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UV light increases vitamin C uptake by bovine lens epithelial cells

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Purpose: To establish whether the oxidation of ascorbic acid (AA) in the aqueous humor may contribute to maintain the high concentration of AA of the anterior eye tissues during oxidative stress.
Methods: Primary cultures of bovine lens epithelial cells (BLEC) were incubated in a medium with the concentration of AA, glutathione (GSH), and cysteine (Cys) found in the aqueous humor. The intracellular concentration of AA was measured over time and compared to that of cultures maintained in the same medium but exposed to UV light.
Results: The uptake of extracellular AA by BLEC was 0.09±0.01 nmol/mg protein/min, but rose to 0.53±0.02 nmol/mg protein/min when cell cultures were exposed to a dose of UV-C light (178μW/cm^2) capable of oxidizing extracellular AA but not intracellular AA. Under the same conditions, intracellular AA was oxidized when cell monolayers were treated with 1,3-bis(2-Chloroethyl)-1-nitrosourea (BCNU), an inhibitor of glutathione reductase and thioredoxin reductase. The uptake of the oxidized form of AA, dehydroascorbic acid (DHAA), was approximately 7 times quicker than that of AA; the uptake and intracellular reduction of DHAA to AA was inhibited by depleting intracellular GSH. Extracellula glucose inhibited the uptake of DHAA by BLEC, and the extent of this inhibition was dependent on the concentration of extracellular glucose.
Conclusions: During mild oxidative stress, AA contained in aqueous humor may provide tissues of the anterior eye with a source of DHAA which is readily taken up and converted to intracellular AA, while intracellular AA is maintained in the reduced status by intracellular antioxidant systems. Since glucose inhibited DHAA uptake, this protective mechanism could be impaired in diabetes.

Enhanced production of reactive oxygen species (ROS) within the eye lens and consequent oxidative damage to the eye lens proteins is thought to be a major mechanism leading to the onset of cataract, the most frequent cause of blindness [1].

ROS can be generated endogenously by several enzymatic systems, or derive from environmental sources. Endogenous sources of ROS include mitochondria, peroxisomes, lipoxygenases, NADPH oxidase, and cytochrome P450. Exogenous sources include ionizing radiation, chemotherapeutics, inflammatory cytokines and environmental toxins [2]. In the eye, an additional source of ROS causally related with the development of cataract is represented by ultraviolet light [3].

The lens, like other organs, is endowed with a complex and efficient system of defense capable of neutralizing oxidative species and to repair, recover, or degrade damaged molecules. These systems include non-enzymatic (glutathione, ascorbic acid, tocopherol, and carotenoids) and enzymatic (superoxide dismutase, glutathione peroxidase, and catalase) antioxidants [1,4]. Ascorbic acid (AA) is present in millimolar concentration in the anterior eye and in the lens, and is thought to act as a filter against diurnal radiation [5-9]. During its antioxidant function, AA is oxidized [1], and its oxidation product, dehydroascorbic acid (DHAA), which is known for being readily absorbed by the lens [10,11], has been detected in normal aqueous humor and lens, and is thought to be toxic to the lens and to induce cataract formation [12]. Within the lens cells, DHAA is known to be efficiently reduced by means of GSH and NADPH dependent mechanisms. These are responsible of preventing its intracellular accumulation and damaging effects [13-16]. Due to the low concentration of GSH and to the absence DHAA recycling enzymes in the aqueous humor, extracellular AA is more prone to oxidation to DHAA than intracellular AA [17,18].

DHAA is known for being transported by proteins of the GLUT family [19], which are expressed in the lens, thus oxidation of the AA contained in the aqueous humor might cause the transfer of DHAA to lens epithelium [20,21]. The present study was undertaken to test the hypothesis that the oxidation of extracellular AA to DHAA, rather than increasing the intracellular concentration of DHAA, provides the lens epithelium with a source of AA due to its rapid reduction within lens epithelial cells.

METHODS

Cell culture: Bovine lenses were obtained from freshly slaughtered animals at a local slaughterhouse (Consorzio Macelli, S. Miniato, Pisa, Italy). Lens capsules were removed and lens capsule explants 2-3 mm wide were placed in 6 well culture dishes, together with 1 ml of RPMI 1640 medium, supplemented with antibiotics (penicillin/streptomycin), glutamine

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2 mmol/l, and 10% fetal calf serum. Cellular outgrowths appeared after 4-6 days, were trypsinized and subcultured after 2 weeks. Unless otherwise specified, experiments were done with cells at passage 7-8.

Enzyme activities: Glutathione reductase and glutathione transferase activities were determined according to Massey and Williams [22] and Habig et al. [23], respectively.

Preparation of DHAA: DHAA was prepared immediately before the assay by exposing a solution of AA (50 mmol/l) in water to liquid bromine; excess bromine was removed by bubbling the solution with N_2 [24].

Determination of AA: AA was determined according to Rose and Bode [25]. Samples to be analyzed were extracted with 50 mM H_3PO_4, centrifuged at 5000x g for 5 min at 4 °C, and then analyzed for their AA content by high pressure liquid chromatography. Aliquots of the supernatants were injected in a C-18 reverse phase column (Resolve, Waters, USA) and determinations were performed by a Beckman Gold HPLC apparatus equipped with electrochemical detector (ESA Coulochem III) with a Model 5011 analytical cell.

Determination of GSH: GSH was analyzed by the enzymic method of Tietze [26] as modified by Baker et al. [27] to adapt it to a microtiter plate reader. Glutathione disulfide was determined by the vinylpiridine method [27,28].

Oxidation of intracellular and extracellular AA by exposure to UV light: Cell cultures were incubated at 37 °C in Dulbecco's phosphate buffered solution (PBS), supplemented with AA, GSH, and cysteine at concentrations reproducing those of aqueous humor (1.1 mM, 10 μM and 10 μM, respectively) [17] and were exposed to a UV source (Osram HNS 5 W/U, emission 178 μW cm^2 at 254 nm at a distance of 40 cm from the source according to manufacturer's information). The conditions of exposure were chosen to reproduce an accidental exposure to one of the occupational sources of UV-C (e.g., germicidal lamp, UV transilluminator).

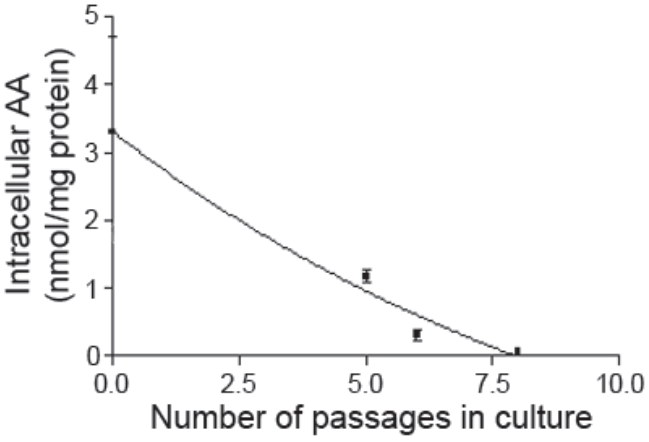

Figure 1. Decline of the content of ascorbic acid during culture of bovine lens epithelial cells. Cell monolayers were maintained in RPMI 1640 medium as described in "methods", and the content of ascorbic acid assessed at passages 0, 5, 6, and 8; the ascorbic acid content of the original explant fragment at plating into the culture dish is reported as passage 0. Each point represents the mean of three independent determinations; the error bars represent the standard deviation. The best fit curve is shown.

Uptake of AA and of DHAA: cell cultures were incubated at 37 °C in Dulbecco's phosphate buffered solution (PBS) containing, unless otherwise specificed, glucose 1 g/l. In some experiments, glutathione reductase and thioredoxin reductase where inhibited by incubating BLEC with 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) 0.3 mmol/l dissolved in ethanol [29], or intracellular GSH was depleted by pretreating cell monolayers with diethylmaleate 1 mmol/l for 60 min. Intracellular content of AA was assayed at time intervals and expressed as nmol/mg protein.

Protein content: Protein content of the samples was determined by the Bio-Rad protein assay kit (Bio-Rad, Milan, Italy).

Statistical analysis: Comparison between different treatments was performed with two way ANOVA. In AA and DHAA uptake experiments, linear regression of the increase of intracellular AA was calculated and the result expressed as slope±standard error.

RESULTS

Culture of epithelial cells: After 3-4 days, cell outgrowth appeared around the original explants. Cell monolayers were trypsinized when reaching 2-3 cm diameter and maintained up to 10 passages. The intracellular content of AA was 3.3±1.4 nmol/mg protein in the explant at plating, and declined to 0.07±0.02 nmol/mg protein at the eighth passage, following a one phase exponential decay curve (half life 7.6, 95%CI -0.4 to 0.3, R^2 0.989, Figure 1), this is in line with the absence of AA in the RPMI 1640 medium. At the eighth passage, in BLEC intracellular GSH was found to be 23±7 nmol/mg protein (18±4 nmol/mg protein in the explant); glutathione reductase and glutathione transferase activities were, respectively, 7.5±1.4 and 64.8±16.1 U/g protein (1.0±0.1 and 56.1±2.3 in the original explant, respectively).

Differential effect of UV light on intracellular and extracellular AA: When BLEC cultures were incubated in PBS containing AA, Cys, GSH, (1.1 mM, 10 μM and 10 μM, respectively), in the presence of glucose 1 mg/ml, the uptake of AA was modest (0.09±0.01 nmol/mg protein/min), unless the cultures were exposed to UV light (178 μW/cm^2), which raised the uptake to 0.53±0.02 nmol/mg protein/min.

The same dose of UV light did not cause oxidation of intracellular AA to DHAA, unless glutathione reductase and thioredoxin reductase were inhibited by BCNU, thus preventing GSH as well as NADPH dependent DHAA reduction (Figure 2).

Characterization of the uptake of AA and DHAA by BLEC: When cell monolayers were exposed to DHAA in the presence of 1 mM extracellular glucose, a concentration dependent, linear increase of intracellular AA was observed (1.1±0.1 nmol/mg protein/min and 2.1±0.2 nmol/mg protein min, respectively, in the presence of extracellular DHAA 0.25 mmol/l and 0.50 mmol/l, $p<0.05$ by ANOVA for the significance of each treatment and for the difference between the two treatments).

The increase of intracellular AA was approximately 7 times slower in the presence of the same concentration of extracellular AA (0.26±0.04 nmol/mg protein/min in the presence of extracellular AA 0.25 mmol/l, $p<0.05$). Upon exposure to extracellular DHAA 0.25 mmol/l, the increase of intracellular AA was concentration-dependently inhibited by extracellular glucose (3.2±0.5 nmol/mg protein/min, 1.6±0.1 nmol/mg protein min, and 0.6±0.03 nmol/mg prorein/min, respectively, in the presence of extracellular glucose 0, 1, and 5 g/l, $p<0.05$ for the difference between treatments). In the presence of extracellular DHAA, the increase of intracellular AA was inhibited (from 2.1±0.3 nmol/min/mg protein to 1.2±0.1 nmol/min/mg protein, $p<0.05$) when intracellular GSH was depleted by diethylmaleate from 20.5 nmol/mg protein to 6.0 nmol/mg protein.

DISCUSSION

The present study shows that a mild oxidative stimulus, corresponding to a short accidental exposure to a UV-C source in an occupational environment, is capable of oxidizing extracellular, but not intracellular AA, and accelerates the transfer of AA from the extracellular to the intracellular compartment via its transient oxidation to DHAA. Considering the relatively large volume of aqueous humor, and the wide contact surface with surrounding tissues, this mechanism might contribute to enhance the content of AA of all the anterior eye tissues in response to oxidative stress. In agreement with Fernando et al. [30] who found that in human lens epithelial cells the intracellular reduction of DHAA to AA depends mostly on the activity of thioltransferase, experiments with diethylmaleate and BCNU showed that this mechanism was dependent on the intracellular concentration of GSH and on the activity of glutathione reductase. In this respect, although the intracellular concentration of GSH and GSH related enzyme in BLEC underwent adaptation to culture conditions, the content of GSH and the activity of glutathione reductase remained in the same range as the original explant, thus confirming the basic requirement to make the GSH dependent recycling of DHAA operative also in vivo.

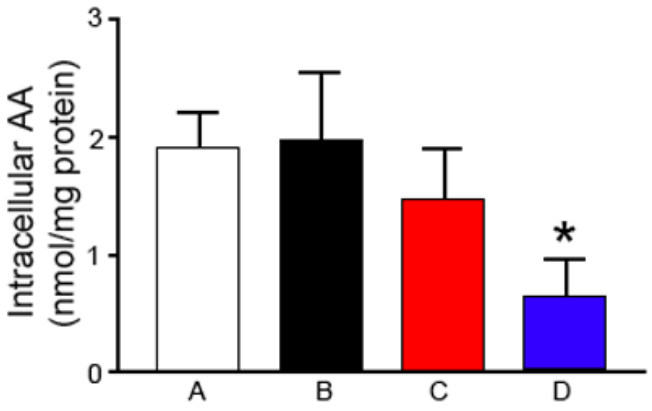

Figure 2. Effect of UV light on intracellular ascorbic acid. Cell monolayers were incubated for 15 min in Dulbecco's phosphate buffered solution containing 1 g/l glucose in the presence of 0.5 mmol/l dehydroascorbic acid. After washing, the monolayers were incubated in fresh Dulbecco's phosphate buffered solution containing glucose alone. Some were treated with 0.3 mmol/l 1,3-bis(chloroethyl)-1-nitrosourea and/or exposed to the UV source (exposure to the UV source was always the last step). Column A is the control and was incubated with dehydroascorbic acid (DHAA) only. Column B was incubated with DHAA and exposed to UV. Column C was incubated with DHAA and then incubated with 1,3-bis(chloroethyl)-1-nitrosourea. Column D was incubated with DHAA, then treated with 1,3-bis(chloroethyl)-1-nitrosourea and exposed to the UV source. At the end of the incubations ascorbic acid content of cell monolayers was determined and expressed as nmol/mg protein. Values presented are the mean of three separate experiments; the error bars represent the standard deviation. The asterisk above bar "D" indicates the value was significantly different from C ($p=0.028$).

Among other antioxidants, AA is particularly concentrated not only in the lens, but in other parts of the eye, including cornea, aqueous humor, tears [31]. AA is thought to act as a scavenger of radical species and of oxidizing radiation, thus its oxidation to DHAA is the consequence of the antioxidant function of this compound within the eye [1].

In the presence of severe oxidative challenge or defect of intracellular antioxidant enzymes, the accumulation of DHAA within the lens is thought to be a crucial step in the development of oxidative stress related diseases, and in particular of cataract [12]. In the present study, we found that extracellular AA is more prone to oxidation than intracellular AA; since aqueous humor contains an elevated concentration of AA (1.1 mmol/l in the bovine eye) [31], mild oxidative challenges brought to this fluid, which lacks efficient DHAA reducing systems, are expected to lead to the formation of a relatively large amount of DHAA. In fact, in the experimental conditions adopted here, approximately 20% of extracellular AA was oxidized without causing any oxidation of intracellular AA.

DHAA is transported easily across the plasmamembranes [19], thus intracellular DHAA reducing systems at the same time are responsible for preventing the oxidation of intracellular AA and for reducing DHAA deriving from the extracellular milieu.

Interestingly, the fact that extracellular glucose, within the range of concentrations that are attained in diabetes, concentration dependently impaired the uptake and intracellular reduction of DHAA suggests an additional mechanism by which diabetes may impair antioxidant lens defense and promote oxidative damage in the eye.

In conclusion, the unique feature of the anterior eye, which has a large extracellular compartment (i.e., aqueous humor) containing a high concentration of AA, but not of GSH or other antioxidants capable of maintaining its reduced status, may represent a specific and short term mechanism for providing the cells of AA trough the uptake of DHAA. While preventing oxidative damage due to the increase of intracellular AA, this is likely to represent a physiological mechanism for enhancing intracellular AA during the exposure to oxidizing agents. Due to the ability of glucose to competitively inhibit the uptake of DHAA, this mechanism might be impaired in diabetes and in conditions, such as aging, in which the de-

cline of intracellular reducing systems based on NADPH and GSH may impair the intracellular reduction of the DHAA originated in the extracellular milieu.

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