Enhancing effects of intracellular ascorbic acid on peroxynitrite-induced U937 cell death are mediated by mitochondrial events resulting in enhanced sensitivity to peroxynitrite-dependent inhibition of complex III and formation of hydrogen peroxide

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A short-term pre-exposure to dehydroascorbic acid (DHA) promotes U937 cell death upon exposure to otherwise non-toxic levels of peroxynitrite (ONOO⁻). Toxicity is mediated by a saturable mechanism and cell death takes place as a consequence of mitochondrial permeability transition. The following lines of evidence are consistent with the notion that the enhancing effects of DHA were related to mitochondrial events resulting in inhibition of complex III upon exposure to otherwise inactive concentrations of ONOO⁻. First, DHA, as well as bona fide complex III inhibitors, similarly enhanced toxicity and subsequent formation of H₂O₂ induced by ONOO⁻ via a rotenone- or catalase-sensitive mechanism. Secondly, bona fide complex III inhibitors were ineffective in DHA-pre-loaded cells. In addition, respiration-deficient cells were resistant to toxicity elicited by ONOO⁻ and

their supplementation with increasing concentrations of DHA, although resulting in the accumulation of vitamin C levels identical with those observed in respiration-proficient cells, failed to affect ONOO⁻ toxicity. Finally, oxygen-consumption experiments demonstrated that pre-exposure to DHA promotes the ONOO⁻-dependent inhibition of complex III. In conclusion, the above results collectively demonstrate that increasing the intracellular accumulation of vitamin C promotes mitochondrial events leading to ONOO⁻-dependent formation of H₂O₂ and resulting in a rapid necrotic response.

Key words: ascorbic acid, cytotoxicity, mitochondrial permeability transition, peroxynitrite (ONOO⁻), reactive nitrogen species, vitamin C.

INTRODUCTION

Ascorbic acid (AsA) and its oxidized form, dehydroascorbic acid (DHA), are taken up by transporters and facilitated diffusion in a cell-type-specific manner [1]. Transport studies have revealed that mammalian cells possess at least two different systems involved in the cellular uptake of vitamin C, a Na⁺-dependent co-transport for AsA [2] and a facilitative glucose transport for DHA [3]. Within the cell, DHA is rapidly converted into AsA via GSH-dependent mechanisms, which can be both chemical [4–6] and enzymic [5,7,8], as well as by GSH-independent mechanisms [9], which are more frequently involved in the reduction of low levels of DHA [10]. A convenient strategy to increase the intracellular levels of AsA therefore involves exposure to DHA, which presents the great advantage of allowing intracellular accumulation of the vitamin in the absence of reactive oxygen species formation. Indeed such an event occurs upon addition of AsA to the cultures [11,12].

It is important to note that cultured cells are normally AsA-deficient, since the vitamin, or DHA, is unstable in solution and, under standard growth conditions, is not added to the culture medium. As a consequence, studies investigating the early effects mediated by a short-term pre-exposure to DHA, or AsA, may mimic a condition of acute supplementation of the vitamin to AsA-deficient individuals. Therefore this model can be used conveniently to obtain information on the impact of reactive species in patients with pathologies associated with a decrease in AsA levels. This is a relevant question, as enhanced oxidative

damage was observed in patients with β -thalassaemia major [13], idiopathic haemochromatosis [14], dietary iron overload [15] and severe/acute inflammation caused by eccentric exercise [16] or sepsis [17] upon AsA supplementation.

We recently focused our attention on the effects of intracellular AsA in cells exposed to peroxynitrite (ONOO⁻), the coupling product of superoxide and nitric oxide, and found that DHA-pre-loaded U937 cells are hypersensitive to its DNA-damaging and toxic effects [18]. Along the same lines, Puskas et al. [19] have shown that Jurkat T-cells exposed to DHA for 20–120 min and then immediately challenged with H₂O₂ were killed more efficiently than cells which did not receive DHA before the oxidant exposure. The same study, however, showed that toxicity can, in fact, be reduced significantly if DHA pre-exposure is prolonged for up to 24 h. Thus it appears that supplementation of AsA to AsA-deficient cells promotes early events increasing the sensitivity to reactive oxygen/nitrogen species and that this process is reversible and followed by a different scenario, allowing the expression of protective responses.

The present study was performed with the aim of gathering more information on the mechanism whereby short-term exposure to DHA enhances U937 cell death mediated by ONOO⁻. It was found that cells with an increased AsA content display remarkable sensitivity to otherwise non-toxic levels of ONOO⁻. These enhancing effects were mediated by a saturable mechanism involving mitochondrial events resulting in potent inhibition of complex III and subsequent generation of H₂O₂ responsible for the ensuing lethal response.

Abbreviations used: AsA, ascorbic acid; calcein-AM, calcein acetoxymethyl ester; DHA, dehydroascorbic acid; DHR, dihydrorhodamine 123; HQNO, 2-heptyl-4-hydroxyquinoline; MPT, mitochondrial permeability transition; Rho, rhodamine 123; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

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MATERIALS AND METHODS

Chemicals

Catalase, antimycin A, rotenone and 2-heptyl-4-hydroxyquinoline (HQNO), as well as most reagent-grade chemicals, were obtained from Sigma-Aldrich (Milan, Italy). Rhodamine 123 (Rho), dihydrorhodamine 123 (DHR) and calcein acetoxymethyl ester (calcein-AM) were from Molecular Probes Europe (Leiden, The Netherlands). FK-506 and cyclosporin A were purchased from Calbiochem (San Diego, CA, U.S.A.) and Sandoz A. G. (Bern, Switzerland) respectively.

Cell culture and treatments

U937 cells were cultured in suspension in RPMI 1640 medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 10 % (v/v) foetal bovine serum (Biological Industries, Kibbutz Beit Haemek, Israel), penicillin (50 units/ml) and streptomycin (50 µg/ml) (Sera-Lab, Crawley Down, U.K.), at 37 °C in T-75 tissue culture flasks (Corning, Corning, NY, U.S.A.) gassed with 5 % CO₂ in air. Respiration-deficient U937 cells were isolated by culturing the cells in RPMI 1640 medium containing 400 ng/ml ethidium bromide, 100 µg/ml pyruvate and 5 µg/ml uridine for 6 days with changes of medium at days 2 and 4. These cells were unable to consume oxygen in response to glucose (5 mM) or to the membrane-permeant NADH-linked substrate pyruvate (5 mM; results not shown).

Synthesis of ONOO⁻ or DHA and treatment conditions

ONOO⁻ was synthesized by the reaction of nitrite with acidified H₂O₂ as described previously [20], and MnO₂ (1 mg/ml) was added to the mixture for 30 min at 4 °C to eliminate the excess of H₂O₂. MnO₂ was removed by centrifugation at 200 g for 3 min and filtration through 0.45 µm pore-size microfilters. The solution was frozen at -80 °C for 24 h. The concentration of ONOO⁻, which forms a yellow top layer due to freeze fractionation, was determined spectrophotometrically by measuring the absorbance at 302 nm in 1.5 M NaOH ($\epsilon_{302} = 1670 \text{ M}^{-1} \cdot \text{cm}^{-1}$). DHA (40 mM stock solution) was prepared immediately before utilization using the method of bromine oxidation of AsA, as described previously [21]. U937 cells (1×10^6 cells/ml) were then loaded with DHA for various time intervals in complete culture medium.

Treatments were performed in 2 ml of pre-warmed saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l NaHCO₃ and 0.9 g/l glucose) containing 5×10^5 cells. The cell suspension was inoculated into 15 ml tubes before the addition of ONOO⁻. ONOO⁻ was rapidly added on the wall of plastic tubes and mixed for few seconds to equilibrate the ONOO⁻ concentration on the cell suspension; to avoid changes in pH due to the high alkalinity of the ONOO⁻ stock solution, an appropriate amount of 1 M HCl was also added.

Cytotoxicity assay

Cells were exposed to ONOO⁻ for 5 min, centrifuged at 200 g for 3 min, post-incubated for 55 min in the appropriate medium and analysed for cytotoxicity using the Trypan Blue dye-exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:1 (v/v) with 0.4 % Trypan Blue and the cells were counted with a haemocytometer.

Measurement of ferricyanide reduction

Ferricyanide reduction was estimated as described previously [22]. The cells were washed three times with saline A, centrifuged at 200 g for 3 min and the pellet was resuspended in 1 ml of

saline A containing 1 mM potassium ferricyanide which was solubilized immediately before use. After 30 min at 37 °C, the cell suspensions were centrifuged at 200 g for 3 min and the resulting supernatants were assayed in duplicate for their ferricyanide content by using 1,10-phenantroline as an indicator and measuring the attenuation at 510 nm ($\epsilon_{510} = 10\,500 \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Rho mitochondrial uptake, DHR oxidation, calcein staining and imaging

ONOO⁻ pre-treated cells (5 min) were post-incubated in the presence of either 10 µM DHR (10 min) or fresh saline A (15 min) in which Rho (11 µM) was added during the last 5 min of incubation. After washing three times, the cells were resuspended in 100 µl of PBS (0.121 M NaCl, 10 mM NaH₂PO₄, 1.5 mM KH₂PO₄ and 3 mM KCl); 20 µl of this cell suspension was stratified on a slide and cellular fluorescence was then imaged using a confocal laser microscope (Bio-Rad DVC 250; Richmond, CA, U.S.A.) equipped with a Hamamatsu 5985 (Hamamatsu Italy, Milan, Italy) chilled CCD (charge-coupled device) camera. Cells were illuminated with the 488 nm line of the argon laser and the fluorescence emitted was monitored at $\lambda > 515$ nm. The laser intensity, the shutter aperture and the exposure/integration settings were kept constant to allow quantitative comparisons of relative fluorescence intensity of cells between treatment groups. Laser exposure was limited to brief image acquisition intervals (≤ 5 s) to minimize photo-oxidation of DHR and/or photo-bleaching of Rho. Confocal images were digitally acquired and processed for fluorescence determination at the single-cell level on a Macintosh G4 computer using the public domain NIH Image 1.63 program (developed at the U.S. National Institutes of Health and available at <http://rsb.info.nih.gov/nih-image/>). Mean fluorescence values were determined by averaging the fluorescence of at least 50 cells/treatment condition/experiment.

For calcein staining, cells (2.5×10^5 cells/ml) were incubated at 37 °C for 15 min in saline A with 1 µM calcein-AM and 1 mM CoCl₂ as described previously [23]. After treatments, the cells were washed, resuspended in 20 µl of saline A and this cell suspension was stratified on a slide. Fluorescence images were captured with a BX-51 microscope (Olympus), equipped with an SPOT-RT camera unit (Diagnostic Instruments). The excitation and emission wavelengths were 488 and 515 nm respectively, with a 5-nm slit width for both emission and excitation. Images were collected with exposure times of 100–400 ms and were digitally acquired and processed for fluorescence determination at the single-cell level on a personal computer using Scion Image software (Scion Corp., Frederick, MD, U.S.A.).

Measurement of oxygen consumption

The cells were washed once in saline A and then resuspended in the same medium at a density of 1×10^7 cells/ml. Oxygen consumption was measured using a Y. S. I. oxygraph equipped with a Clark electrode (model 5300, Yellow Springs Instruments, Yellow Springs, OH, U.S.A.). The cell suspension (3 ml) was transferred to the polarographic cell and the rate of oxygen utilization was monitored under constant stirring for 3 min (basal respiration). The rate of oxygen utilization was calculated as described previously [24].

Statistical analysis

Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by a Dunnett's test. For single

Table 1 Effects of cyclosporin A or FK-506 on ONOO⁻-induced cytotoxicity and mitochondrial depolarization in control or DHA-pre-loaded cells

The cells were exposed for 60 min to 0 or 100 μ M DHA, treated for an additional 5 min in saline A with ONOO⁻ and finally post-incubated for either 10 or 55 min in fresh saline A, in the absence or presence of the indicated concentrations of drugs. Cells post-incubated for 10 min were analysed for mitochondrial membrane potential assessment (Rho was added to the cultures during the last 5 min of incubation). After washing three times, the cells were observed with a confocal microscope and Rho fluorescence was quantified as detailed in the Materials and methods section. Cells post-incubated for 55 min were analysed for cytotoxicity using the Trypan Blue dye-exclusion assay. Results are means $\pm$ S.E.M. for at least four separate experiments.

Treatment	Trypan-Blue-negative cells (% of control)	Rho-derived fluorescence (arbitrary units)
Control cells		
No addition	—	73 $\pm$ 4.4
1 mM ONOO ⁻	52 $\pm$ 4.9	34 $\pm$ 2.5
+ 0.5 μ M cyclosporin A	97 $\pm$ 2.3*	69 $\pm$ 1.7*
+ 1 μ M FK-506	49 $\pm$ 2.8	37 $\pm$ 2.9
40 μ M ONOO ⁻	98 $\pm$ 3.3	71 $\pm$ 5.2
DHA-pre-loaded cells		
No addition	—	76 $\pm$ 6.4
40 μ M ONOO ⁻	55 $\pm$ 1.5	39 $\pm$ 3.8
+ 0.5 μ M cyclosporin A	94 $\pm$ 2.4*	70 $\pm$ 4.5*
+ 1 μ M FK-506	53 $\pm$ 2.7	36 $\pm$ 3.7

* $P < 0.001$ as compared with ONOO⁻-treated cells (unpaired Student's t test).

comparison, the significance of differences between means was determined by Student's t test.

RESULTS

DHA pre-loading sensitizes U937 cells to ONOO⁻-induced toxicity and this response is mediated by a saturable mechanism

In line with our previous results from a study on the mechanism and mode of ONOO⁻-induced U937 cell death [25], we found that loss of mitochondrial membrane potential and toxicity are sequentially observed soon after the addition of 1 mM ONOO⁻ and that both responses are prevented by the mitochondrial permeability transition (MPT) inhibitor [26] cyclosporin A (0.5 μ M) (Table 1). FK-506 (1 μ M), which shares with cyclosporin A the ability to inhibit calcineurin [27], but fails to affect formation of MPT pores [28], did not modify the ONOO⁻-dependent loss of Rho uptake and lethal response (Table 1). It is important to note that, under the above conditions, loss of mitochondrial membrane potential and cell death were not detected after treatment with decomposed ONOO⁻ or with the vehicle (results not shown).

These results strongly suggest that MPT is the cause of ONOO⁻ toxicity. This inference is supported further by results in which MPT pore opening was assessed by monitoring the changes in mitochondrial calcein fluorescence after quenching of the cytosolic and nuclear signal with Co²⁺ [23]. As illustrated in Figure 1, cells exposed to calcein-AM display a uniform, bright fluorescence (Figure 1A). Addition of CoCl₂ during calcein-AM exposure provoked remarkable changes in the fluorescence pattern (Figure 1B) and the resulting punctate fluorescence was caused by the calcein localized in the mitochondrial compartment. Loss of mitochondrial calcein therefore represents a strong indication of MPT pore opening. This event was not observed in untreated cells (Figure 1B), but was clearly detected in cells exposed to 1 mM ONOO⁻ (Figure 1C). The image shown in Figure 1(C) should be compared with that in Figure 1(D), in which the

presence of the cells is demonstrated by darkening the digital image at the expenses of a loss of brightness. Since cyclosporin A (Figure 1E), unlike FK-506 (results not shown), prevented the effects of ONOO⁻, these results provide an additional indication that toxic levels of ONOO⁻ promote MPT-dependent U937 cell death.

Exposure of U937 cells to as low as 40 μ M ONOO⁻, while not causing loss of mitochondrial membrane potential and/or toxicity (Table 1), produced dramatic effects in DHA-pre-loaded cells. The lethal response elicited by a 60 min exposure of DHA-pre-loaded cells to 40 μ M ONOO⁻ and the loss of Rho uptake detected after 15 min of exposure were identical with those observed in naïve cells treated with 1 mM ONOO⁻; furthermore, both events were suppressed by cyclosporin A, whereas FK-506 was inactive. Analyses performed in DHA-pre-loaded cells not receiving additional treatments produced results identical with those obtained with untreated cells. Additional results indicated that, whereas exposure of naïve cells to 40 μ M ONOO⁻ did not cause loss of mitochondrial calcein (Figure 1G), this event took place under conditions in which 40 μ M ONOO⁻ was given to DHA-pre-loaded cells (Figure 1H). The image shown in Figure 1(H) should be compared with that in Figure 1(I), in which the presence of the cells is demonstrated by darkening the digital image. The effects mediated by ONOO⁻ in DHA-pre-loaded cells were prevented by cyclosporin A (Figure 1L), but not by FK-506 (results not shown). Finally, exposure to DHA did not cause loss of mitochondrial calcein when given to the cells in the absence of additional treatments (Figure 1F).

Thus it appears that, as previously observed in cells exposed to intrinsically toxic levels of ONOO⁻, treatment with an otherwise non-toxic concentration of the oxidant promotes MPT-dependent toxicity in DHA-pre-loaded cells.

These findings confirm and extend our previous observation that DHA pre-loading enhances the toxicity mediated by ONOO⁻, and suggest strongly that this response takes place via a mechanism identical with that involved in cell killing induced by a high concentration of ONOO⁻. This inference therefore implies that pre-exposure to DHA results in a leftward shift of the dose-response curve describing the lethal response evoked by ONOO⁻. The results illustrated in Figure 2(A) are indeed consistent with this notion, as it appears clear that DHA pre-loading mediates toxicity in cells treated with non-toxic concentrations of ONOO⁻ and produces minor effects after toxic doses of the oxidant.

The results illustrated in Figure 2(B) show the DHA concentration-dependence of the toxic effects mediated by an otherwise non-toxic dose of ONOO⁻ (40 μ M). It appears that the maximal response is observed using 100 μ M DHA and that no further enhancement of the extent of ONOO⁻-induced cell death is mediated by higher concentrations of DHA. These results should be compared with those shown in Figure 3, indicating that the ability of DHA to promote oxidation of extracellular ferricyanide increases over the concentration range 10–1000 μ M ($K_m = 96.97 \mu$ M). It is important to note that measurement of this activity provides an indirect estimate of the intracellular content of AsA, because the reduction of ferricyanide is mediated by AsA-derived electrons crossing the plasma membrane [29,30].

DHA pre-loading promotes mitochondrial events causally linked to the ensuing lethal response in U937 cells exposed to otherwise non-toxic concentrations of ONOO⁻

The results reported in Table 2 indicate that toxicity was suppressed by catalase (10 units/ml) given 5 min after addition of 1 mM ONOO⁻. Under identical conditions, the boiled enzyme

Figure 1 Effect of cyclosporin A on ONOO⁻-induced MPT in control or DHA-pre-loaded cells

Representative micrographs of U937 cells loaded for 15 min with 1 μ M calcein-AM alone (**A**) or associated with 1 mM CoCl₂ (**B**), washed and then post-incubated in fresh saline A for a further 15 min in the presence of the following additions: 1 mM ONOO⁻ (**C**); 0.5 μ M cyclosporin A/1 mM ONOO⁻ (**E**); DHA-pre-loaded cells in the absence of additional treatments (**F**); 40 μ M ONOO⁻ (**G**); DHA-pre-loaded cells treated with 40 μ M ONOO⁻ (**H**); DHA-pre-loaded cells treated with cyclosporin A/40 μ M ONOO⁻ (**L**). (**D**) and (**I**) show the same images as (**C**) and (**H**) respectively, in which the presence of the cells is demonstrated by darkening the digital image at the expense of a loss of brightness. The micrographs are representative of at least three separate experiments.

was inactive. Formation of H₂O₂ was shown to take place after pulse-treatment with ONOO⁻ using the fluorescent probe DHR (10 μ M), which accumulates in the mitochondria and fluoresces when oxidized by various species, including H₂O₂ [31] and ONOO⁻ [32]. Importantly, these experiments involved a 5 min treatment with ONOO⁻, followed by a 10 min post-incubation in fresh saline A containing the fluorescent probe, a condition that does not allow ONOO⁻ to directly oxidize DHR. The results reported in Table 2 indicate that ONOO⁻ markedly enhances fluorescence above control values and that enzymically active catalase, unlike the boiled enzyme, prevents this response. Importantly, toxicity and formation of H₂O₂ elicited by exposure to 1 mM ONOO⁻ were also suppressed by the addition of 0.5 μ M rotenone, an agent preventing the electron flow through complex I. Inhibitors of complex III, antimycin A (1 μ M) or HQNO (10 μ M) did not modify the above effects induced by 1 mM ONOO⁻, but produced remarkable changes after exposure to 40 μ M ONOO⁻, a treatment that was otherwise non-toxic and unable of eliciting oxidation of DHR. The latter responses were sensitive to both rotenone and enzymically active catalase and their extents were comparable with those observed in cells treated with 1 mM ONOO⁻. Each of the respiratory-chain inhibitors was added 5 min

after addition of ONOO⁻ and produced no effects in the absence of additional treatments (results not shown). These results are therefore consistent with our previous findings [33,34] indicating that ONOO⁻ promotes inhibition of complex III, thereby causing delayed formation of superoxide and H₂O₂, which appears to play a pivotal role in the ensuing lethal response.

The question therefore arises as to whether DHA pre-loading potentiates the mitochondrial effects of ONOO⁻ resulting in enforced formation of H₂O₂. As mentioned above, DHA-pre-loaded cells were viable and the results reported in Table 2 indicate that they were also unable to oxidize DHR. However, when these cells were exposed to 40 μ M ONOO⁻ (which by itself is also inactive; see above), toxicity and subsequent formation of H₂O₂ were found to take place. The extent of these responses was identical with that observed in naïve cells exposed to 1 mM ONOO⁻ or 40 μ M ONOO⁻ associated with either antimycin A or HQNO. Furthermore, these responses were abolished by enzymically active catalase, or rotenone, and were not enhanced further by antimycin A or HQNO. It is important to note that catalase was not effective when added during the 60 min exposure to DHA (results not shown), thus ruling out the possibility that effects are mediated by extracellular H₂O₂ resulting from

Figure 2 Effect of DHA pre-loading on ONOO⁻-induced cytotoxicity

(A) Respiration-proficient (circles) or -deficient cells (squares) were exposed for 60 min to 0 (open symbols) or 100 μ M DHA (closed symbols) and treated for 60 min in saline A with increasing concentrations of ONOO⁻. Cells were then analysed immediately for cytotoxicity using the Trypan Blue dye-exclusion assay. (B) Respiration-proficient (circles) or -deficient cells (squares) were incubated for 60 min in the absence (open symbols) or presence of increasing concentrations of DHA (closed symbols) and treated for an additional 60 min in saline A with 40 μ M ONOO⁻. Cells were then analysed immediately for cytotoxicity using the Trypan Blue dye-exclusion assay. Results in (A) and (B) are means $\pm$ S.E.M. from at least three separate experiments. * P < 0.001; ** P < 0.0001, compared with respiration-proficient cells exposed to ONOO⁻ alone (ANOVA followed by Dunnett's test).

dismutation of superoxides generated by the autoxidation of AsA present as a contaminant of DHA.

These results provide a clear-cut demonstration that DHA pre-loading causes effects identical with those generated by bona fide complex III inhibitors. Although it appears unlikely that inhibition of complex III arises as a consequence of DHA uptake and/or reduction to AsA, we nevertheless performed oxygen-consumption experiments. The rates of oxygen utilization detected in DHA-pre-loaded cells were significantly higher in comparison with those observed in untreated cells (Table 3). Thus the above results are consistent with the possibility that DHA pre-loading does not affect complex III activity, but favours its inhibition mediated by an otherwise inactive concentration of ONOO⁻. Indeed, the results illustrated in Figure 4 indicate that low (40–100 μ M) concentrations of ONOO⁻ effectively reduced the extent of oxygen consumption in DHA-pre-loaded cells, but produced only minor effects in AsA-deficient cells. It is important to note that these experiments were obtained using cell densities one order of magnitude greater than those utilized in toxicity studies and, as a consequence, it may be predicted that the observed effects represent an underestimate of the actual level of

Figure 3 Effect of DHA pre-loading on the ferricyanide reductase activity

Respiration-proficient (closed circles) or -deficient cells (closed squares) were exposed for 60 min to increasing concentrations of DHA, washed with saline A and incubated with fresh saline A supplemented with 1 mM ferricyanide. Formation of ferrocyanide was determined as detailed in the Materials and methods section. Results are means $\pm$ S.E.M. for six to eight separate experiments, each performed in duplicate. * P < 0.001; ** P < 0.0001, compared with control cells (ANOVA followed by Dunnett's test).

Table 2 Effect of catalase and various respiratory-chain inhibitors on ONOO⁻-induced cytotoxicity and DHR-derived fluorescence in control or DHA-pre-loaded cells

The cells were exposed for 60 min to 0 or 100 μ M DHA, treated for an additional 5 min in saline A with ONOO⁻ and finally post-incubated for either 10 or 55 min in fresh saline A, in the absence or presence of the indicated concentrations of drugs. Cells post-incubated for 10 min were analysed for DHR oxidation (DHR was added to the cultures during the last 10 min of incubation). After washing three times the cells were observed with a confocal microscope and DHR fluorescence was quantified as detailed in the Materials and methods section. The number of viable cells found after the 55 min post-incubation was determined with a haemocytometer in the presence of Trypan Blue. Results are means $\pm$ S.E.M. for at least four separate experiments.

Treatment	Number of viable cells ($\times 10^3$)	DHR-derived fluorescence (arbitrary units)
Control cells		
No addition	498 $\pm$ 29	35 $\pm$ 3.4
1 mM ONOO ⁻	262 $\pm$ 15	78 $\pm$ 4.5
+ 10 units/ml catalase	488 $\pm$ 29*	37 $\pm$ 2.7*
+ boiled catalase	259 $\pm$ 38	81 $\pm$ 5.2
+ 0.5 μ M rotenone	485 $\pm$ 45*	38 $\pm$ 1.9*
+ 1 μ M antimycin A	255 $\pm$ 22	80 $\pm$ 3.8
+ 10 μ M HQNO	250 $\pm$ 30	76 $\pm$ 4.1
40 μ M ONOO ⁻	495 $\pm$ 32	39 $\pm$ 3.5
+ 1 μ M antimycin A	256 $\pm$ 15*	75 $\pm$ 5.3*
+ 10 units/ml catalase	480 $\pm$ 24	38 $\pm$ 2.7
+ boiled catalase	261 $\pm$ 29	76 $\pm$ 3.9
+ 10 μ M HQNO	249 $\pm$ 16*	77 $\pm$ 6.2*
+ 10 units/ml catalase	483 $\pm$ 37	35 $\pm$ 3.1
+ boiled catalase	250 $\pm$ 19	82 $\pm$ 5.3
+ 0.5 μ M rotenone	485 $\pm$ 45	38 $\pm$ 1.9
+ 1 μ M antimycin A	479 $\pm$ 33	36 $\pm$ 3.9
+ 10 μ M HQNO	484 $\pm$ 29	34 $\pm$ 2.3
DHA-pre-loaded cells		
No addition	488 $\pm$ 27	32 $\pm$ 3.4
40 μ M ONOO ⁻	239 $\pm$ 26	77 $\pm$ 3.8
+ 10 units/ml catalase	469 $\pm$ 31*	35 $\pm$ 2.7*
+ boiled catalase	247 $\pm$ 20	72 $\pm$ 3.8
+ 0.5 μ M rotenone	475 $\pm$ 33*	36 $\pm$ 4.6*
+ 1 μ M antimycin A	251 $\pm$ 38	75 $\pm$ 3.3
+ 10 μ M HQNO	245 $\pm$ 26	73 $\pm$ 4.7

* P < 0.001 compared with ONOO⁻-treated cells (unpaired Student's t test).

Table 3 Effect of ONOO⁻ on U937 cell-oxygen consumption

The cells were exposed for 60 min to 0 or 100 μ M DHA, washed and suspended in fresh saline A. Cells were analysed for oxygen consumption for 3 min at the beginning of the experiments and after addition of the indicated concentrations of drugs. Results are means $\pm$ S.E.M. for at least three separate experiments.

Treatment	O ₂ (nmol $\cdot$ min ⁻¹ $\times$ 10 ⁷ cells)
Control cells	
No addition	12.7 $\pm$ 0.9
+ 100 μ M ONOO ⁻	11.8 $\pm$ 0.7
+ 1 μ M antimycin A	2.3 $\pm$ 0.4
+ 0.4 mM TMPD/1 mM ascorbate	41.9 $\pm$ 1.3
+ 0.4 mM TMPD/1 mM ascorbate + 1 mM KCN	3.22 $\pm$ 0.8
DHA-pre-loaded cells	
No addition	14.6 $\pm$ 0.5
+ 100 μ M ONOO ⁻	8.51 $\pm$ 0.6
+ 0.4 mM TMPD/1 mM ascorbate	40.4 $\pm$ 1.7
+ 1 mM KCN	3.97 $\pm$ 0.3
+ 250 μ M TTFA	7.76 $\pm$ 1.2
+ 100 μ M ONOO ⁻	4.71 $\pm$ 0.4
+ 0.5 μ M rotenone	1.57 $\pm$ 0.2
+ 6 mM succinate	9.37 $\pm$ 0.5
+ 100 μ M ONOO ⁻	5.60 $\pm$ 0.9

Figure 4 Effect of increasing concentrations of ONOO⁻ on the oxygen consumption of control or DHA-pre-loaded cells

Cells were exposed for 60 min to 0 (open circles) or 100 μ M DHA (closed circles), washed and resuspended in fresh saline A. Cells were analysed for oxygen consumption for 3 min at the beginning of the experiments and after the addition of increasing concentrations of ONOO⁻. Results are means $\pm$ S.E.M. for at least three separate experiments.

inhibition of oxygen consumption taking place in cells analysed for toxicity. The notion that more cells competing for the same amount of ONOO⁻ will suffer a less severe damage was indeed established previously in our laboratory [34]. Thus it appears logical to speculate that the same events observed in low-density culture at a given ONOO⁻ concentration will require higher concentrations of the oxidant in order to take place in high-density cultures. We therefore performed additional oxygen-consumption studies in DHA-pre-loaded cells treated with 100 μ M ONOO⁻, a condition producing a very modest inhibition of oxygen consumption in AsA-deficient cells. It was found that ONOO⁻ caused a 41.7% inhibition of oxygen utilization and that a very similar inhibitory response was observed in complex-II-inhibited

cells [250 μ M thenoyltrifluoroacetone (TTFA); 39.3%] or in cells respiring on succinate (supplemented with 0.5 μ M rotenone and 6 mM succinate; 40.2%). Finally, similar amounts of cyanide (1 mM)-sensitive oxygen consumption were observed upon addition of 0.4 mM *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD)/1 mM ascorbate to antimycin A (1 μ M)-supplemented cells or to DHA-pre-loaded cells exposed to ONOO⁻ (100 μ M) (Table 3). These results are therefore consistent with the notion that low concentrations of ONOO⁻ selectively inhibit complex III in DHA-pre-loaded cells. Complexes I, II and IV appear to be unaffected.

The final set of experiments involved the use of respiration-deficient cells and produced results consistent with the above conclusion. It was interesting to find that respiration-deficient cells were resistant to toxicity elicited by ONOO⁻ and that their supplementation with increasing concentrations of DHA failed to affect ONOO⁻ toxicity (Figures 2A and 2B). This lack of effect, however, was not due to impaired cellular uptake and/or reduction of DHA, since respiration-deficient and -proficient cells supplemented with increasing concentrations of DHA displayed identical abilities to reduce extracellular ferricyanide (Figure 3).

DISCUSSION

The following considerations are necessary for a correct interpretation of the results presented in the present paper. First, it is important to emphasize that our previous work [18] demonstrated that DHA is promptly reduced to AsA and that, at the time of ONOO⁻ exposure, all the vitamin was present in its reduced form. Secondly, exposure to DHA does not appear to cause significant changes in the redox state of U937 cells, since GSH levels [18] and mitochondrial membrane potential (Table 1) were unaffected, formation of H₂O₂ (Table 2) and MPT (Figure 1F) did not take place, and the cells remained viable (Table 2) and capable of proliferating with rates superimposable on those of untreated cells [18]. Thirdly, extracellular formation of H₂O₂, which may occur via dismutation of superoxide generated by autoxidation of AsA present as a contaminant of DHA, did not take place (results not shown).

Thus it appears that intracellular AsA is responsible for the observed enhancing effects of DHA on ONOO⁻-induced cytotoxicity. An additional point to be made is that accumulation of AsA does not cause toxic effects itself, but rather enhances the lethal response evoked by ONOO⁻.

We found that the effects of AsA are dramatic in cells exposed to otherwise non-toxic levels of ONOO⁻ and much less pronounced upon exposure to intrinsically lethal concentrations of the oxidant (Figure 2A). This is consistent with the notion that AsA promotes events favouring the expression of the lethal response evoked by ONOO⁻ and that the same events occur upon exposure to high concentrations of the oxidant. The observation that increasing levels of DHA, while resulting in a progressively greater ferricyanide reductase activity (Figure 3), produced maximal effects at 100 μ M with no further increase at higher levels (Figure 2B), demonstrates that the action of intracellular AsA is mediated by a saturable mechanism.

Comparative studies using naïve cells treated with 1 mM ONOO⁻ and DHA-pre-loaded cells challenged with 40 μ M ONOO⁻, revealed identical responses in terms of mitochondrial depolarization (Table 1) and formation of catalase-sensitive intermediates leading to oxidation of DHR (Table 2); furthermore, both conditions resulted in the same extent of catalase-sensitive cell death (Table 2), mediated by a MPT-dependent

mechanism (Figure 1). The results of additional experiments using various respiratory-chain inhibitors revealed that complex III is a probable site of H₂O₂ formation (Table 2). Consistently, DHA, although efficiently taken up (Figure 3), failed to promote toxicity in respiration-deficient cells (Figure 2). These results therefore confirm our previous findings, indicating that mitochondrially generated H₂O₂ plays a pivotal role in U937 cell death induced by ONOO⁻ [34], and strongly suggest the involvement of an identical mechanism of toxicity induced by low levels of ONOO⁻ in DHA-pre-loaded cells.

Complex III represents 'the weak site' of the respiratory chain and prevention of the electron flow from cytochrome *b*₅₆₂ to oxidized co-enzyme Q leads to formation of superoxide and H₂O₂ [35]. According to the model proposed in the present study, low levels of ONOO⁻ should be able to inhibit complex III in cells supplemented with DHA. Oxygen-consumption studies revealed that, indeed, otherwise inactive concentrations of ONOO⁻ (40–100 µM) produced remarkable effects in DHA-pre-loaded cells (Figure 4). Using specific activators and inhibitors of the different complexes, it was established that complex III was indeed inhibited and the activities of complexes I, II and IV were unaffected (Table 3). Importantly, DHA pre-loading did not itself cause inhibition of oxygen consumption, but rather enhanced the effects of ONOO⁻. The rates of oxygen utilization detected in DHA-pre-loaded cells in the absence of additional treatments were actually significantly higher in comparison with those observed in untreated cells (Table 3). This response may facilitate the ONOO⁻-dependent inhibition of complex III and the mitochondrial formation of H₂O₂ by promoting mitochondrial calcium accumulation. Indeed, we previously reported [36] that pyruvate enhances the extent of oxygen consumption and, in cells exposed to a short-chain lipid hydroperoxide analogue, causes increased formation of H₂O₂ by promoting mitochondrial calcium accumulation, an event favouring radical formation at the complex III level.

It is well established that AsA readily reduces cytochrome *c*, and for this purpose, it is normally used, in high concentrations and in conjunction with TMPD, to achieve full activation of complex IV. It is therefore possible that this represents the mechanism whereby AsA increases oxygen consumption. In cells exposed to ONOO⁻, the AsA-induced reduction of the mitochondrial pool of cytochrome *c* may also affect mitochondrial radical production by backing up electrons in the respiratory chain and reducing the Q pool. Although the redox state of cytochrome *c* was not directly assessed, these are possible mechanisms that need to be investigated carefully.

Since increasing concentrations of ONOO⁻ lead to a progressive impairment in complex III activity [33], we propose that the strong enhancement of the DHR oxidation and lethal responses mediated by intracellular AsA after exposure to low concentrations of ONOO⁻ are mediated by events favouring complex III inhibition. Consistently, the same effects were observed using bona fide complex III inhibitors (Table 2). Along the same lines, the lack of effect of the vitamin after exposure to high levels of ONOO⁻ is a probable consequence of the ensuing, nearly complete inhibition of complex III.

In conclusion, the information provided by the present study allows the identification of the mechanism whereby repletion of AsA to AsA-deficient cells enhances the lethal response mediated by ONOO⁻ [18]. We take these results as an indication that this mechanism might explain the molecular bases for the observed deleterious effects mediated by acute supplementation of AsA in individuals with pathologies associated with a decrease in AsA levels. These effects were observed in patients with β-thalassaemia major [13] and homozygous haemochromatosis

[14], as well as acute inflammation caused by eccentric exercise [16] and sepsis [17].

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