Intracellular ascorbic acid enhances the DNA single-strand breakage and toxicity induced by peroxynitrite in U937 cells

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A well-established protocol to increase the intracellular content of ascorbic acid was used to investigate the effects of the vitamin on DNA single-strand breakage and toxicity mediated by authentic peroxynitrite ($ONOO^-$) in U937 cells. This protocol involved exposure for 60 min to 100 μM dehydroascorbic acid, which was taken up by the cells and converted into ascorbic acid via a GSH-independent mechanism. At the time of exposure to $ONOO^-$, which was performed in fresh saline immediately after loading with dehydroascorbic acid, the vitamin present in the cells was all in its reduced form. It was found that, in cells that are otherwise ascorbate-deficient, an increase in their ascorbic acid content does not prevent, but rather enhances, the DNA-damaging and lethal responses mediated by exogenous $ONOO^-$. These results therefore suggest that acute supplementation of ascorbic acid can be detrimental for individuals with pathologies associated with a decrease in ascorbic acid and in which $ONOO^-$ is known to promote deleterious effects.

Key words: cell death, DNA damage, reactive nitrogen species, vitamin C.

INTRODUCTION

Peroxynitrite ($ONOO^-$) is a powerful biological oxidant produced *in vivo* by the diffusion-limited reaction of superoxide with nitric oxide; its formation seems to be of importance in the pathophysiology of various diseases such as acute endotoxaemia, neurological disorders, atherosclerosis and ischaemia/reperfusion [1,2]. As a strong oxidant, $ONOO^-$ reacts with protein and non-protein thiol residues [3], inhibits some enzymes of the mitochondrial electron transport chain [4], promotes lipid peroxidation [5] and DNA damage [6] and finally leads to mitochondrial permeability transition and cell death [7]. Thus many defence mechanisms have evolved to limit the levels and/or deleterious effects of $ONOO^-$ in biological systems [8–11]. In this perspective, the identification of exogenous molecules counteracting the effects of $ONOO^-$ is of great importance because their supplementation is expected to be beneficial in the pathologies listed. Ascorbic acid (AA) is under consideration as one of these putative agents [8–11].

AA is a major water-soluble antioxidant, found in the aqueous compartments of cells and extracellular fluids, that is capable of scavenging reactive oxygen [12–14] and nitrogen [15–17] species. However, although various papers have shown that AA is an efficient scavenger of $ONOO^-$ in *in vitro* systems [18], it is still unclear whether intracellular AA mitigates the lethal response mediated by $ONOO^-$.

In the present study we used a well-established protocol to increase the intracellular content of AA in U937 cells [19] and investigated the susceptibility of these cells to the deleterious effects mediated by exogenous $ONOO^-$. Surprisingly, intracellular AA was found to enhance the DNA single-strand breakage and toxicity caused by the oxidant.

MATERIALS AND METHODS

Cell culture and treatments

U937 human myeloid leukaemia cells were cultured in suspension in RPMI 1640 culture medium supplemented with 10% (v/v) fetal bovine serum, 50 i.u./ml penicillin and 50 μg/ml streptomycin, at 37 °C in T-75 tissue culture flasks in a humidified of air/CO_2 (19:1) atmosphere.

Dehydroascorbic acid (DHA; 40 mM stock solution) was prepared immediately before use by the oxidation of AA with bromine, as described in [20]. U937 cells (10^6/ml) were then treated with DHA for 60 min in 5 ml of complete culture medium.

$ONOO^-$ was synthesized by the reaction of nitrite with acidified H_2O_2 as described in [3], then stored for 1–2 weeks at −80 °C with negligible change in its concentration. Treatments with $ONOO^-$ were performed in 2 ml of prewarmed saline A (8.182 g/l NaCl/0.372 g/l KCl/0.336 g/l $NaHCO_3$/0.9 g/l glucose). The cell suspension (5×10^5/ml) was inoculated into 15 ml tubes before $ONOO^-$ was rapidly added on the wall of the tubes and mixed for few seconds to equilibrate the $ONOO^-$ concentration on the cell suspension; an appropriate amount of 1 M HCl was also added.

Measurement of ferricyanide reduction

Ferricyanide reduction was estimated as reported by Avron and Shavit [21]. The cells were washed three times with saline A and centrifuged; the pellet was resuspended in 1 ml of saline A containing 1 mM potassium ferricyanide, which was dissolved immediately before use. After 30 min at 37 °C, the cell suspensions were centrifuged and the resulting supernatants were assayed in duplicate for their ferrocyanide content by using 1,10-phenanthroline as an indicator and measuring A_{510} (ϵ_{510} was taken as 10500 $M^{-1} \cdot cm^{-1}$).

Measurement of AA content by HPLC

The cells were centrifuged at 50 ***g*** (1000 rev./min) for 3 min and washed twice with 10 ml of cold saline A; the final pellet was extracted with ice-cold 70% (v/v) methanol containing 1 mM EDTA. The intracellular AA content was measured by HPLC

Abbreviations used: AA, ascorbic acid; DHA, dehydroascorbic acid; RNSF, relative nuclear spreading factor.

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with the UV detection wavelength set at 265 nm, as described in [22]. Because DHA does not absorb at this wavelength, parallel measurements were performed in samples exposed for 10 min to 0 or 10 mM dithiothreitol. The HPLC apparatus consisted of two Model 126 pumps, a PC-8300 solvent programmer, a Model 210 sample injection valve and a Model 166 variable-wavelength UV–visible range detector equipped with a 12 μl flow cell. The column used was a Supelcosil LC-18 (25 cm $\times$ 4.6 mm internal diam.; particle size 3 μm), protected with a 40 μm Pelliguard LC-18 guard column (2 cm $\times$ 4.6 mm internal diam.). The injection volume was 20 μl.

Non-protein thiol assay

Cellular non-protein thiol content was determined as described in [23]. In brief, U937 cells (3×10^6) were washed three times with saline A and centrifuged; the pellet was then resuspended with 300 μl of metaphosphoric acid solution [1.67 % (v/v) metaphosphoric acid/0.2 % EDTA/30 % (w/v) NaCl], kept for 5 min on ice and centrifuged at 10000 ***g*** (14000 rev./min) for 5 min. The non-protein thiol was measured spectrophotometrically at 412 nm in the supernatant by using 5,5′-dithiobis-(2-nitrobenzoic acid) (ϵ_{412} 13600 $M^{-1} \cdot cm^{-1}$). Protein contents were assayed as described in [24], with BSA as standard.

Measurement of DNA single-strand breakage by the alkaline halo assay

DNA single-strand breakage was determined using the alkaline halo assay described in [25] with minor modifications. After treatment, the cells were resuspended at 2.0×10^4 cells/100 μl in 1.5 % (w/v) low-melting agarose in PBS (8 g/l NaCl/1.15 g/l Na_2HPO_4/0.2 g/l KH_2PO_4/0.2 g/l KCl) containing 5 mM EDTA and were immediately sandwiched between an agarose-coated slide and a coverslip. After complete gelling, the coverslips were removed and the slides were immersed in an alkaline buffer [0.1 M NaOH/1 mM EDTA (pH 12.5)], washed and stained for 5 min with 10 μg/ml ethidium bromide.

The ethidium-bromide-labelled DNA was revealed with a DVC 250 confocal laser microscope (Bio-Rad, Richmond, CA, U.S.A.) and the resulting images were taken and processed with a chilled CCD 5985 camera (Hamamatsu Italy, Milan, Italy) coupled with an Apple Macintosh computer using the public-domain NIH Image program (developed at the U.S. National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/).

The number of DNA single-strand breaks was quantified by calculating the nuclear spreading factor values, which are the ratio between the area of the halo (obtained by subtracting the area of the nucleus from the total area of nucleus plus halo) and that of the nucleus, from 50–75 randomly selected cells per experiment per treatment condition. Results are expressed as relative nuclear spreading factor (RNSF) values calculated by subtracting the nuclear spreading factor values of control cells from those of treated cells.

Growth inhibition assay

After treatment, the cells were washed with saline A and resuspended in prewarmed RPMI 1640 culture medium, plated on 35 mm tissue culture dishes and incubated at 37 °C for increasing durations. The number of viable cells was determined with the Trypan Blue exclusion assay.

RESULTS AND DISCUSSION

It is well established that mammalian cells that do not have the capacity to synthesize AA express membrane transport systems that mediate the cellular uptake of the vitamin. One of these systems is represented by facilitated diffusion by the glucose transporter that efficiently takes up DHA, the two-electron-oxidized form of AA, whereas AA itself is a poor ligand for this transporter [26,27]. Once within the cell, DHA is rapidly converted into AA via GSH-dependent mechanisms that can be both chemical [28–30] and enzymic [30–32], as well as by GSH-independent mechanisms [22] that are more frequently involved in the reduction of low levels of DHA [33]. Exposure to micromolar concentrations of DHA therefore seems to be an ideal strategy for increasing the intracellular AA content in the absence of the undesirable parallel effects encountered with AA. Indeed, in solution AA undergoes metal-catalysed oxidation to DHA, with the concomitant formation of superoxides and H_2O_2 [11].

To elucidate the role of intracellular AA in the deleterious effects generated by $ONOO^-$ in U937 cells, we therefore adopted an experimental protocol involving a 60 min loading with DHA in complete culture medium followed by exposure to authentic $ONOO^-$ in fresh saline A. The results in Table 1 indicate that treatment with 100 μM DHA greatly increased the ability of these cells to reduce extracellular ferricyanide, an activity that provides an indirect estimate of the intracellular content of AA because the reduction of ferricyanide is mediated by AA-derived electrons crossing the plasma membrane [19,34]. Consistently, uptake experiments performed under identical conditions revealed that a large amount of AA accumulated in U937 cells (Table 1), leading to an intracellular concentration of approx. 3.2 mM. The observation that dithiothreitol did not significantly alter this response indicates that all of the vitamin was present in its reduced form. Finally, the results illustrated in Table 1 indicate that exposure to DHA did not cause appreciable changes in the non-protein thiol pool, 95 % of which is represented by GSH, a finding consistent with the notion that DHA recycling occurs via a GSH-independent mechanism.

In the next series of experiments the cells were first loaded with 100 μM DHA as described above, subsequently exposed for 30 min to increasing concentrations of $ONOO^-$ in a DHA-free, glucose-containing saline and finally analysed for DNA single-strand breakage with the alkaline halo assay [25]. A visual inspection of typical images obtained after ethidium bromide staining revealed that the size of the haloes was higher in cells treated with 100 μM $ONOO^-$ (Figure 1B) than in sham-treated cells (Figure 1A). Two lines of evidence support the notion that

Table 1 Effects of DHA on AA or non-protein thiol contents and ability to reduce extracellular ferricyanide in U937 cells

U937 cells (10^6/ml) were exposed for 60 min in complete culture medium to 100 μM DHA, after which the cells were washed three times with saline A and incubated with fresh saline A supplemented with 1 mM ferricyanide; the formation of ferrocyanide was then determined. In other experiments, DHA-preloaded cells were analysed for their AA or non-protein thiol contents, as detailed in the Materials and methods section. Abbreviation: n.d., not detectable. Results are means $\pm$ S.E.M. for six to eight separate experiments, each performed in duplicate. $^*P < 0.001$ compared with control cells (unpaired *t* test).

Treatment	Ferricyanide reduced (pmol/min per 10^6 cells)	AA content (nmol/10^6 cells)	Non-protein thiol (nmol/mg of protein)
Control	125 $\pm$ 33	n.d.	22 $\pm$ 1.5
100 μM DHA	337 $\pm$ 35*	2.34 $\pm$ 0.36	20 $\pm$ 2.3

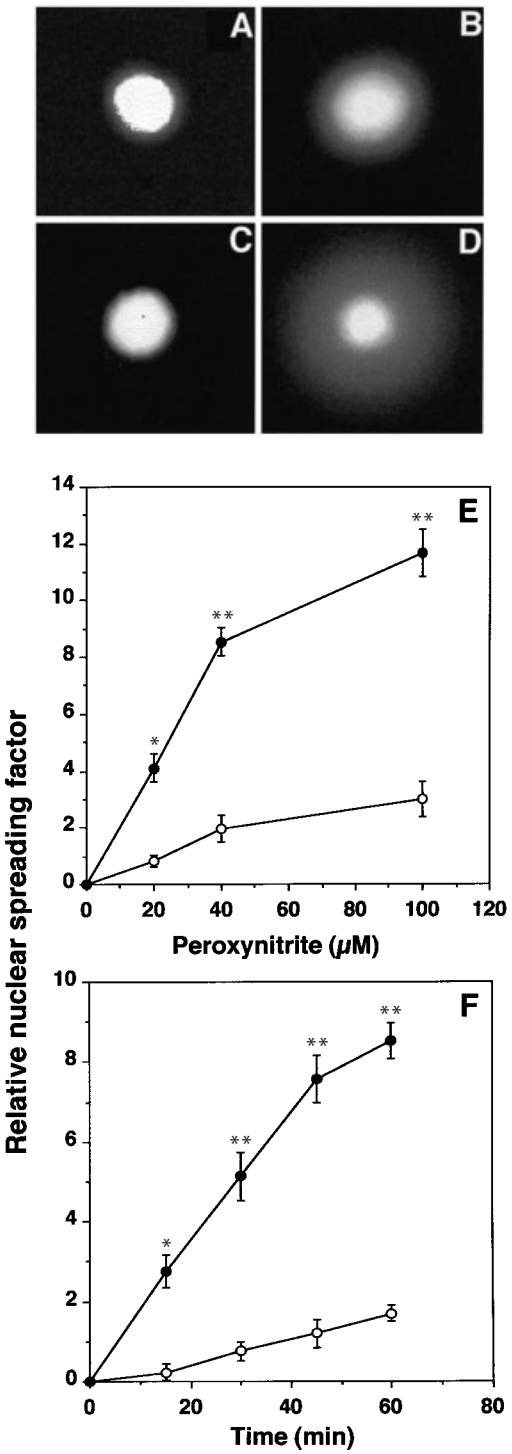

Figure 1 Effect of DHA preloading on ONOO⁻-induced DNA single strand breakage

Representative photomicrographs of U937 cells pre-exposed for 60 min to 0 μM (**A**, **B**) or 100 μM (**C**, **D**) DHA, treated for an additional 30 min in saline A with 100 μM $ONOO^-$ (**B**, **D**) and then analysed by the alkaline halo assay. (**E**) DNA single-strand breakage induced by increasing concentrations of $ONOO^-$ in cells pre-exposed to 0 μM (○) or 100 μM (●) DHA. (**F**) Time dependence of the DNA single-strand breakage generated by 20 μM $ONOO^-$ in control (○) or DHA-preloaded (●) cells. The RNSF values are means ± S.E.M. for three to five separate experiments and were significantly different from those for DNA damage generated by $ONOO^-$ in control cells at the following levels: $^*P < 0.01$; $^{**}P < 0.001$ (unpaired *t* test).

Table 2 Effect of scavengers of $ONOO^-$ or iron chelators on $ONOO^-$-induced DNA single-strand breakage

Control cells or cells preloaded with DHA were treated with the indicated concentrations of $ONOO^-$ in saline A and then analysed for DNA damage as detailed in the Materials and methods section. The conditions were the same as described for Figure 1. Drugs were added to the cultures 5 min before $ONOO^-$. Results are means ± S.E.M. for three to five separate experiments and were significantly different from those for DNA damage generated by $ONOO^-$ alone at $^*P < 0.001$ (unpaired *t* test).

Cells	Treatment	RNSF
Control	400 μM $ONOO^-$	7.89 ± 0.62
	+ 1 mM Trolox	0.97 ± 0.04*
	+ 20 mM L-methionine	0.89 ± 0.03*
	+ 25 μM *o*-phenanthroline	1.12 ± 0.02*
	Decomposed $ONOO^-$	0.62 ± 0.01*
	Vehicle	0.52 ± 0.01*
DHA-preloaded	40 μM $ONOO^-$	8.19 ± 0.52
	+ 1 mM Trolox	1.22 ± 0.02*
	+ 20 mM L-methionine	0.87 ± 0.05*
	+ 25 μM *o*-phenanthroline	1.04 ± 0.04*

Table 3 Effect of scavengers of $ONOO^-$ or iron chelators on $ONOO^-$-induced cytotoxicity

The cells were pre-exposed for 60 min in complete culture medium to 0 or 100 μM DHA and treated for a further 60 min in saline A with the indicated concentrations of $ONOO^-$. Drugs were added to the cultures 5 min before the addition of $ONOO^-$. The cells were then resuspended in prewarmed RPMI 1640 culture medium, plated on 35 mm tissue culture dishes and incubated at 37 °C for 24 or 48 h. Cell numbers were determined with a haemocytometer. Results are means ± S.E.M. for three to five separate experiments and were significantly different at $^*P < 0.001$ (unpaired *t* test).

		10^{-3} × Number of viable cells/ml	
Cells	Treatment	24 h	48 h
Control	No addition	511 ± 36	971 ± 74
	40 μM $ONOO^-$	507 ± 45	980 ± 55
	400 μM $ONOO^-$	207 ± 15*	480 ± 25*
	+ 1 mM Trolox	501 ± 23	895 ± 67
	+ 20 mM L-methionine	498 ± 28	931 ± 22
	+ 25 μM *o*-phenanthroline	495 ± 45	953 ± 19
	Decomposed $ONOO^-$	510 ± 23	935 ± 26
	Vehicle	507 ± 31	981 ± 22
DHA-preloaded	No addition	521 ± 39	958 ± 34
	40 μM $ONOO^-$	203 ± 15*	435 ± 38*
	+ 1 mM Trolox	502 ± 24	899 ± 85*
	+ 20 mM L-methionine	509 ± 20	922 ± 37*
	+ 25 μM *o*-phenanthroline	490 ± 53	943 ± 26*
	400 μM $ONOO^-$	154 ± 19*	217 ± 24*

the observed effects are mediated entirely by $ONOO^-$: (1) cells treated with decomposed $ONOO^-$ or with vehicle produced images similar to those of untreated cells (results not shown, and Table 2), and (2) the $ONOO^-$ scavengers Trolox (1 mM) or L-methionine (20 mM) prevented the DNA cleavage generated by $ONOO^-$ (Table 2). Thus the results shown in Figure 1 are consistent with the notion that $ONOO^-$ induces DNA single-strand breakage in target U937 cells. This DNA-damaging response was markedly increased in DHA-preloaded cells (Figure 1D), whereas no evidence of DNA strand scission was observed after exposure to DHA (Figure 1C). Because the alkaline halo assay allows the detection of DNA damage at the single-cell level [25], we analysed large number of cells (150–300) treated with $ONOO^-$ and found that the DNA-damaging response was

uniformly distributed in target cells, regardless of whether they had been pre-exposed to DHA (results not shown). The concentration dependence and time dependence of the DNA single-strand breakage induced by $ONOO^-$ in untreated or DHA-preloaded cells were investigated next; for this purpose, image analysis was performed on approx. 50–75 cells per treatment condition in three separate experiments. As shown in Figure 1(E), the RNSF values increased progressively in cells exposed to 20–100 μM $ONOO^-$. As expected from the above results, $ONOO^-$ was much more potent in inducing this response in DHA-preloaded cells; under these conditions, the extent of DNA cleavage generated by as little as 20 μM $ONOO^-$ was higher than that mediated by 100 μM $ONOO^-$ in cells that had not been pre-exposed to DHA. The results in Figure 1(F) indicate that the formation of DNA lesions mediated by $ONOO^-$ was first-order with respect to time and that this response, although more pronounced, remained linear in DHA-preloaded cells.

Taken together, these results demonstrate that pre-exposure to DHA markedly enhances the formation of DNA single-strand breaks mediated by $ONOO^-$ in U937 cells. This response does not seem to be restricted to a specific cell population. Because the vitamin was all in its reduced form at the time of exposure to $ONOO^-$, it can be concluded that intracellular AA mediates the enhanced cytotoxic and genotoxic responses. It is also important to note that DHA exposure did not cause obvious signs of toxicity, as measured by visual inspection and by the Trypan Blue exclusion assay (results not shown). In addition, the cells treated with DHA were able to proliferate with kinetics superimposable on that observed in untreated cells (results not shown, and Table 3).

As observed in U937 cells exposed to 400 μM $ONOO^-$, the DNA strand scission induced by a low concentration (40 μM) of $ONOO^-$ in DHA-preloaded cells was abolished by different $ONOO^-$ scavengers (Table 2). Under both treatment conditions the cell-permeant iron chelator *o*-phenanthroline (25 μM) also prevented the $ONOO^-$-dependent DNA-damaging response. This would indicate that iron has a pivotal role in the induction of DNA lesions mediated by $ONOO^-$ in both the absence and presence of AA.

Finally, we performed experiments in which cells pre-exposed to 0 or 100 μM DHA were treated for 60 min in saline A with 40 or 400 μM $ONOO^-$ and were then left to proliferate in fresh culture medium for up to 48 h. Under these conditions, untreated U937 cells seeded at a density of 2.5×10^5 cells/ml exhibited a short lag period and then grew in an exponential fashion. Densities of 5×10^5 and 10^6 cells/ml were found after 24 and 48 h respectively (Table 3). The numbers of viable cells counted at the same time points were similar in cells treated with 40 μM $ONOO^-$ but decreased markedly when the oxidant was given to cells preloaded with DHA. The use of a concentration of $ONOO^-$ (400 μM) that was in itself cytotoxic also caused an increased lethal response in DHA-preloaded cells, although this enhancing effect was less marked than that observed after treatment with 40 μM $ONOO^-$.

It is important to note that the observed toxic effects were not caused by the high local $ONOO^-$ concentrations at the inoculation site; to avoid this problem, the $ONOO^-$ alkaline solution was added on the wall of the test tube, along with an appropriate amount of HCl to maintain a physiological pH; the cell suspension was then vigorously mixed to equilibrate the $ONOO^-$ concentration rapidly in the extracellular milieu. Control experiments were also performed in which $ONOO^-$ and HCl were first added to saline A; this solution was then rapidly poured into tubes containing the U937 cell pellet. Under these experimental conditions, results identical with those reported in Table 3 were obtained (results not shown). That the observed toxic effects were mediated entirely by $ONOO^-$ is emphasized by the observations that cells treated with decomposed $ONOO^-$ or with vehicle remained viable (Table 3) and that the $ONOO^-$ scavengers Trolox (1 mM) or L-methionine (20 mM) prevented the lethal response evoked by $ONOO^-$ (Table 3).

Thus intracellular AA enhances the susceptibility of U937 cells to $ONOO^-$ toxicity. Additional results in Table 3 indicate that *o*-phenthroline abolished the toxic response evoked by the oxidant in control or DHA-preloaded cells.

Taken together, the above findings suggest that U937 cells with increased levels of AA are more susceptible than control cells to the cytotoxic and genotoxic effects mediated by $ONOO^-$. This conclusion is somewhat surprising because AA is known to react with most biologically relevant reactive species, including $ONOO^-$ [12–17]. Although various reports have shown that AA is an efficient scavenger of $ONOO^-$ *in vitro* [18], it is still unclear whether cells with an increased content of AA are more resistant than control cells to the lethal response mediated by $ONOO^-$. Indeed, to the best of our knowledge, only one study [35] has addressed this issue to demonstrate that the vitamin affords cytoprotection when given concomitantly with $ONOO^-$; however, this finding only confirms the notion that AA interacts in solution with $ONOO^-$. The same report demonstrated that pre-exposure to 500 μM AA is also cytoprotective; however, the interpretation of these results is complicated by the fact that high concentrations of the vitamin produce considerable quantities of H_2O_2.

With the use of conditions that avoid these problems, the present study provides information indicating that pre-exposure to DHA, resulting in an accumulation of intracellular AA, enhances the extent of DNA single-strand breakage and cell death caused by exogenous $ONOO^-$. Although the mechanism involved in these responses is currently being investigated, it is important to note that the present study used a protocol involving the repletion of AA to AA-deficient cells; indeed, cells in culture normally contain very little AA because it is extremely labile in solution and, in addition, AA was not added to the culture medium in which U937 cells were grown. Thus the rapid rise in cellular AA levels might promote acute effects such as the reduction of transition metals [11,14] and their mobilization from intracellular stores [36,37], which would increase the $ONOO^-$-dependent damage to target biomolecules.

Although it is 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 suggests that acute supplementation of AA might be deleterious for individuals with pathologies associated with a decrease in AA levels. This seems to be so in patients with idiopathic haemochromatosis and dietary iron overload; indeed, supplementation of their diet with AA was reported to cause serious complications resulting from additional oxidative damage [38]. As final note, an in-hospital depletion–repletion study performed in human volunteers has revealed that AA supplementation promotes a prompt increase in the vitamin content of white blood cells [39]. Neutrophils, monocytes and lymphocytes were saturated at 100 mg daily and contained concentrations of the vitamin at least 14-fold that in plasma. Under these conditions, AA concentrations in the latter two cell types were greater than 3 mM. These levels are remarkably similar to those detected in the present study in U937 cells exposed to DHA.

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