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Full paper

Astaxanthin analogs, adonixanthin and lycopene, activate Nrf2 to prevent light-induced photoreceptor degeneration

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ABSTRACT

Carotenoids, in particular astaxanthin, possess potent antioxidant capabilities. Astaxanthin also induces NF-E2-related factor 2 (Nrf2), which plays a major regulatory role in the antioxidative response. However, little is known whether the carotenoid, by-products of astaxanthin, activate Nrf2. Toward this end, we screened eight astaxanthin analogs for Nrf2 activation in murine photoreceptor cell line, 661 W, by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). In addition, we monitored cell death in 661 W cells pretreated with astaxanthin analogs or only pretreated for 6 h with astaxanthin analogs and then exposed to light. Furthermore, we quantified the reactive oxygen species (ROS) production. Cell death was quantified after light exposure by nuclear staining. Nrf2-controlled genes *Ho-1*, *Nqo-1*, and *Gclm* by qRT-PCR and Nrf2 in the nucleus were upregulated in 661 W cells exposed astaxanthin, adonixanthin, echinenone, and lycopene. Moreover, astaxanthin, adonixanthin, echinenone, β-carotene, adonirubin, and lycopene, but not canthaxanthin, suppressed ROS production and protected cells against light-induced damage. Moreover, pretreatment with adonixanthin or lycopene only before light exposure protected against light-induced cell damage and *Nrf2* silencing canceled these effects. These findings indicate that the more potent astaxanthin analogs, adonixanthin and lycopene, protect against light-induced cell damage through not only an anti-oxidative response but also through Nrf2 activation.

1. Introduction

Carotenoids are organic pigments that commonly present in lobster, salmon, tomatoes, and carrots.[1–3] Among carotenoids, astaxanthin is red-orange color pigment. Astaxanthin executes free radical scavenging, singlet oxygen ($^1O_2$) quenching, anti-carcinogenesis, anti-diabetic, and anti-inflammatory activities.[4–9] It is synthesized by Gram-negative aerobe *Paracoccus carotinifaciens*, and its biosynthesis can be produced on an industrial scales.[10] During this process, *P. carotinifaciens* generates carotenoid analogs such as adonirubin, adonixanthin, β-carotene, canthaxanthin, echinenone, lycopene, and zeaxanthin.[11] It has been reported that these carotenoids have also antioxidative abilities[4,6,12–14]; however, little is known about their protective effects on the central nervous system, particularly in retinal neurons such as photoreceptor cells.

Age-related macular degeneration (AMD) is an ophthalmic disease with visual field defects and accounts for a large percentage of adult vision loss in developed countries.[15,16] AMD mainly causes photoreceptor cell death in the macula. Regarding pathology dynamics, reactive oxidative species (ROS) are produced by routine light exposure, which induces photoreceptor degeneration.[17–20] Therefore, ROS reduction is effective in mitigating AMD, and some antioxidants have been previously reported to have protective effects, as observed in animal experiments. Examples include

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phenyl-*N*-tert-butyl nitrone,[21] dimethylthiourea,[22] and 3-methyl-1-phenyl-2-pyrazolin-5-one (Edaravone).[23] In fact, lutein, zeaxanthin, and *meso*-zeaxanthin are used in the clinical management of eye diseases.[24] Moreover, an Italian clinical study demonstrated that carotenoids enhance visual acuity.[25]

Nuclear factor (erythroid-derived-2)-like 2 (Nrf2) is a transcription factor that regulates the antioxidant responses *via* modulating the expression of phase II enzymes expression such as heme oxygenase-1 (HO-1), NAD (P) H: quinone oxidoreductase (NQO-1), and γ-glutamyl-cysteine ligase (GCL),[26–28] which function as antioxidative or detoxifying enzymes.[29] Although Nrf2 is suppressed by Keap1 and decomposed by proteasome under normal conditions, Nrf2 can dissociate from Keap1 and become activated under stressful conditions. Activated Nrf2 translocates to the nucleus to interact with the Maf protein, which induces transcription of phase II enzymes genes *via* complex binding to the antioxidant response element (ARE).[30] Through this mechanism, Nrf2 has been experimentally shown to exhibit neuroprotective effects on diseases associated with oxidative stress such as brain ischemia/reperfusion injuries, Parkinson's disease, and retinal light-induced cellular damage.[31–33] Recently, it has been reported that astaxanthin activated Nrf2-ARE pathway *in vivo*.[34] However, the degree of contribution and role of Nrf2 activation in conferring neuroprotection and whether other carotenoids including adonirubin, adonixanthin, and, echinenone and so on could activate Nrf2 are not known. Therefore, the purpose of the present study was to investigate Nrf2 activation induced by not only astaxanthin but also precursors of astaxanthin biosynthesis. Moreover, we investigated whether these carotenoids have protective effects on light-induced photoreceptor cell death using the 661 W, murine photoreceptor cell line.

2. Materials and methods

2.1. Materials

Carotenoids; adonirubin, adonixanthin, astaxanthin, echinenone, and zeaxanthin were prepared from *P. Carotinifaciens* extract by JX Nippon Oil & Energy (Tokyo, Japan). β-carotene, canthaxanthin, and lycopene *N*-acetyl cysteine (NAC) were purchased from WAKO (Osaka, Japan), Bardoxolone methyl (BARD) was purchased from Funakoshi Co., Ltd (Tokyo, Japan). NucleoSpin RNA, PrimeScript™ RT reagent Kit (Perfect Real Time) and SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) were purchased from Takara (Shiga, Japan). Hoechst 33342 and propidium iodide (PI) were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Cell culture

661 W cells, a transformed murine cone cell line derived from mouse retinal tumors, were a kind gift from Muayyad R. Al-Ubaidi (University of Oklahoma Health Sciences Center, Oklahoma City, OK, USA). The 661 W cells were maintained in a Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, St. Louis, MO, USA), along with 10% fetal bovine serum (FBS), 100 μg/ml penicillin (Meiji Seika, Tokyo, Japan) and 100 μg/ml streptomycin (Meiji Seika) at 37 °C and 5% CO_2. The cells were trypsinized every 2–4 days.

2.3. Treatment with astaxanthin analogs

Carotenoids except for β-carotene, were dissolved in phosphate-buffered saline (PBS) containing 0.1% dimethyl sulfoxide. On the other hand, β-carotene was dissolved in tetrahydrofuran containing 0.025% dibutylhydroxytoluene before use.

2.4. RNA isolation

661 W cells were seeded at 1.5×10^4 cells/well in 24-well plates and then incubated for 24 h. The entire medium was then replaced with fresh medium containing 1% FBS, then carotenoids were added at 1 h after changing medium. Samples were obtained 6 h after adding agents. RNA was isolated from the cells with a NucleoSpin® RNA kit (Takara, Shiga, Japan) according to the manufacturer's protocol. The RNA concentrations were determined spectrophotometrically at 260 nm by NanoVue Plus (GE Healthcare Japan, Tokyo, Japan). The isolated RNAs were converted to first-strand cDNA using a PrimeScript RT reagent kit (Perfect Real Time; Takara) according to the manufacturer's protocol.

2.5. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis

The level of expression of the mRNAs of the *Ho-1*, *Nqo1*, and *Gclm* genes was determined by Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). qRT-PCR was performed using SYBR Premix Ex Taq™ II (Takara) and TP800 Thermal Cycler Dice Real Time System (Takara) according to the manufacturer's instructions. The PCR primer sequences used were as follows:

For *Ho-1*, 5′
5′-CAAGCCGAGAATGCTGAGTTCATG-3′, and
For *Ho-1* 3′
5′-GCAAGGGATGATTTCCTGCCAG-3′;
For *Nqo1*, 5′
5′-GCGAGAAGAGCCCTGATTGTACTG-3′, and
For *Nqo1*, 3′
5′-TCTCAAACCAGCCTTTCAGAATGG-3′;
For *Gclm* 5′
5′-GCCACCAGATTTGACTGCCTTTG-3′, and
For *Gclm* 3′
5′-TGCTCTTCACGATGACCGAGTACC-3′;
For *Glyceraldehyde-3-phosphate dehydrogenase* (*Gapdh*), 5′
5′-TGTGTCCGTCGTGGATCTGA-3′ and
For *Gapdh*, 3′
5′-TTGCTGTTGAAGTCGCAGGAG-3′

2.6. Nuclear extraction

The 661 W cells were seeded at a density of 3.0×10^5 cells/10 mL in 10 cm dishes. After incubation for 24 h, the medium was changed to DMEM with 1% FBS and incubated for another 1 h, then carotenoids were added at 1 h after changing medium. Six hours after exposure of carotenoids, the cells were sampling and the ProteoExtract® Subcellular Proteome kit (Merck Millipore, Darmstadt, Germany) was used to extract the nuclear proteins following the manufacturer's protocol.

2.7. Immunoblotting

The protein concentration was determined by comparing it to known concentrations of bovine serum albumin using a BCA Protein Assay kit (Thermo Fisher Scientific). For immunoblotting, about 1.5 μg of total protein was diluted in the sample buffer with 20% 2-mercaptoethanol (Wako). Cell lysates were solubilized in sodium dodecyl sulfate sample buffer, separated on 5–20% sodium dodecyl sulfate-polyacrylamide gradient gels, and transferred to polyvinylidene difluoride membrane (Immobilon-P; Merck Millipore). The membranes were blocked by Block One-P (Nakarai Tesque, Inc., Kyoto, Japan) for 30 min at room temperature. Then the membranes were incubated overnight at 4 °C with the primary

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