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# Intracellular dehydroascorbic acid inhibits SVCT2-dependent transport of ascorbic acid in mitochondria

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*Abbreviations:* AA, L-ascorbic acid; Cyt B, cytochalasin B; DHA, dehydroascorbic acid; DTT, dithiothreitol; pCMB, 4-hydroxymercuribenzoic acid; S-Pyr, sulfinpyrazone; TBA, tetrabutylammoniumhydrogensulfate; EB, extracellular buffer; MB, mitochondrial buffer.

**Abstract**

Exposure of U937 cells to low concentrations of L-ascorbic acid (AA) is associated with a prompt cellular uptake and a further mitochondrial accumulation of the vitamin. Under the same conditions, dehydroascorbic acid (DHA) uptake was followed by rapid reduction and accumulation of identical intracellular levels of AA, however in the absence of significant mitochondrial uptake. This event was instead observed after exposure to remarkably greater concentrations of DHA. Furthermore, experiments performed in isolated mitochondria revealed that DHA transport through hexose transporters and $Na^+$-dependent transport of AA were very similar. These results suggest that the different subcellular compartmentalization of the vitamin is mediated by events promoting inhibition of mitochondrial AA transport, possibly triggered by low levels of DHA. We obtained results in line with this notion in intact cells, and more direct evidence in isolated mitochondria. This inhibitory effect was promptly reversible after DHA removal and comparable with that mediated by established inhibitors, as quercetin.

The results presented collectively indicate that low intracellular concentrations of DHA, because of its rapid reduction back to AA, are a poor substrate for direct mitochondrial uptake. DHA concentrations, however, appear sufficiently high to mediate inhibition of mitochondrial transport of AA/DHA-derived AA.

**Keywords:** ascorbic acid, dehydroascorbic acid, SVCT2, ascorbic acid transport.

## Introduction

L-ascorbic acid (AA) concentrations in biological fluids [1] are normally greater than those necessary for its transport through the plasma membrane *via* high affinity/low capacity $Na^+$-dependent cotransporters (SVCT2, more rarely SVCT1) [2-4]. Under these conditions, cells accumulate up to millimolar concentrations of the vitamin, employed to support a variety of physiological reactions [5] and cope with specific stress conditions [6-7]. In principle, AA might then be transported within specific organelles, as the mitochondria, in which AA is expected to promote an array of beneficial effects associated to its well established antioxidant properties [8-9]. In this direction, we recently provided evidence for the expression of a transporter, recognised by anti-SVCT2 antibodies, likely responsible for AA transport in U937 cell mitochondria [10]. Although high Km values are in general predictable on the bases of the low intracellular concentrations of $Na^+$ and $Ca^{2+}$ [11], we recently reported that Km for mitochondrial and plasma membrane SVCT2 are in fact remarkably similar in the U937 cell clone employed in our studies [12]. This high affinity of the mitochondrial AA transporter is coherent with the observation that, in isolated mitochondria, maximal transport activity is observed in nominally $Ca^{2+}$-free medium and in the presence of as low as 1 mM $Na^+$. Recently, the existence of such a transporter has also been demonstrated by other groups by both in silico analysis [13] and experimentally [14].

Oxidation of AA is associated with the formation of dehydroascorbic acid (DHA), that can be either released by the cells [5,15], or more likely readily converted back to AA *via* GSH-dependent and -independent mechanisms [4-5]. Intracellular levels of DHA are therefore kept always very low, but nevertheless several reports proposed its involvement in various biological reactions. For example, it has been suggested that DHA is an inhibitor of the activities of enzymes [16-18] and transcription factors [19], although the information available is insufficient to define the biological relevance of these effects, in particular since the concentrations employed in these studies were far greater than those reasonably achieved inside the cells.

Mitochondrial uptake of DHA through hexose transporters can also take place, as documented by various investigators [8-9,20], but once again the important limitation of its very low cytosolic concentrations should be kept into consideration. The steady-state levels of intracellular DHA may however change under conditions associated with the extracellular conversion of AA to DHA, as it might occur after enforced NADPH-dependent superoxide formation [21-22]. While the relative expression (and activity) of plasma

membrane/mitochondrial hexose transporters and DHA reductive capacities will possibly affect the above events in different cell types, an increase in the intracellular DHA steady-state concentrations should be paralleled by increased chances of DHA-dependent events (unrelated to reduction back to AA) and of its direct mitochondrial clearance.

The overall scenario is therefore complicated by numerous variables that should be taken into consideration, with however a reference point set by the differences in the relative concentrations of AA and DHA in the extra/intracellular compartments. If we focus on intracellular DHA, then we should consider of potential biological relevance only those effects or events requiring the very low concentrations that can be presumably reached.

This premise provides the background for the present study, in which we examined the relevance of the mitochondrial transport of DHA and AA as well as the eventuality of an interaction between DHA and the AA transporter. The complexity of the variables summarised above convinced us to employ once again the U937 cell clone used in our previous studies, for the amount of relevant information available and also because these cells have the advantage of accumulating identical levels of vitamin C after exposure to low micromolar concentrations of AA and DHA [21].

In these cells, we found that DHA is not a relevant substrate for mitochondrial uptake but nevertheless plays a significant role in the regulation of vitamin C mitochondrial accumulation through inhibition of the high affinity AA transporter.

## 1. Materials and methods

*Chemicals.* AA, DHA, DTT, tetrabutylammonium hydrogen sulfate (TBA), EDTA, quercetin, 4-hydroxymercuribenzoic acid (pCMB), sulfinpyrazone (S-Pyr), cytochalasin B (Cyt B), as well most reagent-grade chemicals were purchased from Sigma-Aldrich (Milan, Italy).
Perkin–Elmer Life and Analytical Sciences (Boston, MA) supplied L-[1-$^{14}$C]AA (specific activity 5.35 mCi/mmol), which was dissolved in deionized water containing 0.1 mM acetic acid and stored in multiple aliquots at -20 °C until use [23].

*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% heat-inactivated FBS (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, Corning, NY) gassed with an atmosphere of 95% air-5% $CO_2$. A 10 mM AA 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. Where indicated, NaCl was replaced with choline-chloride. Cells (1 x $10^6$ cells/ml) were treated with AA for 15 min at 37 °C in EB supplemented with 0.1 mM DTT. Stability of AA in EB was assessed by monitoring the absorbance at 267 nm for 15 min ($\varepsilon_{267}$ =14,600 $M^{-1}$ $cm^{-1}$).

*Purification of mitochondria.* Mitochondria were isolated as detailed by Fiorani et al. [24]. Isolated mitochondria were exposed to AA or $^{14}$C-AA in mitochondrial buffer, MB (5 mM Hepes, 210 mM mannitol, 70 mM sucrose, 1 mM Na-EGTA, pH 7.4) and processed as described below. Mitochondria obtained from cells previously exposed to AA were also lysed and processed as indicated for isolated mitochondria.

*Measurement of AA content by HPLC.* After treatments, the cells were washed twice with cold EB and mitochondria with cold MB. The final pellets were extracted with ice-cold 70% (vol/vol) methanol/30% HPLC solution A (10 mM TBA, 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. Where indicated, DTT was omitted. Samples were filtered through a 0.22 µm filter (Millipore, Milan, Italy) and analyzed immediately or

frozen at -80 °C for later analysis. The intracellular AA content was measured by HPLC with the UV detection wavelength set at 265 nm, as described in [25-26].

*Measurement of $^{14}$C-AA*. Isolated mitochondria were incubated at 37 °C in MB supplemented with 30 µM $^{14}$C-AA or 30 µM $^{14}$C-AA in presence of 2.8 U/ml ascorbate oxidase. Uptake was stopped by rinsing the mitochondria twice with ice-cold MB, containing an excess of unlabeled AA. Mitochondria were then dissolved in 1 ml of 1 M NaOH and the incorporated radioactivity was measured by liquid scintillation spectrometry. The $^{14}$C-AA non-specific binding to outer membranes was determined by performing experiments at 0 °C. Under these conditions, radioactivity values detected were as low as those found in mitochondrial preparations exposed to either 0 or cold 30 µM AA.

*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.

## 2. Results

*Mitochondrial accumulation of vitamin C in cells exposed to AA, DHA or the two agents combined*

U937 cells were first treated with a 30 μM concentration of AA, DHA, or the two agents combined, and then processed to obtain the cellular, or mitochondrial, fractions for vitamin C analysis. As expected from our recent studies [27], the cells accumulated identical amounts of vitamin C under these three conditions (inset to Fig. 1A) and, while the mitochondrial accumulation was readily detected after exposure to AA, very low amounts of the vitamin were found in the mitochondria of cells treated with DHA alone (Fig. 1A). Fig. 1A also provides the novel finding that the same poor mitochondrial accumulation of the vitamin is observed after combined exposure to AA and DHA. Having previously shown that, under similar conditions, DHA inhibits cellular uptake of AA [27], we might then conclude that combined exposure to AA and DHA reproduces the conditions of exposure to DHA alone.

These results, once again, raise the important question of why cells exposed to low micromolar concentrations of DHA do not accumulate significant amounts of the vitamin in their mitochondria. More specifically, it appears unclear why under these conditions mitochondria fail to directly take up DHA or, eventually, AA resulting from DHA reduction processes.

In order to address this issue, we investigated the rate of intracellular reduction of DHA. In these experiments, the cells were exposed for increasing time intervals to 30 μM AA, or DHA, and subsequently processed for the assessment of vitamin C accumulation, with or without DTT (10 mM) treatment of the lysates prior to HPLC analysis (Fig. 1B). The results obtained are indicative of an immediate reduction of DHA back to AA, thereby leading to the straightforward conclusion that, under these conditions, direct mitochondrial uptake of DHA is limited by the poor substrate concentrations. Significant mitochondrial accumulation of vitamin C was however detected in cells exposed to very high concentrations of DHA (Fig. 1C), an observation consistent with the possibility of a direct mitochondrial uptake of the oxidised form of the vitamin. Comparison of the results obtained after exposure to AA or DHA provides evidence for an enormous difference in the mitochondrial accumulation of vitamin C associated with the two treatments. As an example, similar mitochondrial accumulation of vitamin C was measured in cells exposed to 10 μM AA or 300 μM DHA, i.e., under conditions in which the overall cellular accumulation of the vitamin was dramatically different (Fig. 1C, inset).

These results indicate that low extracellular concentrations of DHA are associated with rapid uptake and rapid reduction back to AA, so that direct mitochondrial uptake of DHA cannot take place at significant amounts. At increasing extracellular DHA concentrations, however, intracellular steady-state concentrations of DHA progressively increase, thereby enhancing the possibility of its mitochondrial uptake through hexose transporters.

The DHA concentration-dependence for the mitochondrial accumulation of the vitamin in cells exposed to 30 μM AA is illustrated in Fig. 1D. The results obtained were best described by a bimodal curve, with a fast kinetic observed at DHA concentrations $\leq$ 3 μM, and a remarkably slower kinetic at greater concentrations. For a correct interpretation of these results, it is important to examine the outcome of similar experiments however associated with the assessment of the cellular content of vitamin C. The results illustrated in the inset to Fig 1D are in keeping with our recent findings [27], thereby providing evidence for a net diminution of the cellular accumulation of AA at low DHA concentrations. Hence, reduced mitochondrial accumulation observed at low DHA concentrations is most likely due to reduced cellular uptake of AA. The effects observed at greater DHA concentrations, in which the cellular accumulation of the vitamin is increasingly, and eventually entirely based on DHA uptake (inset to Fig 1D) may be explained by DHA-dependent direct or indirect effect on mitochondrial AA transport.

*Accumulation of vitamin C in isolated mitochondria exposed to AA, DHA or the two agents combined*

We performed experiments to investigate the accumulation of vitamin C in isolated mitochondria exposed to 30 μM AA or DHA. Using radiolabelled AA with or without prior exposure to an excess of ascorbate oxidase, we obtained surprisingly similar kinetics of uptake (Fig. 2A). The 5 min exposure time-point was selected to determine that i) AA transport is sensitive to $Na^+$ omission and insensitive to Cyt B (Fig. 2B), thereby implying the exclusive uptake of the reduced form of the vitamin and ii) the amount of the radioactivity associated with exposure to the mixture ascorbate oxidase/$^{14}$C-AA is $Na^+$-independent and sensitive to Cyt B (Fig. 2B), and hence entirely resulting from DHA uptake.

We also performed experiments in which the accumulation of vitamin C was determined in isolated mitochondria exposed to increasing concentrations of AA or DHA. After treatments, DTT (10 mM) was added to the lysates prior to analysis with the HPLC method. The results illustrated in Fig. 2C provide evidence for an identical, DTT-insensitive accumulation of the vitamin in both circumstances. The notion that AA and DHA are taken up by their specific transporters was assessed as in the above experiments (Fig. 2D). Most importantly, experiments

in which AA and DHA were simultaneously added to the mitochondrial preparation demonstrated that the overall mitochondrial accumulation of the vitamin i) is the same as that achieved after exposure to only AA or DHA and ii) is both $Na^+$-independent and sensitive to the inhibitor Cyt B.

The results presented in this section collectively indicate that AA and DHA are efficiently and similarly transported into mitochondria through their respective transporters. In addition, once in the mitochondria, AA is kept in its reduced state and DHA is immediately reduced back to AA. A third important information is that the mitochondrial AA transporter might be susceptible to DHA-dependent inhibition, as previously observed in the case of plasma membrane SVCT2 [27].

*DHA-dependent inhibition of mitochondrial AA transport*

We performed experiments using purified mitochondria exposed to radiolabelled AA (30 μM) alone or associated with various inhibitors. As indicated in Fig. 3A, mitochondrial uptake of the vitamin was suppressed by established inhibitors of plasma membrane SVCT2, as 40 μM pCMB or 200 μM S-Pyr [23,28], and dramatically reduced by a 30 μM concentration of either DHA or quercetin. The ability of the latter to inhibit SVCT2-mediated transport of AA has also been well documented [29-31]. The results of concentration-dependence studies comparing the effects of DHA and quercetin are illustrated in Fig. 3B and provide evidence for a very similar inhibitory response.

We finally performed experiments to determine the reversibility of the effects mediated by DHA on mitochondrial AA transport. For this purpose, the mitochondria were first treated for 15 min with 30 μM DHA, then DHA was removed and the organelles exposed for an additional 15 min to 30 μM radiolabelled AA. As indicated in Fig. 3C, AA uptake was very little inhibited in comparison to the condition of concomitant exposure to AA and DHA. We then repeated the same experiment in mitochondria in which AA exposure was performed after a further 2.5, 5 or 15 min incubation in fresh, drug-free medium. The residual DHA-dependent inhibitory effect on AA transport was lost after only 2.5 min.

Collectively, these results indicate that DHA is a reversible inhibitor of the mitochondrial transporter of AA.

## 3. Discussion

We recently reported that exposure of U937 cells to low micromolar concentrations of AA, or DHA, promotes the accumulation of similar amounts of vitamin C, however with a mitochondrial uptake restricted to exposure to AA [32]. The present study provides results in line with this notion, with the additional demonstration that combined treatment with DHA and AA leads to the same cellular and mitochondrial accumulation of the vitamin observed after treatment with DHA alone (Fig. 1A and its inset). Thus, while cellular uptake of AA appears linked to a rapid transport of the vitamin in mitochondria, the results obtained with DHA alone, or combined with AA, are of more complex interpretation. More specifically, DHA is the species crossing the plasma membrane in both of these conditions [27] and it is unclear why, even at fairly high concentrations (e.g., 30 μM), it is poorly taken up by the mitochondria, either directly or as DHA-derived AA.

In order to address these questions, we performed time-course studies and obtained evidence for a very rapid reduction of DHA, once transported in the cells (Fig 1B). Rapid conversion of DHA to AA has been demonstrated to occur in several other cell types [33-35] and this event is expected to decrease the possibility of a direct mitochondrial uptake of DHA.

A second information derives from studies performed in isolated mitochondria, providing evidence of similar transport kinetics of AA or DHA, both in terms of time- (Fig. 2A and B) and concentration- (Fig. 2C and D) dependence. These experiments also demonstrate that, once in the mitochondria, AA is kept in its reduced state and DHA is instead immediately reduced back to AA (Fig. 2C).

Collectively, the above observations rule out the possibility that lack of mitochondrial accumulation of vitamin C observed in cells exposed to low micromolar concentrations of DHA is due to poor mitochondrial expression of hexose transporters. Rather, it appears that cytosolic levels of DHA, because of its rapid reduction back to AA, are too low to be taken up in significant amounts by the mitochondria. On the other hand, these organelles present a high capacity -albeit low affinity- transport system for DHA (Fig. 2A-D) and exposure of the cells to very high levels of DHA was indeed associated with a mitochondrial accumulation of the vitamin (Fig. 1C). Remarkably, similar mitochondrial accumulation of vitamin C was observed after exposure to 10 μM AA or 300 μM DHA, i.e., conditions in which there was an about 15 fold difference in the overall cellular accumulation of the vitamin (inset to Fig. 1C). Hence, it appears that exposure to these high extracellular DHA concentrations, while associated with rapid uptake

and intracellular reduction, nevertheless results in cytosolic DHA levels that can be taken up by the mitochondria in significant amounts.

There is, however, a more important consideration related to the high intracellular concentrations of DHA-derived AA, which leads to the second question of why aren't these processes associated with the mitochondrial uptake of the reduced form of AA. Intracellular AA should indeed be a substrate of its specific mitochondrial transporters, regardless of whether directly taken up by the cells or resulting from DHA reductive processes. A likely explanation for these results is that the mitochondrial AA transporter is susceptible to DHA-dependent inhibition, as previously observed in the case of plasma membrane SVCT2 [27].

Our results obtained in studies in which the cells were simultaneously exposed to the reduced and oxidized forms of the vitamin (Fig. 1A) are consistent with the possibility of a DHA-dependent inhibition of the cellular uptake of AA. Mitochondrial accumulation of vitamin C in cells exposed to 30 μM AA sharply declined upon addition of 3 μM DHA (Fig. 1D), i.e., under conditions in which the overall cellular accumulation also declined (Fig. 1D, inset). This interesting phenomenon is also based on the reduced cellular uptake of AA mediated by low concentrations of DHA [27]. More complex is instead the explanation of the effects observed at greater DHA concentrations, in which, the accumulation of the vitamin is increasingly, and eventually entirely, based on DHA uptake (Fig.1D, inset). Increased uptake of DHA is associated with an increase in its steady-state concentrations, thereby enhancing the possibility of producing direct effects, as inhibition of mitochondrial AA transport. Clearly, at the highest DHA concentration tested (30 μM), we are back to the same conditions described in Fig. 1A, in which, only DHA enters the cells (because of complete inhibition of AA transport across the plasma membrane, Fig. 3B) and this condition is associated with poor mitochondrial accumulation of vitamin C.

The similarities between the above results and those obtained using isolated mitochondria exposed to the same concentrations of AA or DHA are striking. In both circumstances combined exposure to the two forms of the vitamin caused the accumulation of the same amount of vitamin C observed after exposure to either AA or DHA alone (inset to Fig. 1A and Fig. 2D). Furthermore, the same combined treatment caused a mitochondrial accumulation of the vitamin sensitive to Cyt B and insensitive to $Na^+$ omission, as in the case of exposure to DHA alone (Fig. 2D). Identical results were previously obtained in cell uptake studies [27].

Direct evidence of DHA-mediated inhibition of AA transport in mitochondria is provided by studies using radiolabelled AA: DHA suppressed the uptake of identical concentrations of radiolabelled AA (Fig. 3A), thereby reproducing the effects of other established inhibitors of the

plasma membrane AA transporters [23,28,30], as the thiol reactive agent pCMB, S-Pyr or quercetin. Moreover, DHA and quercetin displayed a remarkably similar concentration-dependence for inhibition of AA transport in mitochondria (Fig. 3B). A final important characterization of the inhibitory effect of DHA on AA mitochondrial transport is represented by the demonstration of its rapid reversibility (Fig. 3C).

While the precise mechanism(s) involved in the above inhibitory effect remains to be defined also for the plasma membrane AA transporter, the possibility that DHA elicits its effects via interaction with critical -SH groups appears plausible in both circumstances. The presence of thiols in SVCT2 is well documented [36-37] and the activity of both the plasma membrane [38] and mitochondrial (Fig. 3B) SVCT2 is indeed susceptible to inhibition by thiol-reactive agents. In addition, DHA is known to react with -SH groups [28] and to inhibit the activity of enzymes containing critical cysteines [17-18].

The above strategy of regulation of vitamin C accumulation is of complex interpretation, as DHA is produced in the presence of oxidants, i.e., conditions in which the cells are expected to benefit of increased cellular and mitochondrial vitamin C, a well established antioxidant [8-9]. Unexpected advantages may however arise after transient inhibition of AA uptake in cells and mitochondria, under conditions in which the vitamin may rather causes deleterious effects, as reduction and mobilization of redox-active metals [39-41]. Furthermore, the vitamin has been reported to enhance superoxide formation in cells producing/exposed to biological hydroperoxides [21,32,42] and to elicit complications in specific human pathologies [43] and conditions [44].

In conclusion, our results indicate that SVCT2 [10] is a major physiological transporter in the mitochondria of the cell type employed in this study, and presumably of other cell types characterized by similar AA transporter densities and DHA reductive capacities. In these conditions, DHA is not a likely precursor for mitochondrial vitamin C accumulation. At low intracellular levels, presumably compatible with those achievable "*in vivo*", DHA may rather function as an inhibitor of mitochondrial transport of AA.

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## Figure legends

**Fig. 1.** Mitochondrial accumulation of vitamin C in cells exposed to AA, DHA or the two agents combined. A) The cells were exposed for 15 min to 30 µM AA, or DHA, either alone or combined, and finally analyzed for vitamin C accumulation (inset) or processed for the isolation of the mitochondrial fraction prior to vitamin C analysis (main graph). 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). B) The cells were first exposed for increasing time intervals to 30 µM AA, or DHA, and then processed for vitamin C analysis, with or without addition of 10 mM DTT to the lysates. Results represent the means ± SD calculated from at least 3 separate experiments. C) The cells were exposed for 15 min to increasing concentrations of AA, or DHA, and then processed for the isolation of the mitochondrial fraction prior to vitamin C analysis (main graph). The inset shows the amount of vitamin C accumulated by the cells after exposure to 10 µM AA or 300 µM DHA. Results represent the means ± SD calculated from at least 3 separate experiments. D) Vitamin C accumulation in the mitochondria of cells exposed for 15 min to 30 µM AA and increasing concentrations of DHA (main graph). The inset shows the results of similar experiments in which the vitamin C content was estimated in total cell extracts (○ ). For comparison, vitamin C content after exposure to DHA alone is also reported ( ● ). Results represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.001$, as compared to exposure to AA alone (two-way ANOVA followed by Bonferroni's test).

**Fig. 2.** Accumulation of vitamin C in isolated mitochondria exposed to AA, DHA, or the two agents combined. A) Time-dependence of accumulation of vitamin C in isolated mitochondria exposed to 30 µM $^{14}$C-AA or 30 µM $^{14}$C-DHA (obtained by addition of an excess of ascorbate oxidase to 30 µM $^{14}$C-AA). Results represent the means ± SD calculated from 3 separate experiments. B) Isolated mitochondria were exposed to radiolabelled AA, or DHA, both at 30 µM, and then analyzed for vitamin C accumulation. Treatments were performed in MB supplemented with Cyt B, or manipulated to replace sodium-EGTA with sodium-free EGTA. Results represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.001$, as compared to the first bar of each set (one-way ANOVA followed by Dunnet's test). C) Concentration-dependence of the accumulation of vitamin C in isolated mitochondria exposed for 15 min to AA, or DHA, with or without addition of 10 mM DTT to the lysates prior to HPLC analysis. Results represent the means ± SD calculated from at least 3 separate experiments. D)

Isolated mitochondria were exposed to 30 μM AA, or DHA, either alone or combined, and finally analyzed for vitamin C accumulation. Treatments were performed in MB, with the manipulations described in (B). Results represent the means ± SD calculated from at least 3 separate experiments. *$P < 0.001$, as compared to the first bar of each set (one-way ANOVA followed by Dunnet's test).

**Fig. 3.** DHA-dependent inhibition of AA uptake in isolated mitochondria. A) Isolated mitochondria were exposed for 15 min to 30 μM $^{14}$C-AA alone, or associated with Cyt B (25 μM), pCMB (40 μM), S-Pyr (200 μM), DHA (30 μM) or quercetin (30 μM) and then processed for the assessment of vitamin C accumulation. 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). B) Isolated mitochondria were exposed for 15 min to 30 μM $^{14}$C-AA, in the absence or presence of increasing concentrations of either DHA or quercetin, and then processed for the assessment of vitamin C accumulation. Results represent the means ± SD calculated from at least 3 separate experiments. C) Reversibility of the DHA-dependent inhibition of mitochondrial AA transport. Mitochondria were treated for 15 min with 30 μM DHA (time -15 min to 0), centrifuged and then exposed for an additional 15 min to 30 μM $^{14}$C-AA, either immediately (time 0) or after a 2.5, 5 or 15 min incubation in fresh medium. After treatments, the radioactivity associated with the mitochondria was measured as described in the Methods section. For comparison, results obtained under conditions of combined exposure to 30 μM $^{14}$C-AA and 30 μM DHA are also included (data in ordinate axis, ▲). Results represent the means ± SD calculated from at least 3 separate experiments.

**Conflict of interest**

All authors declare no conflict of interest related to the present work.

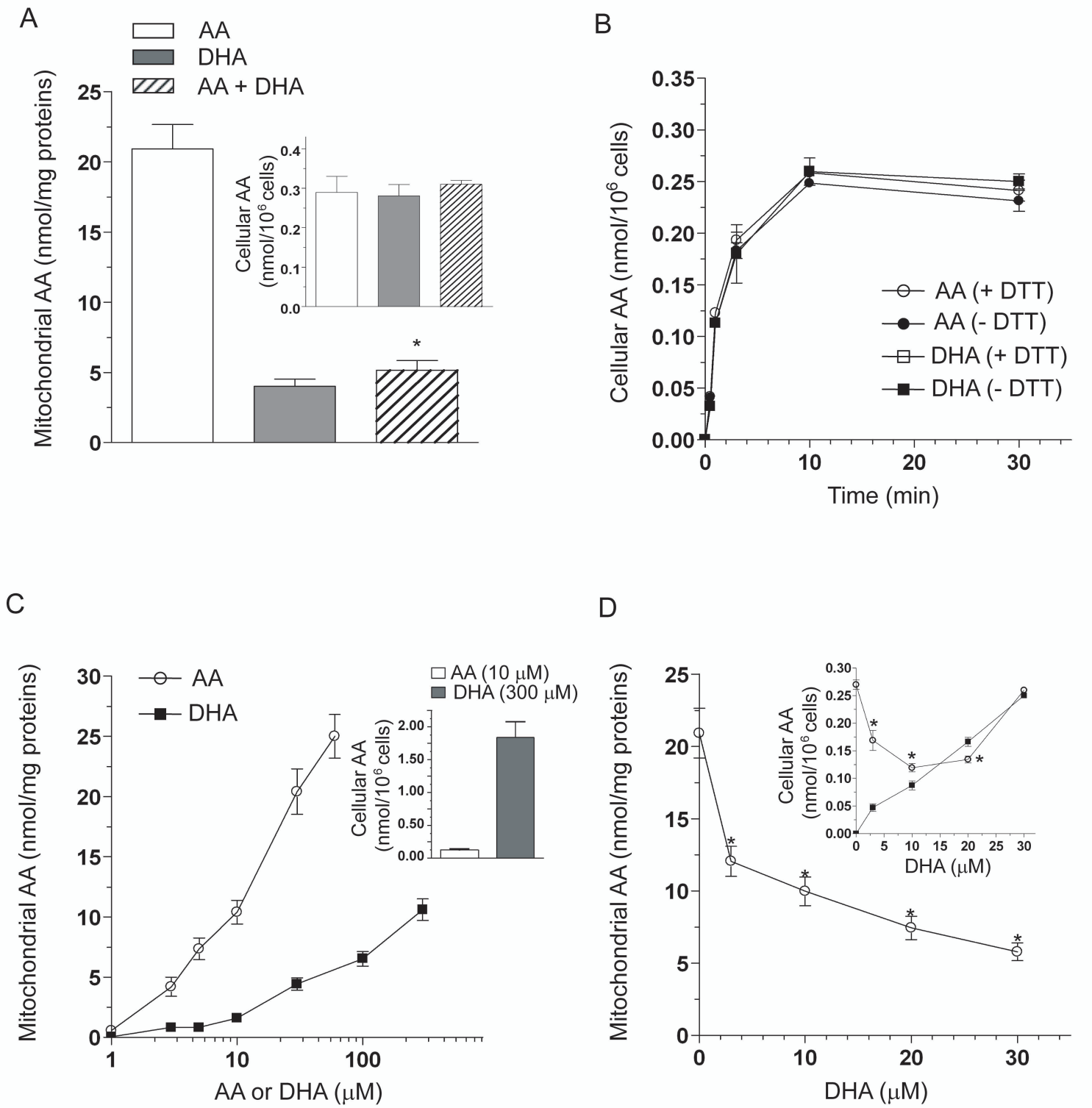

A
AA
DHA
AA + DHA
Mitochondrial AA (nmol/mg proteins)
Cellular AA (nmol/10^6 cells)
*
B
Cellular AA (nmol/10^6 cells)
AA (+ DTT)
AA (- DTT)
DHA (+ DTT)
DHA (- DTT)
Time (min)
C
AA
DHA
AA (10 μM)
DHA (300 μM)
Mitochondrial AA (nmol/mg proteins)
Cellular AA (nmol/10^6 cells)
AA or DHA (μM)
D
Mitochondrial AA (nmol/mg proteins)
Cellular AA (nmol/10^6 cells)
DHA (μM)

Figure 1

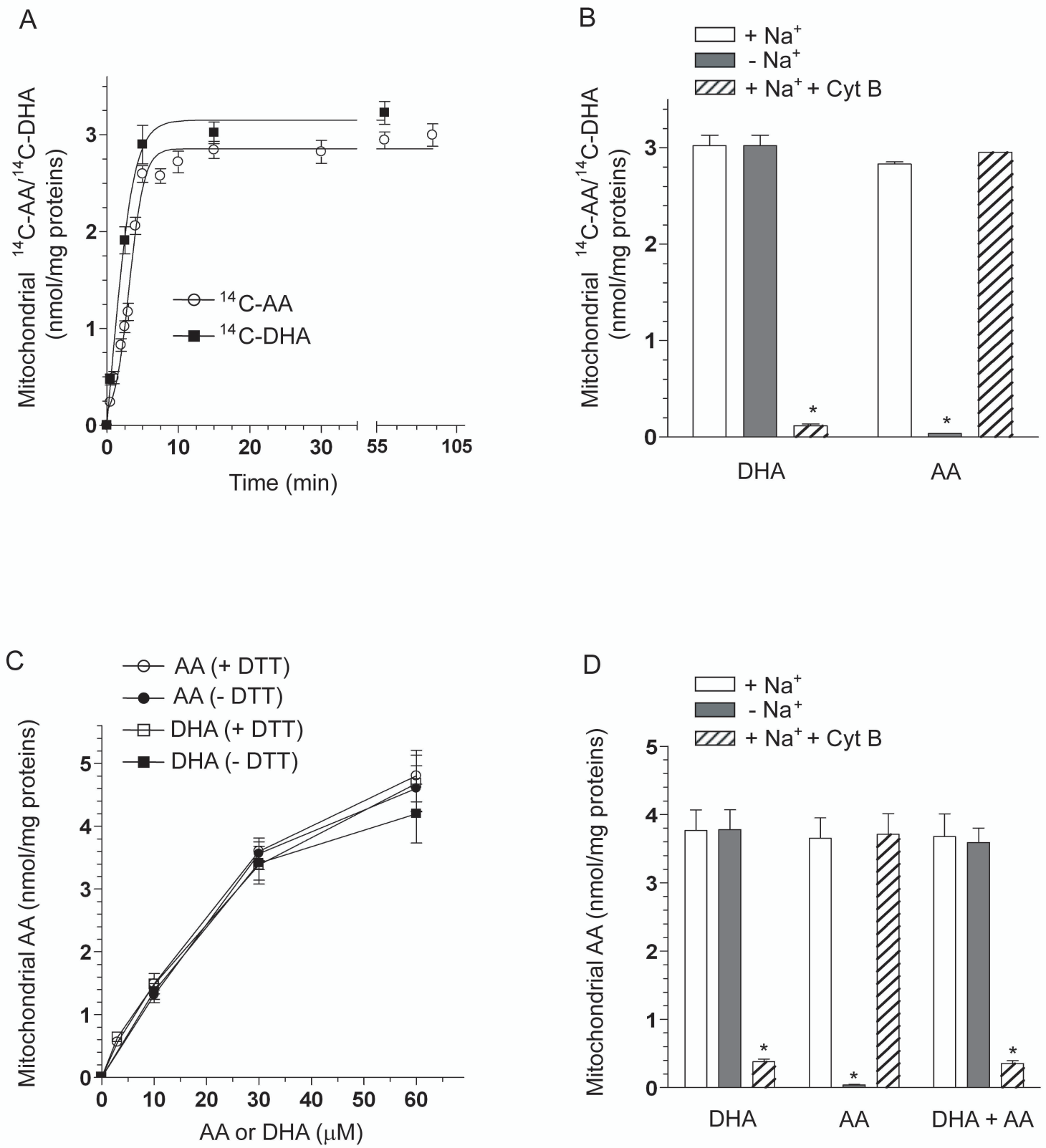
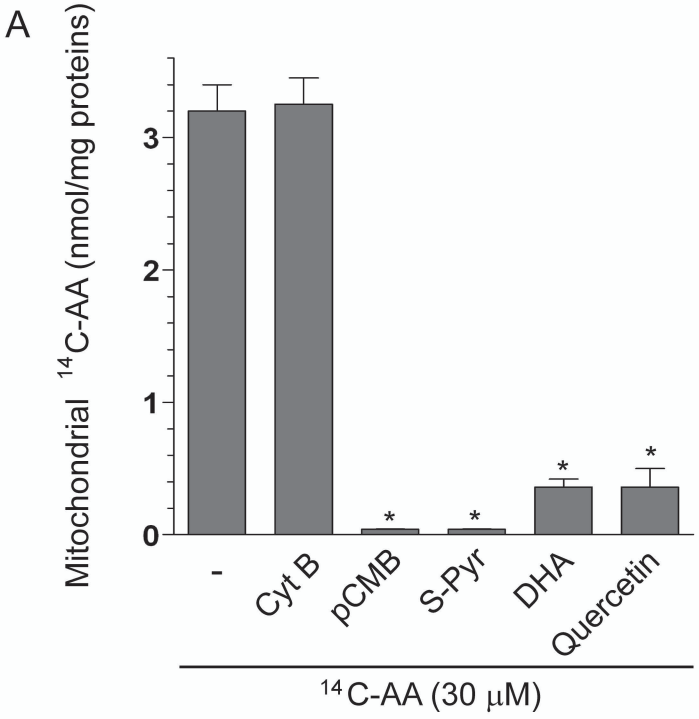
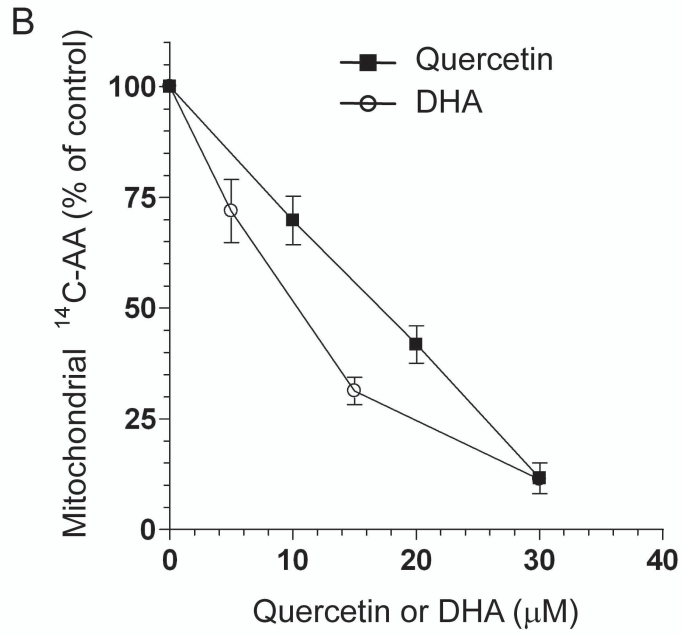

A
Mitochondrial $^{14}$C-AA/$^{14}$C-DHA (nmol/mg proteins)
$^{14}$C-AA
$^{14}$C-DHA
Time (min)
B
+ Na$^+$
- Na$^+$
+ Na$^+$ + Cyt B
Mitochondrial $^{14}$C-AA/$^{14}$C-DHA (nmol/mg proteins)
DHA
AA
C
AA (+ DTT)
AA (- DTT)
DHA (+ DTT)
DHA (- DTT)
Mitochondrial AA (nmol/mg proteins)
AA or DHA (μM)
D
+ Na$^+$
- Na$^+$
+ Na$^+$ + Cyt B
Mitochondrial AA (nmol/mg proteins)
DHA
AA
DHA + AA

Figure 2


A
Mitochondrial $^{14}$C-AA (nmol/mg proteins)
3
2
1
0
*
*
*
*
-
Cyt B
pCMB
S-Pyr
DHA
Quercetin
$^{14}$C-AA (30 μM)


B
Mitochondrial $^{14}$C-AA (% of control)
Quercetin
DHA
100
75
50
25
0
0
10
20
30
40
Quercetin or DHA (μM)

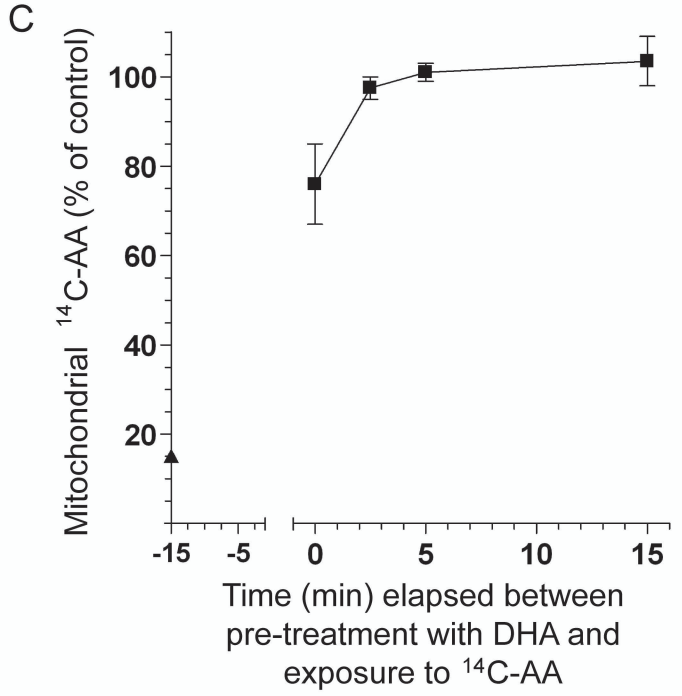

C
Mitochondrial $^{14}$C-AA (% of control)
100
80
60
40
20
-15
-5
0
5
10
15
Time (min) elapsed between
pre-treatment with DHA and
exposure to $^{14}$C-AA

Figure 3