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Phase II enzyme induction by a carotenoid, lutein, in a PC12D neuronal cell line

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ABSTRACT

The mechanism by which lutein, a carotenoid, acts as an antioxidant in retinal cells is still not fully understood. Here, lutein treatment of a neuronal cell line (PC12D) immediately resulted in reduced intracellular ROS levels, implying that it has a direct role in ROS scavenging. Significantly, lutein treatment also induced phase II antioxidative enzyme expression, probably via a nuclear factor-like 2 (Nrf2) independent pathway. This latter mechanism could explain why lutein acts diversely to protect against oxidative/cytotoxic stress, and why it is physiologically involved in the human neural tissue, such as the retina.

1. Introduction

In addition to pharmacotherapeutics, a variety of nutritional interventions are currently under investigation as novel approaches for the suppression of disease progression and/or tissue/organ damage.

Both *in vivo* and *in vitro* experiments suggest that inflammatory processes are involved in disease pathogenesis [1]. Recent studies focused on the link between inflammation and intracellular oxidative stress by reactive oxygen species (ROS). Although ROS are constantly generated in mitochondria and peroxisomes as a result of physiological metabolism [2], excessive ROS may induce inflammation, oxidizing lipids, proteins and nucleic acids [3], thereby causing tissue/organ disorders. Excessive oxidative stress is involved in neurodegenerative diseases [4–6], cardiovascular diseases [7], and cancer [8]. Thus, it is proposed that prevention or inhibition of inflammatory processes can be achieved by suppressing excessive ROS using antioxidative products.

The body produces antioxidative molecules, such as glutathione [9] and bilirubin [10]. Additionally, plant-derived dietary sources of ROS scavengers exist, for example, carotenoids and flavonoids [11–14]. Among these, lutein, a carotenoid, is selectively concentrated in the retina where it forms macular pigment, a physiological component of the eye. The clinical significance of retinal lutein is suggested by human data. Low level of macular pigments, i.e. a low lutein concentration in the macula, is reported to be a risk factor for the development of age-related macular degeneration (AMD), a leading cause of blindness [15]. Moreover, autopsy reports reveal that eyes from AMD patients have ~30% lower levels of macular pigment than age-matched controls [16]. Because the retina has a higher oxygen requirement compared to most other organs [17], it is susceptible to oxidative stress; hence, low levels of retinal lutein could lead to ROS accumulation, resulting in local inflammation and the induction of AMD. Lutein forms a yellow pigment and is thus thought to play a secondary role in filtering blue light in the retina [18]. This mechanism may also protect the retina from light-induced oxidative damage.

Over the past decade, a role for carotenoids and their derivatives in the activation and modulation of transcription systems has been revealed [19,20]. Recently, in inflammatory cells, lutein was reported to induce expression of matrix metalloprotease 9 via a nuclear receptor [21]. Thus, there may be a possibility that lutein acts to reduce oxidative stress not only via ROS scavenging and/or via the physical blockage of blue light. Phase II antioxidant enzymes (including heme oxygenase-1 [HO-1], superoxide dismutase 1 and 2 [SOD1 and 2], catalase, peroxiredoxin 1 [Prdx1], and NAD(P)H dehydrogenase, quinone 1 [NQO1]) are induced in response to stress stimuli, and their expression is regulated by the

Abbreviations: PC12D, rat adrenal pheochromocytoma cell line; ROS, reactive oxygen species; Nrf2, nuclear factor-like 2; HO-1, oxygenase-1; SOD1 and 2, superoxide dismutase 1 and 2; Prdx1, peroxiredoxin 1; NQO1, NAD(P)H dehydrogenase, quinone 1.

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transcription factor, nuclear factor-like 2 (Nrf2) [22]. However, whether lutein can induce gene expression of such an antioxidant defense system is still unknown.

The purpose of this study, therefore, was to investigate the involvement of lutein in the regulation of antioxidative enzyme expression.

2. Materials and methods

2.1. Cell culture

Rat adrenal pheochromocytoma cell line, PC12D, was maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) (D5796) (Sigma–Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS) (Life technologies, Carlsbad, CA, USA), 10% horse serum (HS) (Life technologies), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C under a humidified atmosphere of 5% CO_2. For subculture, PC12D cells were harvested at 80–90% confluence.

2.2. Differentiation into neuronal cells

Cells were seeded on tissue culture plates coated with 0.1% gelatin and containing complete medium without antibiotics. After 24 h, cell culture medium was changed to DMEM containing 0.5% FBS and 50 ng/ml nerve growth factor (NGF) (Upstate Biotechnology, Lake Placid, NY, USA) and maintained at 37 °C for 48 h under a humidified atmosphere of 5% CO_2 to induce differentiation.

2.3. Emulsification of lutein using non-ionic surfactant

Lutein was sourced from lutein-rich marigold extract (LME) containing 85% lutein (provided by WAKASA SEIKATSU, CO., Ltd., Kyoto, Japan). Lutein emulsification was as described elsewhere [23]. Briefly, LME was micellated in an ethanol (Wako pure chemical, Osaka, Japan)/Tween 80 (Sigma–Aldrich) solution (310 μl:10 μl), and concentrated under N_2 gas to make a 500 mM stock solution in 10 μl of Tween 80. The stock solution was serially diluted to make a working solution (50 μM lutein and 0.01% Tween 80 in DMEM with 0.5% FBS without antibiotics), prior to application to PC12D cells. The control vehicle was consisted of 0.01% Tween 80 in DMEM with 0.5% FBS without antibiotics.

2.4. ROS measurement

Intracellular ROS levels were determined using the fluorescent dye, CM-H2DCFDA (Invitrogen, Carlsbad, CA, USA). A 2 mM stock solution of CM-H2DCFDA was reconstituted in anhydrous dimethyl sulfoxide (DMSO Sigma–Aldrich), and 2 μl was added to the culture 30 min before the measurement at each time point. After incubation for 30 min at 37 °C, fluorescence was measured (excitation wavelength, 485 nm; emission wavelength, 528 nm) using the fluorescence microplate reader, Synergy 4 (BioTek Instruments, Inc., Winooski, VT, USA). The area scanning read mode was selected to obtain averaged intensity over the entire area of the well.

2.5. Real-time PCR

Total RNA was extracted from cells using TRIzol reagent (Life technologies). Complementary DNA was synthesized using SuperScript® VILO™ Master Mix (Life technologies) according to the manufacturer's instruction. PCR was performed using the StepOnePlus Real Time PCR system (Life technologies). The mRNA levels were evaluated by the ΔΔCT method, and normalized to GAPDH mRNA. The primers are shown in Supplementary Table 1.

2.6. Whole cell lysate preparation and protein extraction from nuclear and cytoplasmic fractions

Differentiated cells were resuspended in lysis buffer (10 mM Tris–HCl (pH 7.6), 1% TritonX-100, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, 29 mM sodium pyrophosphate decahydrate, 47.6 mM sodium fluoride, 19 mM β-glycerophosphate disodium salt hydrate) containing protease inhibitor cocktail (Complete, EDTA-free; Roche, Mannheim, Germany) and phosphatase inhibitor cocktail 2 and 3 (Sigma–Aldrich), sonicated, and left on ice for 30 min. After centrifugation, the supernatant containing the whole cell lysate was transferred to a new 1.5 ml tube and kept and stored at −80 °C until use.

The nuclear and cytoplasmic fractions were separated using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific Inc., IL, USA) according to the manufacturer's instruction. Protein concentration was determined using the Quick Start™ Bradford 1× Dye Reagent (BIO-RAD Laboratories, Hercules, CA, USA) with Quick Start™ Bovine Gamma Globulin (BIO-RAD) as a standard.

2.7. Immunoblot analyses

Samples of cell extract were separated by SDS–polyacrylamide gel electrophoresis, and the proteins were transferred to a polyvinylidene fluoride membrane (Immobilon-P; Millipore, Bedford, MA, USA) in a Trans-Blot SD Cell (BIO-RAD). The membrane was blocked with blocking buffer (0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl, 0.5% TSA Blocking Reagent [PerkinElmer Life Sciences, Waltham, MA, USA]), incubated overnight at 4 °C with either of the primary antibodies (Supplementary Table 2), and then incubated with horseradish peroxidase-conjugated secondary antibody (1/2,000; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 1 h. The signals were detected using ECL Western Blotting Detection Reagents (GE healthcare Limited, Buckinghamshire, HP8 4SP, UK). Signal intensities were quantified using the ImageJ program [24], and normalized to that of GAPDH. For nuclear and cytoplasmic Nrf2 analyses, protein was loaded at 2.5 and 5 μg, respectively, immunoblotted using Nrf2 antibody (Supplementary Table 2), and nuclear and cytoplasmic Nrf2 were detected and quantified as above.

2.8. Statistical analyses

Values were expressed as the mean ± s.d. ANOVA followed by Tukey's post hoc test was used to assess the statistical significance of the difference among 4 groups (Fig. 1) and Student's *t*-test was used to assess the difference between 2 groups (Figs. 2–4). $P < 0.05$ was regarded as significant.

3. Results

3.1. Lutein reduces ROS in PC12D neuronal cells

To investigate the ROS removal function of lutein, ROS levels were monitored in differentiated PC12D neuronal cells by measuring changes in CM-H2DCFDA fluorescence intensity. Fluorescence intensity was lower in lutein-treated cells than in vehicle-treated cells as early as 1 h after treatment, and remained low for up to 24 h after treatment (Fig. 1). For positive control of the experiment, H_2O_2 was added 5 min before each time point, and the increase in the fluorescence intensity was confirmed. For negative control, no CM-H2DCFDA probe was added. The intensities were shown relative to that of vehicle-treated cells measured 1 h after treatment. The levels were relatively high at earlier time points most probably

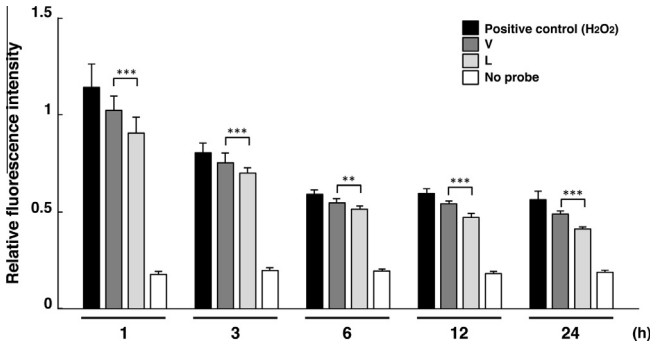

Fig. 1. Lutein reduced ROS levels in PC12D neuronal cells. ROS levels were measured using an oxidative stress indicator, CM-H2DCFDA, in differentiated PC12D cells treated with vehicle (V) or lutein (L). For positive control, H_2O_2 (50 μM) was added 5 min before the measurement. For negative control, no probe of CM-H2DCFDA was added. The levels of ROS relative to the vehicle-treated cells measured 1 h after treatment were shown. At any time points, ROS level of positive control was higher, and that of lutein-treated cells were lower, than the vehicle-treated cells, and the value with no probe showed the lowest level, all in a statistically significant manner analyzed by ANOVA followed by Tukey's post hoc test. $n = 6$. $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., not significant.

due to the medium change-induced stress, but it was statistically significant by the analysis using ANOVA followed by post hoc test that at any time points, positive control showed the highest, vehicle-treated cells showed the second highest, and the lutein-treated cells showed the lowest levels. This result suggests that lutein can scavenge ROS in differentiated PC12D cells.

3.2. Effect of lutein on phase II enzyme mRNA levels

Phase II genes encoding detoxifying and antioxidant enzymes are induced in response to ROS [25]. Thus, we examined the levels of phase II gene mRNAs in lutein- or vehicle-treated differentiated PC12D cells. At 24 h after lutein treatment, the mRNA levels of HO-1, SOD1, SOD2, catalase, Pdrx1, and NQO1 were all higher than in vehicle-treated cells. By contrast, HO-2, which is constitutively expressed under homeostatic conditions and is not a phase II enzyme, was not induced (Fig. 2). Hence, lutein treatment induced phase II enzyme mRNA expression in differentiated PC12D neuronal cells despite the lower levels of ROS in these cells.

3.3. Effect of lutein on phase II enzyme protein levels

To determine whether phase II enzyme protein levels were also higher in lutein-treated cells than in vehicle-treated cells, we performed immunoblot analyses (Fig. 3). The protein levels of HO-1, SOD2, and NQO1 were all significantly higher in lutein-treated cells than in vehicle-treated cells at 24 h, while the levels of HO-2, SOD1, catalase, and Prdx1 remained the same.

3.4. Effect of lutein on the Nrf2 pathway

Phase II enzyme-encoding genes are induced by Nrf2 transcription factor binding to the antioxidant response element (ARE) or the electrophile response element (EpRE) in the promoter regions of these genes [26]. Immunoblot analyses were performed to investigate whether nuclear transport of Nrf2 is induced in differentiated PC12D neuronal cells after lutein treatment. Nuclear Nrf2 levels were unchanged (Fig. 4A) and Nrf2 was virtually undetectable in the cytoplasmic fraction (data not shown) over the time course of lutein treatment. Upstream pathways of Nrf2 (specifically the PI3K/AKT/GSK-3β and MAPKs pathways) can affect nuclear translocation of Nrf2 [27]. Hence, the phosphorylation (activated state) of GSK-3β at Tyr216, MEK1/2, p44/42 MAPK (Erk1/2), and p38 MAPK (p38) was examined in whole cell lysates at 24 h; however, no increase in the ratios of the activated forms were observed (Fig. 4B). Thus, lutein treatment does not appear to activate Nrf2 or its corresponding upstream pathways.

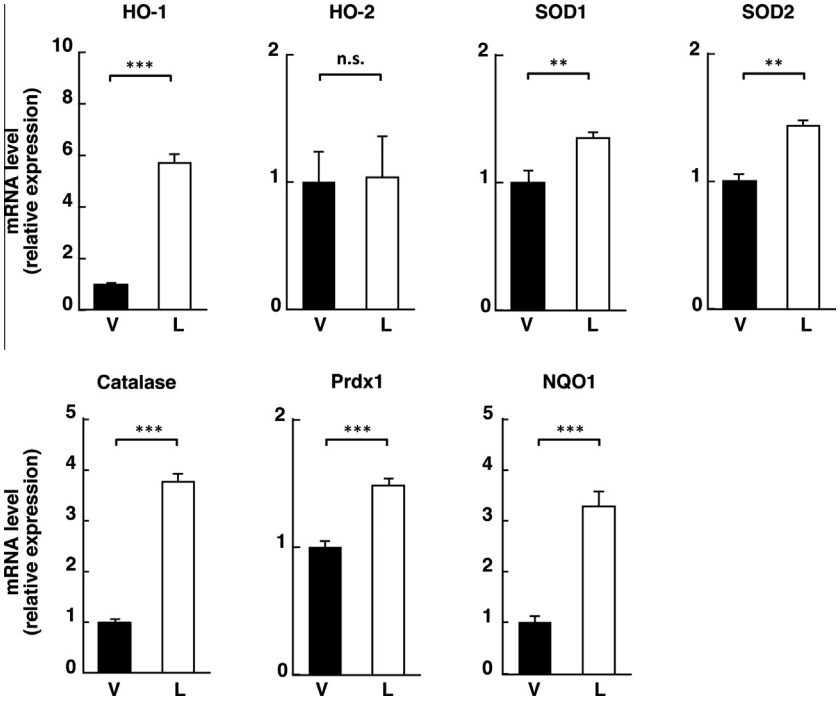

Fig. 2. Effect of lutein on phase II enzyme mRNA levels Real-time PCR showing that phase II enzyme mRNA levels in differentiated PC12D cells were higher 24 h after lutein (L)- than after vehicle (V)-treatment. The mRNA level of HO-2, which is not a phase II enzyme, was not changed. HO-1, Heme oxygenase-1; HO-2, Heme oxygenase-2; SOD1, Superoxide dismutase 1; SOD2, Superoxide dismutase 2; Prdx1, Peroxiredoxin-1; NQO1, NA(D)PH dehydrogenase, quinone 1. $n = 4$. $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., not significant.

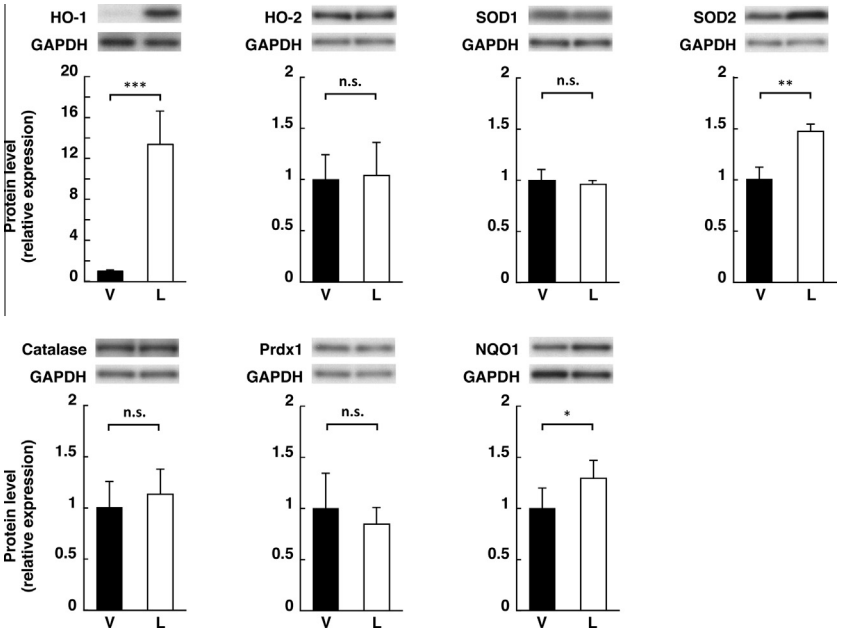

Fig. 3. Effect of lutein on phase II enzyme protein levels Immunoblot analyses revealed that the protein levels of HO-1, SOD2, and NQO-1 in differentiated PC12D cells were higher at 24 h after lutein (L)- than after vehicle (V)-treatment. $n = 4$. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; n.s., not significant.

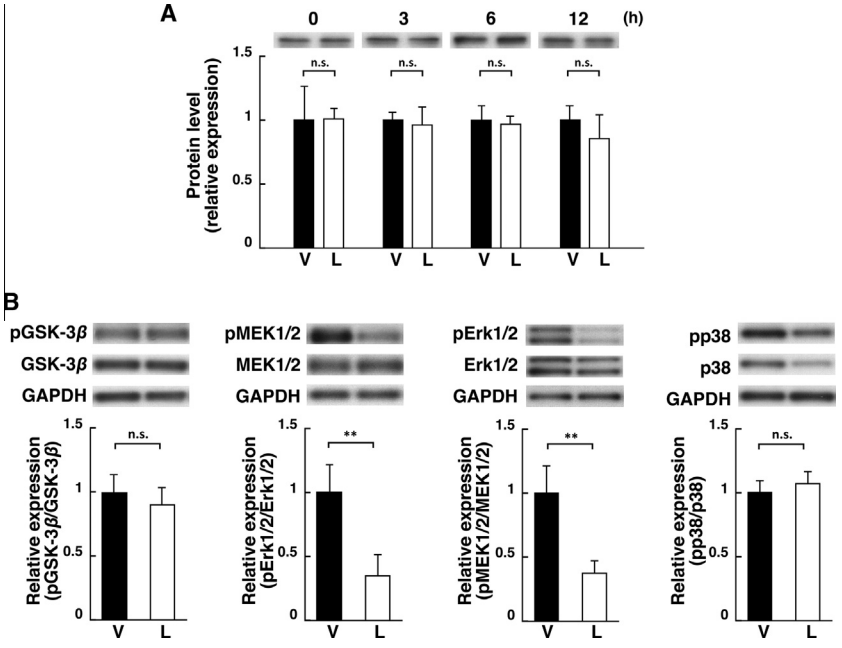

Fig. 4. Effect of lutein on the Nrf2 pathway Immunoblot analyses. (A) There were no differences in the relative nuclear Nrf2 protein levels between lutein (L)- and vehicle (V)-treated differentiated PC12D cells at the time points indicated. (B) Relative protein levels of phosphorylated versus unphosphorylated forms of GSK-3β, MEK1/2, Erk1/2, and p38 in whole cell lysate indicated no increase in the phosphorylated, activated forms of these proteins. Phosphorylation of GSK-3β was checked by detecting phosphorylation at the Tyr216 residue with a phospho-specific antibody. $n = 4$. $^{**}P < 0.01$; n.s., not significant.

4. Discussion

In this study, we demonstrated that an antioxidant, lutein, reduced ROS levels in a neuronal cell culture derived from PC12D cell lines (Fig. 1). Lutein also induced the expression of phase II enzymes (Fig. 2), both at the mRNA and protein levels (Fig. 3). However, interestingly, lutein treatment did not activate the activator of phase II enzyme genes, Nrf2, and its upstream pathways (Fig. 4).

In general, phase II enzymes are upregulated in response to oxidative stress [28], via Nrf2 binding to the ARE [29]. Keap1-mediated proteasomal degradation of cytoplasmic Nrf2 is suppressed by ROS, which disrupt Nrf2-Keap1 binding by modifying cysteine residues in Keap1 [27]. Free Nrf2 translocates to the nucleus, binds to ARE, and initiates transcription of a large array of cytoprotective genes. Previous observations indicated that Nrf2 plays a key role in regulating ARE-driven genes. Overexpression

of a dominant-negative Nrf2 mutant attenuated induction of HO-1 gene expression in response to heme and heavy metals [29], and Nrf2-null mice were more susceptible to oxidative/electrophilic stress-induced liver pathologies than wild-type mice [30]. However, in this study, phase II gene induction was not preceded by an increase in ROS levels, and rather, it was preceded by a decrease in ROS levels. Moreover, basal levels of Nrf2 did not increase in the nucleus in response to lutein treatment, and upstream pathways that induce Nrf2 nuclear translocation were similarly unaffected.

The PI3K/AKT/GSK3β and MAPK pathways are involved in numerous reactions upstream of Keap1 interaction that regulate Nrf2 levels via post-transcriptional modification. In response to oxidative stress, GSK-3β is activated by phosphorylation at Tyr216 and in turn, activates cytosolic Fyn, allowing their nuclear translocation [31,32] and the subsequent phosphorylation of Nrf2 at Tyr568. Nrf2 phosphorylated at Tyr568 is then exported from the nucleus [27], binds to Keap1, and is degraded [33].

Conversely, a member of the MAPK family, Erk1/2, which is activated by MEK1/2, phosphorylates serine/threonine residues on Nrf2 to promote its translocation into the nucleus [34]. Butylated hydroxyanisole (BHA), although it is an antioxidant, activates this pathway, eventually increasing HO-1 expression through the ARE [35]. Meanwhile, another MAPK, p38, phosphorylates Nrf2, resulting in its association with Keap1 thereby preventing its nuclear translocation [27]. p38 overexpression also attenuates the action of sulforaphane, a plant-derived Nrf2 inducer, which activates the ARE-rich enhancer region of HO-1 [36]. Hence, the phosphorylation state of different residues on Nrf2 is a determining factor in the regulation of nuclear Nrf2 levels and subsequent ARE-dependent gene expression.

In this study, the levels of phosphorylated GSK3β, MEK1/2, Erk1/2, and p38 were not increased after lutein treatment. Moreover, phosphorylated and activated MEK1/2 and Erk1/2 were significantly decreased. Because MEK1/2 can be activated by oxidative stress [37], reduction in ROS level by lutein treatment may have reduced the levels of phosphorylated MEK1/2, and subsequently, phosphorylated Erk1/2. Consistently, nuclear Nrf2 levels remained unchanged. These findings suggest a role for lutein in an Nrf2-independent phase II enzyme inducing pathway. We explored the potential involvement of retinoid acid receptor (RAR) activity in this pathway, since RAR is activated by another carotenoid, β-cryptoxanthin [38]. However, the induction of phase II enzyme transcription was unaffected by RAR inhibitors or by its putative co-acting molecule, retinoid X receptor (RXR) (data not shown), suggesting the involvement of other currently undefined pathways.

The potential involvement of another cytoprotective pathway for lutein that induces phase II enzymes, with the potential to reduce oxidative stress and cytotoxic signals, is significant. This finding will pave the way to explaining why lutein is involved in the neural tissue, the retina, and why lutein can protect against both excessive light exposure [39] and diverse oxidative/inflammatory stresses [40,41].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.02.135.

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