Effect of Lutein on Retinal Neurons and Oxidative Stress in a Model of Acute Retinal Ischemia/Reperfusion

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PURPOSE. Retinal ischemia/reperfusion (I/R) occurs in many ocular diseases and leads to neuronal death. Lutein, a potent antioxidant, is used to prevent severe visual loss in patients with early age-related macular degeneration (AMD), but its effect on I/R insult is unclear. The objective of the present study is to investigate the neuroprotective effect of lutein on retinal neurons after acute I/R injury.

METHODS. Unilateral retinal I/R was induced by the blockade of internal carotid artery using intraluminal method in mice. Ischemia was maintained for 2 hours followed by 22 hours of reperfusion, during which either lutein or vehicle was administered. The number of viable retinal ganglion cells (RGC) was quantified. Apoptosis was investigated using TUNEL assay. Oxidative stress was elucidated using markers such as nitrotyrosine (NT) and poly(ADP-ribose) (PAR).

RESULTS. In vehicle-treated I/R retina, severe cell loss in ganglion cell layer, increased apoptosis as well as increased NT and nuclear PAR immunoreactivity were observed. In lutein-treated I/R retina, significantly less cell loss, decreased number of apoptotic cells, and decreased NT and nuclear PAR immunoreactivity were seen.

CONCLUSIONS. The neuroprotective effect of lutein was associated with reduced oxidative stress. Lutein has been hitherto used principally for protection of outer retinal elements in AMD. Our study suggests that it may also be relevant for the protection of inner retina from acute ischemic damage. (Invest Ophthalmol Vis Sci. 2009;50:836–843) DOI:10.1167/iovs.08-2510

Retinal ischemia/reperfusion (I/R) can be a feature of pathologies such as amaurosis fugax and acute angle-closure glaucoma. It can lead to irreversible neuronal damage and visual impairment. Diverse investigations have been carried out to study the effects of I/R damage. In animal models of retinal I/R induced by raising the intraocular pressure (IOP), a loss of retinal ganglion cells (RGC), and an increase in the number of apoptotic nuclei in inner retina have been observed.1 In addition, a significant reduction of b-wave in electroretinogram, indicating an impairment of retinal response, has been shown to occur in I/R retina induced by raised IOP or arterial occlusion.2,3

Reperfusion after ischemia predisposes the retina to oxidative damage. Cellular events such as disruption of ion homeostasis, depletion of adenosine triphosphate store, and glutamate-induced excitotoxicity happen during the challenge of oxidative stress, which lead to production of free radicals.4,5 Excess accumulation of free radicals causes deleterious effects on retinal neurons, including an increased level of lipid peroxidation6–9 and a depletion of endogenous free radical scavengers.10 Conversely, the reduction of free radical formation and oxidative stress have also been shown to retard or prevent neuronal cell death in ischemic retina.11,12 Lutein belongs to the xanthophyll family of carotenoids and is contained in dark green leafy vegetables, such as spinach and kale.13–15 It is characterized by having a hydroxyl group attached to each end of the molecule, making it more hydrophilic; therefore, lutein reacts more strongly with singlet oxygen than other carotenoids.16,17 Lutein is also an efficient pigment for absorbing high energy blue light, as it has a peak absorption at 446 nm.18,19 In ocular tissues, lutein is found in the macula region. The antioxidant property of lutein protects macula and photoreceptors from phototoxicity and oxidative injury.19–22 Lutein has also been shown to improve the visual performance in patients with atrophic macular degeneration.23 It is currently being investigated in the Age-related Eye Disease Study II for its safety and efficacy in reducing the risk of visual loss in age-related macular degeneration (AMD).24

The evidence for the protective effect of lutein on I/R retina is limited. Recently lutein has been shown to attenuate lipid peroxidation and increase endogenous antioxidant capacity after pressure-induced I/R injury.2,3 In this study, only molecular assays were carried out, whereas the specific effects on various retinal cell types were not elucidated. The aim of our present study was to investigate the histologic changes and biological response to lutein in a vascular occlusion I/R mouse model.

METHODS

All chemicals were purchased from Sigma (Sigma-Aldrich Co, St. Louis, MO) unless otherwise stated.

Animals

C57BL/6N male mice (10 to 12 weeks) were kept in a temperature-controlled room with 12-hour light/12-hour dark cycle in the Laboratory Animal Unit of The University of Hong Kong. Animals were divided into two groups: lutein-treated group (n = 7) and vehicle-treated group (n = 11). 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 Faulty Committee on the Use of Live Animals in Teaching and Research in The University of Hong Kong.
Retinal Ischemia/Reperfusion Model and Treatment

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\textsubscript{2}O/30% O\textsubscript{2} for induction, and 1% halothane in 70% N\textsubscript{2}O/30% O\textsubscript{2} for maintenance) and a nylon monofilament suture (Johnson & Johnson, Brussels, Belgium) coated with vinyl polysiloxane impression material (3M Dental Products, St. Paul, MN) was inserted into the internal carotid artery through the external carotid artery. As the ophthalmic artery is one of the bifurcations of internal carotid artery, unilateral ophthalmic artery occlusion could be achieved by the blockade of this vessel. Successful insertion was confirmed by monitoring the relative cerebral blood flow of middle cerebral artery territory using a laser Doppler flowmeter (Perimed, Järfälla, Sweden). Ischemia was maintained for 2 hours with the filament kept inside the internal carotid artery. Reperfusion was then allowed for 22 hours on filament removal.

Lutein Treatment

Lutein (0.2 mg/kg) or vehicle 10% dimethyl sulfoxide (DMSO) at 4 mL/kg (i.e., 0.1 mL/25 g) was administered by intraperitoneal injection 1 hour before and 1 hour after reperfusion.

Tissue Processing

Animals were killed by cervical dislocation. Eyeballs were immediately enucleated and fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; 0.01 M; pH 7.4) overnight at 4°C. Eyeballs were then dehydrated with graded series of ethanol and chloroform, and embedded in paraffin. Cross-sections (7 μm) were cut using a microtome (Microm HM 315R, Heidelberg, Germany).

Histologic Evaluation on Retinal Sections

Retinal sections of lutein-treated and vehicle-treated eyes were deparaffinized and stained with hematoxylin and eosin (H&E). Viable cells in ganglion cell layer (GCL) of the central (100 μm from optic nerve head) and the peripheral retina (100 μm from the peripheral end of retina) were counted. Four pictures, two from central and two from peripheral retina of each retinal section were captured. Four retinal sections for each animal were selected randomly for cell counting. Cells with pyknotic nuclei were defined as dead cells and were excluded in the counting. Retinal swelling in our I/R model has been shown to be due to the increase in thickness of inner retinal layer. Therefore, retinal swelling was assessed by measuring the thickness of inner plexiform layer (IPL) in retinal sections in the present study. For consistency, only retinal sections with optic nerve stumps were used.

Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick-end Labeling (TUNEL)

Apoptosis was examined by TUNEL assay (DeadEnd Fluorometric TUNEL system, Promega, Madison, WI). Sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI) after the reaction. This is to verify that the TUNEL staining is localized in the nucleus. Only sections with optic nerve stumps were used and their images were captured. Six pictures (two from the central, two from the middle, and two from the peripheral retina) were captured from each retinal section. The number of TUNEL-positive nuclei in both GCL and inner nuclear layer (INL) were counted.

Immunohistochemistry

After deparaffinization, antigen retrieval was achieved by incubation with proteinase K. Sections were blocked with normal serum and incubated with primary antibodies: goat anti-calretinin, rabbit anti-PKC (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-calbindin (1:500; Chemicon, Temecula, CA), rabbit anti-neuronal nitric oxide synthase (nNOS 1:1000; Upstate Biotechnology, Lake Placid, NY), rabbit anti-nitrotyrosine (NT 1:200; Upstate Biotechnology) and mouse anti-poly(ADP-ribose) (PAR 1:200; Alexis, Lausen, Switzerland) overnight at 4°C (except calbindin for 72 hours at 4°C). Positive control for nitrotyrosine immunohistochemistry was done by incubating the sections with peroxynitrite (Upstate Biotechnology) according to the manufacturer’s instructions. Signals were visualized by reaction with corresponding secondary antibodies (1:500; Molecular Probes, Invitrogen Corporation, Carlsbad, CA) for 60 minutes at room temperature. The sections were then washed and coverslipped for examination.
that the number of viable cells in both central and peripheral retinas of the lutein-treated I/R eyes was not significantly different from its contralateral control (Fig. 2A).

IPL Thickness
There appeared to be an increased IPL thickness in the vehicle-treated I/R retina compared with its contralateral control (Figs. 1A and 1B); the difference however was not statistically significant (Fig. 2B). There was also no significant difference in the IPL thickness between the lutein-treated I/R retina and its contralateral control (Fig. 2B).

Apoptosis in I/R Retina
Apoptosis-induced neuronal damage in GCL and INL after retinal I/R insult was evaluated by TUNEL staining (Fig. 3 and Fig. 4). In the contralateral control retinas of both vehicle-treated and lutein-treated animals, no TUNEL-positive cells could be found in GCL and INL (Figs. 3A and 3C, and Fig. 4). In the vehicle-treated I/R retina, there was a marked increase in the number of TUNEL-positive nuclei in both GCL and INL (Fig. 3B and Fig. 4) compared with its contralateral control (Fig. 3A and 4). In the lutein-treated I/R retina, there were significantly fewer apoptotic nuclei in the GCL ($P = 0.0005$; unpaired $t$-test) and the INL ($P = 0.01$; unpaired $t$-test) compared with those in the vehicle-treated I/R retina (Figs. 3B and 3D, and Fig. 4).

Other Inner Retinal Neurons
In the normal retina, calretinin is expressed in amacrine cells in the innermost layer of INL and displaced amacrine cells in GCL.\cite{26,27} Our results showed that in the contralateral control retinas (of both the vehicle-treated and lutein-treated animals), the terminals of amacrine cells formed three distinct strata in IPL (Figs. 5A and 5C). The cell bodies were also positively stained with calretinin in both GCL and INL (Figs. 5A and 5C). In vehicle-treated I/R retina, there appeared to be fewer calretinin-positive cells in these two cellular layers (Fig. 5B). Of the three strata in the IPL, staining of the innermost layer appeared weak in the vehicle-treated I/R retina (Fig. 5B). In lutein-treated I/R retina, there appeared to be less attrition of the amacrine cells; the three strata of IPL were intact (Fig. 5D).

In the normal retina, a small population of amacrine cells express nNOS.\cite{28} Our results showed that in the contralateral control retinas (of both the vehicle-treated and lutein-treated animals) sparse nNOS-expressing amacrine cells and displaced amacrine cells were visible in the GCL and INL (Figs. 5E and 5G). In vehicle-treated I/R retina, these nNOS-expressing amacrine cells were rarely seen (Fig. 5F). In lutein-treated I/R retina (Fig. 5H), qualitatively at least, the number of nNOS-positive cells appeared similar to that in the contralateral control retina (Fig. 5G).

In the normal retina, the cell bodies of PKCa-labeled rod bipolar cells were present in the outer border of INL and their axons terminate at the inner border of IPL.\cite{28,29} In the vehicle-treated I/R retina, qualitatively PKC-$\alpha$ immunoreactivity appeared to be less (Fig. 5J) compared with its contralateral control (Fig. 5I). In the lutein-treated I/R retina the PKC-$\alpha$ immunoreactivity also appeared to be less (Fig. 5L) compared with its contralateral control (Fig. 5K).

Calbindin immunohistochemistry was used to identify horizontal cells (Figs. 5M–5P). In the vehicle-treated I/R retina, qualitatively there appeared to be fewer calbindin-positive horizontal cells (Fig. 5N) compared with its contralateral control (Fig. 5M). In contrast, in the lutein-treated I/R retina the number of calbindin-positive cells appeared to be similar (Fig. 5P) to that in its contralateral control (Fig. 5O).

**RESULTS**

Ganglion Cells
Retinal I/R induced a marked neuronal loss in the GCL of both central and peripheral retinas of vehicle-treated animal (Figs. 1B and 1F). The cells were loosely packed and pyknotic. Numerous empty spaces were observed in the cellular layers in both GCL and INL (Figs. 1B and 1F). Quantitative analysis showed that there were fewer viable cells (non-pyknotic nuclei) in the vehicle-treated I/R retina compared with its contralateral control (Fig. 2A; $P < 0.001$; ANOVA). However, there were only a few pyknotic cells and the ganglion cells stayed closely packed in both central and peripheral retinas of lutein-treated eyes (Figs. 1D and 1H). Quantitative analysis showed

Study Design and Statistical Analysis
A blinded approach was used to eliminate any subjective bias during the experimental procedures; this applied to the injections, cell counting and all analyses. Unpaired-$t$ test or one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison test was used for the analysis of results (Prism 4.0, GraphPad Software, Inc., San Diego, CA). Statistically significant difference was set at $P < 0.05$. Error bars represented the SD of the data.

**FIGURE 2.** Quantitative analysis in vehicle-treated and lutein-treated retinas. (A) Number of viable cells in GCL. (B) Measurement of IPL thickness. **$P < 0.001$ (ANOVA followed by Bonferroni multiple comparison test); ns, no significance.**
Nitrotyrosine and PAR Immunoreactivity

NT was used as a marker of oxidative stress. The immunoreactivity of NT was weak in vehicle-treated contralateral control retina (Fig. 6A1). On the other hand, the immunoreactivity of NT was marked in both GCL and INL of vehicle-treated I/R retina (Fig. 6A2). In the lutein-treated contralateral control retina, the immunoreactivity of NT was also weak (Fig. 6A3). However, the NT immunoreactivity in the lutein-treated I/R retina (Fig. 6A4) was qualitatively not different from that in its contralateral control (Fig. 6A3). A positive control of NT labeling in lutein-treated I/R retina was shown in Figure 6A5.

PAR is an indicator of DNA damage. In contralateral control retinas (of both the vehicle-treated and lutein-treated animals) the immunoreactivity of PAR was present inside the cell but not the nuclei (Figs. 6B1 and 6B3). In the GCL and INL of the vehicle-treated I/R retina, PAR immunoreactivity was noted in the cell and also inside the nuclei, which at the same time appeared condensed (Fig. 6B2). In lutein-treated I/R retina, PAR expression was not observed in the nuclei of the GCL and INL (Fig. 6B4).

DISCUSSION

Retinal I/R leads to irreversible neuronal damages, especially the inner retinal neurons.30–32 The possible neuroprotective effect of lutein after I/R injury was explored in the present study.

In rodent models of retinal I/R induced by the raised IOP, apoptosis was observed in both GCL and INL.33–35 Ultrastructural changes in I/R retina have been investigated to confirm the cellular damage of inner retinal neurons. Condensation of nuclear chromatin, accumulation of degenerating organelles and electron-dense bodies in cytoplasm, and shrinkage of nucleoplasm and cytoplasm were observed in GCL and INL of pressure-induced I/R retina in rats.36 In another animal model of I/R induced by the intraluminal method, apoptosis was demonstrated by the presence of TUNEL-positive nuclei in the GCL.37 Additionally, an alteration of synaptic connections of neurons (indicated by the decrease of immunoreactivity of Ves-1/Homer 1c) was also shown in this study.37 In our study, obvious cell loss and apoptotic nuclei were found in GCL and INL. In the past, amacrine cells were found to be affected by I/R injury.3 The immunoreactivity of calretinin-
expressing amacrine cells was greatly reduced and the stratification of IPL was altered after pressure-induced I/R injury in rats. Apart from protein levels, transcription levels in amacrine cells, such as calretinin, paravalbumin, and glycine transporter were also altered during I/R. In our study, we also qualitatively found a reduction in the immunoreactivity of calretinin-expressing amacrine cells. Bipolar cells and horizontal cells, however, in contrast to amacrine cells and RGCs, seemed relatively unaffected by I/R. In our study, we also found very little change in immunoreactivity of PKC- and calbindin were observed after retinal I/R.

Oxidative stress is one of the important factors leading to neuronal cell death in I/R. The high content of polyunsaturated fatty acid in retina makes it vulnerable to oxidative damage. Free radical formation facilitates nitric oxide (NO), which reacts with superoxide to form peroxynitrite, a strong oxidant that causes oxidation and lipid peroxidation, and subsequently damages cellular components. NT is the oxidation product of peroxynitrite reacting with tyrosine residues of proteins. It is therefore regarded as a footprint for NO oxidation and used as one of the markers for oxidative stress. Accumulation of peroxynitrite and free radicals leads to DNA damage of neurons, which then also activates PAR polymerase (PARP) to produce PAR. Indeed, increased PAR formation and NT expression were observed in GCL and INL after I/R injury induced by the intraluminal method in the present study.

Overproduction of NO by nitric oxide synthase (NOS) is recognized as a source of neuronal damage during I/R. nNOS in GCL and INL was observed in retina with ischemia induced by high IOP after 3 days of reperfusion. However, the results are controversial. nNOS immunoreactivity was found to be reduced in rat retina with pressure-induced ischemia after 8 days of reperfusion. Similarly, a decreased nNOS immunoreactivity was also observed after I/R injury for 7 days. We also found that there was an obvious reduction of nNOS expression in 1/R retina. The discrepancy could be due to the temporal effect of I/R injury on nNOS-expressing neurons. Previous studies showed that after I/R injury the number of nNOS- expressing neurons peaked at 3 to 7 days, but declined thereafter. Here, we induced retinal I/R with 2 hours of ischemia and 22 hours of reperfusion, which produced a relatively more severe damage to retinal neurons when compared with the pressure-induced I/R models. Within 24 hours, we observed destructive changes in the retina, including many TUNEL-positive nuclei in both GCL and INL. We speculate that the immunoreactivity of nNOS might be increased several hours after reperfusion and decreased subsequently. In addition to nNOS, activation of inducible NOS (iNOS) might be another possible source of NO overproduction during I/R injury. Further investigation is needed to elucidate the NOS expression in acute I/R induced by intraluminal method.

Application of antioxidants is one of the approaches to reduce damage caused by oxidative stress during I/R. Lutein is a potent antioxidant. It filters harmful short wavelength blue light and protects the retina from oxidative damage. Among the carotenoids, lutein has the highest blue light filtering efficacy in liposomes. Diets rich in antioxidants, including lutein, have been shown to inversely correlate with the prev-
Figure 6. Immunohistochemical staining of nitrotyrosine (NT) (A1–A4) in vehicle-treated and lutein-treated retinas. (A5) Positive control of nitrotyrosine staining in lutein-treated I/R retina. Immunohistochemical staining of poly (ADP-ribose) (PAR) (B1–B4) in vehicle-treated and lutein-treated retinas. PAR expression was present in the condensed nuclei of GCL and INL of the vehicle-treated I/R retina (B2, arrows). Scale bars, 25 μm.
alence of AMD.\textsuperscript{34,35} Also, improved visual function and increased macular pigment optical density in AMD patients given lutein supplements have been demonstrated in clinical trials.\textsuperscript{23,56} However, little information is available on the neuroprotective effect of lutein in retinal I/R. A previous study showed that decreased levels of malondialdehyde (an indicator of oxidative stress) and activated caspase-3, and increased levels of glutathione (indicator of intrinsic antioxidative capacity) were found in lutein-treated I/R retina in rat.\textsuperscript{12} A similar study also revealed that lutein could decrease the I/R damage by lowering cyclo-oxygenase-2 and nNOS level in retina.\textsuperscript{57} These studies were carried out using either biochemical assays or immunoblotting, which could only elucidate the effect of lutein on the whole retina in general. In the present study, we investigated the effects of lutein on I/R retina using histologic and immunohistochemical approaches, which give more information on the effect of lutein on individual cellular layers. Our results suggest that the protective effect of lutein was most prominent in GCL, in which there was decreased cell loss and a reduced number of TUNEL-positive nuclei. A similar protective effect was also observed in the INL. Therefore, lutein might increase the survival of retinal cells by reducing oxidative stress as shown by the marked reduction in NT and PAR expression.

CONCLUSION

The application of lutein protected inner retinal neurons from I/R challenge possibly by reducing the oxidative stress. Thus far, the clinical use of lutein has focused on AMD, a condition that predominantly affects outer retinal elements. Our results suggest that lutein might have a therapeutic role in protecting the inner retinal elements in eye diseases in which acute ischemia is a feature.

References


