Non-transferrin Iron Reduction and Uptake Are Regulated by Transmembrane Ascorbate Cycling in K562 Cells*

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K562 erythroleukemia cells import non-transferrin-bound iron (NTBI) by an incompletely understood process that requires initial iron reduction. The mechanism of NTBI ferrireduction remains unknown but probably involves transplasma membrane electron transport. We here provide evidence for a novel mechanism of NTBI reduction and uptake by K562 cells that utilizes transplasma membrane ascorbate cycling. Incubation of cells with dehydroascorbic acid, but not ascorbate, resulted in (i) accumulation of intracellular ascorbate that was blocked by the glucose transporter inhibitor, cytochalasin B, and (ii) subsequent release of micromolar concentrations of ascorbate into the external medium via a route that was sensitive to the anion channel inhibitor, 4,4′-diisothiocyanatostilbene-2,2′-disulfonate.

Ascorbate-deficient control cells demonstrated low levels of ferric citrate reduction. However, incubation of the cells with dehydroascorbic acid resulted in a dose-dependent stimulation of both iron reduction and uptake from radiolabeled [55Fe]ferric citrate. This stimulation was abrogated by ascorbate oxidase treatment, suggesting dependence on direct chemical reduction by ascorbate. These results support a novel model of NTBI reduction and uptake by K562 cells in which uptake is preceded by reduction of iron by extracellular ascorbate, the latter of which is subsequently regenerated by transplasma membrane ascorbate cycling.

Iron, the most abundant transition metal in mammalian systems, is required for normal metabolic processes spanning molecular oxygen transport, respiratory electron transfer, DNA synthesis, and drug metabolism (1). The tendency of free ferric iron to form insoluble polynuclear aggregates under physiological conditions is typically counteracted through iron sequestration by both proteinaceous and non-proteinaceous chelators in human plasma (1, 2). In addition to the cellular acquisition of iron by the classic transferrin-dependent pathway (2, 3), uptake of non-transferrin-bound iron (NTBI) is well documented (4–13). NTBI uptake may be particularly relevant in the face of iron overload diseases such as hereditary hemochromatosis, hypotransferrinemia, and thalassemia (14–17), in which plasma iron presents in excess of transferrin-binding capacity (18). Under such conditions, NTBI uptake by tissues (e.g. liver, heart, and pancreas (16), but not brain (19)) may serve to “clear” potentially toxic levels of iron from the plasma before damage due to iron-catalyzed oxygen radicals can accumulate (5, 15, 20). However, such iron scavenging may also contribute to the pathophysiology of iron overload disorders (14, 16).

Mechanistically, NTBI uptake depends on (i) iron reduction and (ii) consequent cellular import of ferrous iron (9, 16) by divalent metal ion transporters (e.g. divalent metal transporter, isofrom 1 (21)). Although the requirement for ferrireduction in NTBI uptake is clear (9, 10, 22, 23), the mechanism of reduction remains ill-defined. Most models propose a membrane-bound ferrireductase activity (16) in analogy with the plasma membrane ferrireductase activity of yeast (16) and the recently identified endosomal membrane ferrireductase of the transferrin-mediated pathway (24). Although conceptually appealing, such models ignore the probable involvement of non-enzymatic ferrireduction by endogenous reductants such as superoxide (25) and ascorbate (12) under physiological conditions.

Ascorbate, a two-electron donor and nutrient that must be obtained from exogenous sources in humans, greatly enhances NTBI uptake in cell culture models, predominantly by reducing ferric to ferrous iron (10, 26, 27). The resulting two-electron oxidized form of ascorbate, dehydroascorbic acid (DHA), is rapidly and irreversibly degraded under physiological conditions unless reduced back to ascorbate (28). Intriguingly, there is mounting evidence that extracellular ascorbate can be recycled by DHA import on facilitative glucose transporters (GLUTs), intracellular DHA reduction, and consequent ascorbate release (28, 29).

In this study, we have both extended and linked these previously disjointed observations by demonstrating that (i) K562 cells can indeed recycle extracellular ascorbate via DHA import and reduction, followed by ascorbate export and (ii) this ascorbate cycling is directly linked to NTBI reduction and uptake. Importantly, this mechanism falls under the category of “transplasma membrane electron transport” as the source of reducing equivalents for extracellular ferric reduction is ultimately intracellular. These observations provide new insight into the mechanisms of NTBI reduction and uptake by K562 cells.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all chemicals were from Sigma-Aldrich or Merck (Kilsyth, Victoria, Australia). Microplate assays
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were performed on a Benchmark™ Plus microplate spectrophotometer (Bio-Rad, Regents Park Industrial Estate, New South Wales, Australia) using Nunc 96-well flat-bottom transparent plates.

K562 Cells and Culture Conditions—The human erythroleukemia cell line, K562, was maintained in exponential growth as previously described (30). Prior to an assay cells were harvested and washed three times in cold phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.4). Viable cells were counted using trypan blue exclusion, and paired wells in triplicate in a 96-well plate containing either 25 ml of PBS or MOPS-buffered saline (MBS, 137 mM NaCl, 2.7 mM KCl, 15 mM MOPS-Na+, pH 7.3).

Ascorbate Loading of K562 Cells—Ascorbate loading was performed according to a modification of a previous procedure (30). Briefly, 4.5 ml of PBS-washed K562 cells (8–14 x 10⁶ cells/ml) were aliquotted into 14-ml Falcon tubes. 0.5 ml of freshly prepared DHA at final concentrations of 50–500 μM was added, followed immediately by orbital mixing for 30 min (37 °C) in the dark. The extracellular medium was thoroughly removed by three successive wash centrifugation cycles in ≥100 volumes per wash of ice-cold MBS.

Determination of Intracellular and Extracellular Ascorbate—Ascorbate levels were determined according to a modified enzymatic assay in combination with the ferrocyanide determination assay of Lane and Lawen (30). Briefly, for each unknown ascorbate-containing solution 100–1 ml aliquots were added to paired wells in triplicate in a 96-well plate containing either 25 μl of MBS or 25 μl of 50 units/ml ascorbate oxidase (AO; Caricurbita sp) dissolved in MBS. Plates were then orbitally mixed at 700 rpm at room temperature for 5 min in the dark, following which 50 μl of 3.5 mM ferrocyanide in MBS were added. Plates were then orbitally mixed at 700 rpm at room temperature for a further 5 min in the dark, then ferrocyanide levels determined (30). Ascorbate concentrations were subsequently determined by subtracting the ferrocyanide levels for the AO-containing wells (in which the resident ascorbate had been oxidized from the AO-deficient wells in which the ascorbate was present) for each sample. Note, as each molecule of ascorbate is capable of reducing two ferricyanide molecules (31), the net ferrocyanide levels were divided by a factor of two to determine the net ascorbate concentration. The veracity of the assay was verified by generating standard curves with solutions of known ascorbate concentrations (data not shown).

Intracellular ascorbate was determined by permeabilizing ascorbate-loaded cells with a final concentration of 0.1% saponin (Quijilla bark) plus 1% (v/v) ethanol in ice-cold PBS. Cells were orbitally mixed for 10 min in the cold, then centrifuged at 13,000 g for 5 min in a microcentrifuge maintained at 4 °C. Supernatants were aspirated and ascorbate determined as above.

Ferrereduction Assays—Ferrereduction assays were carried out essentially according to two previously described methods (12, 32). In brief, reduction of extracellular ferric to ferrous iron by K562 cells was assayed colorimetrically in a microplate format following complexing of ferrous iron with the cell-impermeant ferrous chelator, ferene-S (FS; λmax = 593 nm; ε593 = 35.5 mM⁻¹-cm⁻¹) (30) at concentrations of 100–200 μM. Ferric iron was supplied to cells as a freshly prepared ferric citrate chelate (20 μM FeCl3 + 50 μM sodium citrate) (33). Assays were always initiated by the addition of ferric citrate, followed by orbital mixing in an incubator (37 °C) for 60 min. Reactions were stopped by centrifugation of aliquots at 3000 rpm for 5 min in a microcentrifuge maintained at 4 °C. The concentration of the ferrous chelate was then determined spectrophotometrically. "Time zero" readings were always taken by addition of all relevant reagents to cell conditioned medium immediately subsequent to initiation of the assay proper.

Measurement of ⁵⁵Fe Uptake—0.4 ml of MBS washed cells (5 x 10⁶ cells/ml) were aliquotted into 2 ml microcentrifuge tubes. 50 μl of [⁵⁵Fe]ferric citrate containing 0.5 μCi of ⁵⁵Fe as a freshly prepared ferric citrate chelate were added to a final concentration of 1.8 μM FeCl3 + 20 μM sodium citrate in a final volume of 0.5 ml. Tubes were then incubated at 37 °C with orbital mixing for 60 min. Assays were terminated by the addition of 1 ml of ice-cold 'stop' solution (1 mM diethylenetriamine pentaacetic acid in MBS). Cells were sedimented in a microcentrifuge then washed two times with 1 ml volumes of stop solution to remove surface-bound iron (12). Subsequent to the final wash, 0.5 ml of 2% (w/v) SDS in 10% (v/v) MBS was added to the cell pellets. Complete cellular lysis was achieved by heating of the cell suspension at 95 °C for 10 min. Cellular homogenates were then mixed with 5 ml of liquid scintillation fluid (Ultima Gold MV, PerkinElmer) and added to 6-ml plastic scintillation vials. Radioactivity was determined on tritium settings with quench correction in a Wallac 1409 liquid scintillation counter. Iron uptake was calculated from the measured radioactivity following correction for uptake in a paired cell sample that was incubated on ice for 5 min in the presence of 1 μM diethylenetriamine pentaacetic acid.

Data Analysis and Curve Fitting—All curve fitting and hypothesis testing were performed in SigmaPlot® 9.0 (Systat Software, San Jose, CA) or Prism® 5.0 (GraphPad Software, San Diego, CA), respectively. Dose-response data were modeled by single rectangular hyperbolae or simple linear regression. Differences between treatments were analyzed using both one- and two-factor analysis of variances and post hoc tests of significance using GraphPad Prism® 5.0.

RESULTS

K562 Cells Import DHA and Reduce It Intracellularly to Ascorbate—Continuously cultured human cells are typically ascorbate-deficient due to (i) their inability to synthesize the vitamin de novo, (ii) the virtual absence of ascorbate from standard culture media, and (iii) the lability of the vitamin in solution under culture conditions (12, 34, 35). In accordance with this notion, K562 cells cultured as described (see “Experimental Procedures”) demonstrated negligible levels of intracellular ascorbate (Fig. 1). Even when incubated with extracellular ascorbate (0.5 mM) for 30 min these cells failed to show a significant increase in intracellular ascorbate (Fig. 1). However, when incubated with an equivalent concentration of DHA, or an oxidizing mixture of ascorbate plus the membrane-impermeant oxidant ferrocyanide (5 mM), or the enzyme ascorbate oxidase (AO), the cells demonstrated a large increase in intracellular ascorbate (Fig. 1). Because both ferrocyanide and AO
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FIGURE 1. K562 cells rapidly accumulate intracellular ascorbate from extracellular DHA, but not ascorbate. PBS-washed K562 cells (4 × 10⁶ cells/ml) were incubated in PBS with 500 μM DHA. Cells were subsequently exposed to increasing concentrations of DHA (0–100 μM) for a further 60 min. The appearance of extracellular ascorbate was monitored as described under “Experimental Procedures.” The results shown are means of three individual experiments (± S.D.).

oxidize ascorbate completely to DHA (31), these results indicate that K562 cells selectively import the oxidized form of the vitamin and then reduce it intracellularly back to ascorbate. Assuming a mean intracellular volume of 1.65 × 10⁻¹⁰ liter per K562 cell (36), an intracellular ascorbate concentration of ~3 mM was estimated (~5 fmol of ascorbate per cell (Fig. 1)). This entails an accumulation of ascorbate against an apparent concentration gradient of 6:1, a value that compares favorably with previous determinations (34, 37). Importantly, this DHA-dependent increase in intracellular ascorbate was completely abrogated by preincubation of cells with the facilitative glucose transporter inhibitors, cytochalasin B (Fig. 1) and phloretin (data not shown). These data concur with numerous previous findings on DHA uptake by cultured cells (28, 38–40), and thus strongly suggest that K562 cells possess a high capacity DHA-specific uptake route that probably involves GLUT-like transporters. The observation that ascorbate alone failed to significantly increase intracellular ascorbate further suggests that high affinity sodium ascorbate cotransporters, found to specifically import the reduced form of the vitamin in many cell types (28, 41), may not contribute substantially to ascorbate uptake by K562 cells, at least under the present experimental conditions. Moreover, the preferential uptake of the oxidized form of the vitamin by K562 cells suggests that this pathway may be operative under conditions of extracellular oxidative stress, as previously suggested (12, 38).

K562 Cells Release Ascorbate into the Extracellular Medium: the Effect of Exogenous DHA—Many cell types, including leukemic, endothelial, and brain cells, are known to release ascorbate into the extracellular medium following ascorbate loading (12, 28, 29, 37, 42, 43). The ability of K562 cells to facilitate such ascorbate export has, however, not been conclusively demonstrated. The dose-dependent release of ascorbate in response to exogenous DHA was assessed for cells before and after prior ascorbate loading with 0.5 mM DHA. A time course of 60 min was used for these studies as the release of ascorbate was shown to be linear over this period (data not shown). Both control and DHA-loaded cells demonstrated a hyperbolic dose-dependent increase in extracellular ascorbate in response to increasing exogenous DHA concentrations (Fig. 2A). The basal ascorbate release rate for the DHA-loaded cells was vertically shifted upwards by 18 pmol-min⁻¹·(10⁶ cells⁻¹) (Fig. 2, closed versus open circles). Furthermore, the extracellular ascorbate concentrations achieved over the 60-min assay period for a cell concentration of 4 × 10⁶ cells/ml were 0.0–4.4 μM for the control cells and 4.3–10.2 μM for the DHA-loaded cells. The latter is consistent with typical physiological ascorbate concentrations observed in unsupplemented human plasma (20–60 μM (12)).

It was further observed that the addition of AO to either control cells or DHA-loaded cells in the presence of 100 μM DHA completely removed all detectable extracellular ascorbate (Fig. 2A, squares) without affecting intracellular ascorbate (data not shown). Ascorbate release cannot be attributed to cell lysis as the proportion of viable cells before and after the assays was unchanged (data not shown), as previously noted for HepG2 cells (44). Interestingly, the release of ascorbate by DHA-loaded K562 cells was partially inhibited by addition of the cell-impermeant, generic anion channel inhibitor, 4,4'-diisothiocyanatostilbene-2,2'-disulfonate (DIDS) (Fig. 2A, triangles) suggesting that the release of ascorbate may occur partially through DIDS-sensitive plasma membrane anion channels (43). In summary, these results demonstrate that ascorbate-replete K562 cells release physiological amounts of ascorbate into the extracellular medium, and suggest that at least some of the release may occur via DIDS-sensitive anion channels in the cell membrane.
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It should be noted that the accumulation of extracellular ascorbate in response to DHA could be interpreted as being due to extracellular reduction of DHA to ascorbate, as previously claimed for K562 cells (37). To address this issue, we used cytochalasin B to restrict exogenous DHA to the extracellular compartment, allowing us to remove the effect of DHA uptake on extracellular ascorbate accumulation. Identical dose-response studies were performed in the presence of 50 μM cytochalasin B to block the uptake of DHA through facilitative glucose transporters (cf. Fig. 1). As seen in Fig. 2B, blockade of DHA import ablated the dose response to exogenous DHA, strongly suggesting that the appearance of ascorbate in the extracellular medium is dependent on, and preceded by, cellular uptake and reduction of DHA. Importantly, ascorbate release rates were largely unaffected by cytochalasin B (Fig. 2, B versus A, closed circles). Moreover, cytochalasin B was without significant effect on cell-mediated reduction of ferricyanide (data not shown). This indicates, as observed previously for HepG2 cells (44), that cytochalasin B-dependent inhibition of extracellular ascorbate accumulation from DHA cannot be attributed to potential inhibition of transplasma membrane electron transport to extracellular DHA. Thus, the inability of extracellularly restricted DHA to stimulate extracellular ascorbate production (Fig. 2B) argues strongly against the involvement of a cell surface DHA reductase capable of generating extracellular ascorbate in K562 cells.

NTBI Reduction by K562 Cells Is Greatly Enhanced by DHA Uptake and Ascorbate Efflux—Exogenously added ascorbate enhances dietary non-heme iron absorption (45) and NTBI uptake in cultured cells (26, 27), largely through the reduction of ferric to ferrous iron (26). Therefore, the ability of K562 cells to import DHA and release ascorbate might be expected to contribute to the capacity for cell-mediated reduction of NTBI. Consistent with these expectations, May and colleagues (12) demonstrated that U937 cells preloaded with DHA secrete low levels of ascorbate that stimulate reduction of ferric citrate and iron uptake from ferric nitritotriacetate. To test the hypothesis that DHA loading of K562 cells increases NTBI reduction capacity, we assessed the dose response of cell-dependent ferrireduction to exogenous DHA in K562 cells before and after prior DHA loading. Ferrireduction was assessed by determining the appearance of ferrous iron from ferric citrate (a physiologically relevant NTBI chelate (33, 46)) in the presence of FS (see “Experimental Procedures”). A final concentration of freshly prepared 20 μM FeCl₃, 50 μM sodium citrate was used, according to previous recommendations (33). As shown in Fig. 3, concentrations of 100–200 μM FS were found to yield maximal ferrireductase activity for both control (open symbols) and DHA-loaded cells (closed symbols) in the presence of 50 μM DHA and were thus used for subsequent ferrireduction studies. Due to the fact that DHA can spontaneously and non-enzymatically convert to erythroascorbic acid (47) (an oxidizable compound similar in structure to ascorbic acid), all ferrireduction data were corrected for background ferrireduction in the presence of all assay constituents except cells.

DHA loading of ascorbate-deficient K562 cells significantly stimulated the rate of ferrireduction (Figs. 3 and 4). The basal level of ferrireduction displayed by ascorbate-deficient control cells was 1.6 ± 0.2 pmol·min⁻¹·(10⁶ cells)⁻¹ (Fig. 4, open circles) with linearity extending to at least 60 min (data not shown). Remarkably, the basal level of ferrireduction for cells preloaded with DHA was increased 12-fold to 19.1 pmol·min⁻¹·(10⁶ cells)⁻¹ (Fig. 4A, closed circles). Increasing the concentration of exogenous DHA during the ferrireduction assay for both DHA-loaded and control cells resulted in the dose-dependent stimulation of ferrous iron production (Fig. 4A), correlating closely with that for extracellular ascorbate production. The relative
increase in ferrireduction for the control cells was slightly greater than that for the loaded cells (open versus closed circles), suggesting that reduction of ferric citrate becomes saturated at increased levels of DHA loading. The apparent \( V_{\text{max}} \) for ferrireduction (DHA-loaded cells) of 25 ± 1 pmol/min \(^{-1}\) (10\(^6\) cells) \(^{-1}\) is consistent with, though slightly lower than, that found for U937 cells by May and colleagues (12).

Blocking DHA uptake with 50 \( \mu \)M cytochalasin B was sufficient to remove the stimulatory effect of DHA addition for both control and loaded cells (Fig. 4B), suggesting that, in line with the data on ascorbate uptake and release, stimulation of ferrireduction by exogenous DHA is obligatorily dependent on DHA uptake. Importantly, the stimulation appears to be entirely dependent on the release of ascorbate from the cells, rather than the provision of ascorbate-derived electrons to a transmembrane ferrireductase activity, because addition of AO to either control or loaded cells abolished the stimulatory effect of exogenous DHA addition (Fig. 4A, squares). These data support a model of ascorbate-stimulated non-transferrin ferrireduction by K562 cells in which reduction of extracellular ferric citrate is causally linked to ascorbate cycling across the plasma membrane.

**Non-transferrin \( ^{55}\text{Fe} \)-labeled Iron Uptake from Ferric Citrate Is Regulated by DHA Uptake and Ascorbate Efflux**—K562 cells grown replete with iron in the presence of 10% fetal bovine serum (see “Experimental Procedures”) had a mean basal iron content of 390 ± 41 pmol of iron per million cells. On the basis of our observations that K562 cells can recycle ascorbate across the plasma membrane and subsequently reduce extracellular NTBI as a result, experiments were conducted to assess the capacity of this redox cycling to affect NTBI uptake from radio-labeled \( ^{55}\text{Fe} \)-ferric citrate. To remove any iron that may remain bound to the cell surface following assay completion, cells were washed thoroughly with MBS containing 1 mM diethylenetriamine pentaacetic acid (12, 33).

Ascorbate-deficient control K562 cells exposed to 1.8 \( \mu \)M \( ^{55}\text{Fe} \)-ferric citrate in 20 \( \mu \)M sodium citrate (in MBS) at 37 °C for 60 min demonstrated a basal rate of iron uptake of 15 ± 3 pmol/(10\(^6\) cells) \(^{-1}\) (Fig. 5, open circles). Prior loading of cells with DHA, however, increased the basal rate of iron uptake to 31 ± 2 pmol/(10\(^6\) cells) \(^{-1}\) (Fig. 5A, closed circles). This amounts to a doubling of a iron uptake (\( P < 0.001 \)) in response to DHA loading. Moreover, a DHA-dependent increase in iron uptake for both control and DHA-loaded cells was observed (Fig. 5A), mirroring that observed for both ascorbate efflux and ferrireduction (cf. Figs. 2 and 4). In further support of a causal connection between ascorbate efflux, ferrireduction, and \( ^{55}\text{Fe} \)-labeled iron uptake it was observed that, as with the former two, the addition of 50 \( \mu \)M cytochalasin B to the cells largely removed the dose response to exogenous DHA addition (Fig. 5B). Again, this suggests that DHA-stimulated NTBI uptake is dependent on initial DHA import, even for cells already partially loaded with DHA. Interestingly, cytochalasin B did not completely inhibit the dose response of \( ^{55}\text{Fe} \)-labeled iron uptake to exogenous DHA (Fig. 5B). This is most easily understood if one considers that, at an iron concentration of 1.8 \( \mu \)M, the low level of erythroascorbic acid previously observed to be generated spontaneously from DHA (47) would likely be sufficient to reduce a significant fraction of the iron for subsequent iron uptake. This effect, however, would be expected to diminish at increasing iron concentrations.

The addition of AO decreased the DHA-stimulated rate of \( ^{55}\text{Fe} \)-labeled iron uptake almost to the basal rate seen in the absence of prior DHA loading (Fig. 5A, squares). These data clearly demonstrate that, as with reduction of ferric citrate, the DHA-stimulated rate of \( ^{55}\text{Fe} \) is entirely dependent on a direct interaction with ascorbate released from the cell interior.

Importantly, K562 cells imported iron derived from \( ^{55}\text{Fe} \)-ferric citrate predominantly as Fe\(^{2+} \), because addition of the ferrous-specific chelators FS (Table 1) and bathophenanthroline disulfonic acid (data not shown) inhibited \( ^{55}\text{Fe} \) uptake by \( >90\% \) of the DHA-loaded rate. This result is consistent with that previously found for K562 cells in the absence of ascorbate (8).

Table 1 provides a summary of the data obtained on the effect of DHA loading on ascorbate efflux, ferrireduction, and iron uptake. Importantly, K562 cells imported iron derived from \( ^{55}\text{Fe} \)-ferric citrate predominantly as Fe\(^{2+} \), because addition of the ferrous-specific chelators FS (Table 1) and bathophenanthroline disulfonic acid (data not shown) inhibited \( ^{55}\text{Fe} \) uptake by >90% of the DHA-loaded rate. This result is consistent with that previously found for K562 cells in the absence of ascorbate (8).

### TABLE 1

| Treatment | Ascorbate release | Ferrireduction | \( ^{55}\text{Fe} \)-labeled iron uptake |
|-----------|-------------------|----------------|---------------------------------|
| Control   | 0.3 ± 0.6         | 2.0 ± 0.3      | 53.6 ± 1.0                      |
| L (Loaded) | 100.0 ± 4.6       | 100.0 ± 2.0    | 100.0 ± 2.8                     |
| L + AO    | 0.2 ± 0.7         | 2.7 ± 0.5      | 63.0 ± 3.3                      |
| L + DIDS  | 62.1 ± 8.3        | 87.5 ± 1.0     | 76.8 ± 4.3                      |
| L + ferricyanide | 0.7 ± 0.5 | 1.6 ± 0.6 | 57.3 ± 3.8                      |
| L + FS    | 106.1 ± 3.8       | 33.4 ± 8.5     | 8.5 ± 1.1                       |

* The values shown are normalized to the “loaded” values (i.e., “ascorbate release,” 19.0 ± 0.9 pmol/min/million cells, “ferrireduction,” 20.4 ± 0.4 pmol/min/million cells; and \( ^{55}\text{Fe} \)-labeled iron uptake,” 27.9 ± 0.8 pmol of Fe/h/million cells), which are set to 100%.

* NA, not applicable.
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![Diagram](image)

FIGURE 6. Mutually inclusive models for iron reduction and uptake by K562 cells. A, NTBI ferrireduction and ferrous uptake from ferric citrate (Fe{\textsuperscript{3+}}-Cit) may occur by ferric reduction catalyzed by a transplasma membrane ferrireductase (1) that derives reducing equivalents from an unspecified redox couple (R/O) in the cytoplasm. Generated ferrous iron is then transported into the cell via a divalent metal ion transporter (2), such as divalent metal transporter, isoform 1. β, alternatively, NTBI ferrireduction may occur by transplasma membrane ascorbate cycling in which extracellular ascorbate reacts directly with NTBI forming both DHA and ferrous iron. Ferrous iron is imported into the cell as in A (3). Extracellular ascorbate is subsequently regenerated for further ferric reduction events by (i) DHA import on putative GLUT-like transporters (2), (ii) intracellular reduction of DHA to ascorbate (Asc), followed by (iii) release of Asc through as yet unidentified Asc-conductive conduits in the plasma membrane (1).

uptake, as well as the influence of the effectors of ascorbate cycling identified in this study, i.e. cytochalasin B, ascorbate oxidase, and DIDS. Moreover, Table 1 shows that the addition of ferricyanide to DHA-loaded cells led to oxidation of extracellular ascorbate and abrogation of both ferrireduction and the elevated rate of iron uptake (Table 1). Taken together, these data suggest that NTBI iron reduction and uptake by K562 cells are regulated by transplasma membrane redox cycling of ascorbate. To our knowledge this is the first study establishing a basic mechanism of NTBI reduction and uptake by K562 cells from the physiologically predominant form, ferric citrate.

DISCUSSION

The cellular uptake of NTBI is well established as a significant source of cellular iron (4–13, 20). NTBI uptake may serve to either scavenge potentially redox-active iron from the extracellular fluid (e.g. hepatic cells (27)) or to augment the supply of iron for general metabolism and/or erythropoiesis (e.g. K562 cells (11)). A major barrier to our understanding of NTBI uptake is the lack of an adequate mechanism for ferrireduction. Initial ferrireduction is crucial as ferric iron predominates under physiological conditions, yet most cellular iron transport systems show strong preference for the ferrous valency (16). The presumed involvement of a transplasma membrane ferrireductase activity is widely accepted (Fig. 6A). In support of this conclusion, numerous cell types are able to reduce a variety of NTBI ferric chelates in the absence of exogenous reductants (8, 10, 23). However, strong experimental support for exclusive involvement of "enzyme-mediated" ferrireduction is lacking. An alternative paradigm suggests that the release of endogenous reductants, such as superoxide (human bronchial epithelial cells (25)) and/or ascorbate (human U937 cells (12)), into the extracellular medium may contribute significantly to cellular NTBI reduction and uptake under physiological conditions.

In the present study we sought to determine the involvement of ascorbate recycling in NTBI reduction and uptake by human K562 cells, an erythroid precursor-like cell line for which a "classic" transplasma membrane NTBI reductase has been proposed (8). The evidence presented supports a novel model of NTBI reduction and uptake by K562 cells that involves ascorbate recycling and ascorbate-mediated ferrireduction (Fig. 6B). Because cells were able to import iron in a manner inhibitable by cell-impermeant ferrous iron chelators, iron uptake is dependent on the initial adoption of the ferrous state, as previously observed (9, 26, 48).

The specific uptake of DHA via GLUTs 1 and 3, in addition to uptake of ascorbate by sodium ascorbate cotransporters (41), is well established (28). The evidence presented supports a novel model of NTBI reduction and uptake by human K562 cells that involves ascorbate recycling and ascorbate-mediated ferrireduction (Fig. 6B). Because cells were able to import iron in a manner inhibitable by cell-impermeant ferrous iron chelators, iron uptake is dependent on the initial adoption of the ferrous state, as previously observed (9, 26, 48).

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The specific uptake of DHA via GLUTs 1 and 3, in addition to uptake of ascorbate by sodium ascorbate cotransporters (41), is well established (28). The evidence presented supports a novel model of NTBI reduction and uptake by human K562 cells that involves ascorbate recycling and ascorbate-mediated ferrireduction (Fig. 6B). Because cells were able to import iron in a manner inhibitable by cell-impermeant ferrous iron chelators, iron uptake is dependent on the initial adoption of the ferrous state, as previously observed (9, 26, 48).

K562 cells loaded with intracellular ascorbate by incubation with DHA released significant amounts of ascorbate into the extracellular medium. This release of ascorbate was not attributable to simple cellular lysis and was partially inhibited by the cell-impermeant anion channel inhibitor, DIDS. Inhibition of ascorbate efflux from cells by generic anion channel inhibitors has been observed for several other cell types, including rat astrocytes (51), hepatocyte-like HepG2 cells (44), coronary artery endothelial cells (42) and SH-SY5Y neuroblastoma cells (43). The involvement of volume-sensitive ascorbate-conducive channels in the plasma membrane has been suggested (28, 29, 43). Although the molecular identities of such channels remain to be established, our data are consistent with the notion that plasma membrane anion channels participate in ascorbate export by K562 cells. Moreover, our data provide support for the emerging notion that uptake of DHA and release of ascorbate constitute an additional level of transplasma membrane electron transport in which reducing equivalents derived from cellular metabolism may be transferred to the extracellular space for participation in extracellular redox events (29, 42, 44).

Collectively, our results indicate that ascorbate released from cells, following uptake and reduction of DHA, mediates direct reduction of ferric to ferrous iron, the latter of which is then imported. These conclusions are based on the following pivotal observations. First, cells preloaded with ascorbate by incubation with DHA showed a >12-fold stimulation of ferrireductase...
activity and a 2-fold stimulation of iron uptake from $^{55}$Fe-ferric citrate. Furthermore, subsequent addition of DHA to control or loaded cells resulted in a dose-dependent stimulation of both iron reduction and uptake that was inhibitable by cytochalasin B, suggesting response dependence on DHA uptake. Second, both enzymatic (i.e. AO) and chemical (i.e. ferricyanide) oxidation of exported ascorbate eliminated virtually all stimulation, suggesting that stimulation results from a direct chemical reaction between ascorbate and ferric citrate (12, 26). Third, DIDS inhibited ascorbate release, ferrireduction, and iron uptake to a similar degree, suggesting reliance on a common mechanism. Finally, iron uptake was almost completely absent in the presence of cell-impermeant ferrous chelators, suggesting that both the basal and ascorbate-stimulated rates of iron uptake depend on initial adoption of the ferrous state. It should be noted that, although ascorbate can chelate ferric iron and thus exert a mild stabilizing effect in the absence of alternative chelators (52), this cannot explain the inhibition of stimulated iron uptake caused by AO, ferricyanide, and ferrous chelators. Moreover, significant chelation by ascorbate in the presence of a moderately “strong” ferric chelator such as citrate is thermodynamically improbable (26).

We observed a basal level of iron uptake in the absence of ascorbate/DHA of ~15 pmol/h/million cells, similar to that observed by Kovar and colleagues (11). This basal uptake is explained by the low, but non-zero basal rate of ferrireduction observed in the absence of ascorbate/DHA (see Fig. 4A, “0 μM DHA”). However, as the former rate was more than doubled by ascorbate recycling across the plasma membrane, we postulate the existence of at least two mechanistically distinct NTBI ferrireduction/ferrous uptake systems. The first is putatively mediated by an as yet unidentified plasma membrane ferrireductase activity (8, 9) (see Fig. 6A, e.g. DcytB (53–55) and/or members of the Steap family (24, 32, 56)), whereas the second here described is functional only in the presence of redox cycling of the ascorbate/DHA couple (see Fig. 6B).

Because typical plasma ascorbate levels are maintained in the range of 20 – 60 μM (12), while plasma NTBI levels are <1 μM and usually do not exceed 10 – 20 μM (11, 15), there are clearly sufficient ascorbate-derived reducing equivalents to reduce any accessible NTBI (12), even under conditions of iron overload. Interestingly, in silico modeling of iron speciation in human plasma suggests that, in the presence of physiological ascorbate concentrations, NTBI exists largely as low molecular weight ferrous complexes (57). The latter is consistent with the presently proposed mechanism of NTBI reduction and uptake. Thus, processes that maintain extracellular ascorbate can be expected to contribute substantially, if not entirely, to NTBI reduction and iron uptake in vivo.

From a clinical perspective, the combination of increased plasma NTBI in the presence of both ascorbate and molecular oxygen is thought to be responsible for catalyzing the formation of noxious oxygen radicals in iron overloaded subjects by Fenton-type reactions (15, 17, 58). The application of physiological concentrations of ascorbate to human fibroblasts resulted in iron-dependent DNA-strand breakage, intracellular iron mobilization, and increased expression of the intracellular iron sequestration protein, ferritin (58). Interestingly, enhancement of DNA damage was obligatorily dependent on an exogenous source of hydrogen peroxide, suggesting that the toxicity of the ascorbate/iron combination may occur only under conditions of external sources of reactive oxygen species. Such data have prompted the notion of ascorbate as a pro-oxidant in the presence of redox-active transition metals such as iron and copper (59). However, it has also been pointed out that the dynamic equilibrium between ascorbate’s pro- and antioxidant capacities depends on the ascorbate:iron ratio, with increasing antioxidant function at ratios above unity (17, 59). We propose that, under conditions of elevated NTBI levels (e.g. iron overload disorders), ascorbate-cycling-dependent NTBI reduction and uptake may actually serve an iron clearance role (12), because increased NTBI should result in enhanced ascorbate oxidation and DHA production. The increase in DHA production would be expected to increase intracellular ascorbate accumulation and ascorbate release, and thus further iron reduction and uptake.

In conclusion, our results provide support for a novel model of NTBI reduction and uptake by K562 cells in which iron uptake is preceded by iron reduction by extracellular ascorbate, the latter of which is subsequently regenerated from DHA by transplasma membrane ascorbate cycling. This mechanism is likely to be widespread given the apparent ubiquity of both transplasma membrane ascorbate cycling and NTBI uptake. Due to the importance of NTBI uptake in disorders of iron overload (1, 14 –17, 46), this discovery is expected to have profound ramifications for the understanding of iron homeostasis in both human health and disease. An important next step will be to establish the generalizability of the mechanism to other cell types, as well as to identify the protein componentry of the ascorbate-release apparatus.

Acknowledgment—We thank D. Richardson (University of Sydney) for supplying K562 cells.

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