JAK and STAT3, leading to the efficient preservation of rhodopsin protein. Alternatively, ROS may also upregulate STAT3 activation by inducing the expression of an upstream inflammatory cytokine, IL-6, possibly in the Müller glial cells.

Activated STAT3 inhibits rhodopsin expression in the developing retina by transcriptional repression. This mechanism may be also used in the adult retina under normal conditions; however, rapid decreases in rhodopsin during inflammation has turned out to be regulated in a posttranscriptional fashion that involves UPS-mediated protein degradation. In our previous report, the level of activated STAT3 is correlated with the severity of the rhodopsin degradation and visual dysfunction during inflammation, which was demonstrated with the use of retina-specific suppressor of cytokine signaling 3 (SOCS3)-deficient mice. In the absence of SOCS3, a negative feedback regulator of STAT3, UPS-mediated rhodopsin degradation during inflammation is accelerated by excessive STAT3 activation. We have also reported that activated STAT3 induces the putative E3-ubiquitin ligase selective for rhodopsin degradation, Ubr1. This mechanism was also proven with the use of an in vitro system. STAT3 activation induced by IL-6 exposure reduced the rhodopsin levels in an

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932958/ on 06/25/2017)

**FIGURE 2.** (A) Preservation of the ERG response in EIU by lutein. Full-field electroretinogram. (B) Amplitude of the a-wave was decreased during EIU but was clearly preserved by treatment with lutein. (C) The implicit time of the a-wave was not clearly changed. (D) Amplitude of the b-wave was also decreased during EIU but was not significantly altered by lutein. (E) The implicit time of the b-wave was not clearly changed. *P < 0.05. n = 4.

![Image](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932958/ on 06/25/2017)

**FIGURE 3.** Inhibitory effect of lutein on the STAT3 activation induced by EIU. (A, B) STAT3 activation, analyzed by immunoblotting of phosphorylated STAT3, in the retinas with EIU was mostly prevented by treatment with lutein. *P < 0.05. **P < 0.01. n = 9, n = 9, n = 13, respectively.
adult retinal explant culture. This reduction was cancelled by a proteasome inhibitor (MG132), by the introduction of the shRNA against Ubr1, or by a JAK inhibitor, AG490, which inhibits STAT3 activation. In the present study, rhodopsin mRNA expression measured by real-time RT-PCR did not show an alteration after EIU induction, with or without the administration of lutein (data not shown), suggesting that lutein might have inhibited rhodopsin protein degradation through UPS. Because ubiquitin is observed in the OS of rod photoreceptor cells, increased protein modification by ROS may rapidly induce an excessive degradation of rhodopsin through UPS. Moreover, lipid peroxidation was observed in the OS during EIU, which may inhibit normal rhodopsin transfer to the disc membrane. Abnormal distribution of rhodopsin may further induce its degradation.

Alternatively, because various kinds of cytokine signaling is upregulated during inflammation, other kinds of posttranscriptional pathways may be also involved. Given that rhodopsin reduction was prevented by suppressing oxidative stress, other candidate pathways for the rapid decrease in a protein might be excessive protein degradation by ubiquitin-independent proteasome pathways and autophagocytosis.

Induction of GFAP expression during inflammation was also prevented by suppression of STAT3 activation by lutein, consistent with the finding that STAT3 activates gfap transcription. GFAP expression has long been recognized as a marker of pathologic change in Müller glial cells; moreover, it is now clarified that GFAP itself contributes to the pathogenesis of the photoreceptor cells by changing the microenvironment of the retina. Lutein was also effective on Müller glial cells to obtain better visual function during inflammation.

In summary, our findings support the idea that inflammatory reactions threaten visual function by generating oxidative

![Figure 4](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932958/)

**Figure 4.** Inhibitory effect of lutein on ROS accumulation during EIU. ROS, detected by DHE, were upregulated in most of the retinal neural cells in EIU (B compared with A). However, treatment with lutein decreased the level of ROS (C). Fluorescence intensity was measured by ImageJ (D).

![Figure 5](http://iovs.arvojournals.org/pdfaccess.ashx?url=/data/journals/iovs/932958/)

**Figure 5.** Inhibitory effect of lutein on GFAP induction in Müller glial cells. Immunohistochemistry. GFAP expression (A–C) was observed only in glial cell endfeet in control conditions (A) but was clearly upregulated through the columnar cell bodies in EIU (B). However, this change was mostly inhibited after treatment with lutein (C). Nuclear staining of part of this area is shown as a guide to the retinal layers (D).
stress and accelerating cytokine signaling in the retinal neural cells. Treatment with lutein successfully reduced ROS, which prevented the decrease in rhodopsin level, induction of GFAP expression, and visual dysfunction during inflammation. Thus, the administration of an antioxidant, lutein, may be a potential therapeutic approach for neuroprotection against retinal inflammatory diseases.

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