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

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Abstract

Mammalian cells accumulate vitamin C either as ascorbic acid (AA), via Na^+–AA co-transport, or dehydroascorbic acid (DHA, the oxidation product of AA), via facilitative hexose transport. As the latter, unlike the former, is a high-capacity transport mechanism, cultured cells normally accumulate greater levels of vitamin C when exposed to increasing concentrations of DHA as compared with AA. We report herein similar results using the U937 cell clone used in our laboratory only under conditions in which DHA and AA are used at concentrations greater than 50–60 μM. Below 60 μM, i.e. at levels in which AA is normally found in most biological fluids, AA and DHA are in fact taken up with identical rates and kinetics. Consequently, extracellular oxidation of AA switches the mode of uptake with hardly any effect on the net amount of vitamin C accumulated. As a final note, under these conditions, neither AA nor DHA causes detectable toxicity or any change in the redox status of the cells, as assessed by the reduced glutathione/reduced pyridine nucleotide pool. These findings therefore imply that some cell types do not have a preferential route for vitamin C accumulation, and that the uptake mechanism is uniquely dependent on the extracellular availability of AA *v.* DHA.

Key words: Ascorbic acid: Dehydroascorbic acid: Vitamin C uptake: U937 cells

Ascorbic acid (AA), the reduced form of vitamin C, poorly penetrates plasma membranes by simple diffusion[1] and can be taken up by the cells via Na^+–AA co-transporters (SVCT1 and 2)[2–4], with an absolute specificity for the reduced form of the vitamin[4,5]. AA uptake through this high-affinity/low-capacity mechanism is probably of biological relevance, as the transporters are widely expressed in various cell types and the biological fluids normally contain AA concentrations lower than 100 μM[6–8].

Dehydroascorbic acid (DHA) also poorly penetrates plasma membranes by passive diffusion, and its uptake is mainly mediated by facilitative hexose transport[9–14], an event followed by the immediate intracellular reduction back to AA, via reduced glutathione (GSH)- and NAD(P)H-dependent enzymatic and non-enzymatic reactions[15]. As it can be expected from a high-capacity transport mechanism, DHA uptake in cultured cells is dose-dependent up to concentrations in the high micromolar range (e.g. 300–500 μM or more)[16]. These studies, however, do not necessarily imply a physiological relevance for DHA uptake, as its levels in biological fluids are normally very low (1–2 μM)[10]. Furthermore,

even under conditions resulting in extensive extracellular AA oxidation, DHA uptake should be competitively inhibited by the remarkably greater glucose concentrations[8,11,17]. Although these considerations would argue against a physiological relevance of DHA uptake in vitamin C accumulation, numerous studies in fact provide evidence in the opposite direction, and it is now widely accepted that the influence of glucose on DHA uptake is cell type dependent[9,10]. In some cell types, DHA uptake is largely inhibited by physiological concentrations of glucose[8,11,17], in others sensitivity is remarkably lower[18] and still in other cell types hardly any effect of glucose on DHA uptake can be detected[19–22].

In short, although AA uptake through Na^+–AA co-transporters appears of more likely physiological significance, the extensive literature published in recent years nevertheless provides sufficient grounds to attribute a relevance to DHA uptake also through facilitative hexose transport. This second mechanism may even appear more important than the former, on the basis of comparative studies using increasing concentrations of AA and DHA, generally in the high micromolar/millimolar range[16]. The comparison of vitamin C accumulation in

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Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; DHR, dihydrorhodamine 123; DTT, dithiothreitol; GSH, reduced glutathione; RPMI, Roswell Park Memorial Institute.

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cultured cells exposed to physiologically irrelevant AA or DHA concentrations, however, might provide misleading results, as these conditions are obviously ideal for the low-affinity/high-capacity transport of DHA, whereas, at the very best, there is no major advantage for the high-affinity/low-capacity transport of AA. High concentrations of AA may in fact cause detrimental effects associated with the extensive superoxide/H_2O_2 formation[23,24].

We herein report that the U937 cell clone used in our laboratory accumulates more vitamin C when exposed to high DHA v. AA concentrations. Although these results were largely expected, an accurate comparative study in fact revealed identical rates and kinetics of vitamin C accumulation after incubation in glucose-containing media enriched with AA or DHA concentrations lower than 60 μM. It follows that DHA outcompetes with glucose and that enzymatic conversion of AA to DHA switches the mode of uptake without affecting the net amount of vitamin C accumulated. As a final note, under these physiologically relevant conditions, neither AA nor DHA causes detectable toxicity or a change in the redox status of the cells, as assessed by the GSH/reduced nucleotide pool.

Materials and methods

Chemicals

AA, DHA, dithiothreitol (DTT), tetrabutylammonium hydrogen sulphate, cytochalasin B, choline chloride, catalase, ascorbate oxidase as well as most reagent-grade chemicals were purchased from Sigma-Aldrich (Milan, Italy). Dihydrorhodamine 123 (DHR) was purchased from Molecular Probes (Leiden, The Netherlands).

Cell culture and treatment conditions

U937 human myeloid leukaemia cells were cultured in suspension in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (Euroclone; Celbio Biotecnologie, Milan, Italy), penicillin (100 IU/ml) and streptomycin (100 μg/ml; Euroclone), at 37°C in T-75 tissue culture flasks (Corning, Inc., Corning, NY, USA) gassed with an atmosphere of 95% air and 5% CO_2. Fetal bovine serum heat inactivation was performed at 56°C for 30 min. A 10-mM-DHA or -AA stock solution was prepared in saline A (8·182 g/l NaCl; 0·372 g/l KCl; 0·336 g/l $NaHCO_3$; 0·9 g/l glucose; pH 7·4) immediately before use. Cells (1×10^6 cells/ml) were then treated with DHA or AA for 15 min at 37°C in complete RPMI 1640 culture medium or incubation buffer (15 mM-HEPES, 135 mM-NaCl, 5 mM-KCl, 1·8 mM-$CaCl_2$, 0·8 mM-$MgCl_2$), as reported in the legends to the figures. Where indicated, NaCl was replaced with choline chloride in the incubation buffer.

Measurement of ascorbic acid content by HPLC

After the treatments, the cell suspensions were centrifuged, the resulting pellets were washed twice with saline A and extracted with ice-cold 70% (v/v) methanol–30% solution A (10 mM-tetrabutylammonium hydrogen sulphate, 10 mM-KH_2PO_4, 0·5% methanol; pH 6·0) containing 1 mM-EDTA. After keeping for 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 Inc., Milan, Italy) and analysed 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 by Savini *et al.*[25], with minor modifications. The assay involved the use of a 15 cm × 4·6 mm Discovery C-18, 5-μm column (Supelco, Bellefonte, PA, USA) equipped with a Supelguard Discovery C-18 guard column (2 cm × 4 mm, 5 μm). The injection volume was 20 μl. Under these conditions, the retention time of AA was about 4 min. AA concentration was determined from the corresponding calibration curve constructed with the pure chemical dissolved in the extraction solution. Intracellular concentration of AA was calculated using published values for cell volume[26].

Reduced glutathione determination

Cellular non-protein thiol content was determined as described by Beutler[27]. As GSH represents more than 90% of the non-protein thiols, the cellular non-protein thiol content is referred to as GSH here. In brief, cells (4×10^6) were washed three times with saline A and centrifuged; the pellet was then resuspended in 150 μl of a solution containing 1·67% metaphosphoric acid, 0·2% EDTA, 30% NaCl, kept on ice for 5 min and centrifuged for 5 min at 10 000 **g**. The GSH content was measured spectrophotometrically in the supernatant, at 412 nm, using 5,5′-dithiobis(2-nitrobenzoic acid) ($\varepsilon_{412} = 13\,600$/M per cm).

NAD(P)H assay

Intracellular NADH, NAD(P)H, NAD^+ and $NADP^+$ were measured using a modification of the method described by Stocchi *et al.*[28]. After the treatments, the cell suspensions (20×10^6 cells per sample) were centrifuged and the resulting pellets were washed twice with saline A. Pellets were then resuspended in 600 μl of cold 0·25 M-KOH and, after vigorous vortexing, the samples were transferred into Amicon Ultra-4 Centrifugal Filter devices (cut-off 30 000; Millipore Inc.) and then centrifuged for 20 min at 7500 **g** (4°C). A 20% (v/v) of potassium phosphate monobasic (KH_2PO_4; 1 M; pH 6·0) was added to the filtrate and immediately analysed by HPLC, with the UV detection wavelength set at 265 nm. The column used was a 25 cm × 4·6 mm Discovery C-18 (5 μm; Supelco) equipped with a Supelguard Discovery C-18 guard column (2 cm × 4 mm, 5 μm). The injection volume was 200 μl. Under these conditions, the retention time periods of $NADP^+$ and NAD^+ were about 11 and 18 min, respectively, whereas those of NAD(P)H and NADH were about 16 and 20 min, respectively.

Catalase assay

Catalase activity was measured spectrophotometrically in fetal bovine serum following the procedure previously described by Beutler[27].

Cytotoxicity assay

Viability was determined with the trypan blue exclusion assay. In brief, an aliquot of the cell suspension was diluted in the ratio 1:2 (v/v) with 0·4% trypan blue and viable cells (i.e. those excluding trypan blue) were counted with a haemocytometer.

Dihydrorhodamine 123 oxidation

Cells were first exposed for 15 min to 10-μM-DHR in RPMI 1640 complete medium, subsequently treated as indicated in the figure legends and finally analysed for DHR fluorescence with a BX-51 microscope (Olympus, Milan, Italy), equipped with a SPOT-RT camera unit (Diagnostic Instruments; Delta Sistemi, Rome, Italy). The excitation and emission wavelengths were 488 and 515 nm, respectively, with a 5-nm slit width for both emission and excitation. Images were collected with exposure time periods of 100–400 ms, digitally acquired and processed for fluorescence determination at the single-cell level on a personal computer using Scion Image software (Scion Corporation, Frederick, MD, USA). Mean fluorescence values were determined by averaging the fluorescence values of at least fifty cells per treatment condition per experiment.

Statistical analysis

The results are expressed as means and standard deviations. Statistical differences were analysed 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.
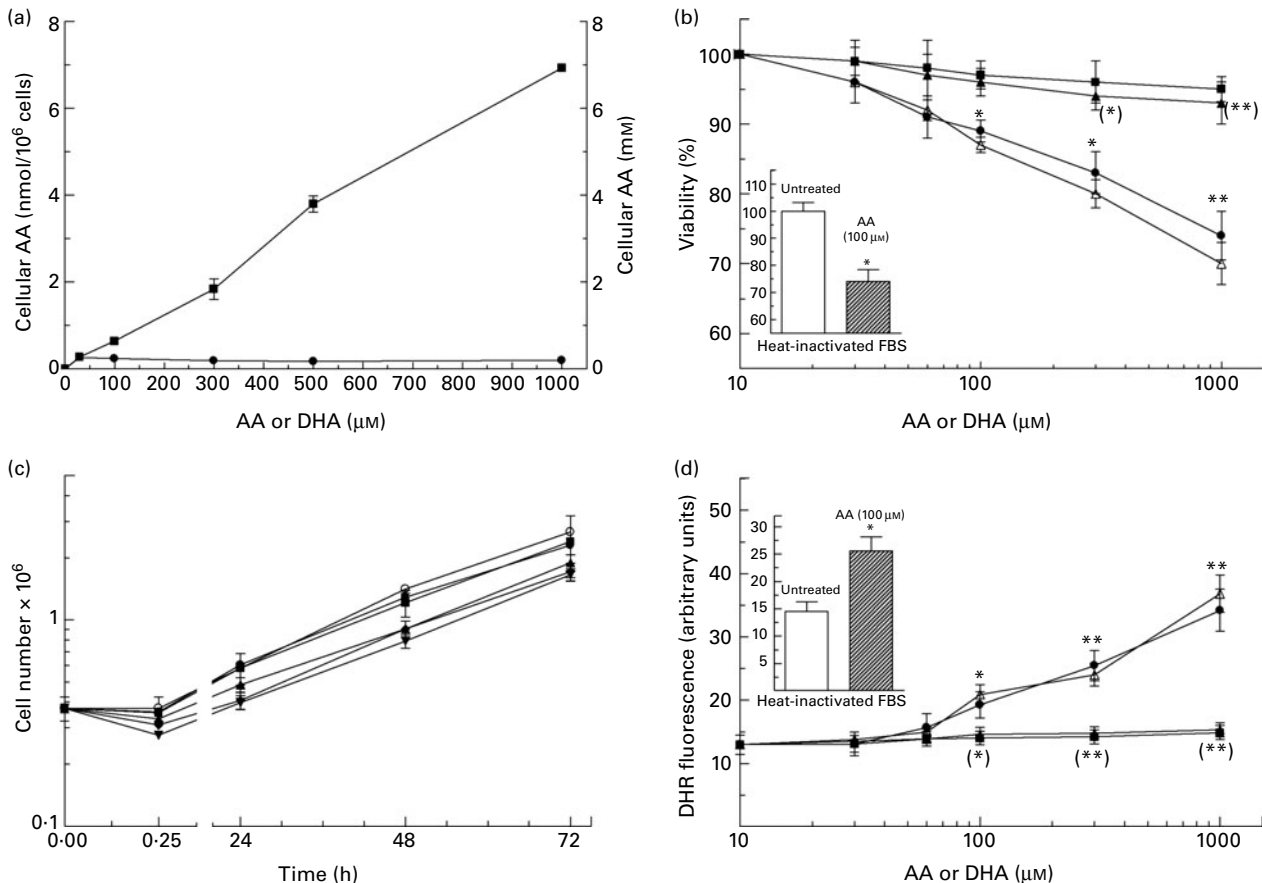
Fig. 1. Ascorbic acid (AA, –●–) and dehydroascorbic acid (DHA, –■–) uptake and the ensuing toxicity. (a) U937 cells were exposed for 15 min to increasing AA or DHA concentrations in complete Roswell Park Memorial Institute (RPMI) 1640 medium and then processed for the assessment of cell-associated AA. (b) Cells were treated as indicated above and analysed for viability using the trypan blue assay. Inset: Cells were treated with 100 μM-AA in RPMI 1640 medium supplemented with heat-inactivated serum and analysed for viability. Mean values were significantly different from those of untreated cells: * $P < 0.01$, ** $P < 0.001$. –▲–, AA + catalase; –△–, AA + boiled catalase. (c) Growth kinetics of cells treated for 15 min with increasing concentrations of AA. –○–, Untreated; –●–, AA (30 μM); –■–, AA (60 μM); –▲–, AA (100 μM); –◆–, AA (300 μM); –▼– AA (1000 μM). (d) Dihydrorhodamine 123 (DHR)-preloaded cells were treated, as detailed in (b) (main graph and inset), and analysed for DHR fluorescence. Values are means, with standard deviations calculated from at least three separate experiments represented by vertical bars. Mean values were significantly different from those of cells treated with AA alone (ANOVA followed by Bonferroni's test): * $P < 0.01$, ** $P < 0.001$. FBS, fetal bovine serum.

Results and discussion

Short-term (15 min) exposure of U937 cells to DHA in complete culture medium leads to extensive accumulation of the vitamin in its reduced form. Approximately 7 mM-AA was indeed detected in cells exposed to 1 mM-DHA and immediately processed with DTT (Fig. 1(a)), and these results were virtually superimposable on those obtained without DTT (data not shown). As expected from previous studies[26,29–31], the net amount of vitamin C accumulated is dramatically lower when DHA is replaced with equimolar concentrations of AA, thereby leading to the conclusion that U937 cells, as numerous other cell types[13,16,31], preferentially take up the oxidised form of the vitamin[17,26,29]. Although this interpretation appears logical on the bases of the results illustrated in Fig. 1(a), the experimental approach suffers from a major drawback, seriously questioning the validity of the above conclusion. Indeed, it is not appropriate to increase AA concentration up to 1 mM, when the latter is normally present at 10- to 20-fold lower concentrations in most biological fluids[6,7]. In addition, and most importantly, the above approach is not correct as AA is taken up by the cells via a low-capacity Na^+–dependent transport, in contrast with DHA entering the cells via high-capacity GLUT.

In other words, the experiments summarised in Fig. 1(a) use non-physiological, but nevertheless ideal, conditions for DHA uptake, whereas, at the very best, there is no advantage for the high AA concentrations when the vitamin is taken up via saturable transport mechanisms.

An additional important consideration further questioning the significance of findings illustrated in Fig. 1(a) derives from toxicity studies, providing evidence of cell lysis after exposure to high AA concentrations (Fig. 1(b)). There was no evidence of trypan blue-positive cells at the end of AA exposure, and cells lysis was inferred from the reduced number of viable (i.e. trypan blue-negative) cells. Although the viability of these residual cells is emphasised by their proliferation rates monitored over 3 d of post-challenge growth (Fig. 1(c)), it appears nevertheless reasonable to conclude that AA uptake is measured in cells recovering from a potentially lethal damage.

Toxicity appears to be largely attributable to extracellular H_2O_2 formation, as previously determined[24], and a catalase-sensitive DHR-derived fluorescence response is indeed readily observed after exposure to AA (Fig. 1(d)). Both events were suppressed by exogenous catalase and unaffected by the boiled enzyme (Fig. 1(b) and (d)). In addition, H_2O_2 formation and toxicity were actually enhanced by heat inactivation of enzyme(s) of the culture medium, presumably catalase contained in the serum (7·32 (SD 0·76) U/ml; insets to Fig. 1(b) and (d)). In remarkable contrast with these findings, there was no evidence of delayed H_2O_2 formation (Fig. 1(d)) or toxicity (Fig. 1(b)) in DHA pre-loaded cells.

Collectively, the above results imply that comparisons between AA and DHA uptake in cultured cells should be restricted to low concentrations. Under the conditions used in the present study, AA concentrations should be below 60 μM in order to prevent confounding toxic effects. These are also physiologically relevant AA concentrations, as

they are commonly found in the bloodstream and other biological fluids[6,7]. Although DHA levels are normally very low[13,26], oxidation of AA may occur in biological fluids, thereby suggesting a physiopathological relevance restricted to findings from uptake studies using DHA concentrations in the low micromolar range.

The results illustrated in Fig. 2(a) are from experiments in which the cells were exposed for 15 min to 0–60 μM-AA or -DHA, and immediately analysed for their AA content,
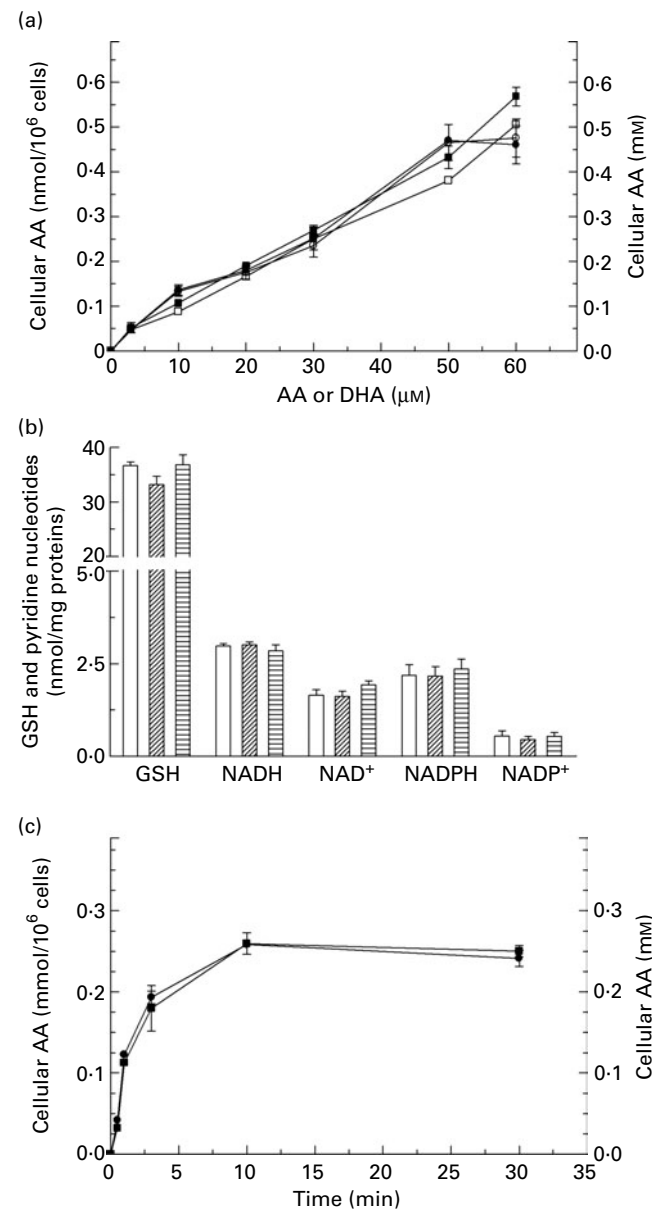

Fig. 2. Characterisation of ascorbic acid (AA) and dehydroascorbic acid (DHA) transport systems. (a) AA content in cells exposed for 15 min to 0–60 μM AA (-○-, -●-) or DHA (-□-, -■-) in complete Roswell Park Memorial Institute 1640 medium. Samples were processed for AA analysis with (-●-, -■-) or without (-○-, -□-) dithiothreitol (10 mM), as detailed in the Materials and methods section. (b) Reduced glutathione (GSH) and pyridine nucleotide levels in cells exposed to 30 μM-AA or -DHA, as indicated in (a). □, Untreated; ▨, AA; ▤, DHA. (c) Time dependence of AA accumulation in cells exposed to 30 μM-AA or -DHA. Values are means, with standard deviations calculated from at least three separate experiments represented by vertical bars.

with or without previous DTT (10 mM) treatment of the cell lysates. There was a linear relationship between the initial AA/DHA concentrations and the fraction of AA associated with the cells, unaffected by DTT and identical under the two conditions. A plateau for AA uptake was observed at 50 μM, under conditions in which cells accumulated a putative intracellular concentration of approximately 0·5 mM.

These results provide evidence for an about 10-fold intracellular accumulation of the reduced form of the vitamin, as in the case of exposure to 30 μM-AA or -DHA, promoting putative 0·25 mM-AA levels. Under these conditions, DHA reduction was not associated with changes in the redox state of the cells, as indicated by the assessment of cellular GSH, NADH, NAD$^+$, NAD(P)H and NADP$^+$ levels (Fig. 2(b)). Along the same lines, cells remained viable by virtue of criteria based on their ability to proliferate with kinetics superimposable on those observed with the untreated cells (data not shown). Identical outcomes are obtained from experiments using AA in the place of DHA (Figs. 1(c) and 2(b)).

Similarities in the low-dose AA and DHA uptake were not limited to the net intracellular accumulation of the vitamin in the absence of obvious signs of toxicity. Indeed, the time dependence of these responses was also similar, with a plateau reached after only 5–10 min (Fig. 2(c)).

Although these results emphasise the existence of similarities in AA and DHA uptake, the underlying mechanisms are in fact remarkably different. It is important to note that, in order to manipulate AA or DHA uptake, it was necessary to use saline (incubation buffer) as a milieu of exposure, in contrast with the experiments illustrated above, performed in complete culture medium. AA uptake was therefore measured under reductive conditions, i.e. in the presence of 0·1 mM-DTT, to prevent extracellular AA oxidation. Interestingly, AA accumulation detected under these conditions (30 μM, Fig. 3) was virtually identical to that measured after incubation in complete culture medium (Fig. 2(a)) and,
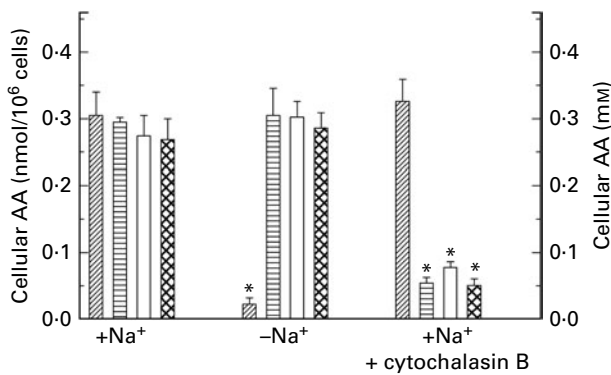
although unaffected by cytochalasin B, a well-established inhibitor of GLUT[25,29,32], was abolished by Na$^+$ omission (replaced with choline)[14,25].

These findings are in line with the notion that AA accumulation resulting from AA exposure is entirely dependent on AA uptake through Na$^+$-dependent transport, with hardly any contribution of DHA uptake, which might take place as a consequence of extracellular oxidation of AA.

Results obtained with DHA (Fig. 3) were also remarkably similar to those illustrated in Fig. 2(a), thereby implying lack of competition between extracellular glucose (10 mM) and DHA (30 μM). Furthermore, AA accumulation resulting from DHA exposure (putative intracellular concentration close to 0·3 mM) was unaffected by Na$^+$ omission and suppressed by cytochalasin B.

These findings therefore indicate that AA accumulation resulting from DHA exposure is entirely dependent on DHA uptake through GLUT, with hardly any contribution of AA uptake, which might take place as a consequence of extracellular reduction of DHA.

In summary, under the conditions used in the present study, AA and DHA are taken up by U937 cells via Na$^+$-dependent transporters and GLUT, respectively, but nevertheless with identical rates and efficiencies. Hence, extracellular oxidation of AA is expected to switch the mode of uptake without affecting the amount of the vitamin taken up by the cells. The results illustrated in Fig. 3 are consistent with this notion, as addition of ascorbate oxidase to cells exposed to AA on the one hand failed to affect the net amount of the vitamin associated with the cells and on the other hand switched the mode of uptake to a mechanism insensitive to Na$^+$ omission and blunted by cytochalasin B. Ascorbate oxidase did not promote effects in cells exposed to DHA.

The switch in the uptake mechanism is of obvious advantage, as it allows AA accumulation independently of the status of the extracellular vitamin C (i.e. reduced v. oxidised forms). It may also be speculated that cells bathed in biological fluids containing greater levels of vitamin C (e.g. >60 μM), may take advantage by a switch from a low- to a high-capacity uptake mechanism of the vitamin. The increased ability of the cells to take up vitamin C would be consistent with its role as an anti-oxidant[33–35] or, more generally, as an agent providing reducing equivalents.

In conclusion, the results presented in the present study indicate that U937 cells take up low micromolar concentrations of vitamin C as AA (via Na$^+$-dependent transporters) or as DHA (via GLUT) with identical rates and kinetics. It follows that the net amount of the vitamin associated with the cells is independent on events, resulting in oxidation of extracellular AA. This information is of potential importance, as U937 cells belong to the monocyte/macrophage lineage and the inflammatory response is associated with the release of oxidants, potentially leading to AA oxidation.

Acknowledgements

Fig. 3. Mechanism of ascorbic acid (AA) uptake in cells supplemented with ascorbate oxidase (AO). AA accumulation was determined in cells exposed for 15 min to: 30 μM-AA alone (▨) or associated with AO (▤), either in Na$^+$-containing or Na$^+$-free incubation buffers; 30 μM-DHA alone (□) or associated with AO (▩), either in Na$^+$-containing or Na$^+$-free incubation buffers. Treatments in Na$^+$-containing incubation buffer were also performed in cells supplemented with cytochalasin B. Values are means, with standard deviations calculated from at least three separate experiments represented by vertical bars. *Mean values were significantly different from those of cells treated in the Na$^+$-containing incubation buffer ($P < 0.001$; ANOVA followed by Dunnett's test).

O. C. O. C. and M. F. designed the study. C. A., A. G and M. F. conducted experiments and analysed the data. The paper was collegially written by all authors. O. C. had the primary responsibility for the final content. The authors declare no conflict of interest.

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