Research Communication

Sodium-Dependent Transport of Ascorbic Acid in U937 Cell Mitochondria

Catia Azzolini[†]
Mara Fiorani[†]
Liana Cerioni
Andrea Guidarelli
Orazio Cantoni[*]

Department of Biomolecular Science, University of Urbino "Carlo Bo", 61029 Urbino, Italy

Abstract

U937 cells exposed to physiological concentrations of ascorbic acid (AA) accumulate the reduced form of the vitamin in the cytosol and even further in their mitochondria. In both circumstances, uptake was dependent on Na^+-AA-cotransport, with hardly any contribution of hexose transporters, which might be recruited to transport the oxidized form of the vitamin. There was an identical linear relationship between the mitochondrial accumulation of the vitamin and the extramitochondrial AA concentration, regardless of whether detected in experiments using intact cells or isolated mitochondria. Western blot experiments revealed expression of both SVCT1 and 2 in plasma membranes, whereas SVCT2 was the only form of the transporter expressed at appreciable amounts in mitochondria. These results therefore provide the novel demonstration of SVCT2-dependent mitochondrial transport of AA and hence challenge the present view that mitochondria only take up the oxidized form of the vitamin. © 2013 IUBMB Life, 65(2):149–153, 2013

Keywords: *ascorbic acid; dehydroascorbic acid; Na^+-dependent transport; vitamin C uptake mitochondria; U937 cells*

Introduction

Ascorbic acid (AA), the reduced form of vitamin C, is taken up by most cell types via a high affinity/low capacity mechanism through Na^+-AA cotransporters (SVCT1 and 2) (1,2). Under these conditions, cells accumulate millimolar concentrations of the vitamin even when exposed to low micromolar concentrations of AA, that is, comparable to those detected in most biological fluids (30–50 μM) (3,4). There are some cells, however, which poorly express SVCTs and therefore potentially accumulate lower amounts of vitamin C. In this perspective, different levels of expression of SVCTs might reflect different cellular requirements of vitamin C and indeed SVCT expression is critically regulated both at the transcriptional and translational levels (5).

An alternative strategy for vitamin C accumulation is based on the uptake of dehydroascorbic acid (DHA) through hexose transporters, however hampered by the very low availability of extracellular DHA, normally present at concentrations below 2 μM (6). On the other hand, under conditions associated with extracellular oxidation of AA, DHA concentrations may locally arise, thereby promoting a potentially effective mechanism for vitamin C accumulation (7).

In summary, while it appears clear that most cell types take up vitamin C as AA, via high affinity/low capacity mechanisms, some cells might under specific conditions take up DHA via a low affinity/high capacity mechanism, however with the important limitation of the low extracellular concentrations of DHA.

There has been some interest (8,9) on the possibility that AA might accumulate in the mitochondria, as its antioxidant activity would be expected to be beneficial for these organelles characterized by the extensive formation of reactive oxygen species. In this perspective, the intracellular environment further polarizes some of the differences noted for the extracellular milieu, with potentially very high concentrations of AA and extremely low levels of DHA.

Abbreviations: *AA, ascorbic acid; DHA, dehydroascorbic acid; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; pCMB, 4-hydroxymercuribenzoic acid; S-pyr, sulfinpyrazone; TBA, tetrabutylammonium hydrogen sulfate.*

© 2013 International Union of Biochemistry and Molecular Biology, Inc.

Volume 65, Number 2, February 2013, Pages 149-153

[†]*Catia Azzolini and Mara Fiorani contributed equally to this work*

**Address for correspondence to: Prof. Orazio Cantoni, Department of Biomolecular Science, University of Urbino, Via Santa Chiara, 27, 61029 Urbino (PU), Italy. Tel: +39-0722-303523. Fax: +39-0722-303521. E-mail: orazio.cantoni@uniurb.it.*

Received 6 September 2012; accepted 29 November 2012

DOI: 10.1002/iub.1124

Published online 3 January 2013 in Wiley Online Library (wileyonlinelibrary.com)

Despite this important limitation, most studies have primarily focused on the mitochondrial uptake of DHA, made possible by the expression of hexose transporters in the mitochondrial membranes (9). Based on the above considerations, however, mitochondrial accumulation of DHA would be restricted to conditions associated with extensive oxidation of cellular AA.

The present study was carried out with the purpose of investigating the existence of Na^+-dependent transport of AA in the mitochondria of cultured mammalian cells.

Materials and Methods

Chemicals

AA, DHA, dithiothreitol (DTT), tetrabutylammonium hydrogen sulfate (TBA), ethylenediaminetetraacetic acid (EDTA), cytochalasin B, choline chloride, 4-hydroxymercuribenzoic acid (pCMB), sulfinpyrazone (S-pyr) 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 (10).

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% foetal 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, Corning, NY) gassed with an atmosphere of 95% air-5% CO_2. A 10 mM AA stock solution was prepared in incubation buffer, IB (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 (1×10^6 cells/mL) were treated with AA for 15 min at 37 °C in IB supplemented with 0.1 mM DTT. Stability of AA in IB 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. (11). 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 High Performance Liquid Chromatography (HPLC)

Cells were washed twice with cold saline solution (8.182 g/L NaCl, 0.372 g/L KCl, 0.336 g/L $NaHCO_3$, 0.9 g/L glucose, pH 7.4) 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. After 10 min at ice bath temperature, 10 mM DTT was added to the samples and centrifuged at 10,000*g* for 20 min at 4 °C. Where indicated, 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 refs. 4,12. Cellular and mitochondrial concentrations of AA were calculated using published values for cell (13) and mitochondrial (14) volumes.

Measurement of ^{14}C-AA Mitochondrial Uptake

Isolated mitochondria were incubated at 37 °C in MB supplemented with 30 μM L-[1-^{14}C]-AA. Uptake was stopped by rinsing the mitochondria twice with ice-cold MB. Mitochondria were then dissolved in 1 mL of 1 M NaOH and the incorporated radioactivity was measured by liquid scintillation spectrometry.

Sub-Cellular Fractionation and Western Blot Analysis

The cells were processed to obtain the mitochondrial and membrane fractions, as described in ref. 15. Equal amounts (30 μg) of the mitochondrial and membrane fractions were resolved in 15% sodium dodecyl sulfate polyacrylamide gel and electrotransferred to polyvinyldiene difluoride membranes. Western blot analyses were performed using antibodies against SVCT1 (N-20: sc-9924) and 2 (S-19: sc-9926), cytochrome *c* and actin (Santa Cruz, Santa Cruz, CA). Details on Western blotting apparatus and conditions are reported elsewhere (15). Antibodies against cytochrome *c* and actin were used to assess the purity of the fractions.

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.

Results and Discussion

The results illustrated in Fig. 1A are from experiments in which the cells were exposed for 15 min to 0–30 μM AA in IB supplemented with 0.1 mM DTT to prevent AA oxidation during treatments. There was a linear relationship between the initial AA concentration and the fraction of vitamin C associated with the cells, unaffected by addition of 10 mM DTT to the lysates prior to analysis. As indicated in the inset to Fig. 1A, AA uptake was both insensitive to cytochalasin B (25 μM), a well established inhibitor of hexose transporters (12), and abolished by Na^+ omission (replaced with coline) (12).

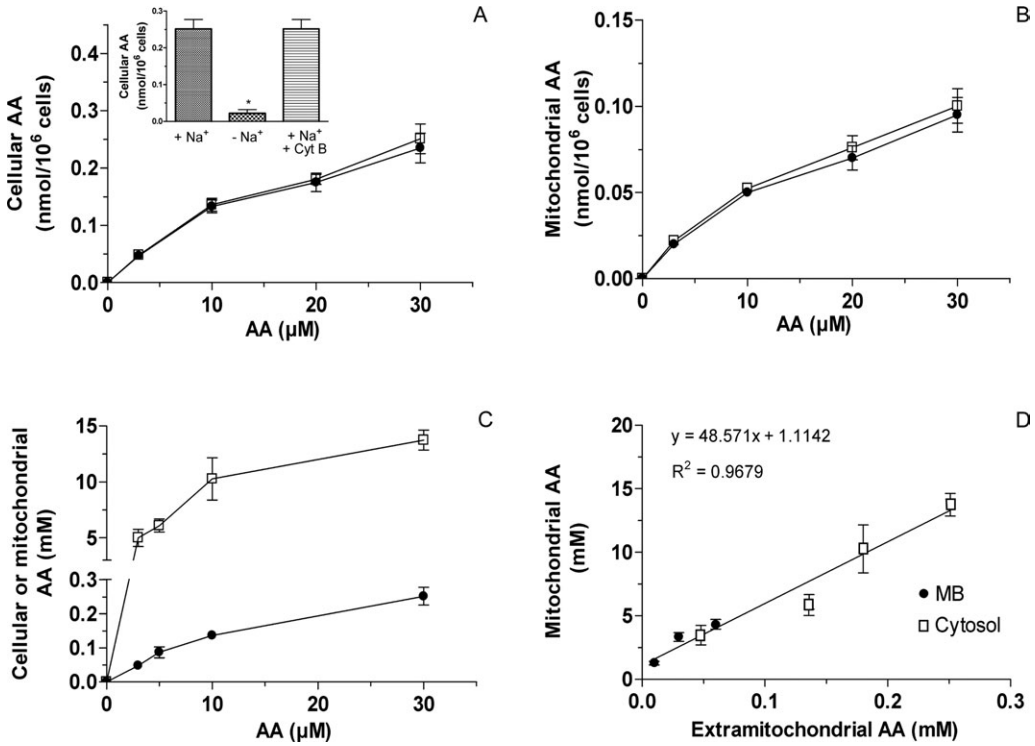

FIG 1

*Cellular and mitochondrial uptake of AA in U937 cells. A: AA content in cells exposed for 15 min to 0–30 μM AA in IB. Samples were processed for AA analysis with (□) or without (●) DTT (10 mM), as detailed in the Materials and Methods. Inset: AA accumulation was determined in cells exposed for 15 min to 30 μM AA. Treatments were performed in IB supplemented with cytochalasin B (cyt B), or manipulated to replace sodium with choline (-Na^+). B: AA content in mitochondria of cells exposed for 15 min to 0–30 μM AA in IB. Samples were processed for mitochondria isolation and AA analysis with (□) or without (●) DTT (10 mM), as detailed in the Materials and Methods. C: Cellular (●) and mitochondrial (□) concentrations of AA in cells exposed to increasing concentration of AA. D: Correlation plot in which mitochondrial AA concentrations are plotted against the extramitochondrial concentration of AA. Experimental results were obtained using both isolated mitochondria (●) and intact cells (□). Results represent the means ± SD calculated from at least three separate experiments. *P < 0.001, compared to cells treated in the Na^+-containing incubation buffer (ANOVA followed by Dunnet's test).*

These results indicate that vitamin C accumulation resulting from exposure to low micromolar concentrations of AA is largely dependent on AA uptake through Na^+-dependent transporters, with hardly any contribution of DHA uptake, which might take place as a consequence of extracellular oxidation of AA. A second important consideration is that intracellular vitamin C is kept in its reduced form under the specific conditions employed.

Results illustrated in Fig. 1B provide evidence for mitochondrial accumulation of vitamin C, that appears to be both proportional to the extracellular concentrations of AA and insensitive to incubation of the lysates with 10 mM DTT prior to analysis. Interestingly, the putative intramitochondrial concentrations of the vitamin were significantly greater than those found in the cytosol (Fig. 1C). As an example, exposure of U937 cells to 10 μM AA resulted in the accumulation of about 150 μM AA and this concentration increased to more than 10 mM in the mitochondrial compartment.

We next performed experiments with isolated mitochondria. These organelles effectively took up AA and, in analogy with the results obtained with intact cells, mitochondrial accumulation of vitamin C was (i) unaffected by prior incubation of the lysates with 10 mM DTT (Fig. 2A); (ii) insensitive to cytochalasin B (Fig. 2B); and (iii) abolished by Na^+ omission (Fig. 2B). As a final note, mitochondrial accumulation of vitamin C was also suppressed by two well established inhibitors of SVCTs, that is, pCMB (16) and S-Pyr (10) (Fig. 2B).

These results provide functional evidence for Na^+-dependent transport of AA in mitochondria, similar to that found in plasma membrane (Fig. 1A and (1,2)). The interdependence between these events is emphasized by the existence of a strong correlation between the intramitochondrial and extramitochondrial concentrations of AA, in both intact cells (Fig. 1B) and isolated mitochondria (Fig. 2A). The linear relationship clearly detected ($R^2 = 0.9679$) despite the different conditions and over a wide range of extramitochondrial concentrations of AA is illustrated in Fig. 1D. These results imply the use of the same transporters, most likely highly expressed in the mitochondrial membranes. The elevated intramitocondrial

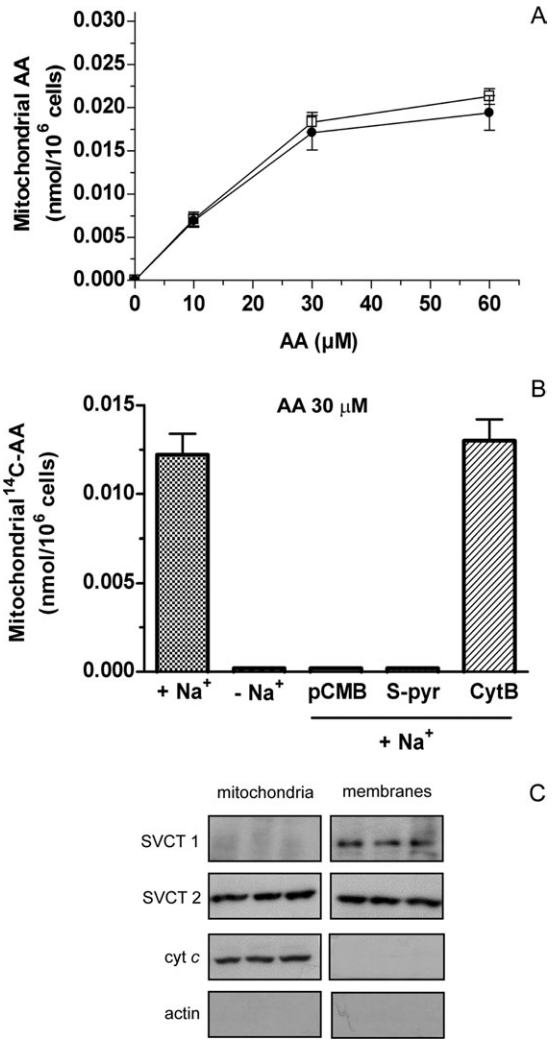

FIG 2

AA uptake in isolated mitochondria is mediated by a Na^+-dependent mechanism. A: AA content in isolated mitochondria exposed for 15 min to 0–60 μM AA in MB. Samples were processed for AA analysis with (□) or without (●) DTT (10 mM), as detailed in the Materials and Methods. Results represent the means ± SD calculated from at least three separate experiments. B: AA accumulation in isolated mitochondria exposed to 30 μM ^{14}C-AA. Treatments were performed in MB supplemented with 40 μM pCMB, 200 μM S-pyr, 25 μM cytB, or manipulated to replace sodium-EGTA with sodium-free EGTA. Results represent the means ± SD calculated from at least three separate experiments. C: Mitochondrial and plasma membrane fractions obtained from three different lots of untreated U937 cells were processed for Western blot analysis using antibodies against SVCT1 and 2. Blots were re-probed for cytochrome c (cyt c) or actin. Similar outcomes were obtained in experiments using two additional cell preparations (not shown).

concentrations of AA appear therefore uniquely dependent on the high cytosolic concentrations of the vitamin achieved during the exposure to low micromolar concentrations of AA.

Figure 2C provides evidence for expression of SVCT2 in both compartments, whereas SVCT1 was uniquely expressed in the plasma membrane. Lack of expression of SVCT1 in mitochondria provides an excellent internal control to rule out the possibility that the observed expression of SVCT2 is in fact attributable to the presence of plasma membrane contaminants. The mitochondrial fraction was associated with the expression of cytochrome *c* in the absence of actin, whereas none of these proteins was detectable in plasma membrane.

Mitochondria are the major source of reactive oxygen species, generated as byproducts of the electron transport chain and producing deleterious effects to various biological targets (17). Not surprisingly, these organelles accumulate various lipid soluble antioxidants and it is still unclear whether the same is true for vitamin C.

Previous studies (9) addressed the possibility of a mitochondrial uptake of the oxidized form of vitamin C and demonstrated that hexose transporters are indeed expressed also in the mitochondrial membranes. We now extend these findings with the demonstration of SVCT2-dependent transport of physiological concentrations of AA in U937 cell mitochondria. While cells grown in culture medium containing very low concentrations of vitamin C likely upregulate high affinity transporters, their overexpression in the mitochondrial membrane argues in favor of an important role of vitamin C in these organelles.

At present, we are unable to predict whether mitochondrial transport of AA is a general phenomenon or is rather restricted to some cell types, as U937 cells. We have indeed to remind that the general view is that DHA -and not AA- is taken up by mitochondria. While this notion is based on a surprisingly limited information (14), failure to detect mitochondrial AA uptake in these studies might relate to existing high mitochondrial AA concentrations.

In conclusion, more work is needed to establish the generality and relevance of the mechanism described in this study. At present, it can only be suggested that, as extensively discussed for plasma membrane transport (4,5,18), mitochondrial uptake of vitamin C may also significantly vary (AA vs. DHA) in different cell types and in different pathological conditions.

References

[1] Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U. V., Chen, X.-Z. et al. (1999) A family of mammalian Na^+-dependent L-ascorbic acid transporters. Nature 399, 70–75.

[2] Liang, W. J., Johnson, D., and Jarvis, S. M. (2001) Vitamin C transport systems of mammalian cells. Mol. Membr. Biol. 18, 87–95.

[3] Lane, D. J. R. and Lawen, A. (2009) Ascorbate and plasma membrane electron transport—enzymes vs efflux. Free Radic. Biol. Med. 47, 485–495.

[4] Azzolini, C., Fiorani, M., Guidarelli, A., and Cantoni O. (2012) Studies with low micromolar levels of ascorbic and dehydroascorbic acid fail to unravel a preferential route for vitamin C uptake and accumulation in U937 cells. Br. J. Nutr. 107, 691–696.

[5] May, J. M. (2011) The SLC23 family of ascorbate transporters: ensuring that you get and keep your daily dose of vitamin C. Br. J. Pharmacol. 164, 1793–1801.

[6] Rumsey, S. C. and Levine, M. (1998) Absorption, transport, and disposition of ascorbic acid in humans. J. Nutr. Biochem. 9, 116–130.
[7] Nualart, F. J., Rivas, C. I., Montecinos, V. P., Godoy, A. S., Guaiquil, V. H., et al. (2003) Recycling of vitamin C by a bystander effect. J. Biol. Chem. 278, 10128–10133.
[8] May, J. M., Li, L., Qu, Z.-C., and Cobb, C. E. (2007) Mitochondrial recycling of ascorbic acid as a mechanism for regenerating cellular ascorbate. Biofactors 30, 35–48.
[9] Sagun, K. C., Cárcamo, J. M., and Golde, D.W. (2005) Vitamin C enters mitochondria via facilitative glucose transporter 1 (Glut1) and confers mitochondrial protection against oxidative injury. FASEB J. 19, 1657–1667.
[10] Qiao, H. and May, J. M. (2009) Macrophage differentiation increases expression of the ascorbate transporter (SVCT2). Free Radic. Biol. Med. 46, 1221–1232.
[11] Fiorani, M., Guidarelli, A., Blasa, M., Azzolini, C., Candiracci, M., et al. (2010) Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid. J. Nutr. Biochem. 21, 397–404.
[12] Savini, I., Duflot, S., and Avigliano, L. (2000) Dehydroascorbic acid uptake in a human keratinocyte cell line (HaCaT) is glutathione-independent. Biochem. J. 345, 665–672.
[13] Perez-Cruz, I., Carcamo, J. M., and Golde, D. W. (2003) Vitamin C inhibits FAS-induced apoptosis in monocytes and U937 cells. Blood 102, 336–343.
[14] Li, X., Cobb, C. E., Hill, K. E., Burk, R. F., and May, J. M. (2001) Mitochondrial uptake and recycling of ascorbic acid. Arch. Biochem. Biophys. 387, 143–153.
[15] Cantoni, O., Tommasini, I., and Cerioni, L. (2008) The arachidonate-dependent survival signaling preventing toxicity in monocytes/macrophages exposed to peroxynitrite. Methods Enzymol. 441, 73–82.
[16] May, J. M. and Qu, Z.-C. (2004) Redox regulation of ascorbic acid transport: role of transporter and intracellular sulfhydryls. Biofactors 20, 199–211.
[17] Orrenius, S. (2007) Reactive oxygen species in mitochondria-mediated cell death. Drug Metab. Rev. 39, 443–455.
[18] Savini, I., Rossi, A., Pierro, C., Avigliano, L., Catani, M. V. (2008) SVCT1 and SVCT2: key proteins for vitamin C uptake. Amino Acids 34, 347–355.