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U937 cell apoptosis induced by arsenite is prevented by low concentrations of mitochondrial ascorbic acid with hardly any effect mediated by the cytosolic fraction of the vitamin

Andrea Guidarelli, Mara Fiorani, Catia Azzolini, Liana Cerioni, Maddalena Scotti, Orazio Cantoni*

Dipartimento di Scienze Biomolecolari, Università degli Studi di Urbino "Carlo Bo", 61029 Urbino, Italy

**Address for correspondence*: Prof. Orazio Cantoni, Dipartimento di Scienze Biomolecolari, Sezione di Farmacologia e Farmacognosia, Università degli Studi di Urbino, Via S. Chiara 27, 61029 Urbino (PU), Italy. Tel: +39-0722-303523; Fax: +39-0722-303521; e-mail: orazio.cantoni@uniurb.it

Abbreviations: AA, L-ascorbic acid; DHA, dehydroascorbic acid; CsA, cyclosporin A DTT, dithiothreitol; EB, extracellular buffer; GSH, tripeptide glutathione; H_2O_2,

hydrogen peroxide; HPLC, high performance liquid chromatography; MB, mitochondrial buffer; MPT, mitochondrial permeability transition; NPSH, non-protein thiol; ROS, reactive oxygen species; SVCTs, sodium-AA co-transporters

Summary

Arsenite directly triggers cytochrome c and Smac/DIABLO release in mitochondria isolated from U937 cells. These effects were not observed in mitochondria pre-exposed for 15 min to 10 μM L-ascorbic acid (AA). In other experiments, intact cells treated for 24-72 h with arsenite were found to die by apoptosis through a mechanism involving mitochondrial permeability transition. Pre-exposure (15 min) to low micromolar concentrations of AA and dehydroascorbic acid (DHA), resulting in identical cytosolic levels of the vitamin, had a diverse impact on cell survival, as cytoprotection was only observed after treatment with AA. Also the mitochondrial accumulation of the vitamin was restricted to AA exposure. An additional indication linking cytoprotection to the mitochondrial fraction of the vitamin was obtained in experiments measuring susceptibility to arsenite in parallel with loss of mitochondrial and cytosolic AA at different times after vitamin exposure. Finally, we took advantage of our recent findings that DHA potently inhibits AA transport to demonstrate that DHA abolishes all the protective effects of AA, under the same conditions in which the mitochondrial accumulation of the vitamin is prevented without affecting the overall cellular accumulation of the vitamin.

Keywords: vitamin C; mitochondria; arsenite; cell death; apoptosis

Running Title: mitochondrial AA prevents apoptosis induced by arsenite

1. Introduction

Arsenic, a widely diffused environmental pollutant, represents a serious threat for the health of millions of people throughout the world [1]. Chronic exposure to arsenical compounds indeed elicits multiple effects in human, including carcinogenesis and toxicity in an array of different tissues and organs [2-6].

Sodium arsenite, often employed to investigate the molecular bases of trivalent arsenic toxicity, causes apoptosis in a variety of cell types [7-12] through a complex mechanism in which at least two components appear of particular importance. The first one is dependent on the ability of arsenite to directly target mitochondria, thereby promoting the formation of reactive oxygen species (ROS) [13-15]. Mitochondrial superoxide may then either react with nitric oxide, to generate toxic peroxynitrite [16], or eventually be converted to H_2O_2 by the manganese superoxide dismutase [16]. Clearly, enforced H_2O_2 formation increases the possibility of escaping catalase- or glutathione peroxidase-dependent metabolism, thereby leading to the formation of toxic hydroxyl radical species. In this perspective, ROS appear of pivotal importance for the geno- and cyto-toxic effects of arsenite [12,14,15,17-19].

The second critical component of the mechanism of arsenite toxicity is related to ability of the metalloid to interact with protein thiols [20-22]. As a consequence, arsenite affects the activity of various enzymes, including some belonging to cellular defences against ROS [22-24]. The tripeptide glutathione (GSH) is therefore of critical importance for the cell and, not surprisingly, the toxicity of arsenite is an inverse function of the GSH pool [12,19,22,25,26].

Clearly the two components of the toxicity mechanisms of arsenite are likely to interact, and eventually synergise, thereby creating a vicious circle leading to cell

damage critically regulated by GSH and, possibly, by the overall antioxidant machinery.

In this direction, L-ascorbic acid (AA) appears as an ideal defensive molecule because it combines a direct scavenging activity [27-30] with other important properties related to GSH [31] and vitamin E [32] recycling. Not surprisingly, AA was found to prevent arsenite toxicity in cultured cells [33] and experimental animals [27-29,34]. AA is directly taken up by the cells *via* sodium-AA cotransporters (SVCT1 and 2) [35-37] or, in alternative, under conditions associated with its extracellular oxidation, taken up as dehydroascorbic acid (DHA) by facilitative hexose transport. Most importantly, cytosolic vitamin C may then be transported in mitochondria as DHA [38-40] or, as recently demonstrated in our [41] and other [42] laboratories, directly as AA.

The aim of the present study was to investigate the specific relevance of mitochondrial *vs* cytosolic AA for the survival of cells exposed to arsenite. Mitochondrial AA is indeed expected to be beneficial for the cells, as arsenite directly targets mitochondria [8,13-15] and elicits ROS formation in the mitochondrial respiratory chain [13,14]. On the other hand mitochondrial GSH, critical for prevention of arsenite toxicity, is synthesised in the cytosol and hence cytosolic AA should also elicit beneficial effects. More generally, the overall redox system of the cell should benefit from an increase in the cytosolic concentration of an antioxidant as AA.

2. Experimental Procedures

2.1 Chemicals

AA, DHA, dithiothreitol (DTT), Hoechst 33342, as well most reagent-grade chemicals were purchased from Sigma-Aldrich (Milan, Italy). FK506, was purchased from Calbiochem (San Diego, CA). Cyclosporin A (CsA) was purchased from Novartis (Bern, Switzerland). MitoTracker Red CMXRos was 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 units/ml) and streptomycin (100 µg/ml) (Euroclone), at 37 °C in T-75 tissue culture flasks (Corning Inc., Corning, NY, USA) gassed with an atmosphere of 95% air-5% CO_2.

A 10 mM AA or DHA stock solution was prepared in extracellular buffer, (EB, 15 mM Hepes, 135 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgCl_2$, pH 7.4) immediately before utilization. Cells (1 x 10^6 cells/ml) were treated with AA supplemented with 0.1 mM DTT or DHA for 15 min at 37 °C in EB. Stability of AA in EB was assessed by monitoring the absorbance at 267 nm for 15 min (ε_{267} =14,600 $M^{-1}cm^{-1}$).

2.3 Cytotoxicity Assay

After treatments with arsenite, the number of viable cells was estimated with the trypan blue exclusion assay. Briefly, an aliquot of the cell suspension was diluted 1:2 (v/v) with 0.4% trypan blue and the viable cells (*i.e.*, those excluding trypan blue) were counted with a hemocytometer.

2.4 Apoptosis Detection

After treatments, the cells were incubated for 5 min with the cell-permeable DNA dye (Hoechst 33342, 10 μmol/l) and then visualized with a fluorescence microscope to assess nuclear morphology (chromatin condensation and fragmentation). Cells with homogeneously stained nuclei were considered viable.

Apoptotic DNA fragmentation in individual cells was also detected using the comet assay [43]. After the treatments, the cells were resuspended at 2.0×10^4 cells/100 μl in 1.0% low-melting agarose in phosphate buffer saline containing 5 mM EDTA and immediately pipetted into agarose-coated slides. The slides were immersed in ice-cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% sarkosyl, 5% dimethyl sulfoxide, and 1% Triton X-100, pH 10.0) for 60 min. The slides were placed on an electrophoresis tray with an alkaline buffer (300 mM NaOH and 1 mM EDTA) and left for 20 min to allow the DNA to unwind; electrophoresis was then performed at 300 mA for 20 min in the same alkaline buffer maintained at 14 °C. The slides were then washed and stained with ethidium bromide. The DNA was visualized with a fluorescence microscope. Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/ treatment condition/experiment.

2.5 Measurement of Mitochondrial Membrane Potential

Cells were pre-loaded with or without vitamin C, exposed for 5 min to 50 nM MitoTracker Red CMXRos and finally treated for 16 h with 2.5 μM arsenite. After treatments, the cells were washed three times and 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 x/0.55 objective lens. The excitation and emission wavelengths were 545 and 610 nm, respectively, 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). Mean fluorescence values were determined by averaging the fluorescence values of at least 50 cells/treatment condition/experiment.

2.6 Measurement of Oxygen Consumption

Cells were pre-loaded with or without vitamin C and treated for 16 h with 2.5 μM arsenite. After treatments, the cells were washed once in saline A (8.182 g/l NaCl, 0.372 g/l KCl, 0.336 g/l $NaHCO_3$, and 0.9 g/l glucose) 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 [44].

2.7 GSH assay

Cellular non-protein thiol (NPSH) content was determined as described in [45]. Since GSH represents more than 90% of the NPSH, the latter will be referred to as GSH. In brief, cells (4×10^6) were washed three times with saline A, centrifuged and the pellet was subsequently resuspended in 150 μl of a metaphosphoric acid solution [1.67% (v/v) metaphosphoric acid/0.2% EDTA/30% (w/v) NaCl]. After a 5 min incubation in an ice-bath, the cell suspension was centrifuged at 10,000 × *g* for 5 min. The NPSH content was measured spectrophotometrically in the supernatant, at 412 nm, using 5,5′-dithiobis(2-nitrobenzoic acid)($\varepsilon 412 = 13{,}600\ M^{-1}\ cm^{-1}$).

2.8 Sub-Cellular Fractionation and Western Blot Analysis

The cells were processed to obtain the mitochondrial fractions, as described in [46]. Equal amounts (25 μg) of the mitochondrial and cytosolic fractions were resolved in 10% sodium dodecyl sulfate polyacrylamide gel and electrotransferred to polyvinyldiene difluoride membranes. Western blot analyses were performed using antibodies against cytochrome c, Smac/DIABLO and HSP-60 (Santa Cruz, Santa Cruz, CA). Details on Western blotting apparatus and conditions are reported elsewhere [47]. Antibodies against actin and HSP-60 were used to assess the purity of the fractions and the equal loading of the lanes.

2.9 Purification of Mitochondria

After treatments, cells were processed for mitochondria purification as described in [48]. Briefly, Cells were suspended in ice-cold mitochondrial buffer (MB, 5 mM Hepes; 210 mM mannitol; 70 mM sucrose; 1 mM EGTA; pH 7.4) containing 10 μg/ml digitonin and mechanically homogenized with a glass potter. The homogenate was centrifuged at 750 × *g* for 20 min at 4 °C to pellet cell debris and generate a crude cytoplasmic fraction. Mitochondria were then pelleted by centrifuging the cytoplasmic fraction at 10,000 × *g* for 30 min at 4 °C.

2.10 Measurement of AA Content by High Performance Liquid Chromatography (HPLC)

After treatments, the cells were washed twice with cold EB and the AA content was measured either in cells or in mitochondria. The cellular or mitochondrial pellets were extracted with ice-cold 70% (v/v) methanol/30% solution (10 mM tetrabutylammonium hydrogen sulfate, 10 mM KH_2PO_4, 0.5% methanol, pH 6.0) containing 1 mM EDTA and 10 mM DTT. After 10 min at ice bath temperature, the samples were centrifuged at 10,000 × *g* for 20 min at 4 °C. Samples were filtered through a 0.22 μm filter (Millipore

Corporation, Billerica, MA, USA) and either 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. [49], 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 AA dissolved in the extraction solution.

2.11 Statistical Analysis

The results are expressed as means ± SD. Statistical differences were analyzed by one-way ANOVA followed by Dunnett's test for multiple comparison or two-way ANOVA followed by Bonferroni's test for multiple comparison. A value of $P < 0.05$ was considered significant.

3. Results

The aim of this study was to determine the specific relevance of mitochondrial *vs* cytosolic AA for the survival of cells exposed to arsenite. For this purpose, we decided to use low concentrations of AA ($\leq$ 30 μM), comparable to (or even lower than) those normally found in plasma and other biological fluids [50,51]. It is important to use low concentrations of AA because cultured cells grown in AA-free medium overexpress Na^+-dependent AA transporters [52,53]. We also used a cell line, U937 cells, well characterised in our laboratory in terms of AA transport across the plasma and mitochondrial membranes [41,54], that presents some important characteristics that will be indicated below.

3.1 Arsenite Directly Targets Mitochondria Via a Mechanism Sensitive to AA

We first made a simple experiment to ask the question of whether intramitochondrial AA is able to prevent the direct effects of arsenite in these organelles. For this purpose, mitochondria were isolated from U937 cells and subsequently exposed for 5 min to 0 or 30 μM AA and then for 25 min to 20 μM arsenite. The results obtained after analysis of residual mitochondrial cytochrome c and Smac/DIABLO are illustrated in Fig. 1 and demonstrate that AA prevents the direct mitochondrial effects mediated by arsenite. Fig. 1 shows the results obtained after normalization of the outcome of three separate experiments.

3.2 U937 Cell Death Induced by Arsenite

U937 cells were exposed to increasing concentrations of arsenite and the number of viable cells estimated after 24, 48 and 72 h of growth. As indicated in Fig. 2A, there was a dose-dependent reduction in cell number at these time points, possibly reflecting inhibition of cell proliferation and/or cell death. We next focused on the effects mediated by 2.5 μM

arsenite and detected after 16 h of exposure a severe inhibition (33 % decrease) of oxygen consumption (Fig. 2B). After 16 h, we also detected a decline in mitochondrial membrane potential (as measured by the MitoTracker Red fluorescence assay, Fig. 2C) sensitive to the MPT inhibitor CsA [55]. Interestingly, CsA also abolished the toxic effects of arsenite (Fig. 2D), thereby implying an involvement of MPT-dependent cell death in these effects. The specificity of the effects of CsA was established using FK506, which shares with CsA the ability to inhibit calcineurin, but fails to affect the formation of MPT pores [56]. As indicated in Fig. 2C and D, FK506 failed to recapitulate the effects of CsA.

The above findings are therefore indicative of MPT-dependent toxicity, most likely associated with apoptosis, as indicated by the results obtained with the chromatin fragmentation (Fig. 2E) and Comet (Fig. 2F) assays, providing evidence for apoptotic DNA fragmentation also sensitive to CsA and insensitive to FK506.

3.3 Vitamin C Affords Cytoprotection

Cells were exposed for 15 min to 10 μM AA, a condition associated with the accumulation of 0.126 nmol AA x 10^6 cells (Fig. 3A), and subsequently treated with 2.5 μM arsenite, as in the experiments reported in Fig. 2A. Further analyses unravelled a potent inhibitory effect of AA on the loss of oxygen consumption (Fig. 2B), mitochondrial membrane potential (Fig. 2C) and number of viable cells (Fig. 2D), as well as on apoptotic DNA fragmentation (Figs. 2E and F) induced by arsenite.

3.4 Cytoprotection is Mediated by Intramitochondrial Vitamin C

In order to address the question of the specific role of mitochondrial *vs* cytosolic AA in the prevention of arsenite toxicity, we took advantage of our previous observation that cells pre-loaded with low concentrations of AA or DHA accumulate identical amounts of vitamin C, however with a mitochondrial uptake restricted to exposure to AA [41]. We

first confirmed these findings and found that indeed exposure to 10 μM AA or DHA promotes the accumulation of identical amounts of vitamin C (Fig. 3A); in addition, we found that significant mitochondrial uptake of the vitamin is only detected after AA exposure (Fig. 3B) and that neither of the two forms of the vitamin elicited significant changes of the GSH pool (Fig. 3C). We therefore asked the question of whether DHA preloading recapitulates the protective effects of AA in the arsenite toxicity paradigm. As indicated in Fig. 3D, DHA (1-30 μM)-derived intracellular vitamin C failed to prevent arsenite (2.5 μM) toxicity under the same conditions in which toxicity was instead abolished by preloading with 10 μM AA, or significantly reduced by as low as 1 or 3 μM AA. Furthermore, DHA (10 μM) failed to recapitulate the protective effects of AA on the loss of oxygen consumption (Fig. 2B), mitochondrial membrane potential (Fig. 2C) and apoptosis elicited by exposure to arsenite (Fig. 2D-F).

We finally performed experiments to determine the rate of disappearance of vitamin C in cells pre-loaded with 10 μM AA and detected an about 60% residual fraction after 6 h of post-treatment incubation (Fig. 4A). Loss of the mitochondrial fraction of the vitamin was instead more rapid with an almost complete disappearance detected after 6 h. Cell viability was then assessed in cells harvested at specific time points after AA preloading and subsequently exposed for 48 h to 2.5 μM arsenite. Interestingly, cytoprotection progressively decreased in cells post-incubated in fresh medium after AA exposure and was basically lost at the 6 h time point (Fig. 4B).

These and the above results are consistent with the notion that vitamin C-dependent cytoprotection is mediated by a fraction of the vitamin localised in the mitochondria. The cytosolic fraction of the vitamin instead appears ineffective in the toxicity paradigm under investigation.

3.5 DHA Abolishes the Protective Effects of AA

The results illustrated in Fig. 3A indicate that combined exposure to 10 μM DHA and 10 μM AA promotes the accumulation of the same amount of vitamin C observed after separate treatment with each of the two forms of the vitamin. Furthermore this treatment failed to affect the GSH pool, as observed after separate exposure to either of the two forms of the vitamin (Fig. 3C).

These findings are therefore in keeping with our previous studies providing evidence that DHA is an inhibitor of SVCT2-dependent AA transport [57] and indeed, as observed with the sole DHA, exposure to the cocktail AA/DHA was not associated with the mitochondrial accumulation of the vitamin (Fig. 3B).

These results therefore provide the rationale for testing the specific role of intramitochondrial *vs* cytosolic vitamin C in preventing the toxic effects of arsenite based on the simple addition of 10 μM DHA during preloading with 10 μM AA. As illustrated in Figs 2B-F, DHA indeed abolished the protective effects afforded by AA in diverse toxicity paradigms, thereby implying once again that the mitochondrial fraction of vitamin C is entirely responsible for cytoprotection.

4. Discussion

The well established notion that cell death induced by arsenite is critically regulated by the formation of secondary reactive oxygen species [12,14,15,18,19] has provided the ground for studies investigating whether antioxidants afford protection in toxicity paradigms associated with exposure to the metalloid. In general, cytoprotection was observed with several antioxidants [12,15,22,26], including AA alone [22,29,33,58], or combined with vitamin E [22,27,28,34], thereby providing the rational for supplementing antioxidants, AA in particular, to individuals environmentally or occupationally exposed to arsenic.

The present study was focused on the identification of the relevant subcellular fraction of AA in cytoprotection. An initial, critical consideration for the discussion of the results herein presented is on the concentrations of the vitamin employed in our experiments, in absolute terms very low, or in any case lower than that normally found in most biological fluids [50,51]. One may actually argue that we are dealing with "scorbutic" concentrations of vitamin C, which however would be a mistake if we consider that cells grown in culture, *i.e.*, virtually in the absence of vitamin C, dramatically upregulate the expression of SVCT2 [52,53]. Hence, even under conditions of exposure to low levels of AA, cultured cells accumulate significant amounts of the vitamin against its own concentration gradient. This is particularly true for the U937 cells clone employed in our experiments, in which AA transport has been extensively characterised [54,57,59], and the mitochondrial expression of SVCT2 initially identified [41].

The present study provides three major findings: 1) intramitochondrial AA inhibits the direct mitochondrial effect mediated by arsenite; 2) intramitochondrial AA prevents U937 cell apoptosis induced by arsenite; and 3) cytosolic AA fails to afford cytoprotection in the same toxicity paradigm. Mitochondrial AA therefore represents the critical cytoprotective fraction of the overall amount of the vitamin associated with the cell,

thereby providing evidence for a very important biological role for the mitochondrial SVCT2 [41].

Arsenite has been reported to induce apoptosis through the activation of the mitochondrial pathway, where a key step is represented by the release of factors as cytochrome c [7,10,15]. In our hands, arsenite promoted release of cytochrome c and Smac/DIABLO in naïve isolated mitochondria but failed to do so in AA-enriched mitochondria (Fig. 1). In this direction, it is important to note that the intramitochondrial concentration of AA (3.36 nmol/mg proteins) is similar to that reached in the mitochondria of cells exposed to as low as 3 μM AA (4.2 nmol/mg proteins) [41].

These experiments allow us to conclude that low and physiologically attainable concentrations of mitochondrial AA prevent the direct mitochondrial effects of arsenite.

We also confirmed the relevance of the mitochondrial pathway of apoptosis in intact U937 cells exposed to arsenite. In particular, evidence of MPT-dependent toxicity was obtained through the assessment of a CsA-sensitive loss of mitochondrial membrane potential and viability detected after 16 (Fig. 2C) or 48 h (Fig. 2D) of exposure to arsenite, respectively. Evidence of apoptosis was instead obtained by visual inspection of the cells as well as by the determination of apoptotic DNA fragmentation with two distinct assays (Fig. 2E and F). An interesting finding of the present study is that pre-exposure of the cells to 10 μM AA was as effective as CsA in preventing arsenite toxicity (Fig. 2D-E). In addition, AA also prevented the early effects elicited by arsenite, as inhibition of oxygen consumption (Fig. 2B) and loss of mitochondrial membrane potential (Fig. 2C).

Together, these results indicate that low concentrations of AA inhibit the early mitochondrial effects of arsenite in intact cells, thereby preventing arsenite-dependent apoptosis.

The next step was therefore to establish the relevance of mitochondrial AA in the protective response under investigation. In order to address this question, we took

advantage of our previous findings indicating that AA and DHA are taken up by the U937 cell clone employed in our laboratory at a similar rate but with a remarkably different subcellular distribution [41]. In particular, we reported that the mitochondrial accumulation of the vitamin was restricted to exposure to AA [41]. While this apparent dichotomy is still under investigation, it appears that at these low concentrations DHA is rapidly converted to AA, thereby preventing the possibility of its direct mitochondrial uptake. In this perspective, steady-state DHA cytosolic levels might be too low to be cleared in significant amounts by the mitochondria. It is indeed important to keep in mind that DHA is transported through a high capacity transport system however characterised by a low affinity. On the other hand, we have reasons to believe that DHA levels might in fact be high enough to trigger other effects, in particular inhibition of mitochondrial SVCT2, as we recently reported for the plasma membrane counterpart [57]. A comparison of the effects of AA and DHA may therefore provide important clues for the definition of the specific relevance of mitochondrial *vs* cytosolic vitamin C in preventing arsenite toxicity.

Results illustrated in Fig 2 and 3 definitely make a strong case for a critical role of the mitochondrial fraction, since an all or nothing response is observed at identical overall levels of cellular accumulation, with cytoprotection being restricted to conditions associated with significant mitochondrial uptake of AA (Fig. 2B-F). An additional important consideration is that the cytoprotective concentrations of AA were remarkably low: while significant effects were also detected with 1 μM AA, three times more AA almost completely prevented arsenite toxicity (Fig. 3D). Under these conditions, AA does not autoxidise [54] and, as expected, did not affect the GSH pool (Fig. 3C). The results obtained with DHA are also indicative of a poor relevance of the cytosolic fraction of the vitamin (Fig. 2B-F and Fig. 3D), thereby further suggesting that the critical events associated with prevention of arsenite toxicity take place in a non cytosolic compartment.

DHA reduction was not associated with a decline in cellular GSH (Fig. 3C), thereby implying the involvement of GSH-independent DHA reductases [49,60]. This is also an important observation, as a decrease in the GSH pool would be expected to enhance arsenite toxicity [22,25]. Such a result would have then introduced a complicating factor for the experimental outcomes illustrated in Fig. 3D.

A second approach providing results consistent with the above conclusions is based on experiments in which AA and DHA were concomitantly administered to the cells prior to arsenite exposure. DHA, by inhibiting AA uptake, prevented all the effects linked to intracellular AA, including its uptake in mitochondria (Fig. 3B) as well all the protective effects of the vitamin on the early (Fig. 2B and C) and late (Fig. 2D-F, Fig. 3D) effects elicited by arsenite.

Finally, the same conclusions can be reached through the analysis of the results illustrated in Fig. 4, showing the impact of time-dependent loss of mitochondrial or cytosolic AA after AA pre-loading and incubation in AA-free medium on arsenite toxicity: loss of cytoprotection occurred in parallel with loss of mitochondrial AA, even under conditions in which significant amounts of the vitamin were still retained in the cytosol. Seen in a different perspective, these results also indicate that the lethal response evoked by arsenite involves some early (mitochondrial) event taking place in the first 2-3 h. Hence, the possibility of prevention of arsenite toxicity is most likely confined to these very early times of exposure, despite the late onset of cell death (apoptosis).

In conclusion, the present study confirms the notion that AA is a potent inhibitor of arsenite toxicity and provides compelling evidence for a pivotal role of the mitochondrial fraction of the vitamin in this protective response. Under standard conditions, mitochondrial AA may represent a primary defence for the cell, as previously established for GSH [12,19,27,61]. Conditions associated with loss of mitochondrial AA may therefore lower the threshold of susceptibility to arsenite toxicity. In a different

perspective, an elevation in mitochondrial AA may instead lower the risk of toxicity in individuals environmentally or occupationally exposed to arsenic. Future studies will determine the impact of mitochondrial AA on the lethal response mediated by a specific form of trivalent arsenic, As_2O_3, highly effective for the treatment of haematological malignancies [62,63] as well as other types of tumours [64-66].

Conflict of interest

The authors declare that they have no conflict of interest.

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Legends to the figures

Fig 1. Direct effects of arsenite in isolated mitochondria are prevented by AA.

Mitochondria were isolated from logarithmically growing U937 cells, exposed for 5 min to 0 or 30 μM AA in MB and finally treated for 25 min with 20 μM arsenite. After treatments, mitochondria were lysed and the resulting lysates processed for Western blot analysis using antibodies against cytochrome c, Smac/DIABLO, HSP-60 (loading control) and actin (cytoplasmic marker). Blots shown are representative of 3 separate experiments with similar outcomes. The relative amounts of cytochrome c and Smac/DIABLO were quantified by densitometric analysis and the results obtained in these experiments are expressed as optical density integration (means ± SD calculated from at least 3 separate experiments). *$P < 0.01$, as compared to untreated cells, (*)$P < 0.01$, or cells treated with arsenite (one-way ANOVA followed by Dunnet's test).

Fig 2. Arsenite promotes mitochondrial permeability transition-dependent U937 cell apoptosis via a mechanism sensitive to low concentrations of AA.

(A) Counts of cells exposed for 24, 48 or 72 h to increasing concentrations of arsenite. Results represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.01$, **$P < 0.001$ as compared to untreated cells (two-way ANOVA followed by Bonferroni's test). (B-F) Cells were exposed for 15 min to 10 μM AA, DHA, or the two agents combined, and subsequently treated for 16 (B and C) or 48 h (D-F) with 2.5 μM arsenite. In some experiments cells were also exposed to CsA (0.5 μM, 15 min), or FK506 (1 μM, 15 min), prior to incubation with arsenite. Cells were analysed for oxygen consumption (B), MitoTracker Red CMXRos-fluorescence (C), number and viability (D) and detection of apoptosis (E and F). Representative micrographs of Hoechst 33342-loaded nuclei (inset to panel E) and microgel-electrophoresed U937 DNA (inset to panel F)

after treatment for 48 h with 0 or 2.5 μM arsenite. Apoptotic cells display a pear-shaped morphology. Results represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.01$, as compared to untreated cells, (*)$P < 0.01$, or cells treated with arsenite (one-way ANOVA followed by Dunnet's test).

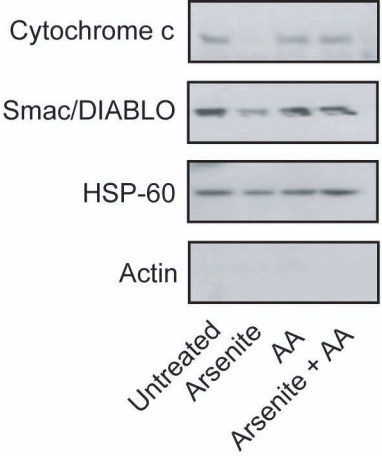
Fig 3. Relationships between mitochondrial content of AA and cytoprotection against arsenite.

Cells were exposed for 15 min to 10 μM AA, DHA, or the two agents combined, and analyzed for vitamin C accumulation (A) or processed for the isolation of the mitochondrial fraction prior to vitamin C analysis (B). Results represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.001$ as compared to the sample exposed to AA (first bar) (one-way ANOVA followed by Dunnet's test). Cells receiving identical treatments were also analyzed for their GSH content and are expressed as % of untreated cells (22 $\pm$ 1.5 nmol/mg protein, C). (D) Cells were exposed for 15 min to increasing concentrations of AA (open circles), or DHA (open squares), subsequently incubated in culture medium with 2.5 μM arsenite for 48 h and finally analyzed for toxicity (count of viable cells). Treatment with arsenite alone caused an about 50% loss of the number of viable cells and pre-exposure to as low as 10 μM AA prevented this effect. Data represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.01$, **$P < 0.001$ as compared to cells treated with arsenite (two-way ANOVA followed by Bonferroni's test).

Fig 4. Time dependent loss of mitochondrial AA is associated with a progressive decline of resistance to arsenite toxicity.

(A) Cells were exposed for 15 min to 10 μM AA and analyzed for AA cellular (open circles) and mitochondrial (open squares) content, either immediately or after various time

intervals of incubation in AA-free culture medium. Results represent the means ± SD calculated from at least 3 separate experiments. (B) Cells were first exposed for 15 min to 0 or 10 μM AA and subsequently incubated for increasing time intervals in AA-free culture medium. At the indicated times, aliquots of the cells were treated with 2.5 μM (circles) arsenite and after 48 h analysed for toxicity. Results represent the means ± SD calculated from at least 3 separate experiments. Data represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.01$, **$P < 0.001$ as compared to cells treated with arsenite (two-way ANOVA followed by Bonferroni's test).


Cytochrome c
Smac/DIABLO
HSP-60
Actin
Untreated
Arsenite
AA
Arsenite + AA

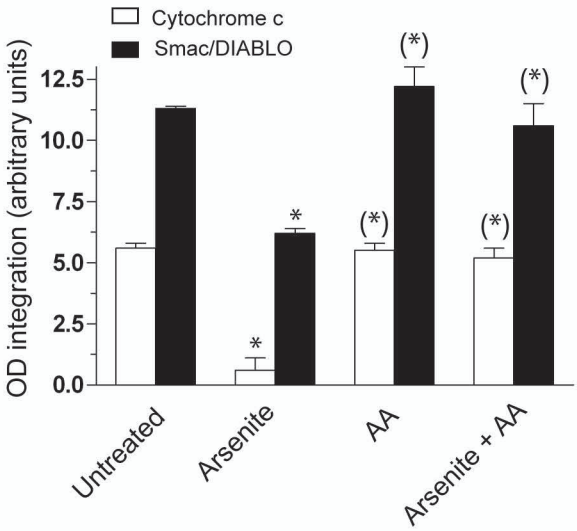

Cytochrome c
Smac/DIABLO
OD integration (arbitrary units)
12.5
10.0
7.5
5.0
2.5
0.0
*
*
(*)
(*)
(*)
(*)
Untreated
Arsenite
AA
Arsenite + AA

Figure 1

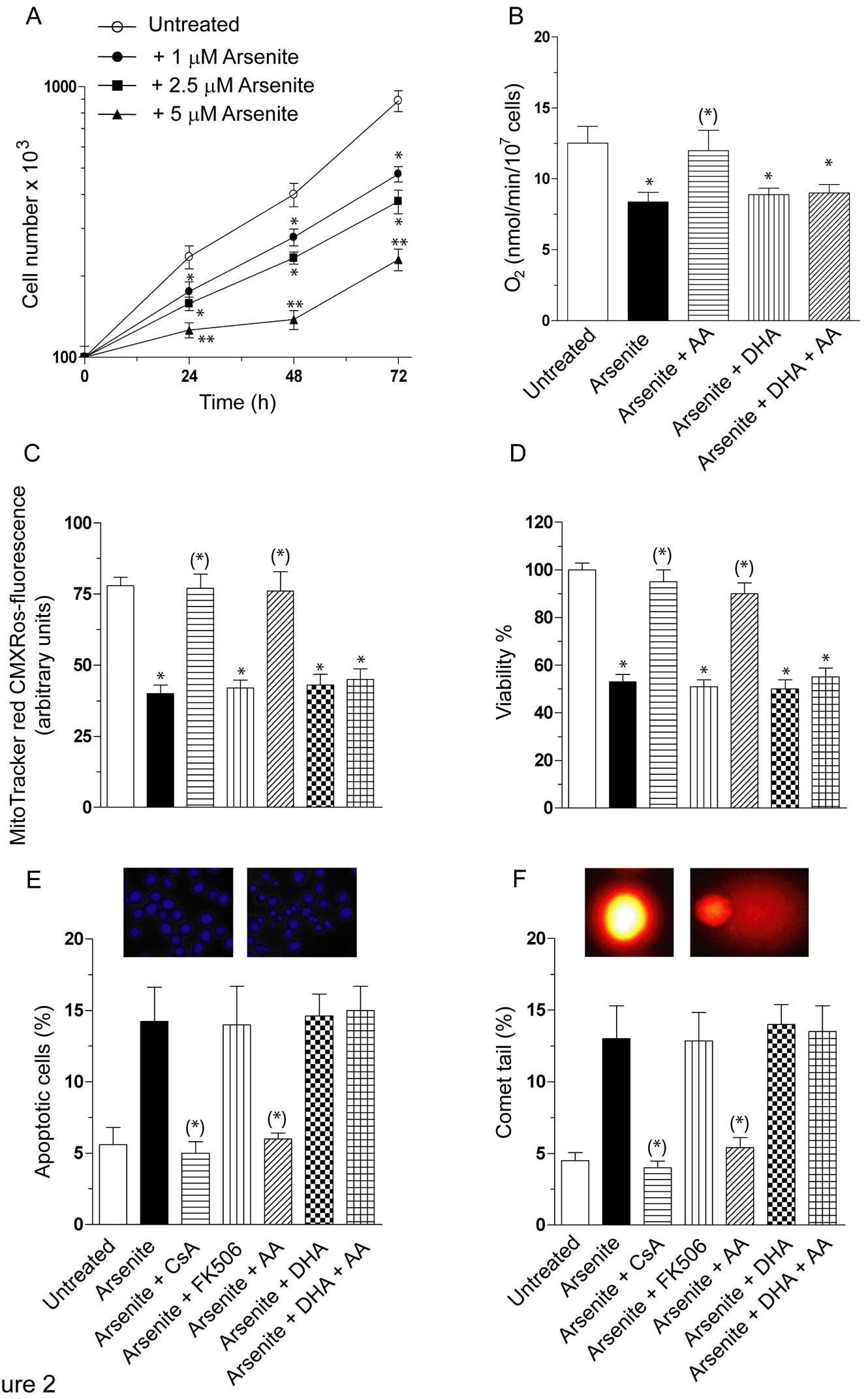

A
Untreated
+ 1 μM Arsenite
+ 2.5 μM Arsenite
+ 5 μM Arsenite
Cell number x 10^3
1000
100
0
24
48
72
Time (h)
B
O2 (nmol/min/10^7 cells)
20
15
10
5
0
(*)
Untreated
Arsenite
Arsenite + AA
Arsenite + DHA
Arsenite + DHA + AA
C
MitoTracker red CMXRos-fluorescence (arbitrary units)
100
75
50
25
0
(*)
D
Viability %
120
100
80
60
40
20
0
E
Apoptotic cells (%)
20
15
10
5
0
(*)
Untreated
Arsenite
Arsenite + CsA
Arsenite + FK506
Arsenite + AA
Arsenite + DHA
Arsenite + DHA + AA
F
Comet tail (%)
20
15
10
5
0
(*)
Untreated
Arsenite
Arsenite + CsA
Arsenite + FK506
Arsenite + AA
Arsenite + DHA
Arsenite + DHA + AA

Figure 2

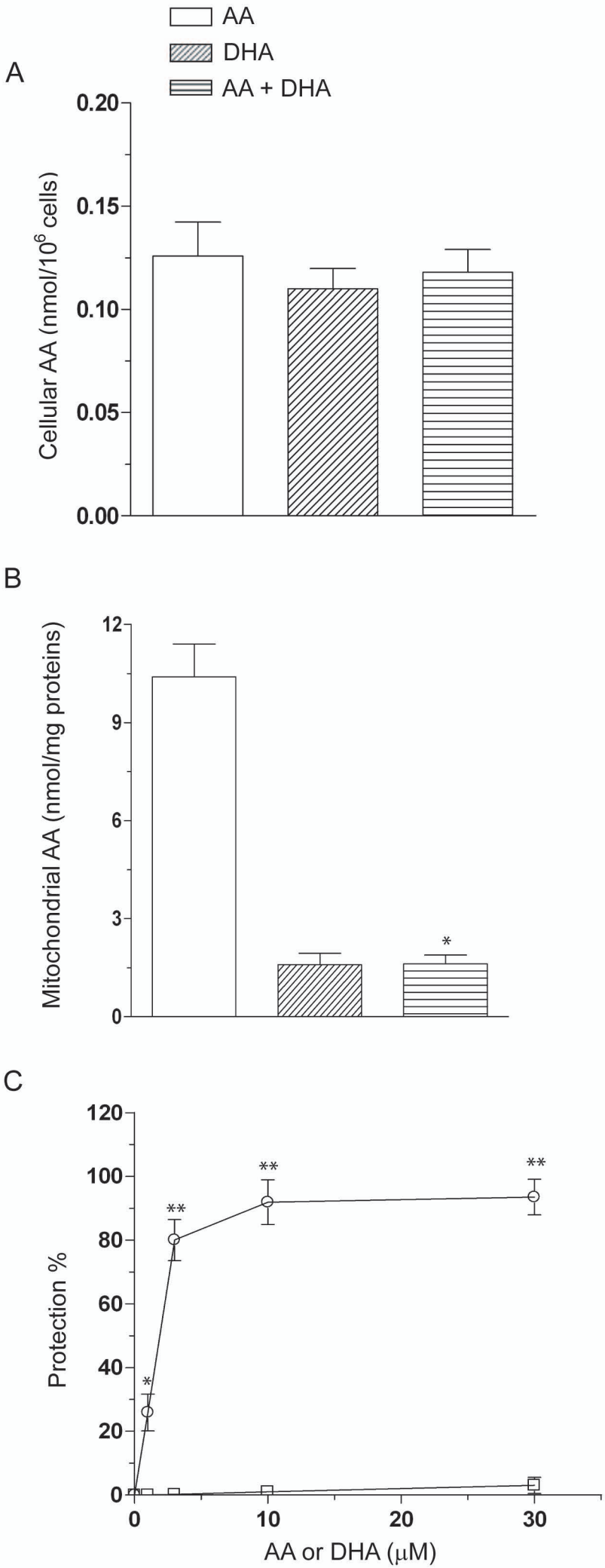
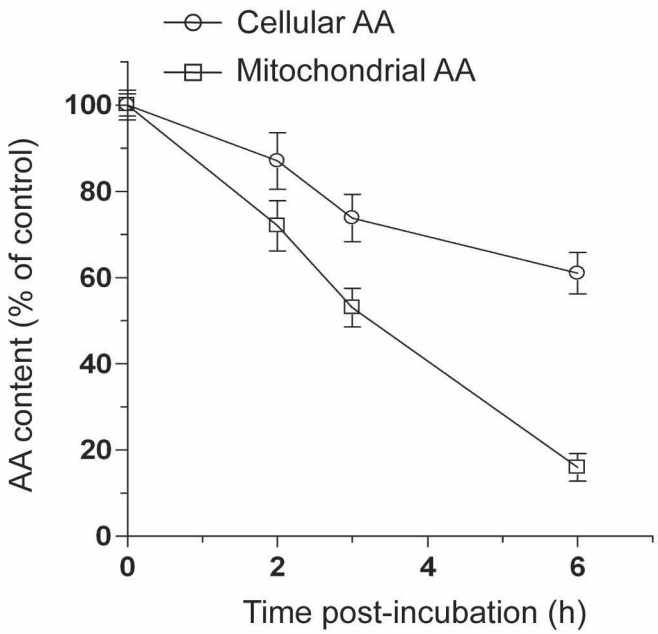

A
AA
DHA
AA + DHA
Cellular AA (nmol/10^6 cells)
0.20
0.15
0.10
0.05
0.00
B
Mitochondrial AA (nmol/mg proteins)
12
9
6
3
0
*
C
Protection %
120
100
80
60
40
20
0
**
**
**
*
0
10
20
30
AA or DHA (μM)

Figure 3

A


Cellular AA
Mitochondrial AA
AA content (% of control)
100
80
60
40
20
0
0
2
4
6
Time post-incubation (h)

B

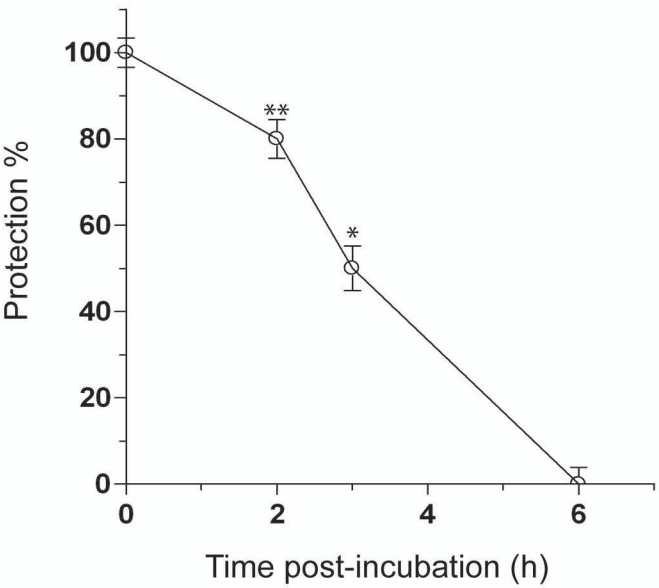

Protection %
100
80
60
40
20
0
**
*
0
2
4
6
Time post-incubation (h)

Figure 4