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Superoxide dictates the mode of U937 cell ascorbic acid uptake and prevents the enhancing effects of the vitamin to otherwise nontoxic levels of reactive oxygen/nitrogen species☆

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Abstract

Exposure of U937 cells to low micromolar levels of ascorbic acid or dehydroascorbic acid, while resulting in identical ascorbic acid accumulation, is unexpectedly associated with remarkably different responses to exogenous oxidants. We observed that otherwise nontoxic levels of hydrogen peroxide, *tert*-butylhydroperoxide or peroxynitrite promote toxicity in cells preloaded with ascorbic acid, whereas hardly any effect was detected in cells pretreated with dehydroascorbic acid. Further experiments performed with peroxynitrite in cells preloaded with ascorbic acid provided evidence for a very rapid nonapoptotic death, preceded by early Bax mitochondrial translocation and by mitochondrial permeability transition. The notion that conversion of extracellular ascorbic acid to dehydroascorbic acid prevents the enhancing effects on oxidant toxicity and nevertheless preserves the net amount of vitamin C accumulated was also established using ascorbate oxidase as well as various sources of superoxide, namely, xanthine/xanthine oxidase or ATP-driven NADPH oxidase activation. These findings suggest that superoxide-dependent conversion of extracellular ascorbic acid to dehydroascorbic acid represents an important component of the overall survival strategy of some cell types to reactive oxygen/nitrogen species.

Keywords: Vitamin C uptake; Superoxide; Reactive oxygen/nitrogen species; Cell death; U937 cells

1. Introduction

L-Ascorbic acid (AA) is a water-soluble vitamin involved in a wide variety of biochemical functions, including regulation of the redox status of the cells. Because of its low redox potential, AA is normally classified as a wide-spectrum antioxidant, i.e., a scavenger of peroxides, hydroxyl radicals, superoxide ($O_2^{\cdot -}$) and peroxynitrite ($ONOO^-$) [1,2]. Although these antioxidant properties are well established by an overwhelming literature, solid experimental evidence nevertheless documents opposite effects, at least restricted to specific cell types and/or conditions [2].

High levels of AA may indeed promote indirect toxic effects *via* autoxidation in the extracellular milieu, an event associated with extensive $O_2^{\cdot -}$ formation promptly followed by spontaneous/superoxide dismutase (SOD)-driven conversion to hydrogen peroxide (H_2O_2) [3–6]. These events, however, should not bear important consequences in normal subjects as redox cycling of the vitamin is limited by the reducing environment of the bloodstream. Furthermore, plasma levels of the vitamin are normally lower than those required to elicit toxicity. Hence, $O_2^{\cdot -}/H_2O_2$ formation associated with conditions leading to enforced oxidation of extracellular AA is most likely tightly controlled by the potent antioxidant defense (both enzymatic and nonenzymatic) comprising several components of the plasma and blood cells.

AA may however promote additional harmful effects in cells preloaded with the vitamin and immediately exposed to reactive oxygen/nitrogen species [7–11]. These observations are in obvious contrast with the antioxidant function of the vitamin, but nevertheless deserve further attention, as acute supplementation of AA might be deleterious for individuals with pathologies, or conditions, associated with enhanced formation/reduced scavenging of reactive oxygen/nitrogen species.

Vitamin C is normally found in plasma and in other biological fluids as AA at concentrations of approximately 50 μM [12,13]. Cell may take up AA *via* high-affinity/low-capacity sodium-AA cotransporters (SVCTs) [14]. Extracellular AA, however, may generate the

Abbreviations: AA, ascorbic acid; AO, ascorbate oxidase; CsA, cyclosporin A; DHA, dehydroascorbic acid; DHE, dihydroethidine; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; H_2O_2, hydrogen peroxide; MPT, mitochondrial permeability transition; $ONOO^-$, peroxynitrite; SOD, superoxide dismutase; SVCTs, sodium-AA cotransporters; *tert*B-OOH, *tert*-butylhydroperoxide; X, xanthine; XO, xanthine oxidase.

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ascorbyl free radical, which then dismutates to dehydroascorbic acid (DHA). While DHA is normally detected at very low (1–2 μM) levels in most biological fluids, its formation is nevertheless of critical importance, as it can be avidly taken up by the cells through facilitative hexose transport [15]. Furthermore, DHA concentrations might significantly increase under conditions associated with $O_2^{\cdot-}$ release, as it might occur in inflamed tissues. This notion was established in neutrophils, whose activation markedly enhanced their own AA accumulation, taken up in the form of DHA through glucose transporters [16]. In addition, under the same conditions, neighboring cells also accumulated more AA, once again as DHA through glucose transporters. This route of uptake is in general employed by most cell types [17–19] and indeed presents the major advantage of being mediated by a high-capacity mechanism associated with the immediate intracellular reduction back to AA.

In a recent study [20], we employed U937 cells to compare the uptake rates of physiologically relevant concentrations of AA vs. DHA, i.e., under conditions minimizing factors potentially affecting the overall results. A good example in this direction is provided by the use of high concentrations of AA or DHA, which obviously favors the high-capacity mechanism [20]. We obtained somewhat unexpected results providing evidence for identical rates and kinetics of vitamin C accumulation, so that enzymatic (i.e., mediated by ascorbate oxidase, AO) conversion of AA to DHA switched the mode of uptake (SVCTs vs. glucose transporters) without affecting the net amount of AA accumulated. Additional relevant information is that none of the two loading procedures was associated with detectable signs of toxicity and that hardly any change was detected in the redox status of the cells, as determined by the GSH and NADH/NADPH pool.

The present study was performed with the aim of assessing in this specific cell system, and using the same exposure paradigm, the impact of AA vs. DHA loading on toxicity elicited by a subsequent exposure to various oxidants. The results obtained indicate that otherwise inactive concentrations of H_2O_2, *tert*-butylhydroperoxide (*tert*B-OOH) and $ONOO^-$ promote toxicity in cells preexposed to physiological levels of AA. Surprisingly, however, the enhancing effects were not detected in cells preexposed to DHA concentrations associated with the accumulation of identical levels of AA. Similar results were obtained using AA in combination with AO. We subsequently demonstrated that $O_2^{\cdot-}$, regardless of whether generated by xanthine (X)/xanthine oxidase (XO) or by activation of NADPH oxidase, recapitulates all the effects mediated by enzymatic conversion of AA to DHA. In this perspective, the switch in the uptake mechanism is of obvious advantage for the cells which, while accumulating identical levels of vitamin C, nevertheless fail to acquire a hypersensitive phenotype to reactive oxygen/nitrogen species. Hence, $O_2^{\cdot-}$ is a master regulator of the uptake mechanism of vitamin C and, in the specific cell system employed in this study, dictates conditions associated with a safe accumulation of the vitamin.

2. Materials and methods

2.1. Chemicals

*tert*B-OOH, H_2O_2, X, XO, AO, cytochalasin B, ATP, SOD, catalase and the remaining chemicals were from Sigma-Aldrich (Milan, Italy). Cyclosporin A (CsA) was purchased from Novartis (Bern, Switzerland). FK-506 was from Calbiochem (San Diego, CA, USA). Apocynin was purchased from R&D Systems (SPACE, Milan, Italy). Dihydroethidine (DHE) and MitoTracker Red CMXRos were purchased from Molecular Probes (Leiden, the Netherlands).

2.2. Cell culture and treatment conditions

U937 human myeloid leukemia cells were cultured in suspension in RPMI 1640 medium (Sigma-Aldrich, Milan, Italy) supplemented with 10% fetal bovine serum (Euroclone, Celbio Biotecnologie, Milan, Italy), penicillin (100 U/ml) and streptomycin (100 μg/ml) (Euroclone) at 37°C in T-75 tissue culture flasks (Corning, Corning, NY, USA) gassed with an atmosphere of 95% air–5% CO_2.

Stock solutions containing AA, DHA, *tert*B-OOH, H_2O_2, ATP and catalase were freshly prepared in saline A (8.182 g/L NaCl; 0.372 g/L KCl; 0.336 g/L $NaHCO_3$; 0.9 g/L glucose, pH 7.4) immediately before utilization. Apocynin was dissolved in dimethyl sulfoxide (DMSO). At the treatment stage, the final DMSO concentrations were never higher than 0.05%. Under these conditions, DMSO was not toxic, nor did it affect the cytotoxic properties of *tert*B-OOH, H_2O_2 and $ONOO^-$.

Cells (1×10^6 cells/ml) were exposed for 15 min to AA or DHA in complete RPMI 1640 culture medium or in incubation buffer (15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$), as indicated in the text or figure legends. In selected experiments, NaCl was replaced with choline-chloride in the incubation buffer. Importantly, 0.1 mM dithiothreitol (DTT) was added to the incubation buffer employed in experiments measuring AA uptake. Stability of AA (3 μM) in the above incubation buffers was assessed by monitoring the absorbance at 267 nm for 15 min (ε_{267}=14,600 M^{-1} cm^{-1}). Stability of AA (3 μM) in culture medium was assessed by high-performance liquid chromatography (HPLC) analysis, after appropriate processing of the samples, as described below.

$ONOO^-$, synthesized as previously described [21], was rapidly added on the wall of the plastic tubes and mixed to equilibrate the $ONOO^-$ concentration on the culture medium. To avoid changes in pH due to the high alkalinity of the $ONOO^-$ stock solution, an appropriate amount of 1.5 N HCl was also added to the wall of the tubes prior to $ONOO^-$. Treatments with H_2O_2, *tert*B-OOH or $ONOO^-$ were performed in saline A at a density of 2.5×10^5 cells/ml.

2.3. Measurement of AA content by HPLC

After treatments, the cells were washed twice with cold saline A; the final pellet was extracted with ice-cold 70% (vol/vol) methanol/30% solution A (10 mM tetrabutylammonium hydrogen sulfate, 10 mM KH_2PO_4, 0.5% methanol, pH 6.5) containing 1 mM ethylenediaminetetraacetic acid. After 10 min at ice bath temperature, 10 mM DTT was added to the samples and centrifuged at 10,000*g* for 20 min at 4°C. Where indicated, treatment with DTT was omitted. Samples were filtered through a 0.22-μm filter (Millipore, Inc., Milan, Italy) and analyzed immediately or frozen at −80°C for later analysis. AA content was measured by HPLC with the UV detection wavelength set at 265 nm, as described by Savini et al. [22], with minor modifications. The assay involved the use of a 15-cm×4.6-mm Discovery C-18, 5-μm column (Supelco, Bellefonte, PA, USA), equipped with a Supelguard Discovery C-18 guard column (2 cm×4 mm, 5 μm). The injection volume was 20 μl. Under these conditions, the retention time of AA was about 4 min. AA concentration was determined from the corresponding calibration curve constructed with the pure chemical dissolved in extraction solution. Intracellular concentration of AA was calculated using published values for cell volume [23].

2.4. Cytotoxicity assay

Cells were preexposed to AA or DHA and subsequently treated with H_2O_2, *tert*B-OOH and $ONOO^-$ in saline A. The number of viable cells was estimated with the trypan blue exclusion assay after 60 min of incubation. Briefly, an aliquot of the cell suspension was diluted 1:2 (vol/vol) with 0.4% trypan blue, and the viable cells (i.e., those excluding trypan blue) were counted with a hemocytometer.

2.5. Measurement of $O_2^{\cdot-}$ formation

Cells were first exposed for 15 min to 10 μM DHE in RPMI 1640 complete medium. Cells were then washed twice with saline A and finally treated as detailed in the legend to the figures. After treatments, the cells were washed twice, resuspended in 20 μl of saline A and stratified on a slide. Fluorescence images were captured with a BX-51 microscope (Olympus, Milan, Italy) equipped with a SPOT-RT camera unit (Diagnostic Instruments, Delta Sistemi, Rome, Italy) using an Olympus LCAch 40×/0.55 objective lens. The excitation and emission wavelengths were 544 and 612 nm with a 5-nm slit width for both emission and excitation. Images were collected with exposure times of 100–400 ms, digitally acquired and processed for fluorescence determination at the single-cell level on a personal computer using Scion Image software (Scion Corp., Frederick, MD, USA). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition/experiment.

Extracellular $O_2^{\cdot-}$ formation was determined spectrophotometrically at 550 nm by measuring the SOD-sensitive ferricytochrome *c* reduction as detailed by Markert et al. [24].

2.6. Measurement of mitochondrial membrane potential

Cells were preloaded with AA or DHA and treated for 3 min with $ONOO^-$ in 35-mm tissue culture dishes containing an uncoated coverslip. Subsequently, the cells were postincubated for a further 7 min with various additions and 50 nM MitoTracker Red CMXRos. Under these conditions, U937 cells rapidly attached to the coverslip. After treatments, the cells were washed three times and analyzed with a fluorescence microscope. The excitation and emission wavelengths were 545 and 610 nm, respectively, with a 5-nm slit width for both emission and excitation. Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition/experiment.