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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.
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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.

2.7. Subcellular fractionation and Western blot analysis

After treatments, the cells were processed to obtain the cytosolic and mitochondrial fractions, as described in Ref. [25]. Equal amounts (25 μg) of the mitochondrial and cytosolic fractions were resolved in 10% sodium dodecyl sulfate polyacrylamide gel and electrotransferred to polyvinylidene difluoride membranes. Western blot analyses were performed using antibodies against Bax, actin and HSP-60 (Santa Cruz, Santa Cruz, CA, USA). Details on Western blotting apparatus and conditions are reported elsewhere [25]. Antibodies against actin and HSP-60 were used to assess equal loading of the lanes.

2.8. Statistical analysis

The results are expressed as means±S.D. Statistical differences were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons. A value of $P<.01$ was considered significant.

3. Results

3.1. $O_2^{\cdot -}$-dependent oxidation of extracellular AA switches the mode of uptake with hardly any effect on the net amount of vitamin C accumulated

Consistently with our recent findings [20], exposure (15 min) of U937 cells to low AA or DHA concentrations promotes identical accumulation of the vitamin in its reduced form, i.e., unaffected by DTT (10 mM) treatment of the resulting lysates prior to analysis (inset to Fig. 1). Hence, addition of AO to cultures exposed to AA failed to affect the net amount of the vitamin accumulated (Fig. 1), but nevertheless promoted a switch in the uptake mechanism, which became insensitive to Na^+ omission and acquired sensitivity to cytochalasin B, an inhibitor of glucose transporters [22]. Interestingly, an identical response was mediated by X/XO (0.1 mM/10 mU/ml), a well-established $O_2^{\cdot -}$-generating system, whereas hardly any effect was detected upon addition of H_2O_2 (50 μM).

The next series of experiments addressed the issue of whether similar results are obtained upon receptor-mediated activation of NADPH oxidase, a condition associated with $O_2^{\cdot -}$ formation. For this purpose, we employed a high concentration of ATP (3 mM), which promotes detectable $O_2^{\cdot -}$ formation also in cell types characterized by low NADPH oxidase expression. This notion was established in cells preloaded with the fluorescent probe DHE (Fig. 2A), responding to ATP with a fluorescence response sensitive to 10 μM apocynin, an inhibitor of NADPH oxidase. Evidence for extracellular $O_2^{\cdot -}$ release can be more readily inferred from the outcome of experiments obtained with membrane-impermeant ferricytochrome *c* (Fig. 2B), providing results in line with those reported in Fig. 2A.

We then moved to AA uptake studies and found that ATP recapitulates all the effects observed under conditions of supplementation of either AO or X/XO (Fig. 2C). Indeed, while not affecting the net amount of vitamin C accumulated by the cells, ATP promoted a clear shift in the uptake mechanism, which lost sensitivity to Na^+ omission and acquired sensitivity to cytochalasin B. These events were prevented by apocynin as well as by SOD, thereby implying a role for NADPH oxidase-derived $O_2^{\cdot -}$.

The results thus far presented indicate that vitamin C accumulation is based on the use of Na^+-dependent transport of extracellular AA and imply that glucose transporters are recruited under conditions associated with extracellular $O_2^{\cdot -}$ formation/release and parallel AA oxidation to DHA.

3.2. Consequences of the switch in the uptake mechanism

We tested whether the switch in the uptake mechanism is functional to a more general defensive strategy, preventing potentially harmful effects of the vitamin often observed after AA accumulation and immediate exposure to reactive oxygen/nitrogen species [7–11]. In order to address this issue, we first selected nontoxic/marginally toxic concentrations of H_2O_2, *tert*B-OOH or $ONOO^-$ and employed identical conditions to treat cells preloaded with either AA or DHA, both at 30 μM. Interestingly, the results illustrated in Fig. 3A–C provide evidence of toxicity in cells preexposed to AA, whereas marginal effects — or more often hardly

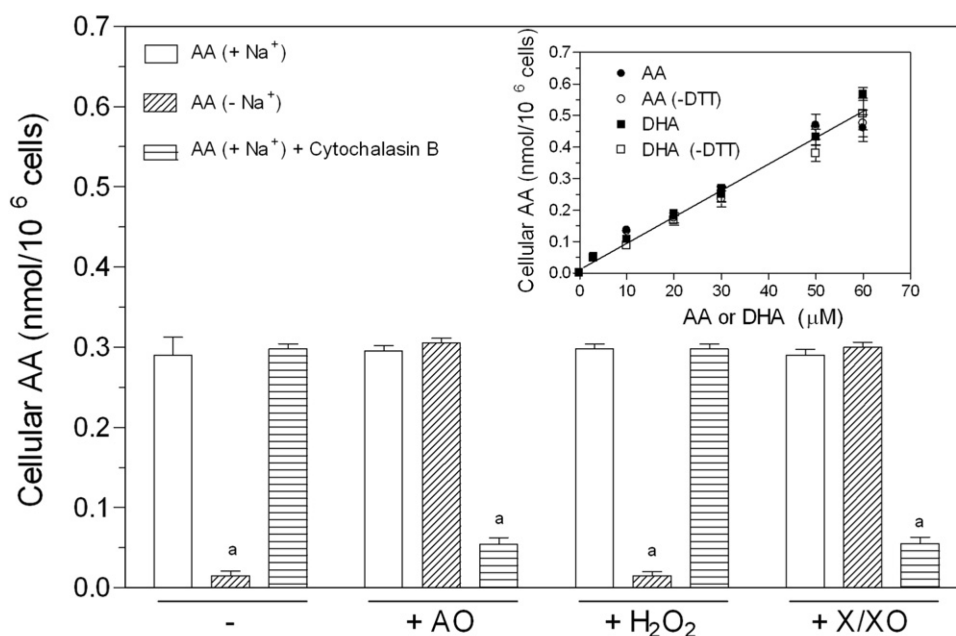

Fig. 1. Mechanism of AA uptake in U937 cells supplemented with AO, H_2O_2 or X/XO. AA accumulation was determined in cells exposed to 30 μM AA alone or associated with 2.8 U/ml AO, 50 μM H_2O_2 or 0.1 mM/10 mU/ml X/XO. The same treatments were also performed in incubation buffer supplemented with 25 μM cytochalasin B or manipulated to replace sodium with choline. Inset: AA content in cells exposed for 15 min to 0–60 μM AA (circles) or DHA (squares). Samples were processed for AA analysis with (closed symbols) or without (open symbols) DTT (10 mM), as detailed in the Materials and Methods section. Results represent the means±S.D. calculated from at least three separate experiments. Statistically significant differences ($P<.01$) are represented by "a" when compared to the conditions involving incubation of the cells in the Na^+-containing buffer (AA+Na^+). Comparisons are within the experimental groups identified in the abscissa axis (ANOVA followed by Dunnett's test).

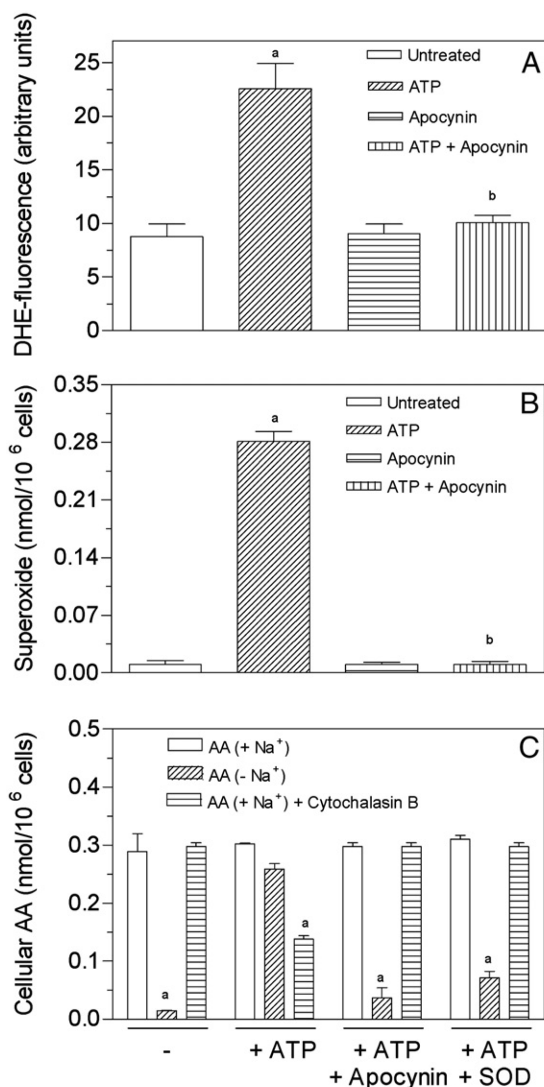

Fig. 2. ATP-dependent superoxide formation impacts on the mode of vitamin C uptake. (A) Cells were preloaded with DHE and subsequently exposed for 5 min to 3 mM ATP in the absence or presence of 10 μM apocynin. The DHE-derived fluorescence response, indicative of superoxide formation, was then determined as detailed in Materials and Methods. (B) Cells were exposed for 5 min to 3 mM ATP in the absence or presence of 10 μM apocynin and assayed for superoxide formation with ferricytochrome *c* assay. Formation of ferrocytochrome *c* resulting from superoxide-driven ferricytochrome *c* reduction was then assessed spectrophotometrically. (C) AA accumulation in cells exposed to 30 μM AA in the absence or presence of ATP alone or associated with either apocynin or 10 U/ml SOD. The same treatments were also performed in incubation buffer supplemented with cytochalasin B or manipulated to replace sodium with choline. Results represent the means±S.D. calculated from at least three separate experiments. Statistically significant differences ($P<.01$) are represented by "a" when compared to untreated cells (A, B) or to cells incubated in the Na^+-containing buffer ($AA+Na^+$) within each of the experimental groups identified in the abscissa axis (C). "b" is used in panels (A) and (B) to indicate statistically significant differences ($P<.01$) in comparison to cells treated with the sole ATP (ANOVA followed by Dunnett's test).

any effect — were observed in DHA-preloaded cells. These results are intriguing as exposure to AA or DHA leads to identical levels of AA accumulation (inset to Fig. 1). Furthermore, AA or DHA, in the absence of additional treatments, neither affected viability (Fig. 3D) nor produced detectable changes in the redox status of the cells, as previously reported [20].

Additional experiments (Fig. 3D) performed with $ONOO^-$ indicate that DHA fails to promote effects at concentrations below 30 μM and that the enhancing effects of AA are maximally induced at 3 μM, a concentration 10-fold lower than that employed in experiments illustrated in Fig. 3A. It is important to note that cells surviving these treatments did not undergo delayed toxicity and in fact proliferated with kinetics superimposable on those of untreated cells (Supporting Fig. 1).

A mechanism saturating at concentrations as low as 3 μM rules out the involvement of enforced $O_2^{\cdot-}/H_2O_2$ formation associated with AA autoxidation in the above responses. While this event appears unlikely for several reasons (see below), we nevertheless performed additional experiments showing that AA, at 3 μM, is stable in the different buffers and solutions employed in this study. In particular, a 15-min incubation of AA (3 μM) in the same Na^+-containing buffer or growth medium employed in uptake studies (Fig. 1) leads to a recovery of >96%. Similar results were obtained after incubation of the vitamin in Na^+-free buffer. Interestingly, this last condition is not permissive for vitamin cellular uptake (Fig. 1), and indeed, we did not detect enhanced $ONOO^-$ toxicity (not shown). Hence, extracellular AA fails to promote $ONOO^-$ toxicity.

A role for AA autoxidation in the $ONOO^-$-dependent lethal response also appears to be unlikely on the bases of results from experiments showing lack of detectable effects in response to either catalase (10 U/ml, not shown) or desferrioxamine (100 μM, not shown) during exposure to AA (3 μM). Along the same lines, the effects of the vitamin were not mimicked by concentrations of H_2O_2 in the range 1–30 μM (not shown). These concentrations of H_2O_2, far greater than those that might possibly arise from AA autoxidation, were not directly toxic for the cells and failed to promote enhanced susceptibility to subsequent exposure to $ONOO^-$.

These results therefore rule out the possibility that the enhancing effects of AA are linked to its autoxidation during the preloading phase and in fact imply a role for intracellular AA. As DHA is equally taken up by the cells but nevertheless fails to enhance the toxic effects of $ONOO^-$, extracellular oxidation of the vitamin might therefore represent an effective strategy to prevent potentially harmful effects and preserve its intracellular accumulation.

The results illustrated in Fig. 3D, showing that the enhancing effects of AA are suppressed by AO under the same conditions in which the intracellular AA concentration is unaffected (Fig. 1), are consistent with this notion. Likewise, Fig. 3E shows that toxicity is blunted by either ATP or X/XO, once again under conditions in which the switch in the uptake mechanism (i.e., from Na^+-dependent/cytochalasin B-insensitive to Na^+-independent/cytochalasin B-sensitive) was induced without affecting the net amount of the vitamin taken up (Figs. 1 and 2C). Interestingly, under both conditions, the switch in the uptake mechanism (Figs. 1 and 2C) and the loss of the ability to enhance $ONOO^-$ toxicity were sensitive to supplementation of SOD. Fig. 3E also shows that the effects mediated by ATP, unlike those mediated by X/XO, were abolished by apocynin, thereby emphasizing the specificity of the effects of the latter as an NADPH oxidase inhibitor. As a final note, SOD or apocynin, either in the absence or presence of ATP or X/XO, failed to promote effects in cells exposed to $ONOO^-$ alone. Lack of toxicity was also detected with ATP or X/XO and $ONOO^-$.

The results illustrated in Fig. 4A indicate that $ONOO^-$ causes cytosolic loss and mitochondrial accumulation of Bax in cells preexposed to AA, whereas hardly any effect was observed in DHA preloaded cells (Fig. 4A). In contrast, $ONOO^-$, AA or DHA alone failed to affect the subcellular localization of Bax. Fig. 4B shows that mitochondrial Bax translocation induced by $ONOO^-$ in AA-supplemented cells is sensitive to addition of X/XO during vitamin preloading. SOD prevented the protective effects of X/XO, and these agents, neither separately nor combined, produced effects in cells exposed to the sole $ONOO^-$.

As indicated in Fig. 5A, U937 cell death induced by $ONOO^-$/AA was inhibited by CsA (0.5 μM), an inhibitor of mitochondrial permeability transition (MPT) [26], thereby implying that toxicity

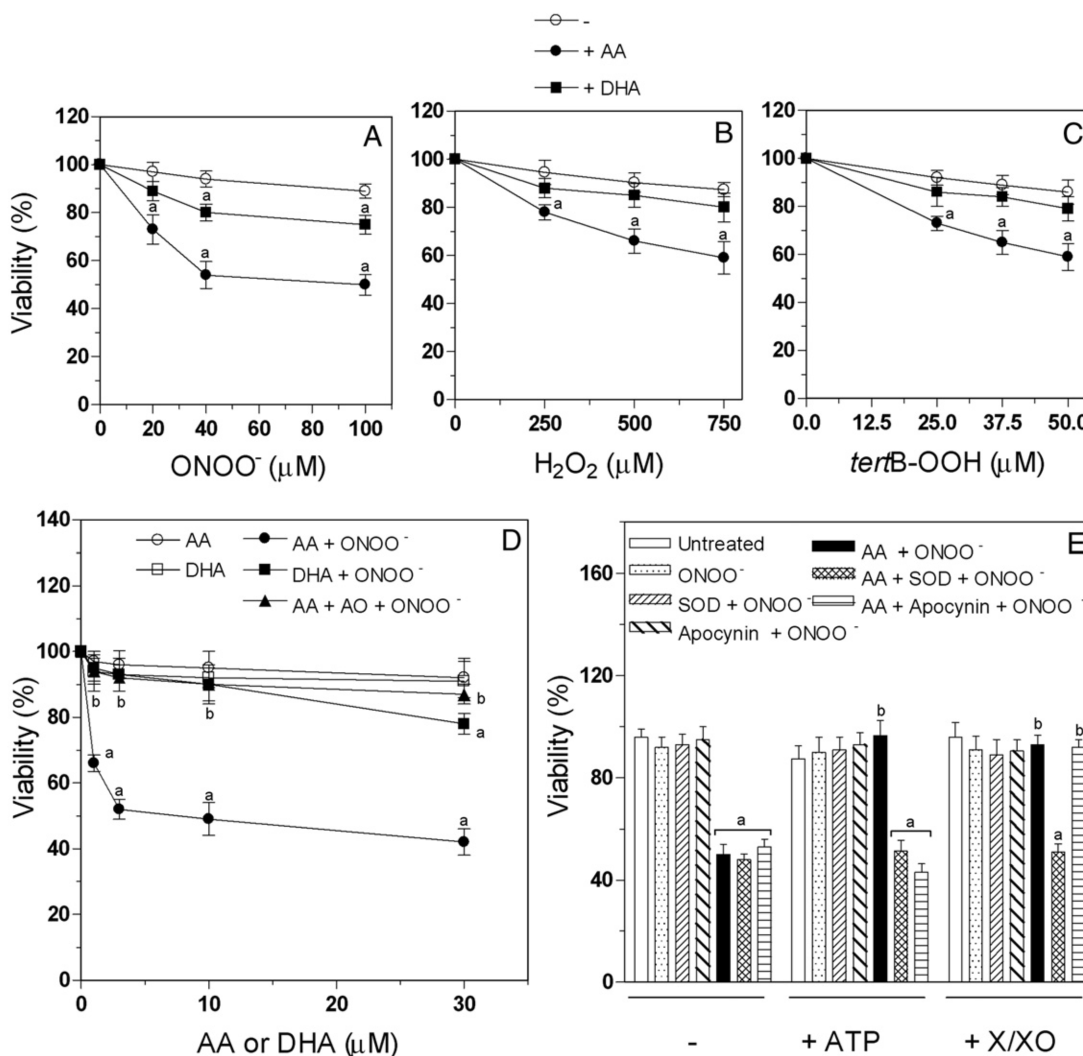

Fig. 3. Otherwise inactive concentrations of different oxidants promote toxicity in cells preexposed to AA: no effect of DHA and prevention of the enhancing effects of AA by AO, ATP or X/XO. Cells incubated for 15 min without or with 30 μM AA or DHA were subsequently exposed for 60 min in fresh saline A to increasing concentrations of $ONOO^-$ (A), H_2O_2 (B) or *tert*B-OOH (C). The number of viable cells was then counted with the trypan blue exclusion assay. (D) Cells were preexposed to increasing concentrations of AA or DHA (both in the absence or presence of AO) and analyzed for viability after further incubation (60 min) in fresh saline A without or with 40 μM $ONOO^-$. (E) Cells were preexposed to 0 or 3 μM AA alone or associated with either SOD or apocynin, both in the absence or presence of either ATP or X/XO. Cells were then treated with $ONOO^-$ and finally analyzed for viability as indicated above. Results represent the means±S.D. calculated from at least three separate experiments. Statistically significant differences (P<.01) are represented by "a" when compared to untreated cells and "b" when compared to cells preexposed to AA and subsequently treated with $ONOO^-$ (ANOVA followed by Dunnett's test).

takes place *via* an MPT-dependent mechanism. Consistently, cells were not rescued by FK-506 (1 μM), which shares with CsA the ability to inhibit calcineurin but fails to affect the formation of MPT pores [27]. In addition, toxicity was preceded by a CsA-sensitive decline in mitochondrial membrane potential, as measured by MitoTracker Red CMXRos uptake (Fig. 5B). Interestingly, toxicity (Fig. 5A), as well as the decline in mitochondrial membrane potential (Fig. 5B), was prevented by X/XO *via* a SOD-sensitive mechanism, and CsA once again prevented these events elicited by SOD. X/XO, with or without SOD, did not affect mitochondrial membrane potential in cells exposed to the sole $ONOO^-$ (not shown), an observation in line with the results obtained in experiments measuring cell viability (Fig. 3E).

Collectively, the above results indicate that otherwise inactive concentrations of different oxidants, in particular $ONOO^-$, promote MPT-dependent toxicity in cells preloaded with AA. In contrast with these findings, DHA failed to enhance oxidant toxicity under the same conditions in which identical levels of intracellular vitamin C were accumulated. Hence, different manipulations causing extracellular AA oxidation, in particular associated with extracellular $O_2^{\cdot-}$ formation, failed to affect intracellular vitamin C accumulation, but nevertheless were not permissive for enhanced susceptibility to oxidant toxicity.

4. Discussion

AA displays both antioxidant [1,2] and prooxidant [3–6] properties. Indeed, while the former activities are more prominent and more frequently described, it appears now clear that the vitamin may in fact enhance, at least under specific conditions, the susceptibility of the cells to reactive oxygen/nitrogen species *in vitro* [7–11] as well as *in vivo* [28–30]. The present study puts more weight on this notion by showing that short-term exposure to AA concentrations comparable or even lower than those normally detected in most biological fluids promotes a lethal response after exposure to otherwise nontoxic levels of different inorganic and organic hydroperoxides (Fig. 3A–D). This is an important observation since these hydroperoxides include H_2O_2 and $ONOO^-$, commonly generated at low levels under

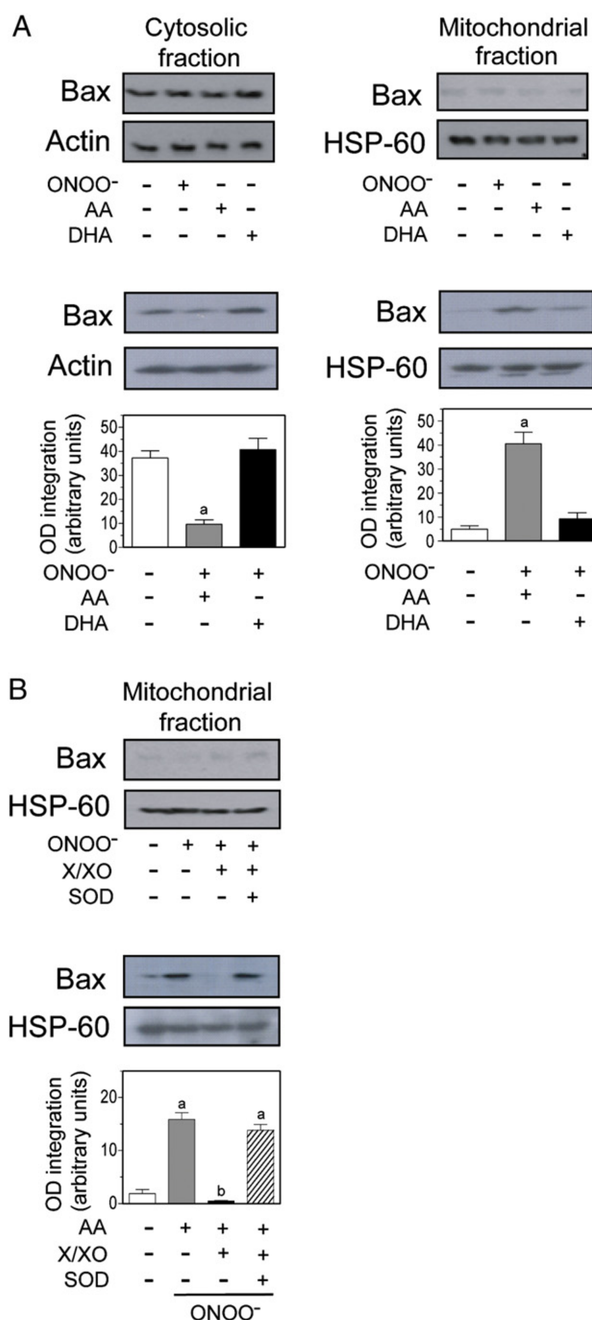

Fig. 4. Otherwise inactive concentrations of $ONOO^-$ promote mitochondrial Bax translocation in cells preexposed to AA: no effect of DHA and prevention of the enhancing effects of AA by X/XO. (A) Cells preexposed to 3 μM AA or DHA were incubated for a further 10 min in fresh saline with 40 μM $ONOO^-$ and finally processed to obtain the mitochondrial and cytosolic fractions for Western blot analysis using an antibody against Bax. In some experiments, cells were exposed to AA, DHA or $ONOO^-$ separately and then processed as detailed above. The blots were then washed and reprobed for actin or HSP-60. (B) Cells were preexposed to AA alone or associated with X/XO, both in the absence and presence of SOD. Cells were then treated for 10 min with 40 μM $ONOO^-$ and finally processed to obtain the mitochondrial fraction for Western blot analysis using an antibody against Bax. Also shown is the effect of $ONOO^-$ alone or associated with X/XO, both in the absence and presence of SOD. The blots were then washed and reprobed for HSP-60. Blots are representative of three separate experiments with similar outcomes. The relative amount of Bax protein was quantified by densitometric analysis of the blots and expressed as optical density integration. Statistically significant differences ($P<.01$) are represented by "a" when compared to untreated cells and "b" when compared to cells preexposed to AA and subsequently treated with $ONOO^-$ (ANOVA followed by Dunnett's test).

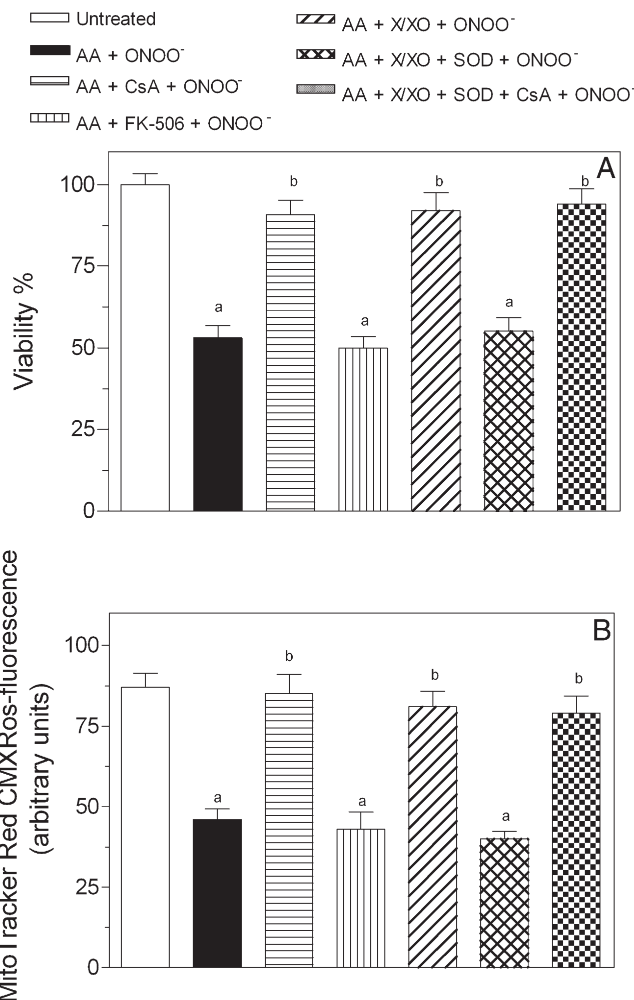

Fig. 5. Otherwise inactive concentrations of $ONOO^-$ promote toxicity preceded by loss of mitochondrial membrane potential in cell preexposed to AA: prevention by CsA or X/XO. Cells were first exposed to AA (3 μM), both in the absence and presence of 0.5 μM CsA, or 1 μM FK-506 and subsequently treated for 10 (MitoTracker Red CMXRos-fluorescence, B) or 60 min (toxicity, A) with 40 μM $ONOO^-$. In other experiments, preexposure to AA was performed in the presence of X/XO, with or without SOD. After treatments, the cells were processed as indicated in Materials and Methods. Results represent the means±S.D. calculated from at least three separate experiments. Statistically significant differences ($P<.01$) are represented by "a" when compared to untreated cells and "b" when compared to cells preexposed to AA and subsequently treated with $ONOO^-$ (ANOVA followed by Dunnett's test).

physiological conditions and to a remarkably greater extent under pathological (e.g., inflammatory) conditions [31–33].

We previously obtained similar results with the same cell type and $ONOO^-$, however, using a loading procedure which involves exposure to 100 μM DHA [10,11]. This paradigm had the advantage of increasing intracellular AA content in the absence of the undesirable parallel effects encountered with the metal-catalyzed oxidation of extracellular AA and was indeed commonly employed to test the effects mediated by intracellular AA in different cell types, including U937 cells [10,11,34,35]. On the other hand, such a DHA concentration is obviously nonphysiological, two orders of magnitude greater than that normally found in biological fluids [13,17], and associated with the accumulation of a very high intracellular concentration of AA [10,23,34,35]. Hence, the results obtained were interpreted as indicative of a potentially relevant biological effect, however, obtained in a physiopathologically irrelevant context.

Our recent work [20] on U937 cell AA uptake has however provided novel information allowing us to address the same issue in a more relevant setting. The present study reproduces some of the above effects using very low AA concentrations, taken up by the cells *via* SVCTs (Fig. 1 and Ref. [20]), thereby strongly enhancing the probability of a physiopathological relevance of the effects under investigation.

These observations bear important implications, as it will be further discussed below, and appear even more intriguing in that the effects of AA were described under the same conditions in which identical DHA concentrations, taken up by hexose transporters, result in identical AA accumulation (inset to Fig. 1A and Ref. [20]) but nevertheless fail to promote oxidant toxicity (Fig. 3A-D). Although presently unable to provide a straightforward explanation for this discrepancy, we suspect that the key of the enigma is on the different subcellular distribution of the vitamin. In other words, AA preloading might lead to greater AA accumulation than DHA preloading within the same subcellular compartment(s) in which critical events promoting $ONOO^-$ toxicity take place.

Our working hypothesis, based on previous results obtained with DHA [10,11], is that a likely critical compartment is represented by the mitochondria. Indeed, high DHA concentrations (e.g., 100 μM) produce effects superimposable on those reported in this study using 3 μM AA through a mechanism involving enforced mitochondrial formation of reactive oxygen species [10,11]. The present study also provides information pointing to mitochondria as the site in which critical effects of AA are mediated, as toxicity was dependent on MPT (Fig. 5) and preceded by early Bax mitochondrial translocation (Fig. 4).

When (and if) this hypothesis will be confirmed, then the challenge will be to identify the cause of the different subcellular distribution, which obviously appears as an apparent paradox as DHA, in particular at very low concentrations, is immediately converted to AA within the cells (inset to Fig. 1 and Refs. [20,34,35]).

Independently of the underlying mechanism(s), however, the above unexpected dichotomy between cellular content of AA and the ensuing effects is suggestive of an ingenious strategy to avoid the potentially harmful effects associated with AA uptake under the same conditions in which reactive oxygen and nitrogen species are being formed. Supportive evidence in this direction is given by the outcome of a variety of approaches in which extracellular oxidation of low micromolar levels of AA, regardless of whether elicited enzymatically (i.e., by AO, Fig. 3D), chemically (i.e., by X/XO, Fig. 3E) or *via* receptor-mediated activation of NADPH oxidase (i.e., by ATP, Fig. 3E), was invariably associated with prevention of the enhancing effects on $ONOO^-$ toxicity. Most importantly, all the above conditions were associated with a switch in the uptake mechanism, now entirely mediated by hexose transporters, with hardly any consequence on the net amount of vitamin C accumulated (Figs. 1 and 2C). Hence, the vitamin was taken up as DHA, and the potentially deleterious effects of direct AA uptake were prevented.

It should also be emphasized that extracellular AA oxidation elicited by X/XO, as earlier documented [16,36], was in fact mediated by $O_2^{\cdot -}$, and indeed, the ensuing prevention of Bax mitochondrial accumulation (Fig. 4B), decline of mitochondrial membrane potential (Fig. 5B) and loss of viability (Fig. 5A) were sensitive to supplementation of SOD. This notion was validated using ATP to promote NADPH oxidase-dependent $O_2^{\cdot -}$ release. Inhibition of $O_2^{\cdot -}$ formation or SOD-mediated scavenging was once again associated with prevention of the switch in the uptake mechanism (Fig. 2C) and loss of the resulting cytoprotection (Fig. 3E).

It is important to note that the above discussion is based on the assumption that AA promotes its effects on $ONOO^-$ toxicity intracellularly, with any contribution of events associated with AA autoxidation, i.e., involving extracellular formation of $O_2^{\cdot -}/H_2O_2$. This notion is however supported by several lines of evidence. In the first place, it is important to keep in mind that interactions between extracellular AA and $ONOO^-$ are impossible, as the two treatments were performed sequentially in two different milieus. Secondly, a role for $O_2^{\cdot -}/H_2O_2$ during AA exposure is unlikely for the following reasons: (a) It is in contrast with the observation that the enhancing effects of AA on $ONOO^-$ toxicity are maximal at 3 μM (Fig. 3D). As we recently reported [20], the deleterious effects mediated by AA autoxidation under the specific conditions employed in this study are a direct function of AA concentration, at least up to 1 mM. (b) It is in contrast with the observation that $O_2^{\cdot -}$ during AA exposure actually prevents toxicity arising in response to otherwise nontoxic levels of different oxidants (Figs. 3–5). In addition, regardless of whether generated *via* NADPH oxidase activation or by X/XO, $O_2^{\cdot -}$ in the absence of AA failed to promote toxicity upon further exposure to $ONOO^-$ (Figs. 3E and 4). Identical results were obtained using reagent H_2O_2 in the place of the $O_2^{\cdot -}$-generating systems (not shown). (c) The enhancing effects of AA on $ONOO^-$ toxicity are insensitive to supplementation of catalase or desferrioxamine during vitamin preloading. (d) AA is not taken up by the cells in Na^+-free medium (Fig. 1) and, under the same conditions, fails to enhance $ONOO^-$ toxicity (not shown). (e) AA, at the concentrations producing its maximal effects, was stable in each of the incubation buffers employed.

The above observations/considerations therefore converge in the conclusion that the enhancing effects of AA are associated with intracellular events, critically dependent on the mode of vitamin C (AA vs. DHA) uptake. Hence, $O_2^{\cdot -}$ appears to be a master regulator of AA vs. DHA uptake [16,36], and conditions associated with $O_2^{\cdot -}$ formation critically regulate the proposed survival strategy.

U937 cells are a tumor cell line, and our results are of potential importance in the field of tumor biology, with potential implications for the treatment of some forms of cancer (e.g., leukemia, multiple myeloma) in which specific antineoplastic drugs are being considered for their use in combination with AA [37–39]. More studies are clearly needed in this direction in order to determine whether a switch in the uptake strategy might influence the susceptibility of specific tumor cells to combined chemotherapy. On the other hand, U937 cells are a promonocytic cell line sharing numerous characteristics with other cells belonging to the monocyte/macrophage lineage [40–43], thereby suggesting putative strategies that macrophages might adopt to cope with AA under inflammatory conditions in which extensive $O_2^{\cdot -}$ release is associated with enforced formation of H_2O_2 and $ONOO^-$.

While these possible implications can be experimentally tested, it is important to keep in mind that the present study used a protocol involving repletion of AA to AA-deficient cells. Indeed, cells in culture normally contain negligible amounts of AA for the following two reasons: (a) the vitamin is not synthesized by the cells [44], and (b) the vitamin is extremely labile in solution and is not normally added to the standard growth medium. While it is always difficult to extrapolate the significance of experimental results obtained with cultured cells to draw conclusions relevant to situations *in vivo*, the information provided by the present study should be taken into consideration to provide an explanation for the deleterious effects observed after acute supplementation of vitamin C to patients with pathologies associated with dysregulated formation/scavenging of reactive oxygen/nitrogen species. This seems to be so in patients with idiopathic hemochromatosis and dietary iron overload experiencing serious complications from additional oxidative damage [45,46]. Similarly, vitamin C supplementation was found to be deleterious for patients suffering from glucose-6-phosphate dehydrogenase deficiency [30] or sepsis [47], and to transiently increase tissue damage and oxidative stress in an

acute human inflammatory model associated with eccentric exercise [28,48].

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