Two distinct uptake mechanisms for ascorbate and dehydroascorbate in human lymphoblasts and their interaction with glucose

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In diabetes, a major cause of mortality is from cardiovascular causes, and low levels of antioxidants such as vitamin C have been associated with such complications. Leucocyte ascorbic acid status can reflect total body stores but the mechanisms that mediate the uptake of ascorbic acid (AA) or dehydroascorbic acid (DHA) in human lymphoid cells are undefined. We have investigated the uptake of AA and DHA with mass assays in human lymphoblasts by using HPLC, with precautions to prevent the oxidation of AA and to take into account the instability of DHA in buffers. Human lymphoblasts exhibit distinct uptake mechanisms for both AA and DHA, with $V_{\max}$ values of 1.35 ± 0.14 and 29.0 ± 5.8 nmol/h per 10^6 cells and K_m values of 23.5 ± 6 and 104 ± 84 μ M respectively. The AA uptake was Na^+ -dependent and inhibitable with ouabain, whereas DHA uptake was independent of Na^+ and ouabain-insensitive. Both uptake mechanisms were inhibited by phloretin or cytochalasin B. AA uptake was decreased significantly (by $13 \pm 2\%$) only at extra-

cellular glucose concentrations of 20 mM ($P < 0.05$). In contrast, glucose competitively inhibited DHA uptake with a K_i of 2.2 mM so that DHA uptake was decreased by glucose even in the physiological range. Phorbol esters stimulated AA but not DHA uptake; this was abolished in the presence of extracellular reductant, indicating that AA was converted to DHA before uptake occurred. Prolonged increased glucose levels (20 mM) led to a decrease in the $V_{\max}$ of DHA uptake. At concentrations of plasma AA or DHA, the AA uptake mechanism might be nearly half-saturated but the DHA mechanism has enormous spare capacity. This allows for cellular uptake and regeneration of AA from DHA derived from oxidative stress. In diabetes, high glucose levels might impair DHA uptake acutely by competitive inhibition or by down-regulation of uptake with chronic glucose exposure, leading to an impaired ability to store and recycle oxidized AA.

INTRODUCTION

Cardiovascular disease remains a major cause of mortality in diabetes, especially in those with nephropathy. Although it is recognized that the pathogenesis of atherosclerosis is multifactorial, recent hypotheses implicating the damaging effect of reactive oxidative free radicals have been proposed [1]. *In vivo*, several free-radical scavenging systems exist, and ascorbic acid (AA) is an important component of these defences against oxygen toxicity and lipid peroxidation [2] by decreasing the production of atherogenic oxidized low-density lipoproteins and facilitating the recycling of lipophilic anti-oxidants such as α -tocopherol. Epidemiological studies have revealed associations between a low AA status and higher rates of cardiovascular disease such as stroke and coronary heart disease [3,4].

In diabetic patients, a consistent finding is a lowered AA status, as measured by levels in both plasma and leucocytes [5–8]. A small study had also indicated that leucocyte dehydroascorbic acid (DHA) uptake is decreased in diabetic patients [6] mainly by a decreased $V_{\max}$ of the transport system, although the mechanism was not examined. AA has an important role in collagen synthesis and platelet function [9], and changes in the polyol pathway in diabetes might affect AA status [10]. The important role of AA in collagen and proteoglycan metabolism [11–14] suggests that a relative deficiency could lead to alterations in aryl sulphatase B activity and decreased sulphation of glycosaminoglycans, and hence altered basement membrane charge, permeability and function. This could provide a basis for the pathogenesis of

diabetic complications involving the cardiovascular and renal systems. In addition, the role of AA in modulating endothelial function [15,16] and in inhibiting the rate-limiting step of cholesterol synthesis, 3-hydroxy-3-methylglutaryl-CoA reductase [17], might indicate a role in the pathophysiology of cardiovascular disease. The effect of AA on improving whole-body glucose disposal underscores its involvement in modulating insulin action in both normal and diabetic subjects [18].

With the introduction of a specific and sensitive assay for AA by using HPLC [19,20] and the realization that conditions for sample preparation and storage are important for the accuracy of assays [20], recent research has focused on the mechanisms that mediate the uptake of DHA and AA [21–26]. Characterization of uptake processes has been performed predominantly on neutrophils, myeloid cells and fibroblasts. The levels in mononuclear cells might be higher than in myeloid cells [27] and the excellent correlation of leucocyte AA with the total body pool of AA suggests the it might be useful for non-invasive monitoring of AA status *in vivo*. However, the uptake mechanisms in lymphoid cells have not been extensively studied.

There is controversy over whether uptake into myeloid cells and fibroblasts is mediated as AA itself [21,22] or as DHA [24–26], or whether these cells could possess both uptake mechanisms [23]. In studies where precautions had been taken to prevent the oxidation of AA [24–26], no uptake has been demonstrated. Also, the different methods used in generating DHA and the known instability of this substance in solution might have accounted for some of the differences described in

Abbreviations used: AA, ascorbic acid; DHA, dehydroascorbic acid; DTT, dithiothreitol; HBS, Hepes-buffered saline.

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these studies. The interaction of these uptake mechanisms with glucose and other hexoses is also debated, some authors finding non-competitive and competitive inhibition of AA uptake [21] and others finding competitive inhibition of DHA uptake [24]. These relationships between AA or DHA uptake and glucose level are important because they might have a bearing on the cellular total AA status in poorly controlled diabetes. A decreased ability of cells to take up and store AA would result in an increased urinary loss of this important antioxidant.

In view of the importance of the leucocyte as an index of AA status, and the possibility that this is affected by hyperglycaemia, we have evaluated the uptake of both DHA and AA in human lymphoblasts, with precautions to prevent the oxidation of AA and with corrections to account for DHA hydrolysis in buffers. The AA in cells was determined by using a specific and sensitive HPLC method. Two distinct uptake mechanisms for AA and DHA were demonstrated, with differences in sensitivity to extracellular Na^+ and ouabain and to inhibition by glucose. The DHA uptake mechanism, being relatively unsaturated in normal circumstances, might be especially important in the recycling of AA when cells are subjected to oxidative stress. Impairment of this process in hyperglycaemia might thus account for the depletion of cellular AA stores that has been documented in diabetes [6–8].

METHODS

Materials and cell culture

AA, non-esterified fatty acid free BSA, glutamine, penicillin and streptomycin, D-mannitol, cytochalasin B, phloretin, ouabain, PMA, reduced glutathione, cysteine and RPMI 1640 medium were from Sigma Chemicals Ltd. (Poole, Dorset, U.K.). Foetal calf serum was from Advanced Protein Products, Birmingham, U.K. RPMI 1640 growth medium contained 2 mM glutamine, 15% (v/v) foetal calf serum, 5 mM glucose, 10^5 i.u./l penicillin, 100 mg/l streptomycin and buffered with 24 mM NaHCO_3 [pH 7.4 in air/ CO_2 (19:1)]. HPLC-grade acetonitrile was from Fisons, (Loughborough, Leics., U.K.).

Human lymphoblasts were from normal non-diabetic subjects and were immortalized by Epstein–Barr virus as previously described [28]. They were cultured in RPMI 1640 growth medium and maintained at a density between 0.25×10^6 and 10^6 cells/ml by regularly changing the medium every 2 days. Fresh stocks of cells were recovered from liquid nitrogen storage every 2 months to ensure consistency in uptake studies.

Mass transport assays for AA and DHA

Approximately 5×10^6 cells were used for each assay. These cells were recovered from the RPMI 1640 growth medium, washed twice in Hepes-buffered saline (HBS) composed of (in mM) NaCl 140, KCl 5, CaCl_2 1.8, MgSO_4 0.8, glucose 5, Hepes 10, NaH_2PO_4 1 with the pH adjusted to 7.4. The cells were then pre-warmed in HBS for 5 min. Na^+ -free HBS contained *N*-methylglucamine and KH_2PO_4 in place of NaCl and NaH_2PO_4 . The correct amount of AA or DHA (both freshly prepared) was then pipetted into tubes containing known numbers of cells and incubated for appropriate lengths of time. Uptake was terminated by plunging the tubes into ice, spinning the cells down on a microcentrifuge and washing the cells three times in PBS. In preliminary studies we established that three washes (over 2 min) were sufficient to deplete extracellular AA or DHA to negligible levels. We had also evaluated the amount of AA that could have leaked out of the cells during these washes and this was negligible unless cells were left in PBS for 30 min, by which time a 10% loss was

incurred. After the last wash, cells were lysed in 0.5 ml of 20 g/l metaphosphoric acid/0.54 mM EDTA [29] to stabilize the AA and DHA, and precipitated proteins were centrifuged down [29]. The supernatants were stored at -70°C until analysis, which was usually within 1 week. We had also established that this method of preserving AA and DHA was effective for periods of up to 6 weeks.

A fresh solution of AA (100 mM) was prepared before each uptake experiment, and incubations with AA also contained 0.1 mM dithiothreitol (DTT) to prevent the oxidation of AA [24–26]. In the presence of DTT, AA remained stable in HBS for over 2 h. Thus, for uptake experiments with AA, the extracellular concentration was predictable over this period, and DTT was always present to prevent oxidation of the substrate.

For DHA studies this substrate was prepared fresh before each experiment owing to its instability. Bromine oxidation of AA, as described by Washko et al. [22], was performed with 10 μl of bromine solution added to 2 ml of 20 mM AA. Bromine was removed by bubbling nitrogen gas through the solution. This DHA solution was stable when kept in the dark on ice for up to 1 h, but we routinely used only solutions prepared within 30 min. During DHA uptake experiments, the level of DHA decayed rapidly from irreversible hydrolysis, and thus its concentration could not be predicted easily from the known amounts added at the onset of incubation. We therefore measured the half-life of DHA at different concentrations of this substrate (see the Results section). This enabled a reliable estimate of average DHA concentrations when known amounts were added to the HBS, by integrating the area under the curve. No DTT was used in the HBS incubations for DHA uptake studies.

Incubations were performed with inhibitors such as cytochalasin B, phloretin and ouabain (all at 100 μM). Cells were always pre-incubated with inhibitors for 15 min at 37°C before addition of the AA or DHA. In experiments with PMA, 100 nM was added to cells 20 min before the AA or DHA.

Measurement of AA and DHA

Measurements of AA were performed with isocratic HPLC, on a Whatman Partisil 10 SAX anion-exchange column, with acetonitrile/50 mM KH_2PO_4 (3/1, v/v) as the mobile phase as previously described [19]. Elution of the 50 μl sample loop was set at a flow rate of 1.5 ml/min; under these conditions the retention time of AA was typically 2.5 min. AA was detected by an on-line Waters 486 tunable absorbance detector set at 270 nm with data stored for further analysis with the Waters Millenium 2010 program. Fresh AA standards were always used and were checked several times a day. Calibrations were linear in the range 10–200 ng AA. DHA was not measurable by this method because its absorbance maximum was at 227 nm. DHA was therefore reduced to AA by neutralizing the metaphosphoric acid in samples with KOH to pH 7.5 and adding DTT to a final concentration of 10 mM. Reduction was complete after 10 min (on ice). We checked all these processes by using a known amount of AA, which was then oxidized to DHA with bromine. Subsequent reduction with DTT recovered 98% of the original amount of AA. Thus DHA could be calculated as the difference between the measured AA and total vitamin C (AA and DHA) in specimens. The coefficients of variation for AA and DHA measurements were both less than 2%.

Statistics

Data were analysed with an Oxstat statistics package (Microsoft Corporation, Reading, Berks., U.K.). Means and S.D. are reported, and comparisons were by Student's *t* test. Two-tailed *P*

values less than 0.05 were taken as significant. Curve fits were performed by using P-fit (Biosoft Corporation, Cambridge, U.K.). These parameters were also checked with another non-linear optimization algorithm [28].

RESULTS

AA uptake in lymphoblasts was linear for up to 2 h, and concentrations of AA in the HBS at 37 °C remained stable over this period. Thus AA uptake experiments were performed over 1 h. However, DHA was very unstable and decayed exponentially when added to HBS at 37 °C so that DHA uptake by the cells was not linear over the 20 min. We therefore measured the half-life of DHA at different starting concentrations of the substrate, to obtain a relationship between DHA concentration and its decay rate constant k (Figure 1). Higher concentrations of DHA between 1 and 2 mM were more stable, with slower decay rates.

Figure 1 Relationship of the DHA decay rate constant (k) in HBS at 37 °C to the original concentration of DHA in the solution

Means $\pm$ S.D. are plotted where the S.D. exceeds the size of the symbol. On the right-hand scale is shown the average achieved DHA concentration over 20 min when the depicted original DHA concentration on the x-axis was added.

Figure 2 DHA uptake after correction for loss of DHA during incubation

When the DHA loss during incubations was calculated from Figure 1 and appropriate amounts of DHA were added every 2 min, the concentration of DHA (■, right axis) remained constant over 20 min. This enabled a demonstration that lymphoblast DHA uptake (○, left axis) remains linear over this period of time.

Figure 3 Concentration dependence of AA uptake with levels of AA up to 500 μ M

A typical kinetic uptake curve for one lymphoblast line (solid line) comprises the sum of a saturable Michaelis-Menten curve and a passive diffusional component (dotted line).

Figure 4 Concentration dependence of AA uptake at high concentrations of substrate (up to 6 mM AA)

Means $\pm$ S.D. for three experiments are plotted.

By using this relationship it was possible to predict the mean concentration of DHA over a 20 min period by integrating the area under the concentration-time curve; these actual figures are used for the kinetic analyses of DHA uptake in the cells. Moreover we confirmed the validity of this approach by replacing the hydrolysed DHA (as calculated from the relationship in Figure 1) every 2 min and showed that DHA remained stable over a 20 min period, resulting in a linear DHA uptake in the lymphoblasts (Figure 2). The relationship between initial DHA concentration and the average achieved DHA concentration in HBS over 20 min at 37 °C is illustrated in Figure 1.

Most (98 %) of the cellular vitamin C was in the form of AA, even when DHA was the transported substrate. DHA was thus rapidly reduced to AA within the cells. The lymphoblasts transported AA in a saturable fashion (Figure 3) so that uptake was virtually saturated at 200 μ M AA. The uptake relationship could be best described by a Michaelis-Menten curve superimposed on a passive diffusional component (Figure 3). At higher concentrations of AA (between 1 and 6 mM; Figure 4), uptake remained linear and parallel to the passive diffusional component, with no evidence of a low-affinity uptake mechanism

Figure 5 Concentration dependence of DHA uptake, illustrating a typical kinetic uptake curve (solid line) for a lymphoblast line

This is the sum of a saturable Michaelis–Menten curve and a passive diffusional component (dotted line).

Table 1 Kinetic parameters of AA and DHA uptake mechanisms in lymphoblasts

Results are means $\pm$ S.D. ($n = 5$).

Mechanism	$V_{\max}$ (nmol/h per 10^6 cells)	K_m (μ M)	Passive diffusion component (nmol/h per 10^6 cells per mM)
AA uptake	1.35 ± 0.14	23.5 ± 6.0	0.27 ± 0.15
DHA uptake	29.0 ± 5.8	104 ± 84	20.5 ± 11.2

in these cells. When the uptake of DHA was examined at different concentrations of DHA (Figure 5), the total uptake was best described as the sum of a saturable Michaelis–Menten curve and a passive diffusional component. Higher concentrations of DHA (above 1 mM) could not be studied owing to a toxic effect on the cells. The uptake parameters of AA and DHA are compared in Table 1. The $V_{\max}$ of DHA uptake is approx. 20-fold higher than that of AA in these cells, and the affinity of the uptake mechanism for AA is higher than that for DHA. The diffusional component for DHA is greater than that for AA, and this is not surprising because DHA is a more lipophilic molecule.

We examined the effect of various reducing agents on the uptake of AA (100 μ M), to evaluate whether DTT had any non-specific effects on AA uptake. All solutions with reducing agents were freshly prepared and contained DTT, reduced glutathione or cysteine (all at 100 μ M). Uptakes of AA were normalized to 100% in cells without addition of reductants. The AA uptake in the absence of reducing agents ($100 \pm 7\%$, $n = 6$) was not different from that in the presence of DTT ($98 \pm 4\%$), reduced glutathione ($102 \pm 7\%$) or cysteine ($101 \pm 3\%$). Thus the presence of DTT had no non-specific toxic effect on the cellular uptake of AA.

To study the characteristics of uptake to assess whether distinct mechanisms existed, AA and DHA concentrations near the $V_{\max}$ values were chosen (100 μ M and 1 mM respectively, unless otherwise stated). Table 2 demonstrates that in Na^+ -free HBS or after preincubation with 100 μ M ouabain, uptake of AA was

Table 2 Effect of extracellular Na^+ depletion or incubation with 100 μ M ouabain on AA or DHA uptake in human lymphoblasts

Means $\pm$ S.D. for three experiments are shown.

Condition	Uptake (nmol/h per 10^6 cells)	
	AA	DHA
Control	0.34 ± 0.02	28.8 ± 7.1
Na^+ -free medium	0.02 ± 0.005	23.0 ± 1.8
Plus ouabain (100 μ M)	0.12 ± 0.006	28.8 ± 1.7

Figure 6 Lineweaver–Burk plots of saturable component of DHA uptake, derived from cells co-incubated with either 5 (▲) or 20 (○) mM D-glucose

The different slopes of the plots with the same y -intercept indicate competitive inhibition of DHA uptake by glucose.

very significantly impaired, whereas these conditions had no significant effect on DHA uptake. Both cytochalasin B and phloretin inhibited AA uptake by $50.9 \pm 3.0\%$ ($n = 4$) and $85.0 \pm 5.2\%$ ($n = 3$) respectively ($P < 0.001$ for both). DHA uptake was inhibited by cytochalasin B by $91.8 \pm 4.2\%$ ($n = 3$, $P < 0.001$) and phloretin by $89.6 \pm 6.0\%$ ($n = 3$, $P < 0.001$). These transport inhibitors are non-specific and could inhibit a variety of glucose transporters [21].

Owing to the association of a depleted AA status with diabetes, we examined the effects of D-glucose on these two different transport mechanisms. At glucose concentrations ranging from 1 to 10 mM glucose, AA uptake was unchanged (results not shown). However, at 20 mM glucose there was only a $13 \pm 2\%$ ($n = 4$, $P < 0.05$) inhibition of AA uptake compared with the uptake at 5 mM glucose. Thus glucose levels similar to those in poorly controlled diabetes had only a marginal effect on AA uptake in these cells.

In contrast, D-glucose had marked effects on the uptake of DHA. If uptake of DHA with an extracellular glucose of 1 mM was taken as 100%, then at 5, 10 and 20 mM glucose, DHA uptake was $85 \pm 17\%$, $77 \pm 15\%$ and $56 \pm 13\%$ respectively ($n = 3$, $P < 0.01$). The mode of inhibition of DHA uptake by D-glucose was analysed further by measuring DHA uptake at different DHA concentrations when co-incubated with either 5 or 20 mM D-glucose. Lineweaver–Burk plots of the saturable component of DHA uptake (Figure 6) demonstrated that glucose was a competitive inhibitor of DHA uptake, with no change in

Table 3 Effect of phorbol ester (PMA, 100 nM) on AA and DHA uptake, in the absence or presence of 0.1 mM DTT

Uptakes in both control and PMA-treated cells were performed without DTT. Means $\pm$ S.D. for three experiments are shown.

Condition	Uptake (nmol/h per 10^6 cells)	
	AA	DHA
Control	0.33 ± 0.02	28.9 ± 7.0
PMA	0.55 ± 0.11	24.3 ± 2.6
PMA + DTT	0.39 ± 0.02	24.3 ± 2.7

the $V_{\max}$ of uptake but a significant increase in the K_m , so that glucose decreased the affinity of the transporter for DHA. This was confirmed in two other separate experiments. The K_i of glucose was calculated to be 2.2 mM.

The chronic effect of incubation with D-glucose on DHA uptake was investigated by culturing cells in RPMI 1640 growth medium with added glucose (to a final concentration of 20 mM). An osmotic control was performed with mannitol to replace any added glucose. After 2 weeks, cells were recovered and the $V_{\max}$ of DHA uptake was determined. Cells chronically exposed to high glucose concentrations (20 mM) had a decreased DHA uptake of 17.9 ± 1.8 nmol/h per 10^6 cells compared with 27.9 ± 3.4 nmol/h per 10^6 cells in control cells cultured in 5 mM glucose ($n = 4$, $P < 0.002$). The cells cultured in the mannitol osmotic control medium had a DHA uptake rate of 28.8 ± 4.2 nmol/h per 10^6 cells (not significantly different from controls).

Further evidence for the existence of two uptake mechanisms in these lymphoblasts was obtained with the response of these cells to phorbol esters. Phorbol esters have been documented to increase AA uptake by cells, owing to the production of oxidants from cell activation [22]. Human lymphoblasts incubated with PMA (100 nM) for 20 min before the determination of AA or DHA uptake demonstrated an enhancement of AA uptake by $62 \pm 19\%$ ($P < 0.01$; Table 3) but no significant change in the DHA uptake rate. This would be expected if the extracellular AA had to be converted to DHA before uptake occurred. Addition of a reductant (0.1 mM DTT) to the HBS together with the PMA abolished much of the increase in AA uptake caused by the phorbol ester, but had no effect on DHA uptake (Table 3). This provides further evidence in favour of two uptake mechanisms, with the DHA uptake being particularly important during conditions of oxidative stress where AA is oxidized to DHA extracellularly.

DISCUSSION

Diabetes is associated with a depleted AA status [5–8]. Because of the possible role played by AA in a variety of physiological processes, including the synthesis of collagen, aryl sulphatase B activity and the sulphation of proteoglycans, platelet function, the rate-limiting step of cholesterol synthesis (3-hydroxy-3-methylglutaryl-CoA reductase), endothelial function and even insulin action [9–18], it has been postulated that AA could have important roles in the pathogenesis of cardiovascular and renal disease [1–4]. It is therefore important to understand the mechanisms underlying the cellular AA economy: impairment of uptake processes could lead to eventual cellular depletion because extracellular AA that could not be stored would be excreted via the kidneys.

Measurements of AA have previously been hampered by techniques that lacked sensitivity and specificity (reviewed in [20]). This has been revolutionized by the introduction of HPLC [19,20]. In addition, many previous studies had not made any corrections for the instability of DHA, so that kinetic parameters quoted might have been overestimated [30]. Even in studies that have used DTT in incubations [24–26], DHA was generated by using ascorbate oxidase rather than the more reliable bromine oxidation method [22,23] but no corrections were made for the decay of DHA in incubation buffers at 37 °C. Furthermore uptake studies involving AA might have been confounded by the presence of trace amounts of DHA [21–23]. However, in the present study the use of different reductants did not affect AA uptake substantially and it is unlikely that DTT had any deleterious effect on AA uptake.

In the present studies AA uptake was always performed in the presence of DTT, so that this was the only species of vitamin C present. We also took precautions to calculate the decay rates of DHA at different incubation concentrations. This enabled the definition of two distinct uptake mechanisms for AA and DHA in lymphoblasts, in contrast with previous studies that have demonstrated only AA or DHA uptake separately [21,22,24–26]. One other study has documented the co-existence of two distinct transporters in myeloid cells and fibroblasts [23]. In the human lymphoblasts, the two different uptake mechanisms show differing sensitivity to extracellular Na^+ and ouabain. The DHA uptake was not sensitive to extracellular Na^+ depletion, in contrast with the marked sensitivity of AA uptake. This has been described recently in HL-60 cells and myelocyte cell lines [23,26]. Ouabain might be inhibitory to AA uptake because of its decreasing the trans-epithelial Na^+ gradient. Furthermore the DHA uptake had a higher capacity and a lower affinity than the AA uptake mechanism. Thus at plasma concentrations of AA ranging from 30 to 100 μM [3,6,7] and of DHA ranging from 10 to 30 μM [7], the AA uptake would be operating at 50–90% full capacity but DHA uptake would only be at approx. 10% full capacity. The absolute rates of uptake of DHA exceed that of AA by 10–15-fold even at these plasma concentrations of substrate. These kinetic parameters would be especially useful when oxidative stress leads to the oxidation of AA to DHA in the extracellular fluid, leading to enhanced DHA uptake, and this is then rapidly reconverted to AA within the cell by DHA reductase [23].

The present K_m results for AA and DHA uptake contrast with some of the values obtained in the literature (reviewed in [30]). In many studies of AA uptake, no extracellular reductant was used. Also, some of the higher supraphysiological K_m values obtained for DHA uptake might have reflected the instability of DHA in buffers [30]. The K_m for DHA uptake mediated by GLUT-1 (60 μM) [25] is in the range that we have observed for DHA uptake in lymphoblasts, although the role of this transporter in lymphoblast DHA uptake has not been evaluated.

On the basis of these studies, hyperglycaemia in diabetes might have a variety of effects on AA status within cells. The uptake of AA itself might not be affected to a great extent, but it does not account for much of the total uptake of vitamin C. We demonstrated a small inhibitory effect of 20 mM glucose on AA uptake. The major effect of glucose is as a competitive inhibitor of DHA uptake (increased K_m with no change in $V_{\max}$), with an inhibitory constant in the physiological range of plasma glucose. Furthermore diabetes is associated with increased oxidative stress [7,31], and increased extracellular generation of DHA would necessitate the use of the DHA uptake mechanism to gain access to intracellular DHA reductase for recycling of oxidized AA. An additional effect is that chronic high glucose levels could

down-regulate the $V_{\max}$ of DHA uptake independently of any osmotic effect. This would further impair DHA uptake and would be relevant in chronic poorly controlled hyperglycaemic patients, in whom one previous study had demonstrated an impaired $V_{\max}$ for DHA [6]. These combined effects on DHA uptake would have an impact on the ability of cells to recycle any oxidized AA via DHA uptake and intracellular reduction, and would lead to cellular AA depletion over a period of time.

In conclusion, we have used mass assays of AA and DHA uptake to define two distinct uptake mechanisms for these vitamin C forms. DHA uptake was especially important in recycling oxidized AA generated by cell activation or oxidative stress. Thus AA depletion could occur in uncontrolled diabetes owing to increased oxidative stress and the inability of cells to recycle AA because of the acute and chronic effects of glucose on DHA uptake.

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