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Lutein and zeaxanthin supplementation reduces photo-oxidative damage and modulates the expression of inflammation-related genes in retinal pigment epithelial cells

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Abstract

Oxidative damage and inflammation are related to the pathogenesis of age-related macular degeneration (AMD). Epidemiologic studies suggest that insufficient dietary lutein and zeaxanthin intake or lower serum zeaxanthin levels are associated with increased risk for AMD. The objective of this work is to test the protective effects of lutein and zeaxanthin against photo-oxidative damage to retinal pigment epithelial cells (RPE) and oxidation-induced changes in expression of inflammation-related genes. To mimic lipofuscin-mediated photo-oxidation *in vivo*, we used ARPE-19 cells that accumulated A2E, a lipofuscin fluorophore and photosensitizer, as a model system to investigate the effects of lutein and zeaxanthin supplementation. The data show that supplementation with lutein or zeaxanthin in the medium resulted in accumulation of lutein or zeaxanthin in the RPE cells. The concentrations of lutein and zeaxanthin in the cells were 2–14-fold of that detected in the medium, indicating that ARPE-19 cells actively take up lutein or zeaxanthin. As compared with untreated cells, exposure of A2E-containing RPE to blue light resulted in a 40–60% decrease in proteasome activity, a 50–80% decrease in expression of CFH and MCP-1, and an ~ 20-fold increase in expression of IL-8. The photo-oxidation-induced changes in expression of MCP-1, IL-8 and CFH were similar to those caused by chemical inhibition of the proteasome, suggesting that inactivation of the proteasome is involved in the photo-oxidation-induced alteration in expression of these inflammation-related genes. Incubation of the A2E-containing RPE with lutein or zeaxanthin prior to blue light exposure significantly attenuated the photo-oxidation-induced inactivation of the proteasome and photo-oxidation induced changes in expression of MCP-1, IL-8, and CFH. Together, these data indicate that lutein or zeaxanthin modulates inflammatory responses in cultured RPE in response to photo-oxidation. Protecting the proteasome from oxidative inactivation appears to be one of the mechanisms by which lutein and zeaxanthin modulate the inflammatory response. Similar mechanisms may explain salutary effects of lutein and zeaxanthin in reducing the risk for AMD.

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Keywords

Lutein; zeaxanthin; RPE; photo-oxidation; proteasome; inflammation

Introduction

Age-related macular degeneration (AMD) is a multifactorial disease and a leading cause of blindness in industrialized countries. In addition to aging, genetic background and cigarette smoking, dietary factors also contribute to the onset and progression of AMD [1, 2]. A growing number of studies indicate that dietary lutein and zeaxanthin play significant protective roles against visual loss from AMD. Epidemiological evidence for these protective effects was first obtained from a large case-control study, which showed that individuals with high dietary intakes and high serum levels of lutein and zeaxanthin have a much lower rate of exudative AMD [3]. Results from many, but not all, subsequent epidemiologic and case-control studies support the conclusion that the risk for onset and progression of AMD is inversely related to lutein and zeaxanthin concentrations in the diet, plasma, and macular pigment [3–8]. Lutein is found in a broad spectrum of foods, such as corn, a variety of fruits, and green vegetables [9, 10]. In comparison, the dietary sources of zeaxanthin are limited. The major sources of zeaxanthin are egg yolks, corn, corn meal, Japanese persimmons, orange peppers and leafy greens [9, 10]. In typical American diets, the contents of lutein are ~ 5 times that of zeaxanthin. The ratio of lutein to zeaxanthin in human blood is similar to that found in typical American diets, indicating that lutein and zeaxanthin are equally absorbed by humans. However, it appears that zeaxanthin preferentially accumulates in the retina, particularly in the macular region [11]. The overall ratio of lutein to zeaxanthin in the whole retina is ~2:1, but the ratio of lutein to zeaxanthin in the central macula (0 to 0.25 mm eccentricity) is less than 1:2. The differential uptake and retentions of lutein and zeaxanthin in the retina may be related to the expression of their binding proteins in the retina [12, 13]. Roughly 50% of the zeaxanthin within the macula is the meso- isomer of zeaxanthin, a metabolite of lutein, indicating that dietary supplement of lutein could also increase meso-zeaxanthin concentrations in the macula [14]. Levels of lutein and zeaxanthin and their metabolites in the retina can be detected non-invasively by measuring the macular pigment optical density. An increase in dietary intake of lutein and zeaxanthin would increase the macular pigment optical density and provide better protection against photo-oxidation [15, 16].

Oxidative stress, particularly lipofuscin-mediated photo-oxidative damage, contributes to the onset and progress of AMD [17]. Thus, it is thought that lutein and zeaxanthin in the retina may protect against AMD by two different mechanisms: blocking harmful blue light and quenching reactive oxygen species. Both lutein and zeaxanthin absorb blue light, the most phototoxic visible light to which the retina is routinely exposed. Studies in quail provide direct evidence that long-term zeaxanthin supplementation results in an increase in retinal zeaxanthin concentrations and provides a protective effect against light-induced photoreceptor death [18, 19]. In rhesus monkeys, supplementation with lutein or zeaxanthin after long-term xanthophyll deficiency protected the fovea from blue light damage [20]. Lutein and zeaxanthin are also efficient quenchers of singlet oxygen and related reactive oxygen species [21, 22]. HPLC measurement of macular pigments in post-mortem retinas of normal and AMD patients demonstrates that elevated levels of lutein and zeaxanthin are associated with reduced odds ratios for AMD [23]. Protective effects of lutein and zeaxanthin against oxidative damage initiated by acute exposure to blue-light has also been demonstrated [20].

Recent studies indicate that innate immunity and inflammation are related to AMD pathogenesis [24, 25]. The evidence for the involvement of innate immunity and inflammation in AMD pathogenesis includes accumulation of immunoglobulin and complement components in drusen [26–28], the association between genetic variants of complement factor H, factor B, C2, C3, factor I and risk for AMD [29–37], and elevated serum CRP levels in AMD patients [38–40]. Emerging evidence indicates dietary lutein and zeaxanthin have anti-inflammation functions, including a reduction of serum levels of CRP and sICAM [41–44]. It is plausible that dietary lutein and zeaxanthin reduce the risk for AMD via modulating ocular or systemic inflammation. However, the mechanisms for such functions remain to be elucidated.

The ubiquitin-proteasome pathway (UPP) is the major non-lysosomal proteolytic pathway within cells [45–47]. Proteins destined for degradation are first conjugated with a polyubiquitin chain by the sequential action of three classes of enzymes: E1, E2 and E3. The ubiquitin-protein conjugates are then recognized and degraded by a large protease complex called the proteasome [46, 48]. The UPP has been involved in a myriad of cellular processes [47, 49], including regulation of immune response and inflammation [50, 51]. Dysfunction of the UPP has been implicated in the pathogenesis of many degenerative diseases such as Alzheimer's disease [52], Parkinson's disease [53], diabetic retinopathy [54] and cataract [55, 56]. A fully functional UPP is required for cells to cope with various stresses, including heavy metals [57], amino acid analogs and oxidation [58]. However, an extensive oxidative insult also impairs the function of critical components of the UPP [59–64]. Oxidative inactivation of the proteasome not only results in accumulation of damaged proteins [56], but also impairs cell signaling process [65].

Oxidative stress and inflammation are interrelated. Whereas oxidative stress triggers inflammatory responses [66, 67], inflammation also enhances the production of reactive oxygen species. Our recent work indicates that oxidative inactivation of the proteasome is a mechanistic link between oxidative stress and increased production of IL-8 in cultured RPE [68]. Since the RPE is a major ocular source of pro-inflammatory mediators and a primary target of photo-oxidative insult, oxidative impairment of the UPP in RPE may contribute to ocular inflammation and AMD-related lesions. To explore the mechanisms by which lutein and zeaxanthin may reduce the risk for AMD, we evaluated the effects of lutein and zeaxanthin supplementation on photo-oxidation induced impairment of the UPP in cultured RPE and the consequent inflammatory response. The data indicate that supplementation with lutein or zeaxanthin to cultured RPE ameliorates photo-oxidation-induced inactivation of the proteasome and partially reverses photo-oxidation-induced changes in expression of some inflammation-related genes.

Materials and Methods

Materials

Lutein crystals were obtained from Kemin (Des Moines, IA USA). Zeaxanthin crystals were obtained from DSM (Basel, Switzerland) or purchased from Sigma Aldrich (St. Louis, MO, USA). Cell culture supplies were obtained from Invitrogen (Carlsbad, CA, USA). The DuoSet ELISA kits for human MCP-1 and human IL-8, were obtained from R&D Systems (Minneapolis, MN, USA). Mouse monoclonal antibody (capture antibody) to human CFH was purchased from Abcam (Cambridge MA, USA) and goat-polyclonal antibody (detecting antibody) to human CFH was purchased from EMD Chemicals (Gibbstown, NJ, USA). All other reagents were obtained from Sigma Aldrich (St. Louis, MO, USA).

Exposure to A2E and blue light

ARPE-19 cells were grown to confluence and then cultured in DMEM with 10% heat-inactivated fetal calf serum and 0.1 mM nonessential amino acid solution with or without 10 μ M A2E for 10 days. The medium with fresh A2E was changed twice a week. To determine the protective effects of lutein and zeaxanthin, the cells that had accumulated A2E were incubated with or without 10 μ M lutein or zeaxanthin for another 3 days in the absence of A2E. Stock solutions of lutein and zeaxanthin were prepared in DMSO in a concentration of 3.3 mM. The stock solutions were first diluted in fetal bovine serum (FBS) and then mixed with DMEM to a final concentration of 10 μ M lutein or zeaxanthin and 10% FBS. All cells, including the controls, received same amount of DMSO (0.03%) and FBS (10%). After washing twice with complete medium with 10% FBS to remove unincorporated lutein or zeaxanthin, cell cultures were transferred to PBS with calcium, magnesium and glucose and were exposed to 430 nm light delivered from a tungsten halogen source (430 nm $\pm$ 20; 10 minutes; 2.62 mW/cm²). The cells were then incubated for an additional 6 hours in DMEM with 1% FBS. After collection of the media, cells were washed twice with cold PBS and then the dishes were placed on ice and the cells were harvested with a cell scraper. Cells that had neither accumulated A2E nor been exposed to blue light were used as controls. The control cells were treated in the same manner as the cells that were exposed to A2E and blue light. Levels of MCP-1, IL-8 and CFH in the medium were determined by ELISA. The latter were performed according to the manufacturer's instructions. Total RNA was also isolated from the cells for the quantitation of mRNA levels of MCP-1, IL-8 and CFH.

Lutein and zeaxanthin determination

To determine the intake of lutein and zeaxanthin by ARPE-19, confluent monolayers of ARPE-19 cells were incubated with 1 and 10 μ M lutein or zeaxanthin in the medium containing 10% FBS for 3 days. After washing three times with DMEM containing 10% FBS, the cells were then collected and levels of lutein or zeaxanthin in the cells were determined by HPLC method as described previously [69]. The presence of FBS, which contains lipoproteins, in the washing solution helps to carry these carotenoids and reduce non-specific adherence of these carotenoids to the cell membrane. However, we cannot rule out the possibility that some of the carotenoids detected the cells was due to non-specific adherence to the cell membrane.

Proteasome activity assay

ARPE-19 cells were lysed in 25 mM Tris-HCl buffer, pH 7.6. The chymotrypsin-like activity of the proteasome was determined using the fluorogenic peptide succinyl-Leu-Leu-Val-Tyr-amidomethylcoumarin (LLVY-AMC) as a substrate [70]. The mixture, containing 20 μ l of cell supernatant in 25 mM Tris-HCl, pH 7.6, was incubated at 25 °C with the peptide substrate (LLVY-AMC at 25 μ M) in a buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA, 1 mM EGTA, 3 mM NaN₃ and 0.04% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). The final volume of the assay was 200 μ l. Rates of reactions were measured in a temperature-controlled microplate fluorometric reader. Excitation/emission wavelengths were 380/440 nm. Proteasome activity was defined as the portion of peptidase activity in the cell extracts that was inhibited by 20 μ M MG132, a potent proteasome inhibitor. Ubiquitin conjugates in the cell lysate were determined by Western Blotting with antibodies specific to ubiquitin and levels of β -actin were used as a protein loading control.

Results

Cultured ARPE-19 cells actively uptake lutein and zeaxanthin

To determine the potential mechanisms by which lutein and zeaxanthin protect the RPE, we investigated uptake of lutein and zeaxanthin by cultured RPE. ARPE-19 cells cultured with normal DMEM contained no detectable lutein or zeaxanthin. When cells were cultured in the presence of 1 or 10 μM lutein for 3 days, concentrations of lutein in the cells increased to 805 or 2233 ng/mg protein (~ 14 and $\sim 39 \mu\text{M}$), respectively. When the cells were cultured in the presence of 1 or 10 μM zeaxanthin for 3 days, the concentrations of zeaxanthin in the cells increased to 296 or 1389 ng/mg protein (~ 5 and $\sim 24 \mu\text{M}$), respectively. The concentrations of lutein and zeaxanthin in the cells were at least 2–14 fold higher than the concentrations in the medium, suggesting that the RPE cells either actively take up or trap lutein and zeaxanthin. Furthermore, a small amount of putative metabolites of lutein or zeaxanthin were detected in RPE cells, but not in the medium (Fig. 1, Compare panels B to A and D to C). These metabolites of lutein or zeaxanthin in RPE had similar, but not identical, absorbency spectra of lutein or zeaxanthin (Fig. 1, panels E and F), suggesting these putative metabolites are isomers of lutein or zeaxanthin.

Based on isomer spectra and retention times, lutein M1 could be assigned as oxo-lutein, lutein M2 as 13-cis isomer of lutein and lutein M3 as 9-cis isomer of lutein. The oxo-product was not observed for zeaxanthin and the 13-cis and 9-cis isomers of zeaxanthin corresponded to M1 and M2, respectively.

Supplementation with lutein and zeaxanthin prevents photo-oxidative inactivation of the proteasome

The UPP is the primary proteolytic system in RPE, with important roles in many cellular functions. Although a fully functional UPP is required for cells to recover from oxidative stress, the UPP itself is also a target of severe oxidative stress. We previously demonstrated that the proteasome is one of the components of the UPP that are vulnerable to oxidative inactivation [64]. Impairment of the UPP in the RPE alters the expression of AMD-related genes, such as VEGF, MCP-1 and IL-8 [68, 71, 72]. To begin to decipher how carotenoids may protect the proteasome from photo-oxidative inactivation, we tested the effects of lutein or zeaxanthin on photo-oxidative inactivation of the proteasome in cultured RPE. As shown previously [64], exposure of A2E-containing RPE cells to blue light resulted in $\sim 60\%$ decrease in proteasome activity (Fig. 2A). However, if A2E-containing RPE cells were incubated with 10 μM lutein or zeaxanthin prior to blue light exposure, the inactivation of proteasome was substantially attenuated (Fig. 2A). Whereas incubation with 10 μM lutein almost completely blocked the photo-oxidative inactivation of the proteasome, the same concentration of zeaxanthin only provided 50% of the protection (Fig. 2A). The greater protective effect of lutein, as compared to zeaxanthin, may be related to its higher cellular accumulation. As presented in the above paragraph, when incubated with the same level of lutein or zeaxanthin for the same period, the RPE cells accumulated nearly twice the amount of lutein as that of zeaxanthin. For example, after incubation with 10 μM lutein or zeaxanthin for three days, concentrations of lutein in RPE cells reached $\sim 39 \mu\text{M}$, whereas concentrations of zeaxanthin in the cells were only $\sim 24 \mu\text{M}$. Consistent with the role of the proteasome in the degradation of ubiquitinated proteins, photo-oxidation induced inactivation of the proteasome was associated with an increase in levels of ubiquitin conjugates (Fig. 2B, compare lanes 2 with 1). Supplementation of the A2E-containing cells with lutein or zeaxanthin prior to blue light exposure also attenuated the photo-oxidation-induced accumulation of ubiquitin-proteins conjugates in the cells (Fig 2B, compare lanes 3 and 4 with 2). The limited accumulation of ubiquitin conjugates in the presence of lutein or zeaxanthin may be due to less oxidative stress or greater proteasome activity. Together,

these data indicate that accumulation of lutein and/or zeaxanthin in RPE provides a protection against photo-oxidation, including protecting the proteasome from photo-oxidative inactivation.

Supplementation with lutein and zeaxanthin prevents photo-oxidation-induced alteration in expression of inflammation-related genes

Since the proteasome in RPE plays an important role in regulating the expression of inflammation-related genes [68, 72], we hypothesized that photo-oxidative inactivation of the proteasome would alter the expression of inflammation related genes and the inflammatory response. Similar to the results of chemical inhibition of the proteasome, exposure of A2E-containing RPE to blue light decreased the expression and secretion of MCP-1 (Fig. 3) and increased the expression and secretion of IL-8 (Fig. 4). To investigate the roles of lutein and zeaxanthin in modulating the inflammatory response to photo-oxidation, we supplemented A2E-containing RPE cells with lutein or zeaxanthin prior to blue light exposure and determined their effects on photo-oxidation-induced expression of these inflammation-related genes. The data showed that supplementation with lutein or zeaxanthin blocked the A2E and blue light-induced decrease in MCP-1 expression (Fig. 3A) and secretion (Fig. 3B). The extents of the protection of lutein and zeaxanthin against A2E and blue light-induced decline in expression and secretion of MCP-1 were comparable to their ability to protect the proteasome from inactivation. Supplementation with lutein or zeaxanthin also suppressed the photo-oxidation induced increase in expression and secretion of IL-8 (Fig. 4). It appears that zeaxanthin was more effective than lutein in suppressing photo-oxidation induced expression and secretion of IL-8, although it was less effective in protecting the proteasome from inactivation. The data indicate that lutein and zeaxanthin may regulate the expression of IL-8 by other mechanisms in addition to protecting the proteasome from photo-oxidative inactivation.

Supplementation with lutein and zeaxanthin prevents photo-oxidation-induced down-regulation of CFH

Recent studies indicate that over-activation of the complement system is related to the pathogenesis of AMD [73]. It is known that oxidative stress, including A2E-mediated photo-oxidative stress, triggers complement activation and renders RPE vulnerable to complement attack [66, 74]. CFH is a negative regulator of the alternative pathway of complement activation; it binds to activated C3 (C3b) and promotes its proteolytic inactivation by complement factor I [75, 76]. Oxidative activation of the complement may be related to decreased levels of activity of CFH. It has been reported that oxidative stress suppresses the secretion of CFH by RPE cells [77]. Here we determined the effects of lutein and zeaxanthin on photo-oxidation-induced down-regulation of CFH. Exposure of A2E-containing cells to blue light resulted in a 60–70% reduction in mRNA levels of CFH (Fig 5A.) and >80% decrease in levels of secreted CFH (Fig 5B). Supplementation of lutein or zeaxanthin to A2E-loaded cells prior to blue light exposure partially prevented the decrease in the expression of CFH (Fig 5A). However, supplementation of lutein or zeaxanthin did not prevent the photo-oxidation-induced decline in the secretion of CFH (Fig. 5B). It is possible that different regulatory steps of CFH production, such as transcription, translation, modification or secretion could be damaged by photo-oxidation, and each of these steps may have different vulnerability to photo-oxidative stress. Since lutein and zeaxanthin cannot completely blocks photo-oxidation under these experimental conditions, supplementation with lutein or zeaxanthin may alleviate the effects of photo-oxidation-induced damage to some, but not all, of these steps, and have different effects on levels of CFH mRNA and secreted protein.

Proteasome inhibition decreases the expression and secretion of CFH

Previous studies indicate that proteasome inactivation is involved in the oxidation-induced changes in expression and secretion of MCP-1 and IL-8 [68, 71, 72]. To investigate whether the oxidation-induced down-regulation of CFH is also related to proteasome inactivation, we determined the effects of proteasome inhibition on expression and secretion of CFH. As shown in Figure 6, incubating ARPE-19 cells with MG132 or epoxomicin, two cell permeable proteasome inhibitors, resulted a significant decrease in mRNA levels (Fig 6A) and secreted protein levels (Fig 6B) of CFH. These data indicate that the proteasome is also involved in regulating the expression of CFH and that oxidative inactivation of the proteasome appears to be one of the mechanisms by which photo-oxidation- reduces the expression and secretion of CFH.

Discussion

Oxidative stress and inflammation are interrelated biological events [78] and both are implicated in the pathogenesis of AMD [67, 79, 80]. There is a vicious cycle in which oxidative stress triggers inflammatory responses, and inflammation also enhances the production of reactive oxygen species, all of them causing oxidative damage [81, 82]. Due to its high metabolic rate and age-related accumulation of lipofuscin, the RPE is a primary target of photo-oxidative damage in the eye [17]. The RPE is also a major source of cytokines that regulate inflammatory response in the retina [68, 83, 84]. The vicious interaction between oxidative stress and inflammatory responses in RPE may contribute to the onset and progression of AMD. Any approach that breaks the vicious cycle between oxidative stress and inflammation in RPE could be a potential strategy for prevention or treatment of AMD.

Results from this study show that photo-oxidative stress inactivates the proteasome in RPE and subsequently alters the expression of inflammation-related genes, such as down-regulation of MCP-1 and CFH and up-regulation of IL-8. These data are consistent with our previous work which indicates that inactivation of the proteasome is a mechanistic link between oxidative stress and altered inflammatory responses [68, 71, 72]. Furthermore, the data indicate that supplementation with lutein or zeaxanthin can partially break the vicious cycle between oxidative stress and inflammatory response in RPE cells via protecting the proteasome from inactivation. The effects of lutein and zeaxanthin in protecting RPE from photo-oxidative damage and their roles in modulating inflammation-related genes may be one of the mechanisms by which dietary lutein and zeaxanthin reduce the risk for AMD.

Photo-oxidative inactivation of the proteasome was consistent with previous reports that the proteasome is a target of oxidative stress [64, 65, 68, 70, 85]. Since the proteasome is involved in regulating the activation of NF- κ B, a master regulator of inflammation processes [86], inactivation of the proteasome alters the expression of inflammation-related genes [68, 72, 87, 88]. The effects of oxidative stress on MCP-1 and IL-8 expression appear to depend on the extent of oxidative stress. Mild oxidative stress may promote the expression of MCP-1 and IL-8 by stimulating NF- κ B activation [89, 90]. However, extensive oxidative stress may inactivate the proteasome and subsequently inhibit NF- κ B activation and result in down-regulation of MCP-1 (Fig. 3). Chemical inhibition of the proteasome also reduced the expression of MCP-1 in RPE [72, 87, 91]. Extensive oxidative stress may inactivate the proteasome and subsequently activate the p38 MAP kinase, and thus promoting the expression of IL-8 [68, 71]. It was also reported that oxidative stress suppresses the expression of CFH in RPE [77, 92]. However, the underlying mechanism for the oxidative regulation of CFH expression in RPE remains to be elucidated. We speculate that inactivation of the proteasome by extensive oxidative stress may be involved in this

process, as proteasome activity is required for activation of many transcription factors [93, 94].

It has long been suspected that accumulation of lutein and zeaxanthin in the retina may protect the retina by filtering high-energy blue light and/or by quenching reactive oxygen species [95]. The highest concentration of lutein and zeaxanthin was detected in the Henle fiber layer in the foveal region, where the concentrations of these carotenoids can be as high as 1 mM. The anatomical localization and deep yellow color of carotenoids may reduce the exposure of photoreceptor and RPE to blue light and subsequently reduce the photo-mediated production of reactive oxygen species, such as singlet oxygen. Lutein and zeaxanthin are also excellent quenchers of singlet oxygen and triplet states of photoactive molecules [96, 97]. The capacities of lutein and zeaxanthin in quenching singlet oxygen are superior to that of α -tocopherol in a cell free system [97]. The antioxidant function of lutein and zeaxanthin for protecting RPE from photo-oxidation requires their local presentation. Indeed, lutein and zeaxanthin were also detected in human RPE, although at relatively lower levels as compared to neuronal retina [98, 99]. Results from these experiments show that cellular concentrations of lutein and zeaxanthin were 2–14 fold higher than that in the medium, indicating that cultured human RPE actively takes up and accumulate lutein and zeaxanthin upon supplementation (Fig. 1). If RPE cells *in vivo* also actively take up lutein and zeaxanthin, choroidal blood supplies would be a source of these carotenoids. The other sources of lutein or zeaxanthin for RPE may be phagocytosed photoreceptor outer segments that are enriched with these carotenoids [100]. Thus, we hypothesize that high dietary intake of lutein and zeaxanthin may not only increase macular pigment density, but also the concentrations of lutein and zeaxanthin in RPE. However, the concentrations of lutein and zeaxanthin used in this study were substantially higher than the levels that were detected in human RPE/choroids. Although the data obtained from these experiments indicate that accumulation of lutein and zeaxanthin in RPE has beneficial effects against photo-oxidation, the protective effects of lutein and zeaxanthin using this acute cell cultural photo-oxidation model may not actually reflect the processes within the RPE *in vivo*. Future experiments using chronic photo-oxidation model and physiologically relevant concentrations of lutein and zeaxanthin are warranted.

An increasing body of evidence indicated dysregulation of inflammatory response, including improper complement activation is involved in the pathogenesis of AMD [24]. It is also known that dietary lutein and zeaxanthin play a role in modulating inflammatory responses [42–44, 101–103]. However, it remained unclear how dietary lutein and zeaxanthin modulates inflammatory response. Results from this work suggest that protection of the proteasome from oxidative inactivation appears to be one of mechanisms by which lutein and zeaxanthin modulate the inflammatory response to photo-oxidative stress. The proteasome is involved in many aspects of cellular functions. In addition to selective degradation of damaged or obsolete proteins, the proteasome is involved in regulation of signal transduction and expression via controlling the levels of regulatory proteins and transcription factors. For example, proteasome-mediated degradation of inhibitors of NF- κ B is required for activation of the NF- κ B pathway [104–106]. We found that inhibition of the proteasome in RPE suppressed the expression and secretion of MCP-1 and the suppression is related to down regulation of NF- κ B signaling pathway [72]. The down-regulation of MCP-1 may have physiological consequences since MCP-1 knockout mice developed AMD-like phenotypes [107]. We also found the inactivation of the proteasome enhances the expression and secretion of IL-8 by activation of the p38 MAPK signaling pathway [68, 71]. Elevated levels of IL-8 may not only promote inflammation, but also trigger neovascularization, because IL-8 is a neutrophil attractant and a strong pro-angiogenesis factor [108–111]. This study showed that inactivation of the proteasome also contributed to the down-regulation of CFH upon photo-oxidative stress (Fig 6). Although it is unknown at

present how proteasome inhibition suppresses the expression of CFH, it is likely that the proteasome is involved in regulating levels of transcription factors and signaling molecules that control the expression of CFH. The down-regulation of CFH may play a role in complement activation [66] and complement attack of the RPE [74] in response to oxidative stress.

Together, these results suggest that inactivation the proteasome is a mechanistic link between oxidative stress and altered expression of inflammation-related genes. Supplementation of lutein or zeaxanthin in RPE protected the proteasome from inactivation and attenuated the changes in expressions of these inflammation-related genes. This may be one of the mechanisms by which dietary lutein and zeaxanthin modulate ocular and systemic inflammation and reduce the risk for AMD.

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Photo-oxidation inactivates the proteasome in retina pigment epithelial cells (RPE)
Inactivation of the proteasome alters the expression of inflammation-related genes.
RPE cells accumulate lutein and zeaxanthin upon supplementation.
Lutein or zeaxanthin attenuates photo-oxidation-induced proteasome inactivation.
Lutein or zeaxanthin attenuates the expression of inflammation-related genes.

Fig. 1. Cultured RPE cells actively take up lutein and zeaxanthin

Confluent ARPE-19 cells were cultured in media containing 1 or 10 μM of lutein or zeaxanthin for 3 days. The medium was removed and the cells were washed three times with medium containing 10% bovine serum. Then the cells were collected. Levels of lutein and zeaxanthin in the medium and in the cells were determined by reversed phase HPLC with a C30 column. This figure showed the chromatograms (monitored at 450 nm) and spectra of lutein and zeaxanthin in the medium and cells upon supplementation with 10 μM lutein or zeaxanthin for 3 days. Panel A: chromatogram of lutein in the medium; panel B: chromatogram of lutein and its putative metabolites in cells; panel C: chromatogram of zeaxanthin in the medium; panel D: chromatogram of zeaxanthin and its metabolites in cells; panel E: spectra of lutein and its metabolites in cells; panel F: spectra of zeaxanthin and its putative metabolites in cells.

Fig. 2. Accumulation of lutein and zeaxanthin in RPE cells prevents photo-oxidative inactivation of the proteasome in ARPE-19 cells

Confluent cultured ARPE-19 cells were loaded with A2E alone or loaded with A2E and then supplemented with 10 μ M lutein or zeaxanthin for 3 days. The cells were then exposed to blue light for 10 min and harvested. The chymotrypsin-like activity of the proteasome in the cells was determined using a fluorogenic peptide as a substrate (panel A) and levels of ubiquitin conjugates were determined by Western blotting analysis using levels of β -actin as a loading control (panel B). The experiments were repeated twice with triplicates each time. The data in panel A are mean $\pm$ SD of the results from 6 samples in each group. The proteasome activity in control cells (neither treated with A2E nor exposed to blue light) was arbitrarily designated as 100 and the rest were expressed as relative activities. * indicates $p < 0.05$ as compared the control cells. # indicates $p < 0.05$ as compared to cells that were loaded with A2E and exposed to blue light.

Fig. 3. Accumulation of lutein or zeaxanthin in A2E- containing RPE cells attenuates blue light induced down regulation of MCP-1

Confluent ARPE-19 cells that were loaded with A2E alone or loaded with A2E and then incubated with 10 μ M lutein or zeaxanthin were exposed to blue light for 10 min. The cells were then cultured in medium containing 1% FBS for another 6 h and the medium was collected and cells were harvested. Cells that were neither loaded with A2E nor exposed to blue light were used controls. Levels of mRNA for MCP-1 in the cells were determined by real-time quantitative RT-PCR using levels of mRNA for GAPDH as a reference (panel A) and protein levels of MCP-1 in the medium were determined by ELISA (panel B). The relative levels of mRNA for MCP-1 in control cells were arbitrarily designated as 1 and relative levels of mRNA for MCP-1 in treated cells were expressed as fold of that in the control cells. The data are mean $\pm$ SD of the results from 6 samples in each group. * indicates $p < 0.05$ and ** indicates $p < 0.01$ as compared the control cells that were neither treated with A2E nor blue light. # indicates $p < 0.05$ as compared to cells that were loaded with A2E and exposed to blue light.

Fig. 4. Accumulation of lutein or zeaxanthin in A2E- containing RPE cells attenuates blue light induced up-regulation of IL-8

Confluent ARPE-19 cells that were loaded with A2E alone or loaded with A2E and then incubated with 10 μ M lutein or zeaxanthin were exposed to blue light for 10 min. The cells were then cultured in medium containing 1% FBS for another 6 h and the media were collected and cells were harvested. Cells that were neither loaded with A2E nor exposed to blue light were used controls. Levels of mRNA for IL-8 in the cells were determined by real-time quantitative RT-PCR using levels of mRNA for GAPDH as a reference (panel A) and protein levels of IL-8 in the medium were determined by ELISA (panel B). The relative levels of mRNA for IL-8 in control cells were arbitrarily designated as 1 and relative levels of mRNA for IL-8 in treated cells were expressed as fold of that in the control cells. The data are mean $\pm$ SD of the results from 6 samples in each group. * indicates $p < 0.05$ and ** indicates $p < 0.01$ as compared the control cells. # indicates $p < 0.05$ and ## indicates $p < 0.01$ as compared to cells that were loaded with A2E and exposed to blue light.

Fig. 5. Accumulation of lutein and zeaxanthin in A2E- containing RPE cells attenuated blue light induced down-regulation of CFH

Confluent ARPE-19 cells that were loaded with A2E alone or loaded with A2E and then incubated with 10 μ M lutein or zeaxanthin were exposed to blue light for 10 min. The cells were then cultured in medium containing 1% FBS for another 6 h and the media were collected and cells were harvested. Cells that were neither loaded with A2E nor exposed to blue light were used controls. Levels of mRNA for CFH in the cells were determined by real-time quantitative RT-PCR using levels of mRNA for GAPDH as a reference (panel A) and protein levels of CFH in the medium were determined by ELISA (panel B). The relative levels of mRNA for CFH in control cells were arbitrarily designated as 1 and relative levels of mRNA for CFH in treated cells were expressed as fold of that in the control cells. The data are mean $\pm$ SD of the results from 6 samples in each group. * indicates $p < 0.05$ and ** indicates $p < 0.01$ as compared to the control cells that were neither treated with A2E nor blue light.

Fig. 6. Inhibition of the proteasome in RPE cells down-regulates CFH expression

Confluent cultured ARPE-19 cells were incubated in fresh medium in the absence or presence of 10 μ M MG132 or 5 μ M epoxomicin for 8 h. Levels of mRNA for CFH in the cells were determined by real-time quantitative RT-PCR using levels of mRNA for GAPDH as a reference (panel A) and protein levels of CFH in the medium were determined by ELISA (panel B). The relative levels of mRNA for CFH in control cells were arbitrarily designated as 1 and relative levels of mRNA for CFH in MG132- or epoxomicin-treated cells were expressed as fold of that in the control cells. The data are mean $\pm$ SD of the results from 4 samples in each group. * indicates $p < 0.05$ and ** indicates $p < 0.001$ as compared to the controls.