Interaction of respiratory burst and uptake of dehydroascorbic acid in differentiated HL-60 cells

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HL-60 cells differentiated with DMSO increased their rates of uptake of ascorbate when they were activated with PMA. The rates observed after this activation were essentially the same as those with dehydroascorbic acid as the original transport substrate. The effect of activation was sensitive to the antioxidant enzymes superoxide dismutase and catalase. When ascorbate was oxidized *in situ* by chemical or enzymic oxidation, the rates of uptake were similar to those after activation of the cells by phorbol ester; however, in the latter case the extracellular vitamin remained largely in the reduced form and there was very little loss by degradation, whereas after immediate oxidation no more reduced ascorbate could be found outside the cells after a few minutes and a significant part of the total vitamin was lost. The generation of superoxide by xanthine/xanthine oxidase stimulated the uptake of ascorbate much less than the activation by phorbol ester; H₂O₂ was even less effective. Stimulation of the uptake by phorbol ester was also insensitive to GSH, in contrast with stimulation by the chemical oxidation of ascorbate. Stimulation of ascorbate uptake by phorbol ester was sensitive to the respiratory-burst inhibitor diphenyliodonium as well as the

protein kinase C inhibitor staurosporine, indicating the respiratory burst as the cause of stimulation. Activation of the cells by the phorbol ester also stimulated the uptake of dehydroascorbate as the original substrate, in a manner insensitive to antioxidants or inhibitors of the respiratory burst. In all cases the intracellular vitamin was completely in the reduced form. Kinetic characterization by the calculation of maximal velocities and apparent *K_m* values and assaying for the dependence of uptake rates on the ionic milieu and for inhibition by glucose analogues and inhibitors of glucose transport revealed that after treatment with phorbol ester the uptake of total vitamin C in differentiated HL-60 cells was largely due to the low-affinity high-capacity glucose transporter. In contrast, in non-stimulated cells reduced ascorbate was taken up by the Na⁺-dependent high-affinity low-capacity ascorbate transporter. This change was probably due to the oxidation of ascorbate and, simultaneously, the recruitment of additional transporter molecules to the cell surface.

Key words: HPLC-EC, phorbol ester, superoxide, transport, vitamin C.

INTRODUCTION

Neutrophils and related cells of the immune system can accumulate large quantities of ascorbic acid [1–3]. This tendency is greatly enhanced on activation of the respiratory burst owing to preferential uptake of the oxidized form, dehydroascorbic acid (DHA) [4,5]. The uptake of DHA occurs by glucose transporters of the GLUT family [6–9], mainly GLUT-1, which is preferentially expressed in these cells [10–12]. Within the cells, DHA is reduced to ascorbate and is stored in the reduced form [8,13–17].

However, it is not entirely clear whether the stimulation of vitamin C uptake after activation of the cells is entirely due to the oxidation of ascorbate or whether an additional enhancement of the transport capacity for either ascorbate or DHA is also responsible. A close correlation between the activation of neutrophils, the oxidation of ascorbate and the observed uptake rates was demonstrated by Levine and associates [4].

We show here that PMA, a standard activator of the respiratory burst, causes an increase in the uptake rate of reduced vitamin C (ascorbate), by initiating its oxidation by oxygen radicals, as well as an increase in the uptake rates for preformed DHA by enhancing the transport capacity of differentiated HL-60 cells, a tumour model for neutrophilic cells. The two effects occur by different mechanisms, both involving GLUT-type transport. Vera et al. [18] recently reported an increase in the affinity of the glucose transporter for DHA after treatment of

neutrophils with a chemotactic peptide; however, this is a different form of stimulation and its effects are probably not comparable with those obtained by chemical stimulant.

EXPERIMENTAL

Cell culture

HL-60 promyelocytic tumour cells (a gift from Dr. P. Chiba, Institute of Medical Chemistry, Vienna, Austria) were cultured in RPMI-1640 medium supplied with 2 mM glutamine, 50 i.u./ml streptomycin, 0.1 mg/ml penicillin and 10% (v/v) fetal calf serum in a humidified air/CO₂ (19:1) atmosphere. They were maintained in exponential growth by dilution with fresh medium every 2–3 days. Cell viability was determined by Trypan Blue exclusion and was always more than 90%.

Cell volume was measured with a Casy 1 cell counter TTC (Schaefer-Systeme GmbH, Reutlingen, Germany), and a value of 830 fl per cell was obtained (see the HPLC analysis section).

Differentiation

Neutrophilic differentiation of HL-60 cells was performed by incubation with 1.3% (v/v) DMSO for 6 days [19] or with 500 μM dibutyryl cAMP for 4 days [20]. The differentiated state was verified by morphological inspection (results not shown) and by a respiratory-burst assay performed by measuring the super-

Abbreviations used: DHA, dehydroascorbic acid; HCF(III), hexacyanoferrate(III); SOD, superoxide dismutase.

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oxide (O_2^{2-})-dependent reduction of ferricytochrome *c* [19] after stimulation with $1 \mu\text{g/ml}$ PMA [21,22]. A decrease in viability was corrected for in the uptake experiments by measuring the non-specific uptake of ascorbate, which was negligible (see below).

Uptake of vitamin C

The uptake of ascorbate or DHA was measured either with [^{14}C]ascorbate and scintillation counting or by the direct measurement of extracellular and intracellular ascorbate and DHA with HPLC and electrochemical detection. Whereas the former method was used preferentially to obtain kinetic data and to determine the specificity of the uptake, the latter was used to determine the nature of the extracellular and intracellular vitamin. The rates obtained were nearly equal under comparable conditions with both methods.

For the radioactive assay [23,24], cells were harvested by centrifugation at $200 g$ at 37°C for 10 min (1500 rev./min, Eppendorf 5403 centrifuge) and washed twice with prewarmed unsupplemented RPMI medium (this medium is free of vitamin C). They were then resuspended at 37°C in Tris/saline/glucose buffer, consisting of 140 mM NaCl, 20 mM Tris and 5 mM glucose adjusted to pH 7.4 with HCl, at a final density of 5×10^6 cells per $100 \mu\text{l}$. All additions used for the specific experiments were included in this volume. The reaction was started by the addition of [^{14}C]ascorbic acid (240 MBq/mmol) at the specified concentration. For concentrations higher than $200 \mu\text{M}$, $50 \mu\text{M}$ labelled ascorbate was pre-mixed with the appropriate amount of unlabelled substrate and the result was calculated accordingly.

To make DHA the transport substrate, either 5 mM potassium hexacyanoferrate(III) [HCF(III)] or between 1 and 3.5 units of ascorbate oxidase were added. This ensured the immediate quantitative oxidation of ascorbate to DHA, which was verified in parallel experiments by HPLC assay (see below).

After the time chosen, 1 ml of ice-cold buffer was added, followed by $500 \mu\text{l}$ of oil [dibutylphthalate/dioctylphthalate (4:1, v/v)]; the mixture was rapidly centrifuged in an Eppendorf laboratory centrifuge. Supernatant and oil were aspirated; the cell pellet was then dissolved in 0.5 ml of 0.5 M KOH containing 2% (v/v) Triton X-100, heated for 45 min at 60°C , neutralized with $250 \mu\text{l}$ of 1 M HCl, mixed with 4 ml of Beckman ReadySafe cocktail and counted in a Packard Tri-Carb Beta Counter. Appropriate blanks were run without cells or by adding the cells immediately before the addition of the terminating buffer.

Non-specific uptake was accounted for by the addition of a 100-fold excess of unlabelled ascorbate. It was negligible in all cases.

The uptake of DHA was not linear with time, owing to a decrease in the concentration of substrate (see the Results section). The initial velocity was calculated from the slope of the time curve of the uptake. Nevertheless, we observed that, for starting concentrations of $200 \mu\text{M}$ or less, the accumulation within 15 min was a true reflection of the uptake rate and was appropriate for comparative measurements such as the effects of inhibitors or for assaying the nature of the vitamin inside and outside the cells (see below). For practical reasons this parameter was then used in experiments not assigned to obtain the absolute kinetic values such as K_m or $V_{\max}$. All results of experiments performed in this way are therefore given as ascorbate accumulated within a given time or as concentrations.

Nature of vitamin C after cellular uptake

To determine changes in the redox state of the vitamin during the uptake process, 5×10^6 cells were incubated in a total volume of

$500 \mu\text{l}$, together with the addition specified, and the uptake was started by the addition of 100 nmol of ascorbate or DHA, yielding a starting concentration of $200 \mu\text{M}$. DHA was again either generated *in situ* (see above) or produced by the bromination of ascorbate [4] and used freshly. After 15 min the reaction was stopped and the mixture was centrifuged as described above.

An aliquot of supernatant was mixed with an equal volume of 10% (v/v) metaphosphoric acid. The cell pellet was dissolved in 5% (v/v) metaphosphoric acid with an ultrasound pulse from a Branson sonifier and centrifuged at $20000 g$ and 4°C for 10 min. The samples were either analysed immediately by HPLC or frozen at -80°C .

HPLC analysis

The samples were injected into the HPLC apparatus and separated on a Spherisorb C_{18} reverse-phase column by the method of Lykkesfeldt et al. [25]. The signals were produced with a Biometra electrochemical detector containing a glass/carbon electrode against an Ag/AgCl reference set at 600 mV .

To estimate the concentration of DHA, the sample was reduced by the addition of 10 mM dithiothreitol in 0.5 M Tris. After 5 min it was re-acidified with 0.2 M sulphuric acid and chromatographed. DHA was taken as the difference between the concentrations obtained in the presence and in the absence of dithiothreitol [26]. This was verified by the re-addition of ascorbate oxidase, which led to the complete disappearance of the signal. The intracellular concentrations were estimated from the measured cell volume (see the Cell culture section).

Calculations

Values for $V_{\max}$ and K_m under the various experimental conditions were calculated from Lineweaver–Burk plots obtained after assaying the transport rates with a set of different concentrations of ascorbate or DHA. Straight lines were obtained in all cases. For studies with inhibitors, Eadie–Hofstee plots were constructed. The lines themselves were calculated from the respective data sets with GraphPad Prism software. Statistical data are listed as means $\pm$ S.E.M. from at least three independent experiments. The Eadie–Hofstee plots are from single sets of experiments.

Materials

RPMI cell culture medium was from Biochrome-Seromed; glutamine, penicillin, streptomycin were from PAA-laboratories; fetal serum was from GIBCO; [^{14}C]ascorbate was obtained from New England Nuclear; ascorbate oxidase was from Roche-Boehringer (Mannheim, Germany); the HPLC columns were from Supelco and from Waters (pre-column); dibutylphthalate was from Serva. All other chemicals were from Sigma, Merck or Fluka.

RESULTS

Basal uptake rates

Uptake of vitamin C by HL-60 cells was dependent on its oxidation state. Transport of ascorbate occurred via a high-affinity low-capacity pathway (apparent K_m $35.3 \mu\text{M}$, $V_{\max}$ $10.8 \pm 0.3 \text{ pmol/min per } 10^6 \text{ cells}$), that of DHA via a low-affinity high-capacity pathway (apparent K_m 1.095 mM , $V_{\max}$ $740 \pm 51 \text{ pmol/min per } 10^6 \text{ cells}$). In cells differentiated with

Figure 1 Uptake of ascorbate (□, ■) and DHA generated by the oxidation of ascorbate with HCF(III) (△, ▲) or with ascorbate oxidase (○) in resting (□, △, ○) and in PMA-activated (■, ▲) differentiated HL-60 cells

The starting concentration was 100 μ M. Further experimental details are given in the Experimental section. Results are means $\pm$ S.E.M. for five experiments.

DMSO or dibutyl cAMP, both transport rates were lower: $V_{\max}$ values were 5.0 ± 0.2 pmol/min per 10^6 cells for ascorbate and 440 ± 10 pmol/min per 10^6 cells for DHA. The K_m values were the same as for non-differentiated cells.

Effects of PMA

The uptake of ascorbate was stimulated by PMA in differentiated cells. Resting cells showed no response. The uptake rate for ascorbate in the presence of PMA was practically the same as that in the presence of 2 mM HCF(III) or ascorbate oxidase, which corresponds to an uptake of DHA. At a starting concentration of 100 μ M ascorbate, the rate increased from 3.9 ± 1.4 to 45.3 ± 10.8 pmol/min per 10^6 cells with ascorbate oxidase, and to 41.7 ± 12.0 pmol/min per 10^6 cells in the presence of PMA. With both agents present at the same time, the rate increased still further to 71.9 ± 12.5 pmol/min per 10^6 cells (Figure 1). The stimulation of ascorbate uptake by PMA alone was partly inhibited by 50 units of superoxide dismutase (SOD) and completely inhibited by the dismutase together with 100 units of catalase. The stimulation of DHA uptake, in contrast, was not inhibited but was slightly stimulated by the antioxidative enzymes (see below).

A comparison of the kinetic data shows that, whereas the stimulation of ascorbate uptake by PMA was accompanied by an increase in the low-affinity transport, the stimulation of DHA uptake affected only the maximal rate. The K_m was the same as with DHA alone (Table 1).

Almost identical results were obtained when the cells were preloaded with ascorbate. This was achieved by incubation with 200 μ M unlabelled DHA, generated by the bromination of ascorbate, and the subsequent addition of PMA to the uptake mixture, followed by the uptake experiment. The purpose of this experimental setting was to show that the inwards concentration gradient of DHA was not saturated by preloading the cells. Apparently the reduction capacity of the cells was sufficient to permit the accumulation of large amounts of vitamin C (see below).

Table 1 Apparent affinity and maximal velocity of the uptake of ascorbate and of DHA by differentiated HL-60 cells in the resting state and after stimulation with PMA

$V_{\max}$ results are means $\pm$ S.E.M.

PMA	Ascorbate		DHA	
	$V_{\max}$ (pmol/min per 10^6 cells)	K_m (μ M)	$V_{\max}$ (pmol/min per 10^6 cells)	K_m (μ M)
—	5.0 ± 0.2	40.7	440 ± 10	1040
+	442 ± 32	703	899 ± 53	1105

Figure 2 Intracellular concentration (A) and residual concentration in the incubation medium (B) of ascorbate (open bars) and total vitamin C (ascorbate plus DHA) (cross-hatched bars) in differentiated HL-60 cells in the resting state and after activation with PMA 15 min after the start of the incubation with 200 μ M ascorbate, and effects of SOD and catalase (Cat)

The concentrations were determined by HPLC as described in the text. DHA was produced by enzymic oxidation with ascorbate oxidase (Asc-oxidase) or by the addition of HCF(III). Results are means $\pm$ S.E.M. for three experiments.

Nature of intracellular and extracellular vitamin C after 15 min of uptake

After incubation of the cells with 200 μ M ascorbate, almost no change occurred in the medium: the proportion accumulated by the cells was only 1%. No degradation of the vitamin was observed. Within the cells, 1 nmol (of a total of 100 nmol) of ascorbate had accumulated, which was also completely in the reduced state.

When ascorbate was oxidized enzymically or chemically by HCF(III), no more reduced ascorbate could be found outside the

Figure 3 Inhibition of the uptake of 100 μ M ascorbate [– HCF(III)] and DHA [+ HCF(III)] by GSH in differentiated HL-60 cells in the resting state and after activation with PMA

Results are means $\pm$ S.E.M. for three experiments.

cells and the total proportion of the newly formed DHA had decreased to approx. 40%. The intracellular concentration was, as expected, higher than without the oxidants and was entirely in the reduced form; however, the recovery was only 6.5–7 nmol (corresponding to the 10-fold uptake rate expected for DHA; see above), which was 6.5–7% of the total DHA present at the start of the experiment. Thus almost 50% was degraded during the uptake process, a typical indication of the instability of DHA.

However, with PMA the intracellular accumulation was only slightly lower than with the oxidants (approx. 6 nmol) but the extracellular loss was much less. Of the 82 nmol still found in the medium after 15 min, 72 nmol was in the reduced state. Thus only 10% could not be accounted for.

The addition of SOD and catalase completely prevented the oxidation of ascorbate. SOD alone decreased the uptake in the presence of PMA to 45%; in the presence of both enzymes the control value (in the absence of PMA) was obtained (Figure 2). This difference in the accumulated amounts between the use of SOD alone and both enzymes together did not result in a significant difference in the extracellular remainder because it was a very small fraction of the total.

Inhibition of DHA uptake by the reducing agent GSH

When added to the incubation mixture for the uptake of 100 μ M ascorbate, 5 mM GSH did not inhibit uptake, either in the absence or in the presence of PMA. When ascorbate was oxidized with HCF(III), GSH decreased the uptake to the control value with reduced ascorbate in the absence of PMA. Consequently, in its presence, the reductant decreased the rate to that observed with PMA alone (Figure 3). The effects of the antioxidant enzymes were totally unaffected (results not shown).

Influence of the ionic milieu on the uptake of ascorbate and of DHA

As known for other cell types [14], neutrophils among them [27], the uptake of reduced ascorbate is at least partly dependent on the presence of Na^+ ions. Two Na^+ -dependent ascorbate transporters have recently been cloned; at least one of them is expressed in every mammalian cell [28]. Apparently it is also present in HL-60 cells. Only 40% of the rate was observed with choline instead of Na^+ in the uptake medium with a starting concentration of 100 μ M ascorbate (2.2 ± 0.4 pmol/min per 10^6 cells without Na^+ , compared with 5.4 ± 0.4 pmol/min per 10^6 cells

Figure 4 Intracellular accumulation in 10 min (A) and residual extracellular concentration (B) of 200 μ M ascorbate in resting and PMA-activated cells in control experiments, in the presence of xanthine/xanthine oxidase (X/XO), and X/XO together with the antioxidative enzymes SOD and catalase

Results are means $\pm$ S.E.M. for three experiments.

with Na^+). After stimulation with PMA, the difference remained but 90% of the total transport rate was independent of Na^+ (38.6 ± 2.2 compared with 42.9 pmol/min per 10^6 cells). Together with the kinetic data reported above, this argues for the oxidation of ascorbate and not an enhancement of ascorbate transport itself brought about by treatment of the cells with PMA.

Transport of ascorbate in the presence of $\text{O}_2^{\cdot-}$ and H_2O_2

The inhibition of PMA-stimulated uptake by SOD and catalase is evidence for the participation of one or both of the oxygen metabolites. Although 100 μ M xanthine together with 25 m-units of xanthine oxidase was able to stimulate ascorbate uptake, this stimulation was only 2-fold (Figure 4). Moreover, the concentration of extracellular ascorbate decreased much more rapidly than could be accounted for by the cellular uptake. At higher concentrations of xanthine, this degradation levelled off as well as the uptake rate, probably owing to the increase in the concentration of uric acid, which itself acts as an antioxidant (results not shown). As expected, the effect of the enzymically generated $\text{O}_2^{\cdot-}$ was totally blocked by SOD.

When these experiments were performed in the presence of PMA, the $\text{O}_2^{\cdot-}$ -generating mixture had no additional effect on the transport rates.

H_2O_2 , although able to oxidize ascorbate, had even weaker effects on its uptake, and only when it was added after the ascorbate itself. The PMA-stimulated rate was not affected (results not shown).

Figure 5 Inhibition of PMA-stimulated ascorbate uptake (○) by 50 mM *O*-methylglucose (▲) and by 50 mM ethylidene glucose (■)

Analysis was by an Eadie-Hofstee plot.

Figure 6 Inhibition of PMA-stimulated uptake of ascorbate (○, ●) and PMA-stimulated uptake of preformed DHA (△, ▲) by 25 μM genistein

Control rates are indicated by ○, △, and rates in the presence of the inhibitor by ●, ▲.

Inhibition of DHA uptake by glucose analogues

To test whether the PMA-stimulated transport of ascorbate was mediated by a glucose transporter, competitive inhibitors were tested [29]. Indeed, *O*-methylglucose as well as ethylidene glucose competitively inhibited PMA-stimulated uptake (Figure 5). The same was true of genistein, which is a specific inhibitor of DHA uptake via the GLUT-1 transporter in HL-60 cells [30]. The inhibition pattern with this compound was the same for PMA-stimulated ascorbate uptake and for PMA-stimulated DHA uptake (Figure 6). Thus the same glucose transporter (presumably GLUT-1) is apparently responsible in both cases.

In all Eadie-Hofstee plots shown, there is a skew towards higher affinity at low rates. This could be explained by the constant contribution of the high-affinity ascorbate transporter, which is more important at low concentrations (and thus at lower rates).

Table 2 Effects of inhibitors of the respiratory-burst oxidase and of the protein kinase C inhibitor staurosporine on the accumulation within 10 min of ascorbate or preformed DHA by differentiated HL-60 cells in the resting state and after activation by PMA

The starting concentrations were 100 μM. Results are means ± S.E.M.

Addition	Uptake (pmol/10 min per 10 ⁶ cells)	
	Ascorbate	DHA
None	76.5 ± 9.2	688 ± 28
Staurosporine	98.5 ± 15.2	837 ± 26
Diphenyliodonium	78.0 ± 35.1	696 ± 30
PMA	624 ± 55	1000 ± 68
PMA + staurosporine	111 ± 5	1174 ± 63
PMA + diphenyliodonium	126 ± 14	1069 ± 32

Effects of disruption of the PMA-initiated signal chain of the respiratory burst

Neither the uptake of 150 μM ascorbate nor that of DHA was affected by the protein kinase C inhibitor staurosporine [31,32]; the inhibitor of NADPH oxidase, diphenyliodonium [33], also had no effect. The stimulation of ascorbate uptake by PMA was completely reversed by these inhibitors, indicating the involvement of the kinase-mediated signal chain as well as of the oxidase, whereas the stimulation of DHA uptake by PMA was unaffected by the inhibitors (Table 2). Apparently this latter stimulation occurs by a different mechanism, possibly a direct interaction of the phorbol ester with the cell surface. This is seemingly unrelated to the oxidase activity.

DISCUSSION

It is now well established that leucocytes and related cells in culture can efficiently accumulate vitamin C by the rapid uptake of the oxidized form DHA whenever this metabolite is generated. In normal blood plasma (and presumably also in cell culture medium containing the vitamin), DHA is practically non-existent [34], partly because of its inherent instability but also because of its disappearance into the cells, which then store it as reduced ascorbate after fast intracellular reduction, mainly by GSH mediation but also by other mechanisms.

The preferred uptake of DHA occurs mainly via the glucose transporters GLUT-1 and GLUT-3, whichever is available. This might be one reason why in diabetes mellitus the concentration of DHA in plasma increases. DHA at higher concentrations is toxic and might contribute to the symptoms of the disease [35].

It has recently been shown that neutrophils activated by chemotactic peptides increase the rate of DHA uptake by an increase in the affinity for the substrate without a change in the total capacity, an effect that was clearly different from the pure effect of the oxidation of ascorbate [18].

With differentiated HL-60 cells, in which the respiratory burst can be initiated in the same way as in neutrophils, the situation seems to be similar in principle, although quite different in detail. Treatment of these cells with PMA is expected to activate the respiratory burst and to initiate the generation of the O₂^{•-} anion and subsequently of H₂O₂ [4]. Both agents can oxidize ascorbate and they also stimulate its uptake by the cells. However, both oxygen metabolites, when produced by an artificial reaction

or added as a substance, had only sluggish and weak effects on the uptake rate of ascorbate. In contrast, after incubation with PMA there was an immediate increase in GLUT-mediated transport. This could be shown by a marked rise in the rate, by a decrease in the apparent affinity and by the inhibition by glucose analogues. The basal uptake of ascorbate by the Na⁺-dependent pathway remained intact but its contribution decreased to a maximum of 10 % of the total rate.

It is also clear that the oxidation of ascorbate is a prerequisite of transport stimulation because it was completely inhibited by a mixture of SOD and catalase as well as by diphenyliodonium, an inhibitor of NADPH oxidase [33]. Moreover, inhibition by staurosporine of the protein-kinase-C-mediated signal pathway of its activation [31,32] also prevented the stimulatory effect.

At first glance, a straight switch of transporters seemed to be indicated from the kinetic data: the rate observed with ascorbate and PMA was almost the same as that with DHA as the original substrate. However, there was a clear difference: when ascorbate was oxidized by the oxidase enzyme or by the addition of HCF(III), all the substrate was immediately transformed to DHA and a loss of approx. 50 % occurred within 15 min. Moreover, the addition of GSH inhibited this oxidation and thereby the stimulation of the uptake.

An enzymic regeneration of ascorbate from the free-radical state by the membrane-bound ascorbate:free-radical reductase [36] seems to be ineffective under the conditions used, otherwise one would expect a significant part of the ascorbate to remain in the reduced state even in the presence of excess HCF(III). Reduction of this oxidant by intracellular ascorbate via an alternative transmembrane electron transport [37] also seems to have no influence on its effect, because there was no difference between chemical oxidation by HCF(III) and enzymic oxidation by ascorbate oxidase.

With PMA, extracellular ascorbate remained in its reduced form, the loss was only 10 % and GSH was without effect. This means that the kinetic data are misleading: the concentration of DHA never increased to the values assumed for the kinetic comparison with the rate for DHA generated chemically or added as a substance obtained from the bromination of ascorbate.

Indeed, when PMA was added to the transport mixture with enzymically or chemically generated DHA, the stimulatory effect was almost doubled. This extra stimulation was not inhibited by antioxidant enzymes, by GSH or by staurosporine. The transport also used the glucose transporter, as seen from the affinity and from the inhibition by the analogues. Only the maximal rate was doubled and under these conditions can be considered a true kinetic value because DHA was present in its true concentration.

Thus differentiated cells respond to activation by phorbol ester by oxidizing ascorbate via the respiratory burst and the generation of oxygen metabolites, and by simultaneously increasing the transport capacity for the DHA generated. Whereas the respiratory-burst-dependent oxidation follows the canonical protein kinase C signal pathway, the mechanism for the increase of the transport capacity is unclear at the moment but is certainly different. It remains to be seen whether this response is truly a means of enhanced uptake of vitamin C or is a by-product of increased uptake capacity for glucose as a substrate for increased metabolic rates and a source of reducing equivalents for NADPH.

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