Anti-Inflammatory Effects of Lutein in Retinal Ischemic/Hypoxic Injury: In Vivo and In Vitro Studies

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PURPOSE. Lutein protects retinal neurons by its anti-oxidative and anti-apoptotic properties in ischemia/reperfusion (I/R) injury while its anti-inflammatory effects remain unknown. As Müller cells play a critical role in retinal inflammation, the effect of lutein on Müller cells was investigated in a murine model of I/R injury and a culture model of hypoxic damage.

METHODS. Unilateral retinal I/R was induced by a blockade of internal carotid artery using the intraluminal method in mice. Ischemia was maintained for 2 hours followed by 22 hours of reperfusion, during which either lutein (0.2 mg/kg) or vehicle was administered. Flash electroretinogram (flash ERG) and glial fibrillary acidic protein (GFAP) activation were assessed. Lutein’s effect on Müller cells was further evaluated in immortalized rat Müller cells (rMC-1) challenged with cobalt chloride-induced hypoxia. Levels of IL-1β, cyclooxygenase-2 (COX-2), TNFα and nuclear factor– NF-kappa-B (NF-κB) were examined by Western blot analysis.

RESULTS. Lutein treatment minimized deterioration of b-wave/a-wave ratio and oscillatory potentials as well as inhibiting up-regulation of GFAP in retinal I/R injury. In cultured Müller cells, lutein treatment increased cell viability and reduced level of nuclear NF-κB, IL-1β, and COX-2, but not TNFα after hypoxic injury.

CONCLUSIONS. Reduced gliosis in I/R retina was observed with lutein treatment, which may contribute to preserved retinal function. Less production of pro-inflammatory factors from Müller cells suggested an anti-inflammatory role of lutein in retinal ischemic/hypoxic injury. Together with our previous studies, our results suggest that lutein protected the retina from ischemic/hypoxic damage by its anti-oxidative, anti-apoptotic, and anti-inflammatory properties. (Invest Ophthalmol Vis Sci. 2012;53:5976–5984) DOI:10.1167/iovs.12-10007

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Laboratory Animal Unit of The University of Hong Kong. Animals were divided into three groups: sham control group, vehicle-treated group, and lutein-treated group. Unilateral retinal I/R was induced in the right eye with the contralateral eye as control. All the experimental and animal handling procedures were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Committee on the Use of Live Animals in Teaching and Research in The University of Hong Kong (CULATR #164808 and #2493-11).

Animal Model of Retinal Ischemia/Reperfusion

Unilateral retinal ischemia was induced using the middle cerebral artery occlusion model that has been described previously. Briefly, animals were anesthetized (2% halothane in 70% N<sub>2</sub>O/30% O<sub>2</sub> for induction, and 1% halothane in 70% N<sub>2</sub>O/30% O<sub>2</sub> for maintenance) and an 8/0 nylon monofilament (Johnson & Johnson, Brussels, Belgium) coated with vinyl polysiloxane impression material (3M Dental Products, St. Paul, MN) was inserted into the right internal carotid artery (ICA) through the right external carotid artery (ECA). The ICA is one of the bifurcations of the common carotid artery (CCA) and provides blood supply to the cerebral regions. It also provides blood supply to the eye as the ophthalmic artery is a branch of the pterygopatalline artery (PPA), which originates from the ICA. Both the right CCA and right ECA were ligated to avoid anastomoses between the ophthalmic artery and the ECA. Successful insertion was confirmed by monitoring the relative cerebral blood flow of middle cerebral artery territory using a laser Doppler flowmeter (Perimed, Järfalla, Sweden). Ischemia was maintained for 2 hours with the filament kept inside the ICA. Reperfusion was then allowed for 22 hours upon filament removal.

Tissue Processing

Eyeballs were fixed with 4% ice cold paraformaldehyde in PBS (0.01 M; pH 7.4) overnight at 4°C. Eyeballs were dehydrated with graded series of ethanol and chloroform, and embedded in paraffin. Seven-micron thick cross sections were cut through the cornea parallel to the optic nerve using a microtome (Microm HM 315R; Microm, Heidelberg, Germany). Sections containing the optic nerve head were selected for histologic and immunohistochemical investigation.

Histology and Immunohistochemistry

Deparaffinized and rehydrated retinal sections were stained with hematoxylin and cosin (H and E) to reveal the histology. For immunohistochemistry (IHC), sections were subjected to antigen retrieval by incubation with proteinase K (20 μg/mL in 1× PBS) for 4 minutes at room temperature. Sections were blocked with normal goat serum and incubated with antibodies against GFAP (1:200; Dako, Glostrup, Denmark) and glutamine synthase ([GS]1:500; Millipore, Billerica, MA) overnight at 4°C. Signals were visualized by reaction with the corresponding anti-rabbit secondary antibody and anti-mouse secondary antibody (1:500 Molecular Probes; Invitrogen Corporation, Carlsbad, CA) for 60 minutes at room temperature. The sections were then coverslipped for examination. Semiquantitative analysis was used to assess the immunoreactivity as previously described. Briefly, all retinal sections for analysis were processed at the same time in a single round of IHC experiment. After the immunohistochemical procedures, microscopic slides were randomly coded and examined in a blinded approach. IHC scores were given according to the intensity as well as the number of cells stained. Score 1 represented the weakest immunoreactivity while score 5 indicated the highest immunoreactivity. Retinal sections were then decoded and the scores were compared among the experimental groups. Photomicrographs were captured and merged using a light microscope (Eclipse 80i; Nikon, Tokyo, Japan) equipped with a digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI).

In Vitro Model of Cobalt Chloride (II)–Induced Hypoxia in Müller Cell Culture

An immortalized rMC-1<sup>C2</sup> was routinely maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco). Cells were grown in a humidified incubator of 95% air and 5% CO<sub>2</sub> at 37°C. Cells were passaged when 80% confluent.

Before inducing hypoxia, cells were starved in DMEM with 1% FBS for 4 hours. 300 μM of cobalt (II) chloride (CoCl<sub>2</sub>; Sigma-Aldrich, St. Louis, MO) was used to induce chemical hypoxia for 24 hours. Either lutein (2.5, 5.0, 10, and 20 μM) or vehicle (0.01% DMSO) was added to the culture simultaneously during the procedures. For cell viability assay, cells were seeded in 96-well plates at a density of 5000 cells per well in DMEM with 10% FBS for 24 hours. For Western blot experiments, cells were seeded in 6-well plates at a density of 2 × 10<sup>5</sup> cells per well in DMEM with 10% FBS for 24 hours before treatments.

Cell Viability Assay

Cell viability was studied using CellTiter 96 Aqueous Proliferation Assay (Promega, Madison, WI)<sup>20,27,41,42</sup> according to the manufacturer’s protocol. Briefly, rMC-1 cells were treated as described above for 24 hours. After washes with 0.01 M PBS, 20 μL of the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate (PMS) mixture was added to the wells and incubated for 3 hours at 37°C. Absorbance at 490 nm was measured with a microplate reader (ELX 800; BioTek Instruments, Winooski, VT). The results were taken from six individual experiments in triplicates.

Western Blot Analysis

Whole cell lysates were prepared by addition of lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl 1% Triton X-100, 0.1% SDS, 1% Na-deoxycholate). The nuclear extracts were prepared using NE-PER...
Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL). Protein lysate of rMC-1 cells was separated by SDS-PAGE and then transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk, membranes were incubated with primary antibodies: β-actin (1:10,000; Chemicon, Temecula, CA), IL-1β (1:1000; Abcam, Cambridge, MA), cyclooxygenase-2 (Cox-2) (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), phospho-nuclear factor–kappa-B (P–NF-κB) (1:1000; Cell Signaling Technology, CST, Beverly, MA), and Histone H3 (1:1000; Cell Signaling Technology). After secondary antibody incubation, signals were detected by enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Arlington Heights, IL). The signals on the films were scanned and quantified using Image J software (National Institute of Mental Health, Bethesda, MD). The results were taken from five individual experiments with duplicate samples.

Statistical Analysis
A blind approach was used to eliminate any subjective bias during the experimental procedures; this applied to the injections and IHC scoring in immunohistochemistry. Data are presented as mean ± SEM and analyzed using a statistical program (Prism v4.0; GraphPad Software Inc., San Diego, CA). ANOVA followed by Bonferroni multiple comparison test was used for the data analysis of ERG and Western blotting. Kruskal-Wallis followed by Dunn’s multiple comparison test was used to analyze the IHC scores. Statistically significant difference was set at \( P < 0.05 \) versus sham control group.

RESULTS

Functional Analysis Using Electroretinogram
Flash ERGs were recorded in all experimental groups. The vehicle-treated group showed attenuated responses, whereas the lutein-treated group displayed similar responses when compared with the sham control group (Fig. 1A). Amplitudes of a-wave, b-wave, and OPs were measured. In addition, the ratio of b-wave/a-wave amplitude (b/a ratio) was also estimated as it is one of the sensitive parameters to evaluate the functional changes in retinal injury. The amplitudes of a-wave in all animal group did not show any noticeable difference (Fig. 1B; \( P > 0.05 \)). There was a trend of decrease of b-wave amplitude in the vehicle-treated group when compared with that in the sham control and the lutein-treated group, but the difference was not significant (Fig. 1C; \( P > 0.05 \)). In contrast, the b/a ratio and OPs were significantly reduced in the vehicle-treated group when compared with those in the sham control group (b/a ratio: Fig. 1D, \( P < 0.05 \); OPs: Fig. 1E, \( P < 0.01 \)). Lutein treatment showed a significant increase in b/a ratio and OPs amplitude (Figs. 1D, 1E; \( P > 0.05 \) versus vehicle-treated group).

Histology and Immunoreactivity of GFAP and GS in the Retina
Retinal I/R injury induced a marked cell loss in the ganglion cell layer (GCL) and inner nuclear layer (INL) as previously reported (Fig. 2B). Numerous empty space was observed in both layers with loosely packed cells when compared with the sham control retina (Fig. 2A). Many pyknotic nuclei were also present. With lutein treatment, the retina mostly retained its normal morphology, with densely packed cells and much reduced pyknotic nuclei (Fig. 2C). To look for astrocytic and Müller cell gliosis, co-immunohistochemistry was performed with antibodies against GFAP and GS, respectively. In the sham control group, GFAP immunoreactivity was mostly confined to astrocytes in the GCL with an IHC score of 1.8 ± 0.4 arbitrary unit (Fig. 2D). GS immunostaining indicated the location of Müller cells (Fig. 2G). In the vehicle-treated group, GFAP immunoreactivity was significantly increased (Fig. 2E) and was not only confined to the GCL, but also found in the cell processes that traversed the whole retina (IHC score = 3.5 ± 1.4 arbitrary unit, \( P < 0.01 \) versus sham control group, \( n = 8 \) in each group) (arrow in Fig. 2E). Co-immunostaining with GS antibody confirmed that these GFAP-expressing cell processes that traversed the retina were Müller cell processes (arrow in Figs. 2H, K). Most importantly, GFAP immunoreactivity was significantly reduced in the lutein-
Figure 2. Representative photomicrographs showing retinal sections stained with H and E (A–C), GFAP antibody (D–F), and glutamine synthase antibody (G–I). (J–K) represented merged images of GFAP and GS co-immunostaining. Retinal I/R injury induced significant cell loss, evidenced by the presence of empty space and presence of pyknotic nuclei (B). This was prevented with lutein treatment (C). In the vehicle-treated group, GFAP immunoreactivity was not only present in the GCL but also found in the Müller cell processes (arrows), whose identity was confirmed by co-immunostaining with GS antibody (E, H, K). Most importantly, lutein treatment reduced GFAP up-regulation and extent of Müller cell hypertrophy (F, I, L). IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer. Scale bar: 25 µm.
treated group when compared with that in the vehicle-treated group (IHC score 2.1 ± 1.4 arbitrary unit, \( P < 0.05 \), Fig. 2F) while GFAP immunoreactivity was similar between the sham control group and lutein-treated group (1.8 ± 0.4 arbitrary unit versus 2.1 ± 1.4 arbitrary unit, \( P > 0.05 \), Figs. 2D, 2F). GS immunostaining also revealed a much reduced intensity and less hypertrophic Müller cell processes (Figs. 2I, 2L).

**Cell Viability of Müller Cells after Chemical Hypoxia**

To further evaluate lutein’s effect on Müller cells, CoCl\( _2 \) was used to induce hypoxia in rMC-1 cells with or without lutein administration. CoCl\( _2 \)-induced hypoxia led to a change in cell morphology (Fig. 3B) when compared with the normal control (Fig. 3A). Most cells were round in shape and many vacuoles were formed. However, the morphology of the cells after the lutein treatment (10 and 20 \( \mu \)M) was relatively more similar to that in the normal control. This evidence was supported by the cell viability assay in Figure 2F. There was a significant decrease in cell viability in the vehicle-treated cells when compared with that in the normal control (Fig. 3F; \( P < 0.001 \)). However, lutein treatment at both 10 and 20 \( \mu \)M significantly increased cell viability as compared with the vehicle-treated group (Fig. 3F; \( P < 0.05 \) and \( P < 0.01 \) at 10 and 20 \( \mu \)M, respectively).

**Expression of Pro-Inflammatory Factors Induced by Hypoxia in Cultured Retinal Müller Cells**

Exposure of rMC-1 cells to 300 \( \mu \)M of CoCl\( _2 \) significantly up-regulated the expression of pro-inflammatory markers such as IL-1\( \beta \), Cox-2, and TNF-\( \alpha \) (Figs. 4A, 4B). To further examine the anti-inflammatory effects of lutein, rMC-1 cells were treated with CoCl\( _2 \) together with various concentration of lutein. Protein levels of IL-1\( \beta \) and Cox-2 were suppressed by 20 \( \mu \)M of lutein while expression of TNF-\( \alpha \) remained unaffected (Figs. 4A, 4B). Furthermore, CoCl\( _2 \) induced hypoxia increased P–NF-\( \kappa \)B expression in the nuclear extract and its expression was significantly attenuated by lutein treatment (20 \( \mu \)M) (Fig. 5).

**DISCUSSION**

Retinal I/R injury leads to irreversible functional and structural damage in retina. The present study demonstrated that lutein treatment could restore retinal function indicated by ERG response during retinal I/R injury. Moreover, lutein treatment decreased Müller cell gliosis by inhibiting the activation of GFAP in retina, which may contribute to the preserved retinal function. To further investigate the effect of lutein in Müller cells, chemical hypoxia was induced in Müller cell culture. Increased cell viability and decreased levels of NF-\( \kappa \)B nuclear translocation were observed in lutein-treated group. Moreover, lutein treatment decreased the protein expression of COX-2 and IL-1\( \beta \), but not TNF-\( \alpha \). All this evidence suggests that lutein protects the retina functionally and structurally from retinal I/R injury, at least in part, by its anti-inflammatory property.

ERG is a noninvasive objective test to assess retinal function. The a-wave is the response generated by photoreceptors. OPs and b-wave are the responses from the retinal cells at the postreceptoral level.\(^{47}\) It has been suggested that b-wave could be used as an index of retinal ischemia and the amount of reduction in amplitude corresponds to the severity of the damage.\(^{48}\) In an animal model of retinal ischemia, the observed reduction of b-wave correlated to the structural damage in retina.\(^{49}\) Reduction of b/a ratio is another sensitive prognostic sign for ischemic injury in an animal model of retinal ischemia and in human central retinal vein occlusion.\(^{43–46}\) In the present study, no obvious reduction of a-wave was noted in the vehicle-treated animals, indicating no considerable functional deficiency in photoreceptors in our retinal I/R model. This correlated with our previous findings that no apoptotic nucleus was found in the outer retinal layer after ischemic injury.\(^{53}\) A significantly smaller b/a ratio and OPs was observed in the vehicle-treated group implying a functional impairment at the postreceptoral...
level including neurons and glial cells. The functional changes at the postreceptoral level is well correlated to the morphological results in our previous published study, which showed many apoptotic nuclei, decreased expression of cell type-specific markers and decreased cell counts in the inner retinal cells.

In addition, gliosis of Müller cells was found after ischemia, which may also attribute to the decrease of b/a ratio and OPs in the vehicle-treated group. In contrast and most importantly, lutein treatment reversed all the ERG changes associated with I/R injury, indicating its protective role in preventing or minimizing the functional damage caused by retinal ischemia.

Lutein belongs to the xanthophylls family and is the major component of macular pigment. Its potent anti-oxidative property protects the macula from damage by strong energy blue light. High intake of anti-oxidants, including lutein, have been shown to inversely correlate with the prevalence of AMD. In clinical trial studies, improved visual function and increased macular pigment optical density have been shown in AMD patients treated with lutein supplements.
known that lutein is a potent anti-oxidant and protects the retina from oxidative stress. In an animal study of retinal I/R injury, lutein treatment could decrease the level of malondialdehyde (an indicator of oxidative stress) and increase the level of glutathione (an indicator of intrinsic anti-oxidative capacity).\(^3\) We also previously demonstrated that lutein could protect the retina from I/R injury by its anti-oxidative and anti-apoptotic properties.\(^5\) Fewer TUNEL-positive cells and decreased levels of poly(ADP-ribose) (PAR) and nitrotyrosine (NT) were noted in retina of lutein-treated animal. Lutein treatment could also rescue retinal neurons from CoCl₂-induced hypoxic injury in vitro.\(^5\) Treatment of CoCl₂ simulates the situation of hypoxia by modulating similar gene and protein expression as in ischemia.\(^5\) Moreover, lutein is neuroprotective in the retina in various disease models including endotoxin-induced uveitis, streptozotocin-induced diabetes, and light-induced retinal degeneration.\(^7,17\)

An increasing body of evidence shows that lutein has anti-inflammatory effects against injuries in addition to its anti-oxidative and anti-apoptotic properties. Dietary lutein reduces inflammation and immunosuppression-induced by ultraviolet radiation in mice.\(^5\) Treatment of lutein increased survival rate and decreased cell death by inhibiting the NF-kB signaling in an animal model of ischemic stroke in our previous study.\(^5\) Lutein decreased lipopolysaccharides (LPS)-induced inflammation by modulating gene and protein expression of IL-1β, COX-2, TNF-α, and inducible form nitric oxide synthase (iNOS) in mouse macrophage cells.\(^5,54\) In animal models of ocular diseases such as laser-induced choroidal neovascularization\(^5\) and LPS-induced uveitis,\(^5\) lutein treatment suppressed inflammation by inhibiting the activation of NF-kB signaling pathway and subsequent up-regulation of pro-inflammatory molecules.

The current study further demonstrated that the anti-inflammatory effect of lutein in I/R injury in vivo and in vitro. In I/R retina, Müller cell gliosis and hypertrophy are complications that worsen the pathological conditions. Retinal Müller cells exhibit hypertrophic morphology and increased expression of GFAP in ocular diseases such as retinal I/R injury\(^5,57,58\) and diabetic retinopathy.\(^5\) In fact, a Müller cell is one of the primary sources of pro-inflammatory cytokine during injuries. In a hyperglycemic condition that mimics diabetes, increased levels of IL-1β\(^5,60,61\) and TNF-α\(^6\) were observed in rMC-1. In the present study, we further showed that CoCl₂-induced hypoxia led to increased levels of pro-inflammatory molecules in Müller cells that could be suppressed by lutein administration. As postischemic inflammation plays a pivotal role in the pathogenesis of I/R injury, one beneficial effect of lutein in I/R injury would be by decreasing GFAP activation and attenuating the release of pro-inflammatory molecules from Müller cells.

NF-kB is a transcription factor and plays a key role during inflammation.\(^6,62\) It is a dimeric protein and stays at an inactive state in the cytoplasm by associating with its inhibitory unit IkB.\(^6,63,64\) Inflammatory stimuli triggers phosphorylation and polyubiquitination of inhibitory kappa B (IκB), leading to dissociation and degradation of IκB and subsequent translocation of NF-kB from cytoplasm to nucleus. Activation of NF-kB signaling resulted in expression of pro-inflammatory cytokines such as IL-1β, TNF-α, as well as COX-2.\(^5,55,64,66\) Therefore, therapeutic agents that can block or inhibit the NF-kB-related signaling pathway and, consequently, the production of pro-inflammatory cytokine may prevent the injury due to inflammation.\(^58,59,67\) We speculated that lutein inhibits IL-1β and COX-2 expression through the reduction of NF-kB nuclear translocation in rMC-1 cells. Indeed, our findings demonstrated that expression of the active form of NF-kB, P-NF-kB, was up-regulated in nuclear extract after CoCl₂-induced hypoxia, indicating the activation of NF-kB signaling similar to that in human skin keratinocytes (HaCaT cells).\(^7,60\) Most importantly, we found that lutein was able to attenuate P-NF-kB level in the nuclear extract, suggesting that lutein might impose an anti-inflammatory effect through suppressing the NF-kB signaling pathway in Müller cells. Furthermore, we found that lutein suppressed expression of both IL-1β and COX-2 in rMC-1 cells despite increased cell viability upon CoCl₂-induced hypoxia, in agreement with the previous study using the LPS cell model that lutein attenuated pro-inflammatory protein expression via inhibition of NF-kB pathway.\(^5\) Yet, no significant decrease in TNF-α level could be observed after lutein treatment. We speculate that TNF-α production may not solely be dependent on the NF-kB mediated pathway; there may be other pathway(s) that can also regulate TNF-α production. Indeed, Hocarau et al.\(^7\) showed that both NF-kB and the p38 MAP Kinase pathway could be involved in TNF-α secretion.

**Conclusions**

Lutein post treatment prevented the functional impairment due to retinal I/R. It minimized gliosis in Müller cells and also exerted its anti-inflammatory effects by suppressing the...
activation of NF-κB and subsequent production of pro-inflammatory markers, IL-1β and COX-2, in Müller cells.

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References
