MITOCHONDRIAL UPTAKE AND ACCUMULATION OF VITAMIN C: WHAT CAN WE LEARN FROM CELL CULTURE STUDIES?

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Abstract

Cultured cells are normally grown under oxidative conditions and in a growth medium deprived of some essential antioxidants, as ascorbic acid (AA). Most of the cell types overexpress high affinity SVCT2 in culture and hence rapidly accumulate AA, even when added at very low concentrations. The oxidized form of vitamin C, dehydroascorbic acid (DHA), may also be taken up by the cells through facilitative hexose transporters and then rapidly reduced back to AA. The DHA concentrations, however, are normally very low in biological fluids, although an increased local concentration is to be expected next to superoxide releasing cells. These conditions may result in significant DHA transport and inhibition of SVCT2-dependent AA transport. The prompt intracellular reduction of DHA to AA blunts the possibility of its uptake through mitochondrial GLUTs. On the other hand, superoxide released by complex III in the intermembrane space may locally oxidise AA and increase the DHA concentration in the close proximity of the GLUTs, thereby favoring its mitochondrial accumulation. Mitochondrial SVCT2, is susceptible to inhibition by DHA and, as a consequence of the restrictive ionic conditions, may transport AA with low affinity. In some cell types however, may even maintain the same affinity of the transporter expressed in the plasma membrane. DHA is rapidly reduced to AA in the matrix by various mechanisms. It can also accept the electrons released by complex III, thereby contributing to the antioxidant response associated with the uptake and recycling of vitamin C.

I. Introduction

Although widely used in research laboratories from all over the world, cultured cells -in particular tumor cell lines- are often criticized for the poor biological significance of the experimental results they provide. This criticism generally applies to virtually all types of studies, but appears particularly appropriate when the questions addressed are related to physiological, toxicological and pathological issues related to oxidative stress. This statement is based on the simple consideration that maintenance and growth of cells in culture is under basal conditions already associated with an oxidative stress, since the oxygen tension is about ten times higher than the one experienced by the cells *in vivo*. Under these non-physiological conditions, basal rates of mitochondrial superoxide ($O_2^{-\cdot}$) formation are significantly increased by the enhanced availability of molecular oxygen (19,39-40), with the ensuing triggering of pro-survival responses (5,115). The overall scenario is then further complicated by the remarkable differences of these adaptive responses, often based on the cell type, in particular when associated to the genomic instability of cultured tumor cells. Among other considerations, not reported here for the sake of brevity, a final one refers to the composition of conventional growth media used to keep cells in culture, which on the one hand often contain ingredients promoting oxidative stress (39-41) and on the other may not contain all essential components of the antioxidant defense. Good examples are represented by vitamin E and vitamin C (L-ascorbic acid, AA) (39-40,107), normally not added to the culture media because the first one is poorly soluble and the second unstable. Hence, cultured cells lack at least two critical components of the cellular redox system regulation and the compensatory events may not simply involve the amplification of the antioxidant defence.

AA, a water soluble vitamin poorly crossing biological membranes, is normally taken up by the cells through specific transporters and it would make a lot of sense if these cells compensate for the deficiency in vitamin C with the overexpression of these transporters, in particular those with a high affinity. As it will be discussed later on, this is indeed what happens and the over-expression/increased activity of the transporters (73,98,101) introduces an additional complicating factor for the correct interpretation of results obtained in cells supplemented with AA. In addition, we should also consider that similar issues are of potential relevance when considering the distribution of AA in specific sub-cellular compartments. Among these, mitochondria are expected to benefit of the antioxidant function of the vitamin, as these organelles produce large amounts of reactive oxygen species (ROS).

These considerations imply that the signaling revolution imposed by the cell culture conditions should be always kept in mind when translating the outcomes of cell culture experiments

on oxidative stress, and/or on the effect of specific components of the antioxidant defense, to biological concepts relevant for living organisms. At the same time, however, we have nevertheless learned a lot on these issues and we could possibly learn more if the limitations and flaws of the cell culture approach are correctly considered during the design of the experiments and the interpretation of their results.

This review represents an attempt to critically interpret the information of the literature on a controversial issue related to AA uptake in mitochondria, in which most of the information available is largely based on results obtained in cultured cells.

II. Cellular accumulation of vitamin C

Vitamin C is synthesised in the liver of the very large majority of mammals, an information consistent with its relevance in various physiological processes. Many comprehensive reviews published on this issue (11,22,25,58,61,88) very well describe the importance of vitamin C not only as an antioxidant, but also for its role in the regulation of critical cellular functions (e.g., syntheses of collagen, catecholamines, carnitin, cholesterol, aminoacids and certain peptide hormones, vitamin E recycling). Other species, as humans and other primates, as well as guinea pigs, have however lost their capacity to synthesize vitamin C, since they fail to express functional L-gulono-gamma-lactone oxidase (10,64,84). These species are therefore dependent on dietary vitamin C and its insufficient intake is associated with an increased susceptibility to various pathologies (44-45,88,116), and eventually may promote the onset of scurvy, which can even lead to death (88).

Scurvy is however rarely observed and may only take place under conditions in which the plasma concentrations of the vitamin are kept below 10-11 μM (108) for a prolonged period of time. Mild vitamin C deficiency is instead frequently observed in humans. In particular, deficiencies are found in as much as 10% of adults in the industrialized world (102,106,113), with subgroups (e.g., smokers and families with a low socio-economic status) displaying an even higher prevalence (31,66,68,121). An increased incidence of adverse effects associated with mild vitamin C deficiency is therefore expected in a very large population of western countries, with potential long-term consequences associated with increased susceptibility to a variety of diseases (67). Hypoascorbinaemia shares several clinical symptoms with diabetes mellitus, as hypercholesterolaemia, atherosclerosis, microangiopathy, capillary hyperperfusion and haemorrhages (70). These events, blunted by vitamin C supplementation (49), are likely associated to the structural similarities of vitamin C with glucose. Indeed, the ‘latent scurvy’ theory (90) postulates that hyperglycaemia leads to competitive inhibition of AA uptake, thereby inducing intracellular vitamin C deficiency.

Dietary AA is readily absorbed in the gastrointestinal tract, circulates in the blood in a free form, is filtered and may be then reabsorbed in the kidney tubule system (13).

Most of the biological effects mediated by vitamin C occur within the cells, in which its concentrations are remarkably higher (often >1 mM) than those found in the plasma and other biological fluids, normally comprised between 50 and 100 μM (59,63), with some exception, as in the cerebrovascular fluid, in which the concentrations can reach up to 200-400 μM (96-97). The comprehension of the mechanism(s) whereby cells regulate the uptake and accumulation of vitamin C is therefore of pivotal importance for a correct interpretation of the overall effects mediated by the vitamin *in vivo*.

A. *Cellular uptake of the reduced and oxidised forms of vitamin C*

AA, as a consequence of its hydrophilicity, poorly penetrates plasma membranes by simple diffusion and is therefore directly taken up by most cell types *via* two different transporters, SVCT1 and SVCT2 (18,69,74,101,114,120,122). SVTC2 (Fig. 1), detected in the majority of cell types of various organisms, has a high affinity (Km ~10–20 μmol/L) and, for optimal activity, requires the high concentrations of Na^+, Ca^{2+} and Mg^{2+} normally found in the extracellular milieu (35). The physiological importance of SVCT2 is emphasised by the observation that SVCT2 knockout mice die soon after birth (109). SVCT1 (Fig. 1) has a more limited distribution than SVCT2 and is responsible for AA uptake into epithelial cells of the small intestine, liver and kidney (9,56,65,73,118,122). In addition, SVCT1 is also expressed in some epithelia of the reproductive system and brain (114). SVCT1 transports AA with a lower affinity (Km ~100–200 μmol/L) and with a greater capacity than SVCT2, both of which are important characteristics that make it functional for intestinal and renal absorption of the vitamin. Targeted deletion of SVCT1 resulted in accelerated renal excretion and low plasma concentrations of the vitamin, as well as in a ~50% perinatal mortality (13,109,126). These findings emphasise the relevance of SVCT1 and in particular its pivotal role in the intestinal absorption and renal re-absorption of AA.

Cells employ additional strategies for vitamin C uptake and, more specifically, may take up the oxidised form of the vitamin, dehydroascorbic acid (DHA), which, as previously noted for AA, also poorly penetrates the plasma membrane by passive diffusion (63). DHA uptake is mediated by facilitative hexose transport, with the involvement of numerous glucose transporters (14,88,99,122). DHA uptake is followed by its rapid intracellular reduction back to AA *via* NADH, NADPH and GSH-dependent reactions (14,55,64,123). Hence, the mechanism of vitamin accumulation based on DHA transport appears advantageous because of its high capacity and rapid intracellular recycling, which in concert allows a rapid accumulation of large amounts of the vitamin. It is however

important to keep in mind that the use of this strategy is hampered by the extracellular levels of DHA, generally below 2 μM (88), with some variation associated with the release of AA oxidizing species (e.g., $O_2^{\cdot-}$, (85-86)), as it might occur in inflamed tissues. An additional problem may arise from the possible competition for DHA transport through GLUTs by glucose itself, normally present at millimolar concentrations, which however is not always observed. DHA transport may not even be affected at all by the physiological glucose concentrations, as in the case of human erythrocytes (78).

There has been a long debate on the specific relevance of the Na^+-dependent transport of AA *vs* the facilitative diffusion of DHA and the very many experiments performed in cultured cells over the years have provided relevant information for the comprehension of the overall phenomenon of vitamin C transport in various tissues (25,63,88,109). On the other hand, some conflicting information has been produced, when comparative studies were performed using conditions favouring an uptake system *vs* the other.

A typical example, as we recently discussed (4), is represented by the comparison of the amounts of vitamin C accumulated after exposure to increasing concentrations of AA and DHA. The concentrations of the two forms of the vitamin employed in some of these studies were very high, often 1 mM, i.e., remarkably greater than those found in physiological conditions. Furthermore, even using 10 times lower concentrations, the comparison would nevertheless remain poorly physiological, since 100 μM is still very high for DHA. 100 μM AA is instead closer to the concentrations of the vitamin detected in biological fluids, but it may actually be too elevated under conditions in which high affinity transporters with a Km close to 10 μM are over-expressed, as a consequence of the cell growth in absence of the vitamin in the culture medium (101). What is normally observed in these experiments is that exposure to high concentrations of DHA leads to an enormous accumulation of vitamin C, as a consequence of the high transport capacity, with an uptake of the reduced form of the vitamin that in comparative terms appears negligible, since characterised by a low transport capacity. Clearly, these results are potentially misleading and should not be taken as an indication that, in the specific cell type under investigation, the DHA uptake mechanism is preferential over the Na^+-dependent one. The conditions selected are obviously ideal for the high capacity transport of DHA, with very little advantage, if any, for the high affinity/low capacity transport through SVCTs. Comparative studies performed with low concentrations of AA should instead result in significant vitamin C accumulation in SVCT2 expressing cells, with very little vitamin accumulation after treatment with the same low concentrations of DHA. We should also consider that the high concentrations of AA might be toxic for the cells, since these conditions are associated with an extensive formation of ROS generated by AA autoxidation processes (77,105). Uptake studies should not be performed under these conditions, since the active transport of the vitamin is likely hampered

by the insult received by the cells. Furthermore, the process of AA autoxidation results in DHA formation, which might then be taken up by the cells, thereby complicating the interpretation of the experimental results. When using high AA concentrations, one should always use a strategy to keep extracellular vitamin C in its reduced state.

The process of AA autoxidation is a typical cell culture effect (77,105), mediated by the elevated oxygen tension and by the presence of transition metals in the culture media (39-40), normally not observed in the plasma or other biological fluids, because of the low oxygen tension, and the poor availability of free transition metals. In other words vitamin C, at physiological levels, fails to produce a pro-oxidant effect in biological fluids for the same reasons that vitamin C is not a pro-oxidant inside the cells. Things are obviously different when very high concentrations of vitamin C are injected intravenously in an organism, either alone or with other agents, with the purpose of killing cancer cells (23,32,91). Under these conditions, the extensive ROS release is critically associated with the antitumor effects of the vitamin alone or in combination therapy.

The cell culture approach should be therefore carefully employed for the purpose of evaluating the relevance of AA *vs* DHA transport in a specific cell type, especially when using high concentrations of the vitamin. Under these conditions, the remarkably greater accumulation of the vitamin should not lead to the conclusion that the biological relevance of DHA transport through GLUTs is greater than that of AA through SVCTs. In this direction, the evidence that SVCT2 knockout mice present very little AA in their brain and other tissues (109,126), provides a straightforward indication of the biological relevance of this transporter. Hence, SVCT-2 mediated AA transport *in vivo* is of critical importance and cannot be compensated by DHA transport through glucose transporters.

There is, however, a potential biological significance of this second transport mechanism. As mentioned above, normal DHA plasma levels are in the very low micromolar range, although some increase is expected to take place under inflammatory conditions (85-86). Many investigators suggest that activated inflammatory cells promote the release of $O_2^{-\cdot}$ and the ensuing AA oxidation, thereby enhancing the pericellular concentration of DHA, and hence the possibility of its uptake through GLUTs. Furthermore, this event may not only take place in the $O_2^{-\cdot}$ releasing cells, but also in the bystander cells. The cell culture approach has provided abundant information in this direction (26,85-86). Using different cellular systems it was indeed observed that activation is invariably associated with an enhanced cellular accumulation of vitamin C (26,85-86).

While convincing, within the limits of the experimental system in which they were obtained, these results should be considered suggestive of a contribution of the bystander effect *in vivo* although, at least to our best knowledge, this hypothesis has not as yet received a definitive and

univocal demonstration. This mechanism (Fig. 2) may very fulfil the requirements for a greater availability of vitamin C under the same conditions in which it is being oxidised.

III. Mitochondria and vitamin C

We previously mentioned that vitamin C is critical for an array of physiological functions and that its insufficient intake in organisms unable to produce it leads to scurvy. The well-known ability of the vitamin to mediate effects in different sub-cellular compartments raises the problem of the mechanism of vitamin transport in mitochondria and other organelles. At the same time, we should also be aware of the fact that scurvy may be relevant both at the cellular and sub-cellular levels, and eventually play a role in different pathologies (6,70). This important issue has been addressed in several studies and the general idea is that the vitamin uptake is largely based on DHA transport. Hexose transporters were indeed detected in the Golgi apparatus (6,82), lysosomes (2,6,20), nuclear envelop (6,15,70) and endoplasmic reticulum (6,70,83,104). Recent reviews provide detailed information on this important topic (6,70). We would like to focus our attention on mitochondria, in which the process of vitamin C accumulation is expected be particularly important.

Mitochondria are subcellular organelles playing a critical role in a variety of functions, which include energy biogenesis by means of the oxidative phosphorylation (8,81,117), regulation of Ca^{2+} homeostasis (21,92), cell signaling responses affecting various physiological processes (21,92) as well as autophagic (16-17,54,124) and apoptotic pathways (17,53-54,94). Mitochondria also represent the main intracellular source of ROS, in particular $O_2^{-\cdot}$, a partially reduced form of molecular oxygen generated by a one electron reduction reaction (1,42,80). $O_2^{-\cdot}$ formation takes place as a consequence of the electron leak from the mitochondrial respiratory chain, in particular at the level of complex I and III (7,12,24,42-43). Although the specific contribution of each of these events is potentially affected by specific characteristics of the cell type, in particular its metabolic activity, complex III is in general more effective in mediating $O_2^{-\cdot}$ formation than complex I. Under physiological conditions, a significant fraction of molecular oxygen (about 1%, probably less) is converted to $O_2^{-\cdot}$ and roughly 75% of this fraction derives from the Q cycle, during electron transfer to cytochrome c in complex III (42). Importantly, $O_2^{-\cdot}$ produced at the level of complex I is released in the matrix, whereas the bifacial localization of complex III leads to $O_2^{-\cdot}$ release in the same compartment as well as in the intermembrane space (1,53). Once produced, $O_2^{-\cdot}$ may rapidly react with nitric oxide, when available, to generate peroxynitrite (62,87,110), a highly reactive and toxic nitrogen species (87,110). Protonation of $O_2^{-\cdot}$, with the ensuing formation of hydroperoxyl radicals, is also a mechanism potentially resulting in the induction of deleterious effects on various biomolecules (42,53). Finally, and most commonly, $O_2^{-\cdot}$ is converted to H_2O_2 either spontaneously,

or enzymatically (24,43,53), both inside and outside the mitochondria, thereby allowing its interaction in different sub-cellular compartments with transition metals, typically Fe^{2+}, to generate hydroxyl radical species (1,24,53). These species are highly reactive and may damage various biomolecules, in particular lipids, proteins and the DNA (24,43,53,110). Under physiological conditions, mitochondrial ROS instead participate to the complex machinery regulating cell signaling (1,42-43,46), and this is an important reason, among many others, explaining the lack of therapeutic effect, or even the increased incidence of adverse effects, demonstrated by various antioxidants in clinical studies (34).

Mitochondria are therefore continually exposed to a flux of $O_2^{-\cdot}/H_2O_2$ derived from the respiratory chain and other mitochondrial sources not reported here for the sake of brevity (24,42-43), with both physiological and pathological/toxicological implications, thereby implying the existence of an organised and efficient mitochondrial antioxidant defence. Mitochondria indeed contain a network of antioxidant enzymes as glutathione (GSH) peroxidase, oxidized glutathione reductase, manganese-containing superoxide dismutase, catalase and peroxyredoxins (24,43,50-51,53,95,100). The major non-enzymatic antioxidants are GSH, coenzyme A, ubiquinol and vitamin E (24,43,70-72,95). Vitamin C is also expected to be beneficial for the mitochondria, under both physiological conditions and during extensive ROS release.

A. *Mitochondrial accumulation of vitamin C*

Cultured cells cope very well with the elevated oxygen tension and the enhanced ROS release, and indeed survive/proliferate even in the absence of vitamin C. We should therefore recognise that mitochondrial function and integrity is also well preserved under the same conditions. This would imply that vitamin C is not essential for these cells, which can develop alternative/compensatory strategies allowing their survival even under harsh culture conditions. As previously discussed, however, overwhelming information derived from clinical or animal studies demonstrates a severe impact of insufficient AA intake, or cellular uptake, in living organisms (63,70,88,126).

Seen in this perspective, there are very good reasons to believe that mitochondrial AA is also critical for the function and integrity of these organelles *in vivo*.

The available information on vitamin C uptake and accumulation in animal tissue mitochondria is surprisingly limited and some of these studies are about 35 years old. Vitamin C has been detected in rat liver mitochondria and the experimental results were in the range 0.5-1 mM (33,60). Results compatible with these values were obtained in our laboratory, however using mouse liver mitochondria (data not shown). Liver mitochondria isolated from guinea pig displayed an AA content 3 times greater than the above (47). Remarkably higher values (about 50 times) were instead

reported by Ramanathan et al. (93) for rat liver mitochondria. AA was also detected in rat brain mitochondria (119) at levels comparable with those found by most studies in liver mitochondria. Much less AA (about 5 times) was detected in the mitochondria from the mouse skeletal muscle (60) and these results are in keeping with our preliminary findings obtained with the mouse *tibialis anterior* muscle (103). We are not aware of human studies documenting the presence of vitamin C in the mitochondria.

In summary, high levels of vitamin C can be found in the mitochondria isolated from various animal tissues. We previously mentioned that the hydrophilic nature of both AA and DHA is not permissive for their passive diffusion through the plasma membrane, a consideration that holds true also for their transport through the inner mitochondrial membrane. Thus, we are back to the same problem and, at least in principle, the involvement of the same active transporters for AA, and facilitative glucose transporters for DHA, should be taken into account. There are however numerous differences between the context of the plasma and mitochondrial membranes. An important one is related to the AA concentrations of the extramitochondrial milieu (cytosol), in general remarkably higher than those of the extracellular milieu. Why should then mitochondria use a high affinity transport of the reduced form of vitamin C? In principle, it makes very little sense to express a mitochondrial transporter with a Km in the low micromolar range to transport AA from a cytosol containing millimolar levels of the vitamin. Moreover, we can think of additional critical limitations in transport efficiency based on the low cytosolic concentrations of Na^{+}, Mg^{2+} and Ca^{2+} (35,79). Although it would have been worth to at least consider the possibility of the existence of such transporters working with a low affinity, due to the restrictive composition of the cytosol, many scientists working in the field found nevertheless unreasonable this possibility, and focused their attention on the more likely involvement of DHA transport through GLUTs.

B. *Mitochondrial transport of DHA through facilitative hexose transporters*

As previously discussed, DHA is a substrate of hexose transporters and its uptake may represent a possible alternative strategy of vitamin C accumulation in specific cell types. The same transporters, however, are also expressed in mitochondria (6,52,57,70,75), despite the lack of an apparent role of glucose in mitochondrial metabolism. Furthermore, once in the cells, glucose is readily phosphorylated and glucose-6-phosphate is not a substrate for the hexose transporters. This means that DHA transport may well take place in the absence of competing substrates. The second reason favouring the possibility of DHA transport through mitochondrial GLUTs is that this event is not influenced by the ionic microenvironment. Finally, a high capacity transport is expected to be advantageous for providing high levels of the vitamin under conditions of extensive mitochondrial

formation of ROS. The role of GLUT1 as a DHA transporter has also been validated by *in silico* predictions (111).

After the initial demonstration in plant cells (112), experiments performed in cultured mammalian cells have significantly contributed to the demonstration of the physiological significance of mitochondrial DHA transport. These studies provided convincing information on the possibility of mitochondrial DHA transport, but in some circumstances the approach employed was based on the results of comparative uptake studies performed in isolated mitochondria exposed to high concentrations of DHA and AA. We critically considered this approach when the endpoint was cellular uptake (see above), the same is herein being proposed for studies measuring the mitochondrial uptake of the vitamin. More specifically, the use of a high concentration of AA seems appropriate in these studies, as high concentrations of the vitamin are normally found in the cytosol of cells isolated from living organisms. However, the same is not true for the use of high concentrations of DHA, which will never be reached in the extramitochondrial compartments. Indeed, the DHA concentrations are low in the extracellular milieu and most likely even lower in the cytosol, in which it is indeed very rapidly reduced (14,55,60,64,76,88). We could then postulate that there will be more DHA to be transported under conditions of oxidative stress, as discussed above for the plasma membrane, but nevertheless an important piece of the puzzle seems missing. Time course studies of DHA uptake reveal its immediate intracellular reduction back to AA (14,55,60,63-64,76,88,122). As this event is also observed using concentrations of DHA two order of magnitude greater than those found in the extracellular milieu, it appears unlikely that intracellular AA can be oxidised at a rate compatible with the accumulation of cytosolic concentrations of DHA permissive for its mitochondrial uptake.

The scenario is therefore once again complicated by conflicting factors, as the DHA transport appears likely to take place for several reasons, but at the same it remains unclear whether or not there will be enough DHA to be transported. In our opinion, we should not think in terms of cytosolic concentrations, but rather of selective microdomains in which AA is oxidised to generate local concentrations of DHA. Moreover, we should consider likely a competition between reduction and transport of DHA, thereby implying that these microdomains must be located in the close proximity of the DHA transporters.

The hypothesis we can make is that DHA transport in mitochondria is linked to its selective formation in the intermembrane space. As previously discussed, a fraction of the $O_2^{-\cdot}$ generated at the level of complex III is released in the intermembrane space and, under these conditions, AA would be locally converted to DHA and build up local concentrations functional for a significant DHA transport in the matrix (Fig. 3). The ability of AA to reach the intermembrane space has been

previously documented (75). This mechanism may well represent the strategy used to transport appropriate amounts of vitamin C in the form of DHA: an enhanced rate of $O_2^{\cdot-}$ release will be indeed associated to a selective increase of the DHA concentration in the intermembrane space, thereby bursting its uptake through GLUTs.

C. *Mitochondrial transport of AA through SVCT2*

While our previous considerations make an argument against the active transport of AA in mitochondria, a definitive demonstration in this direction has not has yet been provided. Western blot studies for the detection of SVCTs are difficult to perform in the mitochondria isolated from animal tissues, because of the problems encountered to obtain pure mitochondrial preparations (89). In addition, negative results would still leave some doubts since a very low expression of these transporters is eventually to be expected in mitochondria bathed in a milieu containing high levels of AA. Likewise, for these same reasons, transport studies performed in isolated mitochondria may fail to detect significant vitamin uptake over the short incubation time allowed by these experiments.

With these considerations in mind, we can approach this problem indirectly, once again with use of cultured cells, overexpressing active AA transporters in their plasma membrane, when grown under standard conditions, i.e., in the absence of the vitamin. The increased expression of these transporters in mitochondria, coupled with their functional activity, may at least prove that these transporters can indeed be expressed and can be functionally active also in these organelles.

We performed these experiments using U937 cells, which express both SVCT1 and 2 (3,27), and were initially very skeptical on their outcomes since the cytosolic conditions in terms of cation concentrations, in particular Na^+ and Ca^{2+}, are too much different from those of the extracellular milieu. To our surprise, we obtained both immunocytochemical and Western blot evidence of mitochondrial SVCT2 expression, in the absence of detectable expression of SVCT1 (3,27). The image reproduced in Fig. 4 provides evidence for a virtually exclusive expression of SVCT2 in subcellular domains that can be stained by specific mitochondrial probes (27). We reproduced these results in other cell types, e.g., Raw 264.7 and C2C12 myoblasts (103, unpublished data), other investigators detected SVCT2 in HEK-293T cells and reported a similar prevalent mitochondrial expression of SVCT2 (79). Another study (125) employed the same cell line to demonstrate that the high mitochondrial expression of the transporter detected by immunolocalization studies can be confirmed with the use of a novel fluorescent probe detecting SVCT2. These Authors also used HepG2 cells, which express both SVCT1 and SVCT2. SVCT2 was mostly detected in mitochondria, with some minor expression in the plasma membrane. SVCT1 was instead uniquely detected in the plasma membrane.

The first conclusion we can draw from these studies is that SVCT2 is expressed in the mitochondria of various cell types grown in the absence of vitamin C. It is also interesting to observe that there was no evidence for the expression of mitochondrial SVCT1, even in cells expressing this transporter in their plasma membrane.

The obvious question to be asked is then whether the mitochondrial expression of SVCT2 is compatible with its function and the results obtained were once again unexpected. Not only these organelles avidly accumulated the reduced form of the vitamin, but the Km of the transporter was very low, remarkably similar to that measured for the plasma membrane SVCT2 (27). An additional similarity was found in the pH-dependence and positive cooperativity for Na^+ (nH ≅ 2). Strikingly, however, 100 fold less Na^+ was necessary to maximally support AA transport in mitochondria in comparison with the transport through the plasma membrane. A final critical difference was found for the Ca^{2+}/Mg^{2+} dependence; while the plasma membrane SVCT2 required millimolar concentrations of these cations, its mitochondrial counterpart was fully active in their virtual absence. We reproduced some of these results in Raw 264.7 and obtained remarkably similar results (unpublished data). Other studies confirming the existence of a mitochondrial SVCT2 in human HEK-293 cells showed that under intracellular ionic conditions SVCT2 instead behaves as a low-affinity AA transporter (79). Recently, we found that the mitochondrial SVCT2 expressed in C2C12 myoblasts also works with a low affinity (103).

These results therefore lead us to a second conclusion, i.e., that the mitochondrial SVCT2 is functionally active, although its affinity is potentially compromised by the low intracellular concentrations of specific ions. On the other hand, probably as a consequence of the very high requirement of the vitamin in their mitochondria, some cell types, as the U937 cells employed in our studies have evolved a strategy to make this same transporter fully active and with a high affinity by minimizing its cation requirements (27).

The third conclusion we can make is inspired by the combination of some of the above findings. We mentioned that the high affinity SVCT2 of the plasma membrane is less densely expressed than its mitochondrial counterpart. We should interpret this observation as an indication that the cytosolic concentrations of the vitamin may eventually be kept low by the mitochondrial clearance processes, in particular at limiting extracellular levels of the vitamin. We found that this is indeed the case, as a 15 min exposure to a very low concentration of AA, 3 μM, leads to a cellular concentration of about 50 μM and an intramitochondrial concentration of as much as 5 mM (Fig. 5). This means that during uptake the increase in the cytosolic concentration of the vitamin is continuously hampered by its more efficient mitochondrial uptake. Based on these results, we may predict that U937 cells as well as other cultured cells expressing a high affinity mitochondrial

transport of AA manifest a greater need of the vitamin in their mitochondria than in the cytosol. We take these results as indicative of a critical biological relevance of mitochondrial vitamin C, which can be easily tested against specific mitochondrial toxins.

The U937 cell model provides a very interesting system for asking questions on the relevance of mitochondrial *vs* cytosolic AA in the toxic response evoked by specific agents. We recently demonstrated that, in the same U937 cell clone used in the above studies, arsenite promotes toxicity *via* a mechanism mediated by the mitochondrial formation of $O_2^{\cdot-}/H_2O_2$ which triggers downstream mitochondrial permeability transition dependent apoptosis (36-38). All these events were sensitive to either rotenone, a complex I inhibitor, or the respiration-deficient phenotype. A 15 min pre-exposure to 10 μM AA, while producing a very high mitochondrial accumulation of AA/very low increase in the cytosolic concentration of the vitamin, generated the same protective effects (36-37). This experimental system enabled us to link cytoprotection with the mitochondrial fraction of AA, but we could do even more using DHA in the place of AA. DHA, for the reasons that will be detailed below, can be used to enhance the cytosolic fraction of the vitamin with no apparent increased in its mitochondrial content. Under these conditions, the significant elevation of cytosolic vitamin C associated with DHA exposure failed to promote cytoprotection (37).

D. *DHA as an inhibitor of cellular and mitochondrial uptake of AA through SVCT2*

We recently investigated the existence of possible interactions between AA and DHA transport with an initial very simple experiment. The cells were exposed to a single concentration of either AA or DHA, or the two forms of the vitamin in combination (28). The results were surprising since the uptake observed with the combined treatment was identical to that observed with DHA alone. In addition, the uptake was in both circumstances sensitive to cytochalasin B, an inhibitor of DHA transport, and insensitive to Na^+ omission, which inhibits AA transport. This was the first evidence of an inhibitory effect of DHA on AA transport. This notion was then established using different approaches, and led us to formulate the hypothesis that extracellular oxidation of AA may lead to transient and reversible negative modulation of the transport of the reduced form of the vitamin with a parallel switch to the high capacity facilitative transport of DHA. We are currently investigating the mechanism(s) involved in this inhibitory response and more specifically challenging the possibility that DHA elicits its effects *via* interaction with critical thiols of SVCT2. Consistently with this notion are our previous results indicating that the plasma membrane and mitochondrial SVCT2 are both susceptible to inhibition by thiol-reactive agents (29). Furthermore, DHA is known to react with -SH groups and to inhibit the activity of enzymes containing critical cysteines (30).

At the moment, we do not have enough information to determine the physiological relevance of this event, but we can imagine that conditions associated with the bystander effect discussed above will likely result in inhibition of AA transport in SVCT2 expressing cells.

More research is needed to understand the extracellular cross-talk between the two forms of the vitamin but probably even more interesting is the comprehension of these same interactions taking place intracellularly, with a potential impact on vitamin C accumulation in specific subcellular compartments, mitochondria in the first place.

We very well know that exposure of U937 cells to low concentrations of AA (e.g., 3 or 10 μM) is associated with a prompt cellular uptake and a further mitochondrial accumulation of the vitamin. Under the same conditions, however, the uptake of DHA -even when resulting in greater cellular levels of AA- was not associated with a significant mitochondrial uptake (29). This event was only observed after exposure to very high concentrations of DHA, 50 μM or more. We further investigated this issue in both intact cells and isolated mitochondria and concluded that low intracellular concentrations of DHA, because of their rapid reduction back to AA, are a poor substrate for direct mitochondrial uptake. DHA concentrations, however, were sufficiently high to mediate inhibition of mitochondrial transport of DHA-derived AA (29). The increased accumulation of vitamin C observed in cells treated with the very high concentrations of DHA was instead due to its direct mitochondrial uptake.

IV. Conclusions

AA mediates numerous effects in various sub-cellular compartments and its hydrophilic nature implies the expression of an active transporter, typically SVCT2, that mediates high affinity/low capacity uptake through the plasma membrane and in sub-cellular compartments in which it is eventually expressed. An alternative pathway is dependent on the oxidation of AA to DHA, and to its uptake by facilitative hexose transport, once again through the plasma membrane and in different compartments. In this review we focused on the plasma membrane and mitochondrial transport of the vitamin and made an attempt to critically interpret the available information largely based on results obtained in cultured cells. A summary of the mechanisms discussed and proposed is provided in Fig. 6.

Based on the results obtained in our laboratory and on the interpretation of the available information, cellular uptake of vitamin C appears to be mostly dependent on SVCT2-mediated transport of AA, provided that this transporter is expressed in the plasma membrane. Extracellular oxidation of AA to DHA may lead to inhibition of SVCT2-mediated transport of AA and will be permissive for some uptake through GLUTs, especially in cells in which these transporters are highly

expressed. The prompt reduction back of DHA to AA likely prevents the possibility of a direct mitochondrial uptake of DHA, which might eventually inhibit mitochondrial SVCT2, when expressed in these organelles. DHA, however, can also be produced by AA oxidation in the intermembrane space, mediated by the $O_2^{-\cdot}$ generated at the level of complex III and released in this compartment. This event promotes an antioxidant response associated with the scavenging of $O_2^{-\cdot}$ and allows the DHA-dependent inhibition of SVCT2-mediated AA uptake. Most importantly, this mechanism links the rate of DHA uptake with the rate of $O_2^{-\cdot}$ release. Under these conditions, the switch in the uptake mechanism is also advantageous since intramitochondrial DHA is readily recycled back to AA by various mechanisms dependent on GSH and/or other reductants, or through the acceptance of electrons released by complex III (or complex I) of the respiratory chain. This last event clearly prevents $O_2^{-\cdot}$ formation in the matrix.

The scheme depicted in Fig. 6, which obviously needs to be validated by further experimental evidence, nevertheless appears attractive since it provides a likely strategy for the mitochondrial accumulation of vitamin C based on a mechanism associated with an effective antioxidant action exerted in the intermembrane space as well in the matrix. More work is also necessary to understand the complex relationships of these events with other key steps of vitamin C recycling, for example associated with the formation of semidehydroascorbic acid and/or the conversion of latter to either AA or DHA in the intermembrane space. At least in principle, the NADH-semidehydroascorbic acid reductase activity identified in the outer mitochondrial membrane in rat hepatocytes (48) may significantly contribute to events associated with the transport of the reduced form of the vitamin.

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Legend to the figures

Fig. 1 - Vitamin C transport in mammalian cells. AA is actively transported across the plasma membrane by two Na^+/AA cotransporters, SVCT1 and SVCT2, characterised by different affinities and capacities, determined by their specific role, distribution and biological significance. DHA, the oxidised form of vitamin C, is instead transported through facilitative glucose transporters (GLUTs) and, once inside the cell, immediately reduced back to AA. The major limiting factor for DHA transport is represented by its low concentration in biological fluids.

Fig. 2 - Vitamin C uptake based on the bystander effect. The low extracellular concentrations of DHA may be increased under conditions associated with $O_2^{-\cdot}$ formation. Activated cells (e.g., inflammatory cells) undergoing the oxidative burst oxidize extracellular AA to DHA, with an ensuing increase in the extracellular DHA concentration, potentially taken up by the same as well as bystander cells. DHA is then immediately reduced back to AA which eventually exerts antioxidant functions in both cell types.

Fig. 3 – Proposed mechanism for DHA uptake in mitochondria. In the intermembrane space AA is oxidised to DHA by the $O_2^{-\cdot}$ generated at the level of complex III and released in this compartment. DHA is then taken up in the matrix by facilitative diffusion through the GLUTs.

Fig. 4 – Mitochondrial localization of SVCT2 in U937 cells. Co-localization of anti-SVCT2 immunoreactivity with MitoTracker Red CMXRos, a mitochondrial fluorescence probe.
Representative images of cells double-stained for mitochondria (red) and SVCT2-FITC (green). In the merged image, the regions containing both the green and the red signals appear yellow. Adapted by Fiorani et al., Biochim. Biophys. Acta, 1848, 1393-1941, 2015.

Fig. 5 – Sequential transport of AA by the plasma and mitochondrial membrane high affinity SVCT2. U937 cells express high affinity SVCT2 in the plasma membrane and to a greater extent in their mitochondria. As a consequence, exposure to a very low concentration of AA promotes a small increase in the cytosolic fraction of the vitamin and a remarkably greater enhancement in its mitochondrial accumulation.

Fig. 6 – Vitamin C uptake and recycling in cells and in their mitochondria. AA is taken up by the cells and by their mitochondria, provided that SVCT2 is expressed in the respective membranes. When the concentration of DHA is elevated by an increased availability of AA oxidising species, inhibition of SVCT2 activity may be observed. DHA may also be taken up the cells through hexose transporters. This event is however followed by its immediate reduction back to AA, thereby preventing the possibility of a direct DHA mitochondrial uptake. DHA may nevertheless be produced in the intermembrane space, in which AA is oxidised by superoxide released by complex III. DHA can then inhibit mitochondrial SVCT2, or be transported in the matrix, in which it is rapidly reduced back to AA by GSH and or other mechanisms. DHA reduction can also be mediated by electrons, derived from complex III, an event preventing the formation of superoxide in the matrix. The antioxidant function of the vitamin is then associated with the scavenging of superoxide in the intermembrane space and with the prevention of superoxide formation in the matrix.

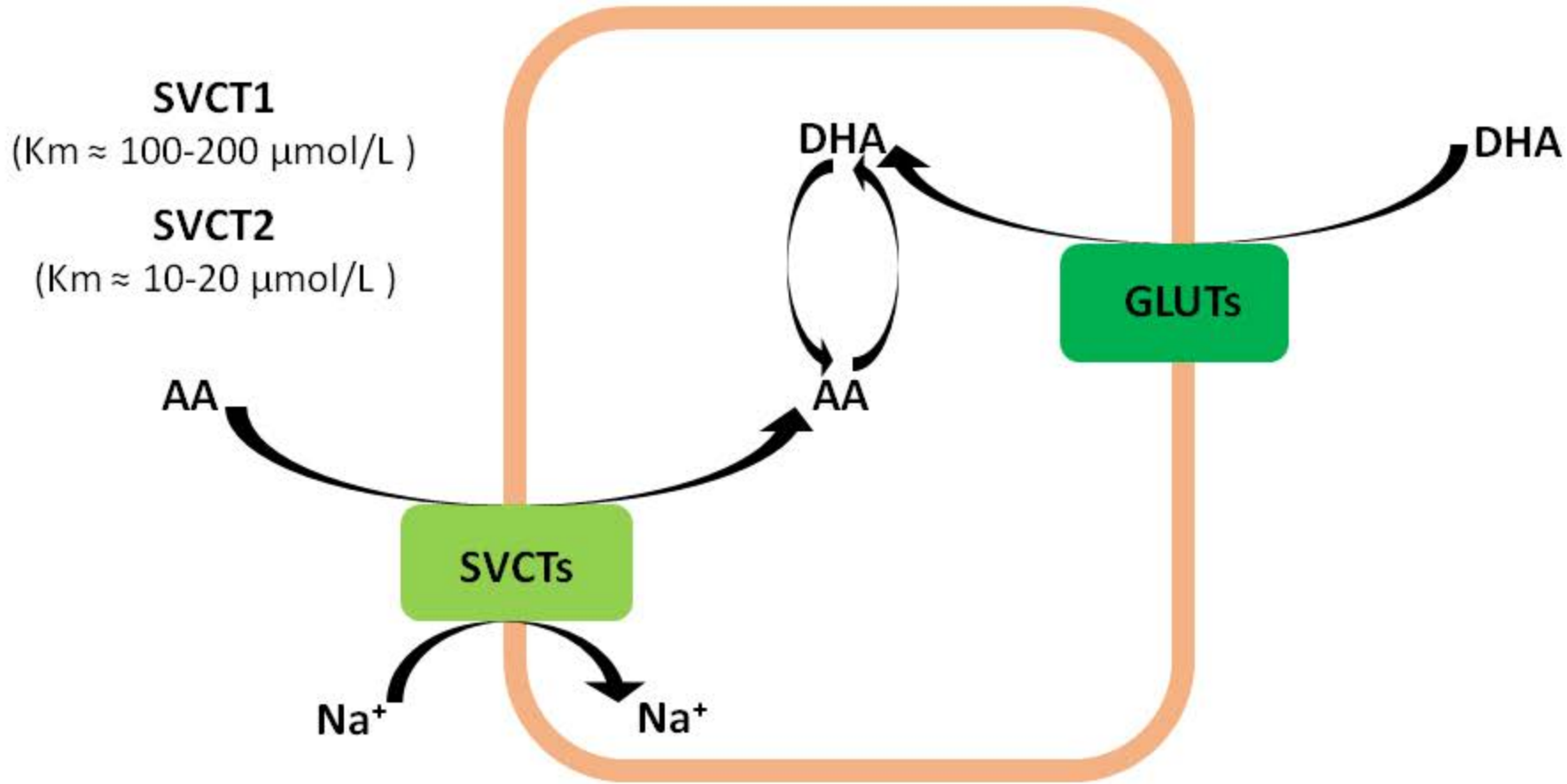
SVCT1
(Km ≈ 100-200 µmol/L)
SVCT2
(Km ≈ 10-20 µmol/L)
DHA
DHA
GLUTs
AA
AA
SVCTs
Na+
Na+

Figure 1

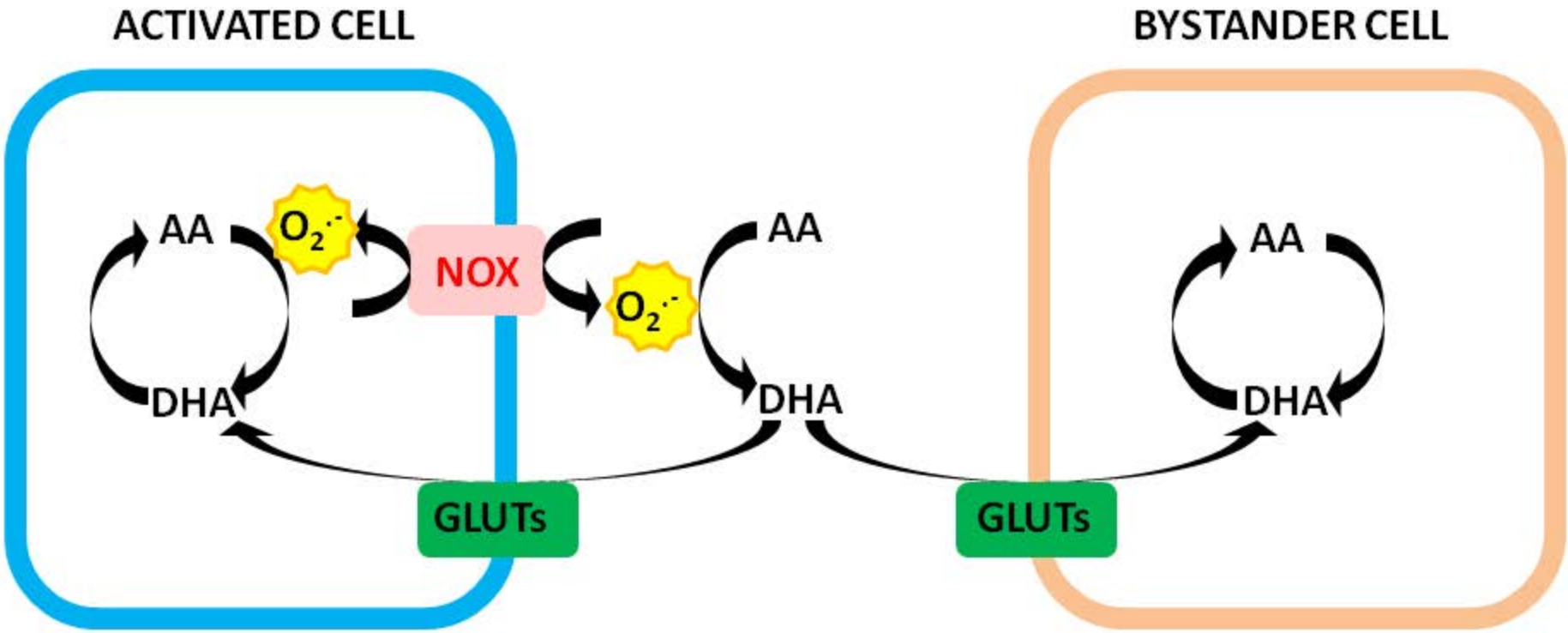
ACTIVATED CELL
BYSTANDER CELL
AA
$O_2^{\cdot -}$
NOX
AA
$O_2^{\cdot -}$
AA
DHA
DHA
DHA
GLUTs
GLUTs

Figure 2

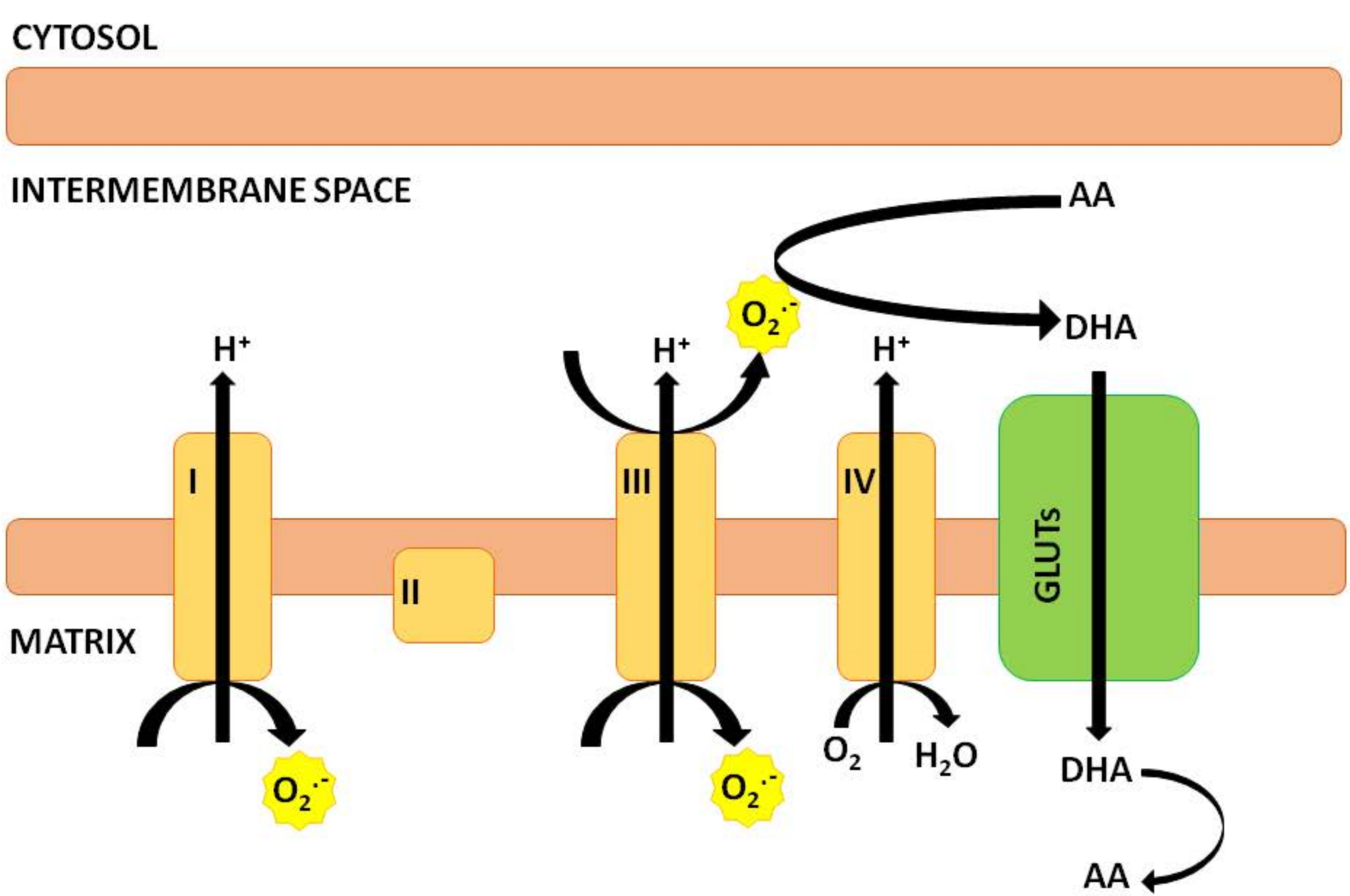
CYTOSOL
INTERMEMBRANE SPACE
AA
DHA
H+
H+
H+
O2·-
I
II
III
IV
GLUTs
MATRIX
O2·-
O2·-
O2
H2O
DHA
AA

Figure 3

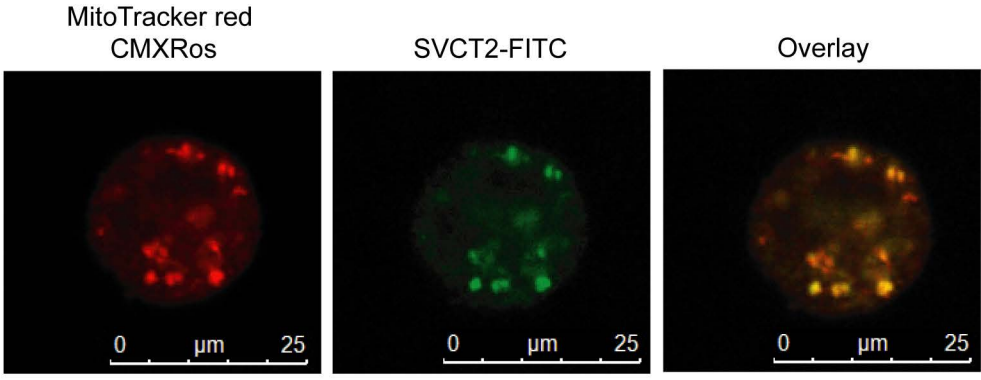
MitoTracker red
CMXRos
SVCT2-FITC
Overlay
0
µm
25
0
µm
25
0
µm
25

Figure 4

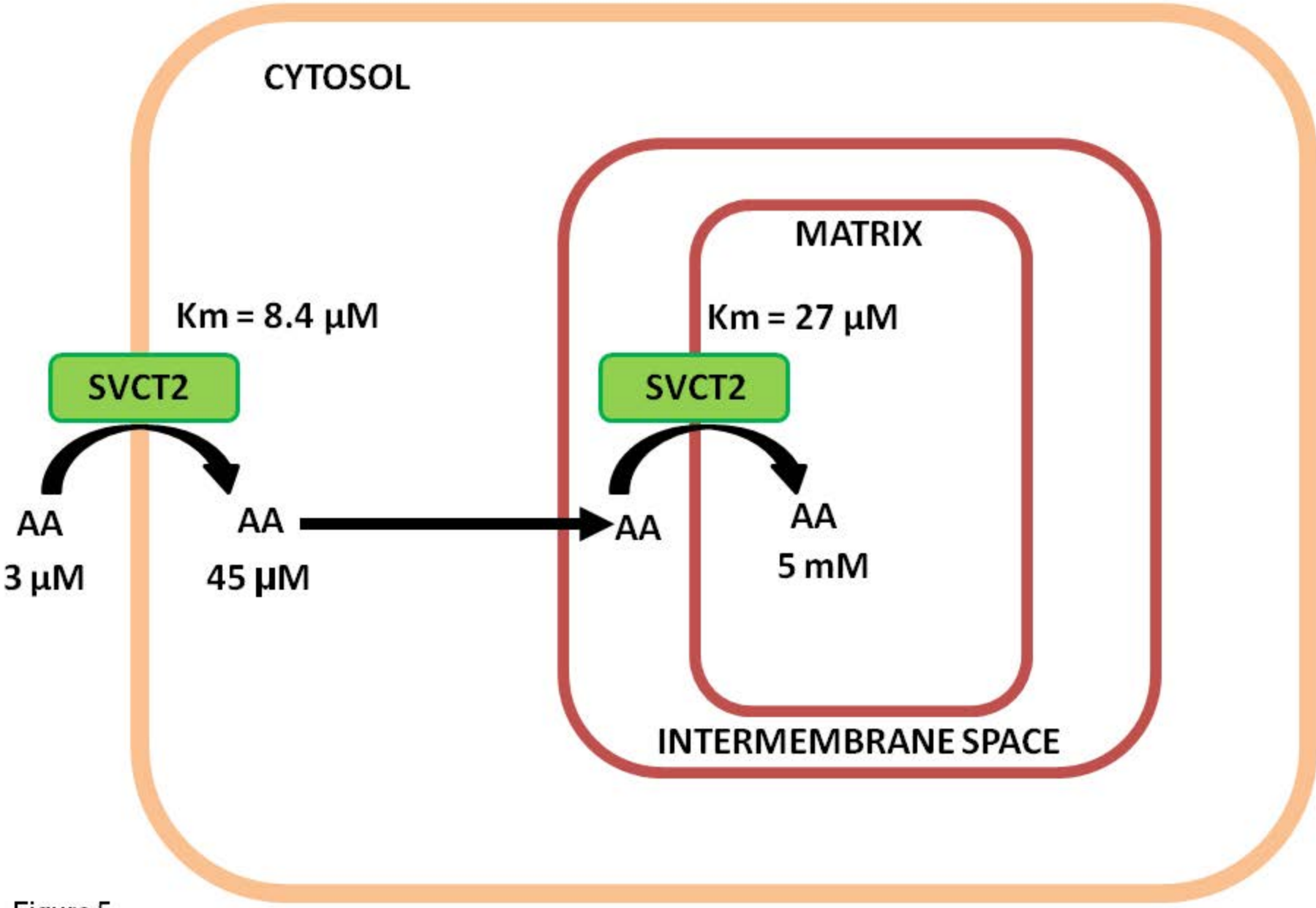
CYTOSOL
MATRIX
Km = 8.4 µM
Km = 27 µM
SVCT2
SVCT2
AA
3 µM
AA
45 µM
AA
AA
5 mM
INTERMEMBRANE SPACE

Figure 5

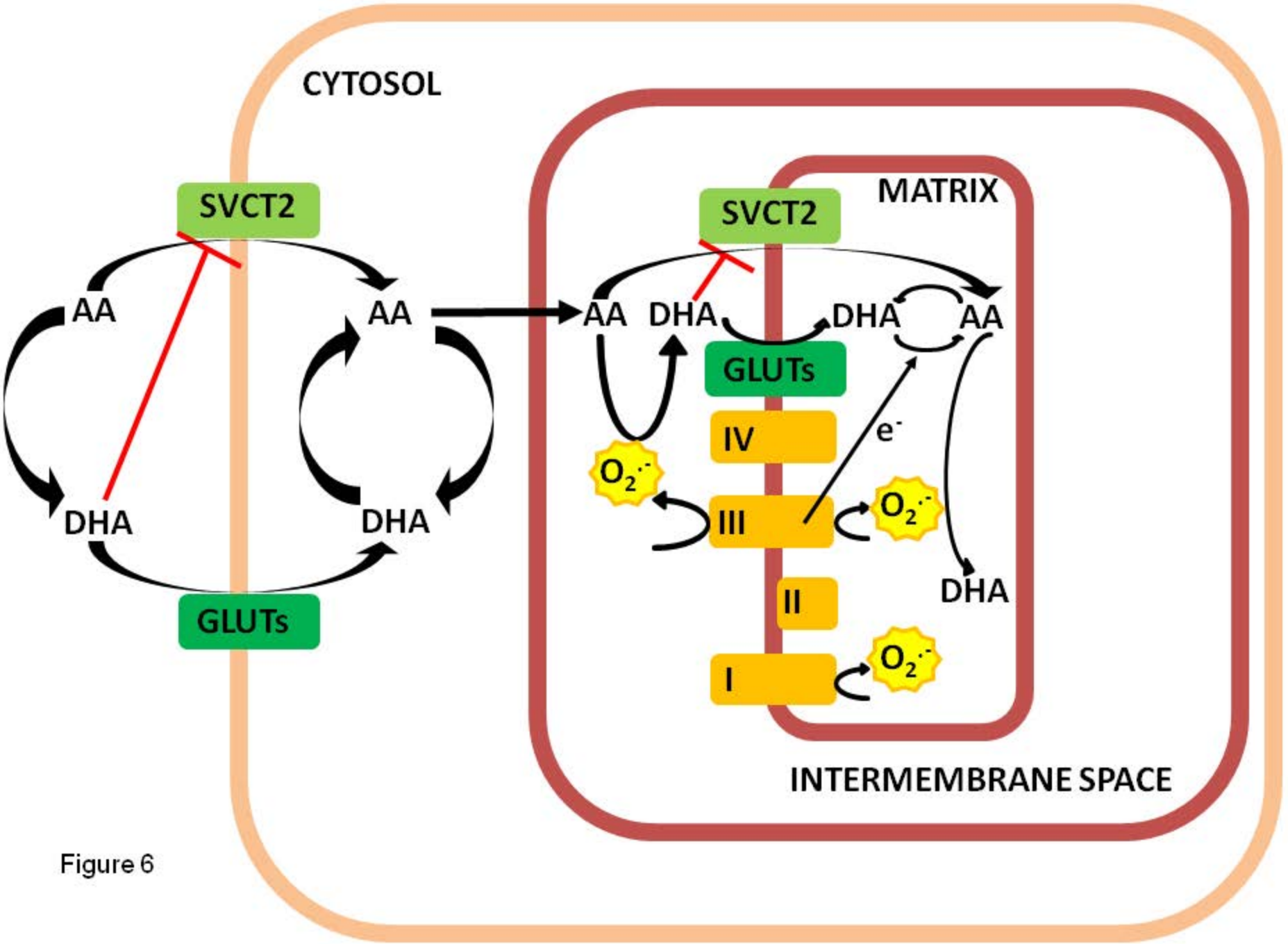
CYTOSOL
SVCT2
AA
AA
DHA
DHA
GLUTs
MATRIX
SVCT2
AA
DHA
DHA
AA
GLUTs
IV
III
II
I
e-
O2·-
O2·-
O2·-
DHA
INTERMEMBRANE SPACE

Figure 6