A role for Na+/H+ exchangers and intracellular pH in regulating vitamin C-driven electron transport across the plasma membrane

Darius J.R. LANE*†, Stephen R. ROBINSON‡, Hania CZERWINSKA‡ and Alfons LAWEN*†

*Department of Biochemistry and Molecular Biology, Monash University, VIC 3800, Australia, †School of Biomedical Sciences, Monash University, VIC 3800, Australia, ‡School of Psychology and Psychiatry, Monash University, VIC 3800, Australia

Ascorbate (vitamin C) is the major electron donor to a tPMET (transplasma membrane electron transport) system that was originally identified in human erythrocytes. This plasma membrane redox system appears to transfer electrons from intracellular ascorbate to extracellular oxidants (e.g. non-transferrin-bound iron). Although this phenomenon has been observed in nucleated cells, its mechanism and regulation are not well understood. In the present study we have examined both facets of this phenomenon in K562 cells and primary astrocyte cultures. Using ferricyanide as the analytical oxidant we demonstrate that tPMET is enhanced by dehydroascorbate uptake via facilitative glucose transporters, and subsequent accumulation of intracellular ascorbate. Additionally, we demonstrate that this stimulation is not due to ascorbate that is released from the cells, but is dependent only on a restricted intracellular pool of the vitamin. Substrate-saturation kinetics suggest an enzyme-catalysed reaction across the plasma membrane by an as-yet-unidentified reductase that relies on extensive recycling of intracellular ascorbate. Inhibition of ascorbate-stimulated tPMET by the NHE (Na+/H+ -exchanger) inhibitors amiloride and 5-(N-ethyl-N-isopropyl)amiloride, which is diminished by bicarbonate, suggests that tPMET activity may be regulated by intracellular pH. In support of this hypothesis, tPMET in astrocytes was significantly inhibited by ammonium chloride-pulse-induced intracellular acidification, whereas it was significantly stimulated by bicarbonate-induced intracellular alkalinization. These results suggest that ascorbate-dependent tPMET is enzyme-catalysed and is modulated by NHE activity and intracellular pH.

Key words: ascorbate (vitamin C), astrocyte, ferricyanide, intracellular pH (pHi), K562 cell, Na+/H+ -exchanger (NHE).

INTRODUCTION

All eukaryotic cells tested possess tPMET (transplasma membrane electron transport) systems that oxidize intracellular electron donors to reduce extracellular oxidants (e.g. non-transferrin-bound iron or ascorbyl radical), although membrane-impermeant FIC (ferricyanide) is frequently used as the analytic oxidant [1-3]. This ubiquitous redox capacity has been linked to a range of cellular functions in mammals including growth, proliferation, differentiation and apoptosis [1-3], iron-reduction prior to uptake [4,5] and regeneration of extracellular ascorbate (vitamin C) [5-7]. Conversely, a deregulation of this system may be relevant to various human disease states including neurodegeneration [8], cancer [9], diabetes [10] and disorders of iron metabolism [11].

Although various intracellular electron donors have been proposed, ascorbate appears to be the major electron donor to tPMET in human erythrocytes [5-7]. Both ascorbate and its two-electron oxidized form, DHA (dehydroascorbate), are also known to stimulate tPMET in nucleated cells, although the mechanism has not been thoroughly established [5]. The proposed mechanisms of this stimulation can generally be divided into those that depend on intracellular or extracellular ascorbate [5]. Derived mainly from studies of human erythrocytes, the major ‘intracellular ascorbate’ model proposes the existence of an as-yet-unidentified transplasma membrane oxidoreductase that utilizes intracellular ascorbate as the proximal source of reducing equivalents [5-7,12,13]. The ‘extracellular ascorbate’ models propose either that ascorbate that is released from cells, or is simply present in the extracellular space as a result of supplementation, is all that is required for the stimulation of FIC reduction [5]. Although Merker et al. [14] have provided strong evidence for the claim that the reduction of extracellular FIC and thiazine dyes derives electrons from a restricted intracellular pool of ascorbate in primary cultures of pulmonary endothelial cells, many studies on ascorbate/DHA-stimulated FIC reduction in nucleated cells have failed to conclusively determine the side of the plasma membrane from which ascorbate exerts its stimulatory effect [5]. That is, the ‘transplasma membrane oxidoreductase’ hypothesis has thus yet to be validated as a general phenomenon for nucleated cells.

The regulation of ascorbate-stimulated tPMET is also not well understood. In human erythrocytes, ascorbate-driven tPMET appears to be sensitive to changes in the resting potential across the plasma membrane [15]. Additionally, studies on tPMET in nucleated cells occurring in the absence of vitamin C have suggested that FIC reduction by cells can be inhibited by pharmacological blockade of NHEs (Na+/H+ exchangers), and that the reduction of FIC can trigger NHE-dependent proton export and intracellular alkalinization [2,16]. This putative association of NHE activity with tPMET suggests a mechanism by which tPMET may contribute to the regulation of mitosis and apoptosis [2], in which NHE activity is crucial [17]. No such regulatory role for NHEs has yet been identified for ascorbate-stimulated tPMET.

The present study examines both the mechanism and regulation of ascorbate-stimulated tPMET in two nucleated cell types: K562

Abbreviations used: AO, ascorbate oxidase; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; DHA, dehydroascorbate; 2-DOG, 2-deoxy-D-glucose; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; FIC, ferricyanide; FOC, ferrocyanide; GLUT, glucose transporter; HBS, Hepes-buffered saline; HNE, Na+/H+ exchanger; pHi, intracellular pH; pHo, extracellular pH; tPMET, transplasma membrane electron transport.

1 To whom correspondence should be addressed (email alfons.lawen@med.monash.edu.au).
cells and primary astrocytes. We demonstrate for the first time
the existence of ascorbate-stimulated tPMET in astrocytes, and
that for both cell types this activity depends only on a restricted
pool of intracellular ascorbate. We also present results for both
cell types that strongly suggest the involvement of an enzyme-
catalysed reaction mechanism that relies on extensive recycling
of intracellular ascorbate. Additionally, we show for the first
time that blockade of NHEs with amilorides potently inhibits
ascorbate-stimulated tPMET, and furthermore that this sensitivity
is diminished by bicarbonate. Finally, by direct manipulation
of pHi (intracellular pH) we also show for the first time that
ascorbate-stimulated tPMET can be modulated by pHi.

EXPERIMENTAL

Materials

Unless otherwise stated, all chemicals were obtained from Sigma–
Aldrich or Merck. Spectrophotometric microplate assays were
performed on a Benchmark™ Plus microplate spectrophotometer
(Bio-Rad) using Nunc 96-well flat-bottom transparent plates,
or Greiner 24-well plates. Fluorescence microplate assays were
performed on a FLUOstar Optima (BMG Labtech) using Greiner
Bio-One 24-well culture plates (astrocytes) or 96-well Microlon F
solid white plates (K562 cells). Orbital mixing of cell suspensions
was performed with a MixMate® orbital mixer (Eppendorf South
Pacific) maintained at 37°C in a thermoregulated incubator/shaker
(Foinoe) set to 37°C.

Cells and culture conditions

Human erythroleukaemia (chronic myeloid leukaemia; K562)
cells were maintained and handled as described previously
[13,18].

Primary astrocyte cultures were prepared from the brains of
newborn Wistar rats and C57BL/6J mice (<24 h old), as described
previously [19,20] and as approved by the animal committee of
the School of Psychology and Psychiatry, Monash University,
Victoria, Australia. Immediately prior to use, the wells in each
plate were washed three times with 1.5–2.0 ml of ice-cold HBS
[Hepes-buffered saline: 134 mM NaCl, 5.2 mM KCl, 1.8 mM
CaCl₂, 0.8 mM MgSO₄ and 20 mM sodium Hepes (pH 7.2 at
37°C)].

Ascorbate-loading of cells

Ascorbate-loading of cells was typically accomplished by
exposing cells to freshly prepared solutions of DHA dimer
for 30 min at 37°C. The reported concentrations of HBS are for
the hydrated monomeric species that is rapidly formed upon
hydrolysis of the crystalline DHA dimer in aqueous solution [21].

In experiments involving astrocytes, cell suspensions were
aspirated and washed three times with 1.5 ml of ice-cold HBS
containing 5 mM D-glucose per well (astrocytes).

FIC-reduction assays

FIC-reduction assays for K562 cells were conducted essentially as
described previously [13]. In the case of cultured astrocytes, cell-

dependent reduction of FIC to FOC (ferrocyanide) was assessed
with ~0.3 ¥ 10⁶ astrocytes in 24-well culture plates. FOC levels
were then determined as described previously [13].

Ferrireduction assays

Ferrireduction assays were also carried out as described
previously [18,22].

Determination of intracellular ascorbate

Intracellular ascorbate levels were determined by either of
two procedures. The first was performed exactly as described
previously [18], and involves the extraction of ascorbate from
cells with 0.1% saponin in HBS, followed by the determination
of the AO (ascorbate oxidase)-sensitive reduction of FIC to
FOC. The second is a modification of the previous procedure
in which, instead of the addition of FIC, ascorbate-containing
saponin extracts are combined with an equivalent volume of a
freshly made solution containing 25% (v/v) acetic acid, 1.65 mM
FeCl₃, and 2.4 mM Ferene-S, resulting in the production of 2
mM Fe²⁺ per mol of ascorbate, with Fe²⁺ levels detected as
their Ferene-S chelate (λ₅₉₅ = 595 nm; ε₅₉₅ = 35.5 mM⁻¹·cm⁻¹
[13]). In both methods, ascorbate levels were interpolated
from ascorbate standard curves constructed in parallel for each
determination. The ascorbate concentrations of the standards
were always determined spectrophotometrically (λ₅₉₅ = 265, ε₅₉₅ =
14.5 mM⁻¹·cm⁻¹ [23]). Both methods provided identical results.

Fluorimetric determination of pHi

pH was determined and followed fluorimetrically on a
FLUOstar Optima, using BCECF [2′,7′-bis(2-carboxyethyl)-5(6)-
carboxyfluorescein] according to a microplate adaptation [24]
of the ratiometric method of Thomas et al. [25], whereby the
ratio of emitted light at 530 nm with sequential excitation at
both 500 nm and 450 nm (i.e. 500/450) is a function of pH
[24,25]. In order to assay pH, cells were initially exposed to
3 μM of the acetoxyethyl ester of BCECF (BCECF-AM;
obtained from Sigma–Aldrich as a 1 mg/ml stock in DMSO)
in HBS containing 5 mM D-glucose for 20 min at 37°C with
orbital shaking in a thermoregulated incubator/shaker in the
dark. Cells were then washed four times as above. The above
BCECF fluorescence ratio was always calibrated to a series of
‘clamped’ pH values (i.e. 6.2, 6.6, 7.0, 7.5 and 8.0) using BCECF-
loaded cells exposed to sodium-free, high-potassium (i.e. 130 mM
KCl) 20 mM Hepes-choline or 15 mM Mops-choline buffers of
the corresponding pH values, additionally containing 10 μM of
the K⁺/H⁺ ionophore nigericin [25]. BCECF fluorescence was
then typically followed over periods of 40–50 min. The pH,
values of the unknown samples were always interpolated from
the resulting standard curves constructed for each experiment.
In experiments involving astrocytes, the calibration buffers also
contained 1.8 mM CaCl₂ and 0.8 mM MgSO₄ to maintain cellular
adhesion to the substrate.

Manipulation of pHi

All pH manipulations were carried out at a constant pH,
(extracellular pH) of 7.2 at 37°C in the dark. A stable baseline
pH was always obtained prior to a 40–50 min ‘treatment phase’
in which various acidifying or alkalinizing regimes were imposed
by medium replacement. As it was important to observe effects
on pH, during the course of cellular FIC reduction, acidifying or alkalinizing medium replacements typically contained 500 μM FIC.

The intracellular acidification of cells was primed by the exposure of BCECF-loaded cells to HBS in which 20 mM NaCl had been isosmotically replaced by 20 mM NH₄Cl during the 10 min prior to the treatment phase. This NH₄⁺-pulse always resulted in a rapid alkaline-shift in pHᵢ (results not shown), as typically observed [26–29]. In the treatment phase the NH₄⁺-containing medium was immediately replaced by an NH₄⁺-free medium. The removal of the NH₄⁺-pulse induces rapid dissociation of accumulated intracellular NH₄⁺ ions into NH₃ and H⁺ ions [29]. The efflux of membrane-permeant NH₃ down its concentration gradient leaves behind an unbuffered H⁺ load in the cytoplasm [26]. In the absence of HCO₃⁻, pHᵢ is typically recovered in a primarily NHE-dependent manner [30]. This NHE-dependent component of pHᵢ recovery is inhabitable by the selective NHE inhibitor, EIPA [5-(N-ethyl-N-isopropyl)amiloride] [31]. Thus, in order to prolong a state of intracellular acidification during exposure to FIC, the replacement medium additionally contained EIPA at a concentration of 5 μM.

The intracellular alkalinization of BCECF-loaded cells was achieved by addition of HBS in which 25 mM NaCl had been isosmotically replaced by 25 mM NaHCO₃ at the beginning of the treatment phase. In order to avoid possible confounding effects of changes in pHᵢ, caused by addition of NaHCO₃, the pH of HCO₃⁻-containing solutions was always adjusted to 7.2 prior to the start of the assay. It is well-established that the entry of HCO₃⁻ into cells by various transport systems, including Na⁺–HCO₃ co-transport [30], typically induces intracellular alkalinization [28,32]. As the primary aim of HCO₃⁻ addition was the attainment of intracellular alkalinization, HCO₃⁻-containing solutions were not pre-equilibrated with CO₂. The rapid hydration of dissolved CO₂ to H₂CO₃ by cellular carbonic anhydrases [33] tends to induce compensatory intracellular acidification [34].

### Protein determination

The protein content of astrocyte cultures was obtained by solubilizing cellular protein with 2% (w/v) SDS and assessing the protein concentration using the microplate adaptation of the BCA (bicinchoninic acid) assay kit for protein determination (Sigma–Aldrich), according to the manufacturer’s instructions. BSA was employed as a standard.

### Data analysis and curve fitting

All curve fitting and hypothesis testing were performed using SigmaPlot® 9.0 (Systat Software) or GraphPad Prism® 5.0 (GraphPad Software) respectively. Dose–response data were modelled either by single rectangular hyperbolae (with or without residual) or simple linear curves. Differences between treatments were analysed using either one- or two-factor ANOVAs with either Bonferroni’s or Dunnett’s post hoc tests of significance using GraphPad Prism® 5.0.

## RESULTS

### Exposure of K562 cells and astrocytes to DHA stimulates tPMET

The cellular reduction of FIC by mammalian cells is known to be stimulated by the exposure of cells to micromolar concentrations of DHA [5–7]. We have previously shown that pre-exposure of K562 cells to a putative DHA-generating mixture of 25 μM ascorbate/1 mM FIC results in a greater than 7-fold increase in FIC reduction capacity [13]. To confirm that this stimulation is actually due to the exposure of the cells to DHA, we have assessed in the present study the stimulatory ability of pre-incubation with genuine DHA in the absence of FIC. Consistent with our original hypothesis [13], we observed a near 10-fold increase in FIC reduction for K562 cells that had been pre-incubated with at least 25 μM DHA (Figure 1A). Intriguingly, a similar stimulation was also observed in both rat and mouse primary astrocyte cultures (Figure 1A). To the best of our knowledge this is the first demonstration of vitamin C-stimulated FIC reduction in astrocytes, and provides further support for the notion [5] that this phenomenon is ubiquitous.

The stimulation of FIC reduction by DHA requires GLUT (glucose transporter)-dependent DHA uptake and intracellular ascorbate accumulation

We next tested the hypothesis that the stimulation of FIC reduction by DHA depends on DHA uptake and accumulation of intracellular ascorbate. To do this we took advantage of the fact that DHA uptake appears to be GLUT-mediated in mammals [35]. For K562 cells we have previously demonstrated that DHA uptake is most probably GLUT-dependent [18,36]. To assess the involvement of GLUTs in DHA uptake by astrocytes we assessed the dose–response effects of two pharmacological modulators of GLUT-mediated transport: cytochalasin B [36–38] and 2-DOG (2-deoxy-D-glucose) [37]. To strengthen our conclusions, the effects of cytochalasin B were also compared with those of its structural analogue, dihydrocytochalasin B, which inhibits cellular motile processes similarly to cytochalasin B, but is ineffectve against GLUT-mediated transport [39]. Similarly, the effects of 2-DOG were compared with the glucose stereoisomer, L-glucose, which is not readily transported by GLUTs [40]. We observed that accumulation of intracellular ascorbate from extracellular DHA by rat astrocytes is sensitive to cytochalasin B, but not dihydrocytochalasin B (Figure 1B), and is sensitive to 2-DOG, but not L-glucose (Figure 1C). As with our previous results on DHA uptake by K562 cells [36], the present results strongly suggest the involvement of GLUTs in DHA uptake by rodent astrocytes.

Given the cytochalasin B sensitivity of DHA uptake, the order of addition of cytochalasin B relative to DHA can be used to determine the dependence of a DHA-mediated effect on DHA uptake [37]. We used this approach to determine the dependence of DHA-stimulated FIC reduction on cellular uptake of DHA. The stimulatory effect of DHA on FIC reduction by K562 cells (Figure 1D) and rat astrocytes (results not shown) was abrogated by cytochalasin B. Instructively, however, cytochalasin B only inhibited FIC reduction when added prior to, but not after, the addition of DHA (Figure 1D). This observation provides strong support for the conclusion that cellular DHA uptake is required for the DHA-mediated stimulation of FIC reduction by both K562 cells and astrocytes.

In addition, we also observed that the DHA-mediated stimulation of FIC reduction in K562 cells (Table 1) and rat astrocytes (results not shown) was entirely specific for the L stereoisomer of DHA. This suggests that the stimulatory effect of DHA is mediated by a stereospecific transport process, such as GLUT-mediated transport, across the plasma membrane.
Pre-incubation of cells with DHA stimulates FIC reduction and is due to GLUT-dependent DHA uptake and accumulation of intracellular ascorbate

Rates of FIC reduction were assessed for (A) K562 cells (empty bars) and primary rat (diagonal hatched bars) or mouse (horizontal hatched bars) astrocytes that had been pre-incubated with the indicated concentrations of DHA. (B and C) Intracellular ascorbate (AA) levels in rat astrocytes that been exposed to the indicated concentrations of (B) cytochalasin B (CB) or dihydrocytochalasin B (H2CB), or (C) 2-DOG or L-glucose, 10 min prior to and during incubation with 400 μM DHA for 30 min at 37°C. (D) Rates of FIC reduction by K562 cells were assessed after pre-incubation with or without 25 μM DHA. In the indicated conditions, CB (25 μM) was either added 10 min prior to and during exposure to DHA and during FIC reduction (CB→DHA), or only during FIC reduction (DHA→CB). Values are means ± S.D. for three experiments. (A) ***P < 0.001 compared with 0 μM DHA. (B and C) *P < 0.05, **P < 0.01, ***P < 0.001 compared with the corresponding value with H2CB or L-glucose respectively. (D) ***P < 0.001 compared with control or CB→DHA.

The stimulation of FIC reduction by DHA is due to intracellular ascorbate

Schweinzer and Goldenberg [41] originally proposed that the vitamin C-driven reduction of FIC by K562 cells depends on a direct reaction of FIC with extracellular ascorbate [41]. However, as the FIC reduction assays employed in their study did not control for the uptake of DHA and formation of intracellular ascorbate [41], which would result from FIC-driven oxidation of ascorbate to DHA [13,42], the origin of the reducing equivalents required for FIC reduction is unclear [5,13]. Despite this, it is well-established that many cells, including both K562 cells [18] and astrocytes [5,35], are capable of releasing ascorbate into the extracellular medium. This leaves the possibility open that the DHA-mediated stimulation of FIC reduction is caused by ascorbate that is released by ascorbate-loaded cells. In order to deconvolute these issues we directly tested the hypothesis that DHA-stimulated reduction of FIC is dependent on extracellular ascorbate.

We first assessed whether cell-conditioned medium from ascorbate-loaded cells had a significant stimulatory effect on FIC reduction (Figure 2A). The results clearly indicate that the cellular release of endogenous reductants (e.g. ascorbate) is not responsible for reducing the majority of the FIC that is reduced, and also that the stimulatory response is dependent on the presence of cells. However, as the ‘extracellular ascorbate’ model proposed by Schweinzer and Goldenberg [41] relies on only low micromolar concentrations of ascorbate that can be enzymatically recycled in the presence of FIC, it remained possible that low catalytic levels of ascorbate were being released that might still contribute to extracellular FIC reduction. To eliminate this possibility, AO was added to the incubation medium during the course of FIC reduction at a final concentration of 10 units·ml⁻¹, in order to selectively and rapidly oxidize any extracellular ascorbate to DHA [18,22]. As shown in Figure 2(B), the presence of AO significantly inhibited the chemical reduction of FIC (0.5 mM) by ascorbate (25 μM; a concentration 5-fold greater than that released by K562 cells [18]) in control reactions conducted in the absence of cells. Importantly, however, the presence of AO had no significant effect on the stimulated rate of FIC reduction (i.e. 962 ± 22 pmol/10⁶ cells per min) by ascorbate-loaded cells (Figure 2B). Conversely, the presence of AO significantly inhibited the stimulated rate of extracellular

Table 1  DHA-mediated stimulation of FIC reduction by K562 cells is specific for L-DHA

FIC reduction was assessed for cells that had been pre-incubated with L- or D-DHA. DHA was formed by complete oxidation of 25 μM L-ascorbate or D-ascorbate with a 40-fold molar excess of FIC (i.e. 1 mM) [13]. Values are means ± S.D. for three experiments. ***P < 0.001 compared with control and D-DHA.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FIC reduction (pmol/min per 10⁶ cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>78 ± 12</td>
</tr>
<tr>
<td>L-DHA</td>
<td>543 ± 9***</td>
</tr>
<tr>
<td>D-DHA</td>
<td>87 ± 3</td>
</tr>
</tbody>
</table>

The stimulation of FIC reduction by DHA is due to intracellular ascorbate

Schweinzer and Goldenberg [41] originally proposed that the vitamin C-driven reduction of FIC by K562 cells depends on a direct reaction of FIC with extracellular ascorbate [41]. However, as the FIC reduction assays employed in their study did not control for the uptake of DHA and formation of intracellular ascorbate [41], which would result from FIC-driven oxidation of ascorbate to DHA [13,42], the origin of the reducing equivalents required for FIC reduction is unclear [5,13]. Despite this, it is well-established that many cells, including both K562 cells [18] and astrocytes [5,35], are capable of releasing ascorbate into the extracellular medium. This leaves the possibility open that the DHA-mediated stimulation of FIC reduction is caused by ascorbate that is released by ascorbate-loaded cells. In order to deconvolute these issues we directly tested the hypothesis that DHA-stimulated reduction of FIC is dependent on extracellular ascorbate.

We first assessed whether cell-conditioned medium from ascorbate-loaded cells had a significant stimulatory effect on FIC reduction (Figure 2A). The results clearly indicate that the cellular release of endogenous reductants (e.g. ascorbate) is not responsible for reducing the majority of the FIC that is reduced, and also that the stimulatory response is dependent on the presence of cells. However, as the ‘extracellular ascorbate’ model proposed by Schweinzer and Goldenberg [41] relies on only low micromolar concentrations of ascorbate that can be enzymatically recycled in the presence of FIC, it remained possible that low catalytic levels of ascorbate were being released that might still contribute to extracellular FIC reduction. To eliminate this possibility, AO was added to the incubation medium during the course of FIC reduction at a final concentration of 10 units·ml⁻¹, in order to selectively and rapidly oxidize any extracellular ascorbate to DHA [18,22]. As shown in Figure 2(B), the presence of AO significantly inhibited the chemical reduction of FIC (0.5 mM) by ascorbate (25 μM; a concentration 5-fold greater than that released by K562 cells [18]) in control reactions conducted in the absence of cells. Importantly, however, the presence of AO had no significant effect on the stimulated rate of FIC reduction (i.e. 962 ± 22 pmol/10⁶ cells per min) by ascorbate-loaded cells (Figure 2B). Conversely, the presence of AO significantly inhibited the stimulated rate of extracellular

© The Authors Journal compilation © 2010 Biochemical Society
Regulation of ascorbate-driven plasma membrane electron-transport

Figure 2  The stimulation of FIC reduction by DHA is not mediated by extracellular ascorbate

(A) Reduction of FIC by K562 cells that had been pre-incubated with 50 µM DHA or cell-conditioned medium from such cells. Rates of reduction of (B) FIC (500 µM) or (C) ferric citrate (100 µM) by K562 cells that had been pre-incubated with 50 µM DHA. In the indicated conditions AO was present at 10 units - m1. The hatched bars indicate the levels of FIC reduction in cell-free medium containing ascorbate (25 µM) with or without AO. AO was added simultaneously with FIC. Rates are expressed as a percentage of the maximum rates of FIC or ferric citrate reduction, which were 962 ± 22 and 45 ± 5 pmol/min per 10^6 cells respectively. (D) Rates of FIC reduction by mouse astrocytes with or without AO that had been pre-incubated with or without 120 µM DHA. Values are means ± S.D. for three experiments. ***P < 0.001 compared with control (B and C) or the respective incubations without DHA (D). AA, ascorbate.

Ascorbate-driven tPMET relies on intracellular ascorbate cycling and enzymatic tPMET

DHA-stimulated FIC reduction by human erythrocytes has previously been observed to be saturable with respect to both intracellular ascorbate and extracellular FIC concentrations, suggesting the contribution of an enzyme-catalysed tPMET reaction [6,12]. We thus established the dose–response behaviour of the reaction for both intracellular ascorbate and extracellular FIC by K562 cells and astrocytes.

When both K562 cells and rat astrocytes with different intracellular ascorbate concentrations, resulting from exposure to different DHA concentrations, were exposed to a single concentration of FIC (500 µM) in the presence of AO, a hyperbolic-dependence of FIC reduction on intracellular ascorbate was observed (Figures 3A and 3B). Based on the total amount of FIC reduced, and the calculated concentrations of intracellular ascorbate, it can be deduced that the cells must have recycled their intracellular ascorbate 3–15 times (K562 cells) and 10–90 times (astrocytes) over the time frame of the assays, with the highest rate of recycling occurring when the intracellular ascorbate levels are lowest (Figures 3A and 3B). A similar reliance on intracellular ascorbate recycling has been noted for ascorbate-loaded human erythrocytes [12] and ascorbate-loaded HL-60 cells [37]. It is likely that the intracellular ascorbate recycling capacity of the cells observed in the present study explains a significant component of the hyperbolic-dependence on intracellular ascorbate levels in both K562 cells and astrocytes. The markedly greater apparent recycling capacity of the astrocyte cultures is also noteworthy, and may be reflective of a greater capacity for ascorbate conservation and/or ascorbate-driven tPMET by these cells.

We next asked whether the DHA-stimulated reduction of FIC in K562 cells and rodent astrocytes is likely to be an
had been pre-incubated with either 25 μM protein (95% confidence interval: 47–55 nmol/min per mg of protein) [12]. Values are the baseline-corrected means ± S.D. for two experiments.

Collectively our results are consistent with an enzyme-catalysed process, as we have recently suggested [5]. To accomplish this, cells were pre-incubated with a single concentration of DHA. AO was also added in all FIC reduction experiments to remove any contribution from ascorbate that had been released from cells. Additionally, time frames for these experiments were kept sufficiently brief such that < 30% of the total FIC present was reduced at the lowest FIC concentrations. In the presence of ascorbate-loaded cells, a saturating hyperbolic-dependence on FIC concentration was observed for both K562 cells and rat astrocytes (Figures 3C and 3D). As indicated above, this type of kinetic behaviour is typically indicative of an enzyme-catalysed reaction. However, it is also possible that this rate-limitation is, at least in part, imposed by the capacity of the cells to recycle intracellular ascorbate. According to this hyperbolic modelling, apparent Michaelis–Menten parameters were determined for both K562 cells ($K_m = 54 ± 4 \mu M$ (95% confidence interval: 47–61 μM) and $V_{max} = 534 ± 10$ pmol/min per 10^6 cells (95% confidence interval: 515–554 pmol/min per 10^6 cells)), and rat astrocytes ($K_m = 59 ± 4 \mu M$ (95% confidence interval: 49–69 μM) and $V_{max} = 53 ± 2$ nmol/min per mg of protein (95% confidence interval: 49–55 nmol/min per mg of protein)). The fact that the apparent $K_m$ values presented here for K562 cells and astrocytes are so similar to each other, and to that for DHA-loaded human erythrocytes [12], suggests a common electron-transfer mechanism.

Collectively our results are consistent with an enzyme-catalysed reaction for an as-yet-unidentified transplasma membrane ascorbate-FIC oxidoreductase activity in both K562 cells and rodent astrocytes that relies on extensive recycling of intracellular ascorbate.

**NHE antagonists inhibit ascorbate-driven FIC reduction**

It has previously been observed that tPMET activity occurring in the absence of vitamin C can be inhibited by pharmacological blockade of NHEs in the plasma membrane using NHE-specific amiloride derivatives [2,16]. To determine whether ascorbate-driven tPMET is also sensitive to blockade of NHEs, we assessed the dose–response effects on tPMET of two classical NHE inhibitors: amiloride and EIPA [43,44]. In K562 cells, amiloride caused a statistically significant ($P < 0.001$) dose-dependent inhibition of ascorbate-driven FIC reduction (Figure 4A), with the 95% confidence interval of 50% inhibition estimated in the range 27–140 μM. As amiloride inhibits NHE activity with IC_{50} values typically ranging from 1 to 100 μM [43,44], these results are consistent with the inhibitory effect on FIC reduction resulting from inhibition of NHE activity. Although amiloride can inhibit NHE activity, it can also inhibit other sodium-dependent transporters (e.g. the Na⁺/Ca²⁺ exchanger and the Na⁺ channel; although with significantly higher IC_{50} values) [43]. In order to increase the specificity of NHE inhibition, we next assessed the effect of EIPA, an NHE-selective amiloride derivative [44]. As the activity of NHEs is more pronounced in the absence of HCO₃⁻ [26,27], we also assessed the sensitivity of ascorbate-driven tPMET to EIPA in the presence of HCO₃⁻. We observed that EIPA also caused a dose-dependent inhibition of ascorbate-dependent FIC reduction in K562 cells (Figure 4B; 95% confidence interval of IC_{50}: 0.3–0.6 μM) and in rat astrocytes (Figure 4C; 95% confidence interval of IC_{50}: 0.07–0.15 μM) that was markedly diminished by the presence of HCO₃⁻. This diminished sensitivity supports the
Regulation of ascorbate-driven plasma membrane electron-transport

Figure 4  Ascorbate-driven FIC reduction is sensitive to NHE inhibitors

The effects of amiloride on FIC reduction by K562 cells that had been pre-incubated with (A) 50 μM DHA, or EIPA on FIC reduction by (B) K562 cells and (C) rat astrocytes with (open symbols) or without (closed symbols) 25 mM HCO$_3^-$ ions. Values are means ± S.D. for three experiments. (A) ***P < 0.001 compared with 0 μM. (B and C) ***P < 0.001 compared with the identical EIPA value in the presence of HCO$_3^-$.

Figure 5  EIPA inhibits pH$_i$ recovery after intracellular acidification

pH$_i$ was monitored over time in ascorbate-free K562 cells (A) and ascorbate-free rat astrocytes (B) that had previously been exposed to an NH$_4^+$ pulse. In both (A) and (B) the arrow indicates the point of medium replacement either with or without 5 μM EIPA, and with (B) or without (A) 500 μM FIC. The baseline values (closed diamonds) represent the pH$_i$ of the control prior to medium replacement (indicated by the arrow). The dotted lines represent the initial rate of pH$_i$ recovery for the indicated treatments. Values are means ± S.D. for three experiments.

NHE antagonists inhibit pH$_i$ recovery after intracellular acidification

The primary biochemical activity of NHEs appears to be sodium-dependent proton export, resulting in intracellular alkalinization [30,31]. This activity is a major contributor to pH$_i$ homeostasis in many cells [30] including astrocytes and numerous cancer cell types [46]. Accordingly, it is well-established that the blockade of NHEs with amilorides causes mild intracellular acidification, inhibits sodium-dependent proton release [16] and inhibits the rate of recovery of pH$_i$ after an intracellular acidification event [27,28].

We confirmed that EIPA causes an inhibition of the rate of pH$_i$ recovery after NH$_4^+$-pulse-induced intracellular acidification in K562 cells (in the absence of FIC; Figure 5A) and in astrocytes (in the presence of FIC; Figure 5B). In the experiment shown in Figure 5(A), the rate of pH$_i$ recovery after removal of the NH$_4^+$ pulse was so rapid that pH$_i$ was almost completely recovered by the time fluorescence readings were initiated (i.e. < 60 s after pulse removal). The rapid recovery of pH$_i$ in K562 cells is consistent with the general observation that NHE1 activity is increased in cancer cells [46]. The occurrence of a significant, but slower, recovery of pH$_i$ in both K562 cells and astrocytes in the presence of EIPA is most likely due to alternative acid-extrusion mechanisms that can operate in the face of NHE inhibition (e.g. a bafilomycin-inhibitable ATP-dependent proton pump in astrocytes [26] and the ubiquitous Na$^+$/HCO$_3^-$ co-transporter [30,46,47]).

It is also worth noting that the addition of FIC causes a slow rise in pH$_i$ in control astrocytes over the time course of the assays (Figure 5B, circles). FIC-induced intracellular alkalinization has previously been noted [2] and can be inhibited by amiloride derivatives [16], suggesting the involvement of NHEs. Consistent with this notion, we observed that the FIC-induced rise in pH$_i$ of ascorbate-free astrocytes is partially preventable by EIPA (results not shown).

The present results suggest that ascorbate-driven FIC reduction can be modulated by blockade of NHE activity in K562 cells and astrocytes, and further that this effect can be attenuated by the presence of HCO$_3^-$ ions. Taken together our results indicate...
that the ascorbate-driven reduction of FIC may be modulated by perturbations in pHᵢ.

As pHᵢ recovery from an NH₄⁺-pulse-induced intracellular acidification was somewhat slower in astrocytes compared with K562 cells (see Figure 5), astrocytes were considered more amenable to an analysis of the effects of a sustained perturbation of pHᵢ over the typical time course of FIC reduction employed here. Therefore astrocytes were used for all subsequent pHᵢ/FIC reduction analyses.

To more directly assess the role of pHᵢ in modulating ascorbate-driven FIC reduction, rat astrocytes were acidified with an NH₄⁺ pulse/pulse-removal with addition of EIPA or alkalinized by exposure to medium containing 25 mM HCO₃⁻ 60 s prior to time-course analyses of pHᵢ. In all experiments pHᵢ was maintained at 7.2 and changes in pHᵢ were monitored over periods of 40 min at 37°C. FIC was always added simultaneously with the medium replacement that initiated the ‘treatment phase’ (see the Experimental section for details). This was done so that: (i) changes in pHᵢ could be observed that occurred during the course of FIC reduction, and (ii) differences in FIC reduction could be assessed directly at the end of the treatment phase.

In nominally CO₂/HCO₃⁻−free HBS, rat astrocytes had an average basal pHᵢ of 6.7 ± 0.03 (n = 14), consistent with that previously observed for cultured astrocytes [26–28]. In the absence of further additions, these basal values typically persisted for approx. 1 h (results not shown). As anticipated from the data on EIPA-mediated inhibition of FIC reduction in the previous section, the exposure of ascorbate-loaded astrocytes to both FIC and EIPA resulted in a mild intracellular acidification compared with ascorbate-loaded control cells that was exacerbated by NH₄⁺ pulse/pulse-removal (Figure 6A). Conversely, the presence of 25 mM HCO₃⁻ caused a marked rise in pHᵢ of more than 0.6 pH units over 40 min in ascorbate-loaded astrocytes (Figure 6B).

Consistent with the hypothesis that ascorbate-driven FIC reduction can be modulated by pHᵢ, the rates of FIC reduction by ascorbate-loaded astrocytes were inhibited by the treatments that resulted in intracellular acidification (i.e. EIPA and NH₄⁺/EIPA) and stimulated by the treatment that resulted in intracellular alkalinization (i.e. HCO₃⁻; Figure 6C). Moreover, the NH₄⁺/EIPA condition, which caused the greatest fall in pHᵢ, also caused the greatest inhibition of FIC reduction. It should be noted that all data shown were corrected for rates of FIC reduction in ascorbate-free control cells. These results strongly suggest that intracellular acidification impairs ascorbate-driven tPMET, whereas intracellular alkalinization is stimulatory. That is, ascorbate-driven tPMET appears to be capable of being regulated by pHᵢ.

It is interesting to note that the addition of FIC appears to cause a very rapid intracellular acidification of ~0.2 pH unit in ascorbate-loaded astrocytes (Figure 6), which is not apparent in ascorbate-free astrocytes (Figure 5B). This effect was not replicated by the addition of FOC (results not shown), suggesting the requirement of both an electron acceptor (i.e. FIC-dependence) and electron donor (i.e. ascorbate-dependence) in mediating these effects. Such considerations indicate that ascorbate-driven tPMET, in addition to being regulated by pHᵢ, may itself be capable of inducing intracellular acidification. Such an effect is consistent with the reaction mechanism recently hypothesized [5], and generally supported by the present study, in which ascorbate-driven tPMET to extracellular FIC results in the initial oxidation of intracellular ascorbate to the ascorbonyl radical concomitant with the liberation of a proton. This apparent perturbation of pHᵢ by ascorbate-driven tPMET should be investigated further in future studies.

DISCUSSION

The mechanism of ascorbate-driven tPMET

Although it is has been documented that the exposure of cells to ascorbate or DHA stimulates their ability to reduce extracellular FIC, the general mechanism underlying this stimulation remains unclear [5]. Thus in the first part of the present study we confirmed that a pre-exposure to genuine DHA markedly stimulates FIC reduction by K562 cells, and demonstrated for the first time this effect in rodent astrocytes. On the basis of our data, this activity appears to depend on sequential uptake of DHA and intracellular accumulation of ascorbate, yet is independent of extracellular ascorbate. The major findings are: (i) pre-incubation of cells with DHA markedly enhances extracellular FIC reduction; (ii) the stimulation of FIC reduction by pre-exposure to DHA...
is dependent on GLUT-mediated DHA uptake and intracellular accumulation of ascorbate; and (iii) extracellular FIC is reduced by electrons derived from a restricted intracellular pool of ascorbate.

Moreover, as the reaction demonstrates saturation kinetics with respect to both intracellular ascorbate and extracellular FIC in K562 cells and astrocytes, an enzyme-catalysed mechanism is highly probable. These kinetic data also reveal that extensive recycling of intracellular ascorbate is required to support the levels of FIC reduction achieved, with astrocytes demonstrating a greater degree of apparent ascorbate recycling. Although the identity of the putative enzyme(s) responsible for electron transfer across the plasma membrane has yet to be identified, it has been suggested that members of the ubiquitous cytochrome b561 family (e.g. Dcyt b) are likely to be involved [5,48].

The identity of the native electron acceptor for this activity has yet to be confirmed, although both non-transferrin-bound iron and extracellular ascorbyl radicals are possibilities [5]. Instructively, the present results strongly suggest that the ascorbate-stimulated reductions of ferric citrate and FIC occur by different mechanisms. Reduction of the former is due to ascorbate release [18], while reduction of the latter is clearly dependent on a restricted pool of intracellular ascorbate (the results of the present study). Therefore as the major physiological form of non-transferrin-bound iron appears to be ferric citrate, the results of the present study argue against the role of ferric citrate as a significant native electron acceptor for ascorbate-driven tPMET. However, the case for extracellular ascorbyl radicals as native electron acceptors remains strong, and, if experimentally demonstrated, would implicate the presently described tPMET system in the regeneration of extracellular ascorbate [5].

A regulatory role for NHEs and pH

In the second part of the present study we investigated the possibility that ascorbate-dependent tPMET may be regulated by NHE activity. It was observed that ascorbate-driven FIC reduction in both K562 cells and astrocytes is acutely sensitive to the classical NHE inhibitors amiloride and/or EIPA. Importantly, the estimated IC50 ranges are suggestive of the involvement of NHEs 1 or 2 [43,44]. To our knowledge, this is the first description of a sensitivity of ascorbate-dependent tPMET to NHE inhibitors.

Consistent with the notion that NHE activity is a major contributor to the maintenance of pH+ in both cancer cells [46] and astrocytes [31], we confirmed that the application of EIPA inhibited the rate of pH+ recovery after an NH+4-pulse-induced intracellular acidification. These results suggest that the inhibitory effect of NHE blockade may be related to perturbations in pH+. This prediction was confirmed by the observation that selective intracellular acidification by NH+4 pre-pulsing with subsequent exposure to EIPA is associated with a significant inhibition of tPMET, whereas selective intracellular alkalinization with HCO−3 is associated with a significant stimulation of tPMET.

The pH+-dependent modulation of ascorbate-driven tPMET proposed here may have multiple origins. One possibility is an impairment of intracellular ascorbate recycling under conditions of intracellular acidification, and a stimulation of ascorbate recycling under conditions of intracellular alkalinization. As indicated above, our results suggest that ascorbate-driven tPMET requires a high degree of recycling of intracellular ascorbate (e.g. 10–90-fold in the case of astrocytes). In support of this concept, Holmes et al. [49] observed that intracellular acidification induced by EIPA impairs the intracellular ascorbate-recycling capacity of coronary artery smooth muscle cells. Therefore it is plausible that the inhibition of ascorbate-driven tPMET by NHE inhibition and/or intracellular acidification may be due to an impairment of ascorbate cycling at more acidic pH+ values. Conversely, the stimulation of tPMET by HCO−3 suggests a greater rate of recycling of intracellular ascorbate at elevated pH+ values.

Our results also suggest that FIC can induce an ascorbate-dependent intracellular acidification. The ascorbate-dependence of this effect suggests that ascorbate-driven tPMET may be the causative factor. Although the origins of this FIC-induced acidification are unknown, a plausible possibility is that the induction of ascorbate-driven electron transfer across the plasma membrane is accompanied by the one-electron oxidation of intracellular ascorbate to the ascorbyl radical and a free proton [5]. The potential physiological implications of this hypothesis (see below) warrant further investigation, but will be difficult without specific inhibitors or knowledge of the proteins responsible for ascorbate-driven tPMET.

The association of ascorbate-driven tPMET with NHE activity and pH+, identified in the present study suggests a mechanism that may link cellular ascorbate metabolism with the regulation of diverse cellular processes that are themselves regulated by changes in pH+ and/or NHE activity. Such processes include the dynamic modulation of pH+, homeostasis, cell-volume sensing and regulation [31] and mitogenic/apoptotic signalling [17,50]. For example, both ascorbate [51] and intracellular acidification [17,50] can cause selective apoptosis in cancer cells. Although the mechanism of ascorbate-dependent induced cell killing is not yet known, it is thought to rely on the induction of oxidative stress [51]: a circumstance under which extracellular ascorbyl radical concentrations would be expected to increase. Thus, in the light of the results of the present study, it might be speculated that an additional mechanism by which ascorbate contributes to the onset of apoptosis in cancer cells could be via intracellular acidification induced by tPMET from intracellular ascorbate to extracellular ascorbyl radicals.

In conclusion, the results of the present study clarify the mechanism of ascorbate-driven FIC reduction by K562 cells, and identify for the first time the existence of this system in rodent astrocytes. This tPMET system appears to: (i) be responsible for >90% of the FIC reduction in K562 cells and astrocytes (in the presence of physiological levels of intracellular ascorbate); (ii) require extensive recycling of intracellular ascorbate; and (iii) be modulated by NHE activity and pH+. Future studies should endeavour to identify both the physiological electron acceptor(s) and protein components of this ascorbate-driven oxidoreductase activity. Once these aspects are established, the physiological significance of the basic regulatory mechanisms identified should become apparent.

AUTHOR CONTRIBUTION

Darius Lane designed, performed and analysed all experiments, and drafted the manuscript. Stephen Robinson supervised Hania Czerwinska and was involved in the writing of the manuscript. Hania Czerwinska was involved in the preparation and maintenance of primary astrocytes. Alfons Lawen supervised Darius Lane, was involved in the analysis of all experiments, and supervised the writing of the manuscript.

FUNDING

D.J.R.L. and A.L. would like to acknowledge the financial support of the Department of Biochemistry and Molecular Biology, Monash University. D.J.R.L. was a recipient of a Faculty of Medicine, Nursing and Health Sciences (Monash University, Victoria, Australia) bridging fellowship.
REFERENCES


Received 8 January 2010/16 March 2010; accepted 22 March 2010
Published as BJ Immediate Publication 22 March 2010, doi:10.1042/BJ20100064

© The Authors. Journal compilation © 2010 Biochemical Society