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Reduced levels of rat lens antioxidant vitamins upon *in vitro* UVB irradiation

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Abstract

Ultraviolet (UV) radiation is one of the major risk factors of cataractogenesis. UV radiation induced damage to the eye lens is believed to be mediated through reactive oxygen species. Antioxidant defense systems, enzymatic and non-enzymatic, resist this damage. In the present study, the levels of rat lens endogenous antioxidants, L-ascorbic acid, α-tocopherol and β-carotene, have been determined by HPLC upon *in vitro* UVB irradiation. UVB irradiation for 24 h (300 nm; 100 μW/cm^2) of three months old rat lens suspended in RPMI medium, leads to 69–89% decrease in endogenous levels of these antioxidants. The addition of ascorbic acid (2 mM), α-tocopherol (2.5 μM) or β-carotene (10 μM), separately to the medium during irradiation significantly prevented the decrease in their endogenous levels, thereby suggesting a protective role for these antioxidant micronutrients against photodamage to the eye lens.

Keywords: Antioxidants; L-Ascorbic acid; β-carotene; HPLC; Rat lens; α-Tocopherol; UVB radiation

1. Introduction

Cataract, the opacification of eye lens, is the leading cause of blindness all over the world. There is an increasing body of evidence that implicate reactive oxygen species (ROS) in cataract formation [1–4]. The overall biochemical and the consequent functional aberrations initiated by ROS are commonly referred to as "oxidative damage". Both, intracellular and extracellular oxidative stress affect the lens *in vivo* [3]. Though, intracellular oxidants are generated as a result of normal metabolic processes, they can also be produced extracellularly by a variety of factors. One such environmental factor implicated in the oxidative damage to the lens [4,5], exposure to which enhances cataract formation, is ultraviolet (UV) radiation [4–6]. Since the lens is exposed to chronic near–UV radiation and enough oxygen and sensitizer molecules are present in the normal lens, photodamage to the lens could thus involve the production of free radicals [3–5]. Generation of oxidants during photooxidation of lens crystallins has been reported [4,5].

The lens epithelium contains systems that are capable of protecting the lens from oxidative insult. The lens defense system against oxidative damage includes both enzymatic (superoxide dismutase, catalase and glutathione redox cycle enzymes) and non-enzymatic factors such as the antioxidant micronutrients (L-ascorbic acid, α-tocopherol and β-carotene) [1–3,7]. Nevertheless, in many types of cataracts and aged lenses this defense system is compromised [2,3,7,8]. The resultant tissue damage is thus determined by the balance between the generation of free radicals and their elimination by the oxidant scavengers. Ascorbate and glutathione (GSH) are present in the lens at millimolar range and their concentrations are decreased in cataractous lenses [3,9]. Though, the presence of carotenoids in the lens is not clear, vitamin E is present in micromolar range [9]. α-tocopherol was found to protect the lens catalase from near-UV damage [10]. Apart from GSH recycling and maintenance of membrane integrity, vitamins C, E and β-carotene, can directly scavenge free radicals [11]. However, the direct effects of UV radiation on lens antioxidant vitamins are not known. Therefore, in the present investigation, we have assessed the endogenous levels of the antioxidants, L-ascorbic acid, α-tocopherol and β-carotene, upon *in vitro* UVB irradiation, in the absence and presence of externally added antioxidants to assess the UVB radiation effects on lens antioxidant micronutrients.

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2. Materials and methods

2.1. Chemicals

L-ascorbic acid, β-carotene, HEPES, α-tocopherol and α-tocopherol nicotinate were from Sigma. RPMI-1640 was from Hi-Media (India) and all other reagents were of analytical grade.

2.2. Lenses

Wistar/NIN rats (3 months old; 240–260 g body weight) were obtained from National Center for Laboratory Animal Sciences, National Institute of Nutrition. Lenses were collected from the overnight fasted male rats by posterior approach [12]. Guidelines of Institutes' Animal Ethics Committee were strictly followed in handling the animals and other protocols. Lens dissection was carried out under dim light at 4°C.

2.3. Irradiation

Irradiation at 300 nm (20 nm band width) was carried out by using a perspex sheet to place the lenses inside the 1cm quartz cuvette and facing the anterior side of the lens towards the light source. Experiments were carried out in RPMI-1640 (without phenol red) containing 10 mM HEPES buffer, at pH 7.2 (1 ml/lens) as described earlier [12,13]. Lenses were pre-incubated in the medium for 2–3 h prior to irradiation. The osmolarity of the medium was adjusted to 295–300 milliosmoles/L with sodium bicarbonate. Lens integrity was not disturbed under these conditions for 24 h [12,13]. The source of UV radiation was 150 W xenon high pressure lamp attached to a double monochromator [12]. The intensity of the incident light falling on the lens surface was 100 $\mu W/cm^2$ and the duration of irradiation was 24 h at room temperature [12]. To study the effect of external antioxidants, freshly prepared solutions of ascorbic acid, α-tocopherol and β-carotene were added to the medium separately [13]. All the solutions were sterile filtered through 0.22 μm filters (Millipore). Lenses kept in dark under the similar conditions served as controls.

2.4. Estimation of vitamins C, E and β-carotene

The levels of L-Ascorbic acid, α-tocopherol and β-carotene were estimated at '0' time and after 24 h, by HPLC (LC-6A pump, SCL-6B system controller & SPD-6AV UV/Vis detector; Shimadzu Co.). For ascorbic acid estimation, control and irradiated lenses were rinsed thrice with distilled water and a 10% (w/v) homogenate was made in 0.35 M perchloric acid. The 10,000 g supernatant was filtered through 0.45 μm filters (Millipore) and loaded onto a 250 × 4.6 mm C_{18} reversed phase (RP) column (Bondapak, 10 μm) according to the method described by Finely and Duang [14]. The detection wavelength was 254 nm. For the estimation of α-tocopherol and β-carotene, lenses were rinsed thrice with phosphate buffered saline, pH 7.4, containing 1% vitamin C and a 10% homogenate was made in the same buffer. An equal volumes of ethanol (containing 0.1% vitamin C) was added to the homogenate and thoroughly vortexed. α-tocopherol and β-carotene were extracted with known amounts of hexane and the solvent phase was evaporated under nitrogen gas. Samples (reconstituted in ethanol) were loaded onto a 100 × 4.6 mm C_{18} RP column (Bondapak, 5 μm) and detected at 292 (for α-tocopherol) or 448 nm (for β-carotene) [15]. α-Tocopherol nicotinate was used as internal control for the recovery of both α-tocopherol and β-carotene. Data were analyzed by student 't' test and $P < 0.05$ was considered as significant.

Table 1
Relative retention time of standard L-ascorbic acid, α-tocopherol, α-tocopherol nicotinate abd β-carotene and their '0' time levels in rat lens

Antioxidant	Relative retention time (min)	'0' time level
L-Ascorbic Acid	2.50	168.32 μg/g tissue
α-tocopherol	5.29	1058.5 ng/g tissue
α-tocopherol nicotinate	7.89	—
β-carotene	10.0	Undetectable

Data represent mean of four values. α-tocopherol nicotinate was used as an internal standard. See materials and methods section for other details.

3. Results

The HPLC analysis indicated that the relative retention times of L-ascorbic acid, α-tocopherol, α-tocopherol nicotinate and β-carotene were 2.50, 5.29, 7.87 and 10.0 min respectively (Table 1). The lenses were processed and antioxidant micronutrients were estimated immediately after lens dissection ('0' time). While the '0' time values for L-ascorbic acid and α-tocopherol are presented in Table 1, basal levels of β-carotene could not be detected under the HPLC conditions used in the study.

UVB irradiation of rat lens for 24 h reduced the endogenous levels of ascorbic acid and α-tocopherol by 69% and 89% respectively, as compared to the control lenses kept in the dark for 24 h under similar conditions (Figs. 1 and 2). As expected, levels of β-carotene could not be detected in both 24 h control and irradiated lenses. However, UVB irradiation at an incident intensity of 100 $\mu W/cm^2$ for 24 h, did not induce any lens opacification as examined by slit-lamp biomicroscope (not shown). Irradiation experiments were then carried out in the presence of externally added antioxidant micronutrients. Separate addition of ascorbic acid (2 mM) and α-tocopherol (2.5 μM) to the medium raised their respective endogenous levels significantly and the overall decrease in their levels on irradiation was only 20–35%, as estimated at the end of 24 h (Figs. 1 and 2). Interestingly, the endogenous levels of β-carotene (27.35 ng/g tissue)

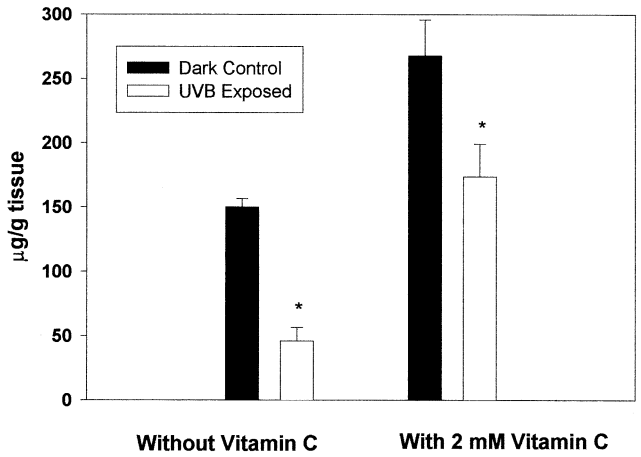

Fig. 1. Rat lens L-ascorbic acid levels on UVB irradiation, in the absence and presence of 2 mM ascorbic acid. Data represent mean ± S.E. of four separate observations obtained for four different sets of irradiation. *Denotes significantly different from the respective dark control.

could be detected when lenses were incubated with 10 μM β-carotene added to the medium externally and upon irradiation for 24 h, however, its levels were reduced by 36% (17.41 ng/g tissue). Exogenously added antioxidants seemed to penetrate into lens because β-carotene was detected when it was added to the medium, otherwise it was undetectable. Therefore, the concentration of these antioxidants remained either same, or higher, to that of control lenses when the lenses were irradiated in the presence of respective external antioxidant than the lenses that were irradiated in the absence of added antioxidants (Figs. 1 and 2). The concentrations of externally added antioxidants used in this study were found to be optimal in protecting the lens macromolecules against *in vitro* UV irradiation [13]. Therefore, no attempts were made with varying concentrations. Moreover, these concentrations are very much in the physiological range [3,10].

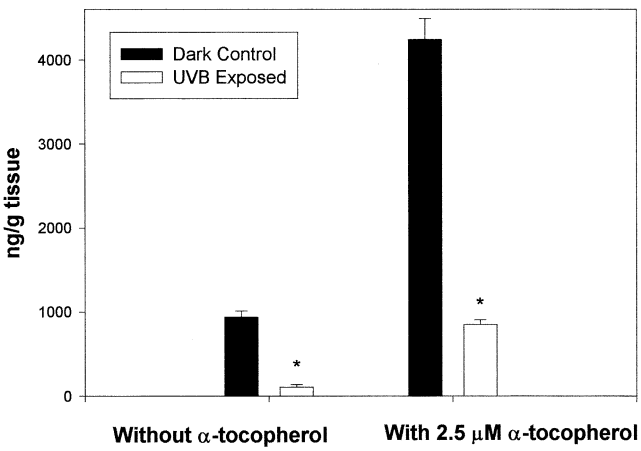

Fig. 2. Rat lens α-tocopherol levels on UVB irradiation, in the absence and presence of 2.5 μM α-tocopherol. Data represent mean ± S.E. of four separate observations obtained for four different sets of irradiation. *Denotes significantly different from the respective dark control.

4. Discussion

Oxidative stress to the lens is a major damaging factor in cataract development [1–5,7,8]. Since the lens is exposed to chronic near-UV radiation and enough oxygen and sensitizer molecules are present in normal lens, the effects of near-UV radiation on lens could involve a variety of free radical reactions [4,5,9,13]. Increased lipid peroxidation under identical experimental conditions was observed earlier and indicate that UVB radiation causes oxidative damage to lens [13]. In addition, UVB irradiation induced alterations in activities and kinetic properties of key enzymes of major metabolic pathways of lens [12,16] and protein cross-links [17]. Moreso, UVB induced damage to lens macromolecules could be prevented when antioxidant micronutrients were present during irradiation [13], and strengthen the theory of oxidative damage to lens by UVB radiation. In this context it is essential to investigate the direct effects of UV radiation on major endogenous antioxidants of lens.

The results of the present study clearly reveal that the levels of the antioxidant micronutrients decreased significantly upon UVB irradiation *in vitro*. There was a small but definite decrease in the basal levels of these antioxidants from ‘0′ time to 24 h. This may be due to the stability of these antioxidants at the ambient temperature and/or involvement of the antioxidants in various reactions in the lens. It is highly probable that UVB radiation, though, over a longer period of cumulative exposure, could lead to decreased antioxidants *in vivo*. In fact, Babu et al., have recently reported that even a short term (1–2 weeks) *in vivo* UVB exposure could result in oxidative stress on rat lens as shown by an increase in lipid peroxidation and H_2O_2 production, and a decrease in glutathione levels in the absence of any lens opacification [18]. These studies together indicate that a compromise in lens antioxidant defense system could be at the bottom in the process that leads to lens opacification. Interestingly, endogenous levels of vitamin C, E and β-carotene were increased many fold above the basal values when the lenses were incubated with external antioxidants. Further, the decrease upon UVB irradiation was only 25–35%, suggesting that by enriching the endogenous antioxidant micronutrient levels we could minimize the UV induced alterations.

Though, antioxidants in the medium can absorb UV light, particularly α-tocopherol (with an absorption maxima at 292 nm), the present experiment is not just a photochemical degradation mechanism of these antioxidants. Hence, the antioxidants detected in the lens is the sum of the residual antioxidants originally contained in the lens and antioxidants penetrated into the lens from medium during 24 h experimental period, even if one considers the above degradation process. Moreover, we have observed similar results, in a separate study, wherein the lens was first incubated for 24 h in antioxidants containing medium and irradiated further for 24 h in the medium without antioxidants considering the time dependent loss in both the groups. It

could also be possible that any of the components in the medium complicate UV induced effects. Therefore, lenses were also irradiated in phosphate buffered saline and modified medium 199 and similar results were obtained [13].

Vitamins C, E and β-carotene have shown protective role against UV damage to lens cytoskeleton proteins [19]. Vitamin E was also shown to prevent cortical cataracts induced by sugar [20]. Photoprotective effect of vitamin E and carotenoids have been well documented [11]. Epidemiological studies also suggest that dietary and metabolic antioxidants, including ascorbic acid, α-tocopherol and β-carotene, play an important role in delaying the cataract formation [1,8]. Lowered serum levels of vitamins C and E and carotenoids as well as reduced intake of these nutrients were associated with higher risk of cataract [21,22]. Hence, the prophylactic and therapeutic role of antioxidant micronutrients against cataract formation is gaining importance [1,2, 8,21]. In this context the results of the present study, which demonstrate that enhancing endogenous antioxidant micronutrient levels could minimize the UVB radiation induced photodamage to the lens, merit attention.

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