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Nitroxide free radicals protect macular carotenoids against chemical destruction (bleaching) during lipid peroxidation

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ABSTRACT

Macular xanthophylls (MXs) lutein and zeaxanthin are dietary carotenoids that are selectively concentrated in the human eye retina, where they are thought to protect against age-related macular degeneration (AMD) by multiple mechanisms, including filtration of phototoxic blue light and quenching of singlet oxygen and triplet states of photosensitizers. These physical protective mechanisms require that MXs be in their intact structure. Here, we investigated the protection of the intact structure of zeaxanthin incorporated into model membranes subjected to oxidative modification by water- and/or membrane-soluble small nitroxide free radicals. Model membranes were formed from saturated, monounsaturated, and polyunsaturated phosphatidylcholines (PCs). Oxidative modification involved autoxidation, iron-mediated, and singlet oxygen-mediated lipid peroxidation. The extent of chemical destruction (bleaching) of zeaxanthin was evaluated from its absorption spectra and compared with the extent of lipid peroxidation evaluated using the thiobarbituric acid assay. Nitroxide free radicals with different polarity (membrane/water partition coefficients) were used. The extent of zeaxanthin bleaching increased with membrane unsaturation and correlated with the rate of PC oxidation. Protection of the intact structure of zeaxanthin by membrane-soluble nitroxides was much stronger than that by water-soluble nitroxides. The combination of zeaxanthin and lipid-soluble nitroxides exerted strong synergistic protection against singlet oxygen-induced lipid peroxidation. The synergistic effect may be explained in terms of protection of the intact zeaxanthin structure by effective scavenging of free radicals by nitroxides, therefore allowing zeaxanthin to quench the primary oxidant, singlet oxygen, effectively by the physical protective mechanism. The redox state of nitroxides was monitored using electron paramagnetic resonance spectroscopy. Both nitroxide free radicals and their reduced form, hydroxylamines, were equally effective. Obtained data were compared with the protective effects of α-tocopherol, which is the natural antioxidant and protector of MXs within the retina. The new strategies employed here to maintain the intact structure of MXs may enhance their protective potential against AMD.

1. Introduction

Many epidemiological studies suggest that the high consumption of lutein and zeaxanthin is associated with a lower risk of age-related macular degeneration (AMD) [1–3]. Only these two carotenoids are selectively accumulated in the membranes of the retina from blood plasma, where more than 20 other carotenoids are available [4,5]. Another carotenoid, meso-zeaxanthin (which is a stereoisomer of zeaxanthin), is converted from lutein within the retina [6]. These carotenoids, named macular xanthophylls (MXs), are accumulated primarily in the region of photoreceptor axons [7,8] but also are detected within photoreceptor outer segments [9,10] and in the retinal pigment epithelium (RPE) [11]. They are thought to combat light-induced damage mediated by reactive oxygen species by absorbing the most damaging incoming wavelength of light prior to forming reactive oxygen species (a function expected of carotenoids in axons) and by chemically and physically quenching reactive oxygen species once they are formed (a function expected of carotenoids in photoreceptor outer segments and retinal pigment epithelium).

Two main functions explain the selective presence of MXs in the retina. One functional hypothesis states that MXs, due to their appropriate location mostly in the outer plexiform layer [7,8], form a

Abbreviations: AMD, age-related macular degeneration; MXs, macular xanthophylls; Zea, zeaxanthin; RPE, retinal pigment epithelium; TCA, tri-chloroacetic acid; BHT, butylated hydroxytoluene

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filter for blue light and block hazardous light before it reaches the potential photosensitizers responsible for photodynamic damage to the retina. Blue-light absorption can be considered an indirect antioxidant action because it prevents the generation of reactive oxygen species that can damage retinal cells. In fact, most ultraviolet light below 300 nm is absorbed by the cornea [12], whereas ultraviolet light in the range of 300–400 nm is blocked by the lens. Nevertheless, some fraction of short wavelength blue radiation reaches the retina and may activate endogenous retinal photosensitizers. Another function of MXs is connected with the necessity of protection against oxidative stress, because oxidative stress, in addition to aging, seems to be a major determinant in the pathogenesis of AMD [13]. MXs may act directly as lipid-soluble antioxidants in retina membranes. The antioxidant role of carotenoids involves both quenching of singlet oxygen and scavenging of free radicals. Unfortunately, the same properties that make carotenoids as beneficial as antioxidants also make them highly susceptible to oxidation. In this study, we evaluate how several types of nitroxide free radicals (synthetic antioxidants) may protect macular carotenoids against chemical degradation.

Carotenoids are known to be effective singlet oxygen quenchers, and their activities are much higher than those of another retinal antioxidant α-tocopherol [14,15]. Singlet oxygen quenching by carotenoids in organic solvents mainly depends on the carotenoid's triplet state energy level and, thus, on the number of conjugated double bonds. Zea, with 11 conjugated double bonds, is an extremely efficient quencher. At room temperature, the singlet oxygen quenching rate constant for Zea in benzene has been found to be 1.2×10^{10} M^{-1} s^{-1} [16]. The rate constant for lutein, with 10 conjugated double bonds, is two times smaller (~0.66×10^{10} M^{-1} s^{-1}). MXs are also capable of quenching excited triplet states of singlet oxygen photosensitizers such as all-trans-retinal [17,18], retinyl ester [19], all-*trans*-retinol [20], cytochrome c oxidase [21], porphyrins [22], melanin [23,24], and lipofuscin [25,26]. Carotenoids can quench singlet oxygen by two different mechanisms. The first mechanism, involving energy transfer and termed physical quenching, is considered the major pathway of singlet oxygen deactivation. According to this mechanism, carotenoid molecules deactivate singlet oxygen to the less reactive triplet ground state. During that process, carotenoid molecules become excited to the triplet state and can return to the ground state, dissipating the energy excess as heat. The benefit of physical quenching is that carotenoids may act without alteration of their own chemical structure. The second mechanism is called chemical quenching. It involves a chemical reaction between carotenoids and singlet oxygen that results in pigment oxidation. This latter process consumes the carotenoids themselves. Chemical quenching has been reported to contribute less than 0.05% to the overall singlet oxygen quenching by carotenoids [27]. Several carotenoid oxidation products have been found in the retina [11,28], indicating that this mechanism can take place in vivo and is responsible for destruction of the pigment molecule.

The conjugated double bond system is primarily responsible for the high chemical reactivity of carotenoids with both singlet oxygen [14,29] and free radicals [30–32]. The selective localization of MXs in domains rich in polyunsaturated phospholipids [33–35], and therefore susceptible to free radical- and singlet oxygen-induced damage, is ideal for their chemical antioxidant action. The scavenging of lipid-derived peroxyl radicals is also an important antioxidant activity of MXs. Carotenoids scavenge lipid peroxyl radicals by forming radical adducts [30], which are less reactive than lipid alkyl peroxyl radicals. Thus, carotenoids are also reported as chain-breaking antioxidants, which delay the oxidation of biomembranes by trapping chain-initiating or chain-propagating peroxyl radicals. This chemical reaction leads to the destruction of carotenoid molecules. The mechanism by which partially consumed carotenoids are replaced or repaired in the human retina is poorly understood.

Both the physical and chemical stability of MXs in the retina are significant factors that allow them to perform their protective action effectively and for a prolonged time. Physical stability is manifested by their very slow removal from the retina, observed after discontinuation of xanthophyll supplementation [36]. MXs are also degraded more slowly than other dietary carotenoids such as carotenes and, thus, are chemically more stable [37,38]. As indicated above, most of the protective actions of MXs in the retina are performed through their physical protective mechanisms, which require that MXs be in their intact structure. Thus, it is timely and important to identify mechanisms whereby the chemical destruction (bleaching) of MXs in retinal membranes can be diminished.

Several studies have postulated that zeaxanthin and α-tocopherol provide synergistic protection of the human retina against chronic oxidative damage caused by photo-induced lipid peroxidation in photoreceptor and RPE cell membranes [39,40]. The proposed mechanism is the inhibition of xanthophyll consumption by α-tocopherol. One could expect that nitroxide free radicals could act in a similar way by sparing zeaxanthin and allowing it to efficiently quench singlet oxygen.

Lipid soluble nitroxide free radicals can be considered synthetic antioxidants. They can act as chain-breaking antioxidants, inhibiting the formation of lipid alkyl radicals. Being radicals themselves, they react with other radicals, which leads to the termination of radical chain reactions. Both nitroxide free radicals and their reduced forms, EPR-silent hydroxylamines, can react with oxygen-centered and carbon-centered radicals and break the radical chain reaction. Nitroxides, in contrast to other antioxidants such as carotenoids and α-tocopherol, do not act as prooxidants. (See Ref. [41]. for a discussion of the prooxidant activities of carotenoids.) They are not depleted during their antioxidant action because their reduction products (hydroxylamines) are also effective antioxidants [42]. It has been reported that carotenoids are less effective in trapping peroxyl radicals then α-tocopherol [43]. On the other hand, lipid-soluble nitroxides provided better protection to polyunsaturated membranes than α-tocopherol [44,45]. The recycling mechanism of antioxidant action of nitroxides through oxoammonium cations and hydroxylamines seems to be a great advantage as compared with traditional antioxidants: carotenoids and α-tocopherol [46].

Here, we investigated how membrane- and/or water-soluble nitroxide free radicals (spin labels) protect the intact structure of a representative MX, zeaxanthin (Zea), incorporated into model membranes subjected to lipid peroxidation. We compared these data to the protective effect of α-tocopherol, a natural antioxidant within the retina. We also tested whether we could detect a synergistic, inhibitory effect on lipid peroxidation when nitroxides and Zea act together. We theorize that new strategies, like the one employed here, aimed at maintaining the intact structure of MXs during oxidative stress, which is implicated in AMD pathogenesis, should enhance their protective potential.

2. Materials and methods

2.1. Reagents

Dilinoleoylphosphatidylcholine (DLPC), dimyristoylphosphatidylcholine (DMPC), 1-palmitoyl-2-arachidonoylphosphochatidyline (PAPC), 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and 1-palmitoyl-2-(16-doxylstearoyl)phosphatidylcholine (16-PC) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Zea was purchased from CaroteNature (Lupsingen, Switzerland), malondialdehyde (MDA) was obtained from Cayman Chemical (Ann Arbor, MI). CTPO (3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrrolin-1-oxyl), TEMPO (2,2,6,6-Tetramethylpiperidine 1-oxyl), TEMPONE (2,2,6,6-tetramethylpiperidone-N-oxyl), and other chemicals, of at least reagent grade, were purchased from Sigma-Aldrich Co. (St. Louis, MO).