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Role of Ascorbic Acid in Transferrin-Independent Reduction and Uptake of Iron by U-937 Cells

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ABSTRACT. The role of ascorbic acid in transferrin-independent ferric iron reduction and uptake was evaluated in cultured U-937 monocytic cells. Uptake of ^{55}Fe by U-937 cells was doubled by 100 μM extracellular ascorbate, and by pre-incubation of cells with 100 μM dehydroascorbic acid, the two-electron-oxidized form of ascorbate. Reduction of extracellular ferric citrate also was enhanced by loading the cells with dehydroascorbic acid. Dehydroascorbic acid was taken up rapidly by the cells and reduced to ascorbate, such that the latter reached intracellular concentrations as high as 6 mM. However, some ascorbate did escape the cells and could be detected at concentrations of up to 1 μM in the incubation medium. Further, addition of ascorbate oxidase almost reversed the effects of dehydroascorbic acid on both ^{55}Fe uptake and ferric citrate reduction. Thus, it is likely that extracellular ascorbate reduced ferric to ferrous iron, which was then taken up by the cells. This hypothesis also was supported by the finding that during loading with ferric citrate, only extracellular ascorbate increased the pool of intracellular ferrous iron that could be chelated with cell-penetrant ferrous iron chelators. In contrast to its inhibition of ascorbate-dependent ferric iron reduction, ascorbate oxidase was without effect on ascorbate-dependent reduction of extracellular ferricyanide. This indicates that the cells use different mechanisms for reduction of ferric iron and ferricyanide. Therefore, extracellular ascorbate derived from cells can enhance transferrin-independent iron uptake by reducing ferric to ferrous iron, but intracellular ascorbate neither contributes to this reduction nor modifies the redox status of intracellular free iron. BIOCHEM PHARMACOL **57**;11:1275–1282, 1999. © 1999 Elsevier Science Inc.

KEY WORDS. iron uptake; ferrireductase; ascorbic acid; calcein; dehydroascorbic acid; ferricyanide; U-937 cells

Whereas transferrin-mediated iron uptake is considered the primary route of iron uptake in most cells, there is also evidence for a transferrin-independent mechanism [1–3]. Uptake of iron in the transferrin-independent pathway is saturable, temperature-dependent, and not inhibited by agents that are known to affect transferrin-mediated iron uptake [1, 3]. Of several possible mechanisms for transferrin-independent iron uptake, one that has received experimental support involves first the binding of ferric iron, typically in the form of a water-soluble chelate, to the cell surface [4]. Ferric iron then is reduced by a cell-surface ferrireductase activity to ferrous iron [5], followed by uptake across the cell membrane. A cell-surface site of reduction is supported by the ability of cell-impermeant chelators of ferrous iron to inhibit transferrin-independent uptake of radioactive ferric iron in some [5, 6], but not all cell types [7]. Additionally, the mild oxidant ferricyanide, which also does not enter cells, inhibits uptake of radioactive iron from ferric iron chelates in K562 [8] and Caco-2 cells [6]. In contrast to iron chelating agents, inhibition by ferricyanide might be due to competition for reducing equivalents available at the cell surface or across the cell membrane. In this regard, both erythrocytes [9, 10] and mononuclear cells [11, 12] long have been known to reduce extracellular ferricyanide. A trans-plasma membrane oxidoreductase enzyme has been proposed to account for this reduction [10, 13]. This raises the possibility that the extracellular reductions of ferric iron and ferricyanide are mediated by the same trans-plasma membrane process, or that they use the same intracellular reductants.

The source of reducing equivalents for cell-surface reduction of ferric iron has not been established. Whereas intracellular reduced pyridine nucleotides are likely donors, ascorbic acid, when present, also might serve to reduce ferric iron. Reduction of extracellular ferric iron chelates by added ascorbate enhances radioactive iron uptake into HeLa cells [5], rabbit reticulocytes [2], and Caco-2 cells [6]. There is less support for intracellular ascorbic acid as a source of reducing equivalents for extracellular ferric iron. For example, Han *et al.* [6] found that incubation of Caco-2 cells with 1 mM DHA†, the two-electron oxidized form of

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†*Abbreviations:* BPS, bathophenanthroline disulfonate; calcein-AM, calcein, acetoxymethyl ester; DHA, dehydroascorbic acid; and KRH, Krebs-Ringer-HEPES.

ascorbate, enhanced cellular uptake of ^{55}Fe from a complex with nitriloacetic acid. The DHA entered cells and was reduced to ascorbate. However, when ascorbate oxidase was added to the incubation medium, the stimulation of radioactive iron uptake by DHA was lost. This suggests that the effect was due to release of ascorbate from the cells, rather than to transfer of electrons from within the cells. On the other hand, intracellular ascorbic acid can serve as a source of electrons for reduction of extracellular ferricyanide [9, 10, 14]. Although it was proposed initially that ascorbate released by erythrocytes was directly responsible for reduction of extracellular ferricyanide [9], subsequent studies have shown that the mechanism involves trans-plasma membrane electron transfer from intracellular ascorbate [10, 14]. Further, ascorbate may be the major source of such electrons within cells that contain the vitamin [15]. Thus, the effects of intra- and extracellular ascorbate might be useful in defining the mechanism of iron uptake and in discriminating between cell-surface ferric iron and ferricyanide reductase activities. To address these questions, we used cultured human U-937 monocytic cells, which lack ascorbate in culture, but which can be loaded readily with the vitamin.

MATERIALS AND METHODS

Materials

Analytical reagents, including ascorbate oxidase (from *Cucurbita* species), BPS, 2,2′-bipyridyl, ferrous ammonium sulfate, and sodium ascorbate were purchased from the Sigma Chemical Co. The Aldrich Chemical Co. supplied the DHA and tridecylamine. The calcein-AM was obtained from Molecular Probes. ^{55}Fe (20.5 μCi/μg) was from Dupont-New England Nuclear.

Cell Culture

U-937-D cells were cultured in RPMI-1640 medium that contained 10% (v/v) fetal bovine serum, 50 μg/mL penicillin, and 100 μg/mL streptomycin [16]. Cells were cultured at 37° in an atmosphere of 95% air and 5% CO_2. All experiments were carried out with exponentially growing cells. HPLC measurements showed that the medium did not contain ascorbic acid, and none was added. In preparation for an experiment, U-937 cells in suspension culture were washed three times by centrifugation at 1000 *g* in 40 mL of KRH buffer. The latter consisted of 128 mM NaCl, 10 mM HEPES, 5.2 mM KCl, 1 mM NaH_2PO_4, 1.4 mM $MgSO_4$, and 1.4 mM $CaCl_2$, pH 7.4. Cells were suspended to the noted concentration in KRH just before an assay.

Preparation of Soluble Ferric Iron Chelates

Ferric iron was solubilized by adding a 5-fold molar excess of either sodium citrate or the disodium salt of nitriloacetic acid to a 1 mM suspension of ferric chloride in KRH. Soluble radioactive ferric iron was prepared by adding 10 μCi of ^{55}Fe to a 25 μM solution of ferric nitriloacetate.

Measurement of ^{55}Fe Uptake

After a preliminary incubation of cells (2×10^6/mL) in 0.5 mL of KRH as noted, ferric nitriloacetate that contained 0.25 μCi of $^{55}Fe^{3+}$ was added to a final concentration of 0.5 μM Fe^{3+}, and the incubation was continued at 37°. At the indicated times, uptake was stopped with the addition of 0.5 mL of ice-cold stop solution that consisted of 1 mM diethylenetriamine pentaacetic acid, 140 mM NaCl, and 10 mM Na_2HPO_4, pH 7.4. The cells were incubated for 10 min on ice and then pelleted in the microfuge. The centrifugation washes were repeated twice more in 1 mL of ice-cold stop solution, and the cells were suspended in 0.1 mL of KRH. The cell suspension was transferred to a scintillation vial and mixed with 0.1 mL of 1% SDS in water. After the addition of 5 mL of aqueous scintillation counting fluid, radioactivity was determined on tritium settings with quench correction in a Packard 2000CA liquid scintillation spectrometer. Uptake of ^{55}Fe was calculated from the measured specific activity of $^{55}Fe^{3+}$ in the incubation medium, following correction for uptake in a paired cell sample that was incubated on ice for 5 min. All cell uptakes were performed in duplicate.

Measurement of Medium and Cellular Ascorbate

The ascorbate content of cells or cell-conditioned buffer was measured by HPLC with electrochemical detection. A cell suspension or the medium from cells was diluted 10-fold in ice-cold 80% methanol that contained 1 mM EDTA. The sample was mixed, placed on ice, microfuged after 5 min, and an aliquot of the supernatant was taken for assay of ascorbate as described recently [17].

Spectrophotometric Assays

The amount of extracellular iron generated by the cell-surface ferrireductase activity was measured in a spectrophotometric assay that is dependent on the color change of BPS when it binds Fe^{2+} [18]. In time-course studies, the absorbance of the BPS-iron complex was followed at 10-sec intervals at 535 nm with stirring in a spectrophotometric cuvette at 37°. All measurements were made using a Beckman DU-640 spectrophotometer that was equipped with a thermostatically controlled cuvette holder. The absorbance in incubations that contained cells was corrected for the absorbance change in a paired sample that did not contain cells. In experiments in which single time points were taken, cells were incubated under the above conditions in a microfuge tube at 37°. At 30 min the cells were pelleted by centrifugation and the optical density of the supernatant was measured at 535 nm. Following correction for the reading in a sample that did not contain cells, the amount of Fe^{2+} generated in the medium was calculated based on an extinction coefficient for the BPS-Fe^{2+} complex of 22.24 $mM^{-1} \cdot cm^{-1}$ [19].

Ferricyanide reduction by U-937 cells was measured as

previously described for human erythrocytes [14]. Following pre-incubations as noted, U-937 cells were washed three times by centrifugation and suspended in KRH that contained 5 mM D-glucose and 1 mM potassium ferricyanide. After 30 min of incubation at 37°, duplicate aliquots of mixed cells and buffer were microfuged for 1 min to pellet the cells, and duplicate aliquots of the resulting supernatant were assayed for ferrocyanide by the method of Avron and Shavit [20], using 1,10-phenanthroline as indicator.

Measurement of a Labile Intracellular Iron Pool

The labile pool of intracellular iron was measured using calcein as described by Breuer *et al.* [21]. Briefly, U-937 cells were incubated at 37° in RPMI medium that contained 125 nM calcein-AM. Preliminary studies, not shown, indicated that the fluorescence increase of calcein-loaded cells was linear from 0.25 to 2.0 $\times 10^6$ cells/mL, so a final concentration of 10^6 cells/mL was used in subsequent studies. After 5 min, the cells were pelleted and washed three times by centrifugation in HEPES-buffered saline that contained 140 mM NaCl and 10 mM HEPES, pH 7.3. The cells were suspended to a concentration of 10^7/mL and stored in the dark at room temperature until use (<15 min). An aliquot of the calcein-loaded cells was diluted in a stirred fluorometric cuvette with buffer and other additives as noted to a concentration of 10^6/mL. After 10 min of incubation at 37° in the dark, with or without ascorbate or DHA as noted, the baseline fluorescence of the sample was measured for 1–2 min in a SPEX Fluorolog spectrofluorometer. Instrument settings were as follows: excitation wavelength, 486 nm; emission wavelength, 517 nm; slit width, 2 mm. Ferric citrate, prepared as described above, was added in the dark to a final concentration of 50 μM, and the change in fluorescence was followed for 4–5 min. Finally, 2,2′-bipyridyl was added from a 10 mM stock solution in DMSO to a final concentration of 50 μM, and the fluorescence was followed for another 4 min.

Analytical and Statistical Methods

Data were expressed as means ± SEM from the indicated number of experiments. Curve-fitting to an hyperbolic model with residual of the data shown in Fig. 4 was carried out using the graphics analysis program FigP (Biosoft). Differences between treatments were analyzed by one-way analysis of variance and post-hoc tests of significance, using the statistical software package Sigmastat 2.0 (Jandel Scientific).

RESULTS

Pre-loading of U-937 cells with 100 μM DHA resulted in a linear, time-dependent increase in the ability of washed cells to take up ^{55}Fe from ferric nitriloacetate complexes (Fig. 1). Inclusion of ascorbate oxidase in the medium decreased this rate by about half. At 10 min, the ascorbate oxidase-inhibited rate was similar to the rate observed in control cells, and the DHA-stimulated rate was similar to that observed with 100 μM ascorbate (Fig. 1). Since ascorbate oxidase remains outside the cells, its ability to inhibit DHA-stimulated iron uptake suggests that the effect was due to ascorbate that had left the cells. In this mechanism, the effect of ascorbate was to reduce chelated ferric iron to ferrous iron, which then was taken up by the cells. To determine whether extracellular reduction of ferric iron was involved, BPS was used both to chelate extracellular ferrous iron and to provide a measure of the extent of such chelation.

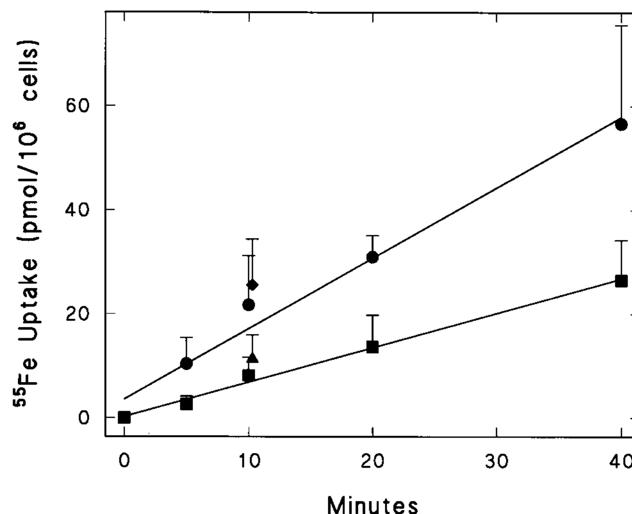

FIG. 1. Stimulation of ^{55}Fe uptake by DHA and ascorbate in U-937 cells. U-937 cells (2 × 10^6/mL) were incubated at 37° in KRH that contained 5 mM D-glucose and the following additions: none (▲); 100 μM DHA (●); 100 μM DHA and 4 U/mL ascorbate oxidase (■); or 100 μM ascorbate (♦). After 10 min, ^{55}Fe as ferric nitriloacetate was added to a final concentration of 0.5 μM iron, and uptakes were carried out for the indicated times as described in Materials and Methods. Results are shown as means ± SEM from 4 time-course experiments, and from 6 determinations each for control and ascorbate-incubated cells.

The time-dependence of the ability of BPS to bind ferrous iron that was generated in the presence of U-937 cells was measured in control cells and in cells that had been pre-loaded with DHA. After washes to remove any remaining DHA or ascorbate, the cells were incubated with BPS and ferric citrate in the presence or absence of ascorbate oxidase, as shown in Fig. 2. U-937 cells had a low but linear rate of ferrous iron generation that was accelerated markedly by pre-loading with DHA. However, the effect of DHA loading on generation of the BPS-Fe^{2+} complex was almost abolished by the presence of ascorbate oxidase in the incubation medium. These results again suggest that extracellular ascorbate was responsible for the observed effect. This was tested directly by measuring the ascorbate content of cells and medium after DHA loading.

U-937 cells took up DHA from the medium and reduced

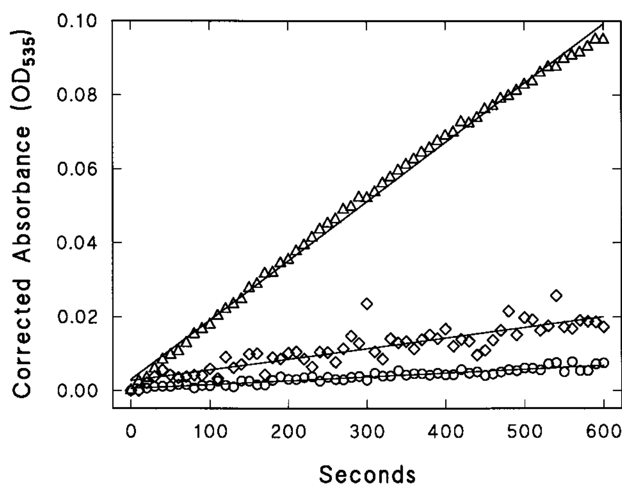

FIG. 2. Effects of DHA-loading on the time course of BPS-Fe^{2+} formation by U-937 cells. Washed U-937 cells (4×10^6/mL) were incubated at 37° in KRH that contained 5 mM D-glucose and either no additions (○) or 200 μM DHA (△, ◇). After 15 min of incubation, the cells were washed 3 times in 1 mL of KRH and suspended in a spectrophotometric cuvette to 2×10^6 cells/mL in KRH that contained 5 mM D-glucose, 20 μM ferric citrate, and 40 μM BPS, in the absence (○, △) or presence (◇) of 2 U/mL ascorbate oxidase. Incubations were carried out at 37° with stirring, and the absorbance at 535 nm was followed at 10-sec intervals. These data are from a single experiment representative of 3 that were performed, corrected at each time point for readings in a blank sample without cells, and fit to a linear model.

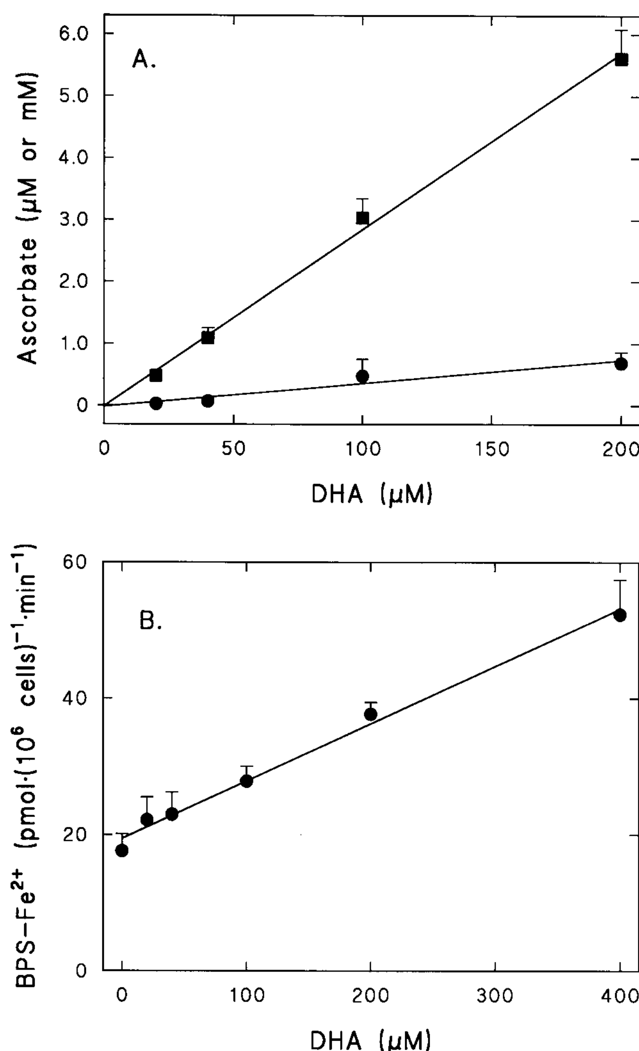

FIG. 3. Concentration-response study of DHA loading on intra- and extracellular ascorbate and on BPS-Fe^{2+} complex formation. *Panel A*: Cells were loaded for 15 min with the indicated concentration of DHA as described in the legend to Fig. 2, washed, and incubated for another 30 min at 37° in KRH that contained 5 mM D-glucose. An aliquot of the incubation buffer was sampled for assay of ascorbate, and the cells were washed twice in KRH and taken for assay of intracellular ascorbate. Data are shown as means ± SEM from 3 experiments, plotted as intracellular ascorbate in units of millimolar (■) or as extracellular ascorbate in units of micromolar (●). *Panel B*: BPS-Fe^{2+} complex formation was measured in the incubation buffer during the final 30 min of incubation under the same conditions as described for Panel A, except that 20 μM ferric citrate and 40 μM BPS were present. Data are from 4 experiments. In both panels, the data are fit to linear models as depicted by the solid lines.

it to ascorbate, as deduced from the concentration-dependent increase in the ascorbate content of the cells (Fig. 3A). Ascorbate was also released into the medium by the cells, as also shown in Fig. 3A. It should be emphasized that the intracellular ascorbate concentrations are expressed in units of millimolar in Fig. 3A, whereas those of ascorbate in the medium are expressed in units of micromolar. Intracellular ascorbate accumulation was a linear function of increases in the initial extracellular DHA concentration, reaching a concentration of about 6 mM at 200 μM initial extracellular DHA. These results show that the cells can reduce DHA to ascorbate, and that they lose small, but detectable, amounts of the reduced form into the medium over a 30-min period. As shown in Fig. 3B, the release of ascorbate into the medium was associated with a corresponding linear increase in the appearance of the BPS-Fe^{2+} complex when BPS and ferric citrate were present outside DHA-loaded cells. The effects of DHA loading on the kinetics of extracellular ferric iron reduction were evaluated further with the studies shown in Fig. 4. Incubation of U-937 cells with increasing amounts of ferric citrate resulted in a biphasic response (Panel A), in that the greatest relative accumulation of the complex occurred at concentrations of ferric citrate of 50 μM and below, with a nearly linear increase at higher concentrations. Pre-loading of cells with DHA increased the extent of complex formation at each ferric citrate concentration, but did not affect the shape or the half-maximal point of the curve. When fit to an hyperbolic model with residual, the apparent Michaelis–Menten parameters were: control, $K_m = 4 \pm 2$ μM, $V_{max} = 23 \pm 2$ pmol · (10^6 cells)$^{-1}$ · min^{-1}, residual = 0.06; and DHA-loaded, $K_m = 7 \pm 2$ μM, $V_{max} = 50 \pm 3$ pmol · (10^6 cells)$^{-1}$ · min^{-1}, residual = 0.05. Increasing amounts of BPS resulted in a plateau of the response that also was increased by pre-loading the cells with DHA, but again DHA pre-loading did not affect the half-maximal point of

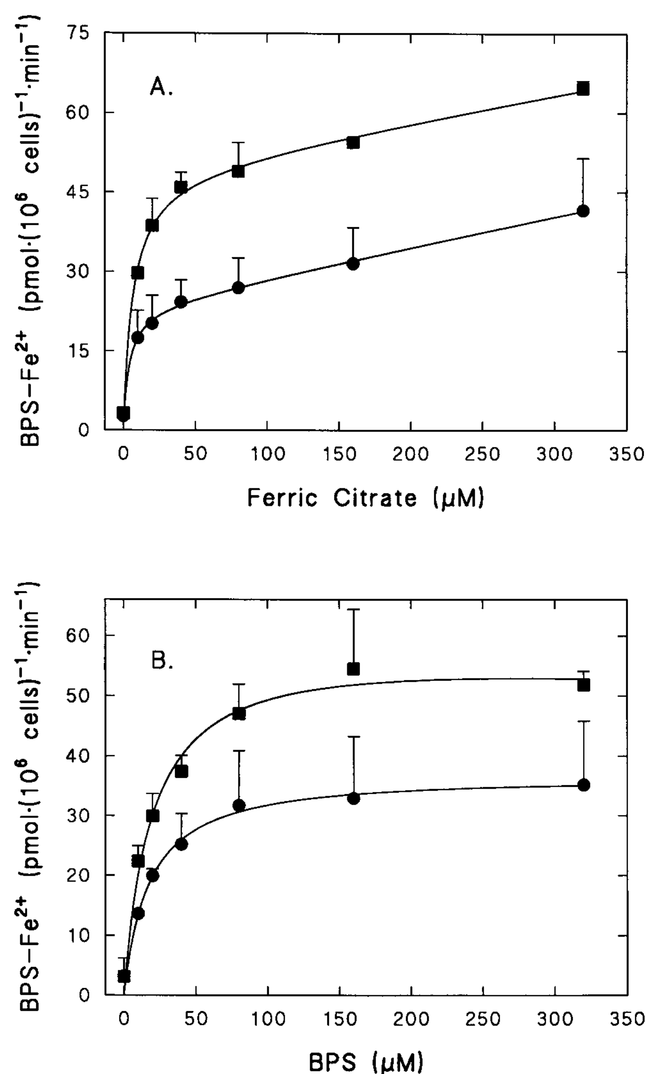

FIG. 4. Effects of DHA loading on the concentration-response curves for ferric citrate and BPS. Cells were incubated without (●) or with (■) 200 μM DHA as described in the legend to Fig. 2, washed, and suspended to 4 × 10^6 cells/mL for measurement of $BPS\text{-}Fe^{2+}$ complex formation. *Panel* A: response of $BPS\text{-}Fe^{2+}$ complex formation to increasing concentrations of ferric citrate at 80 μM BPS. *Panel B*: effects of increasing concentrations of BPS at 40 μM ferric citrate on complex formation. The results in both panels have been fit to an hyperbolic model with residual, as indicated by the solid lines. Results are shown for N = 2 as means ± range (Panel A, squares; Panel B, circles), and for N = 3 as means ± SEM (Panel A, circles; Panel B, squares).

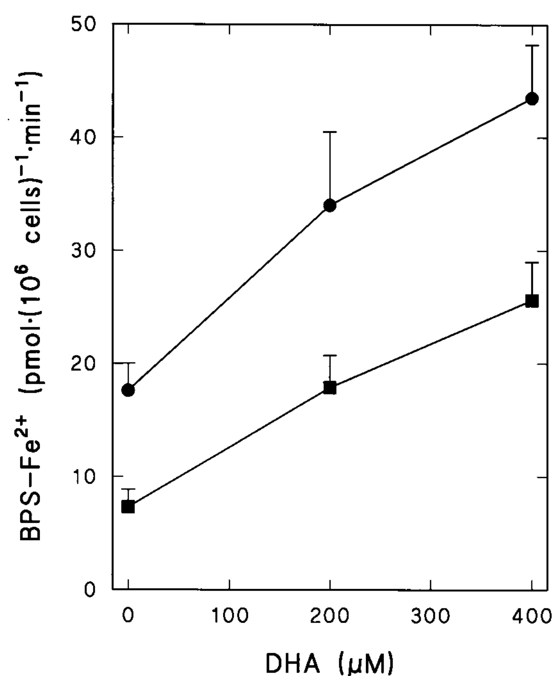

FIG. 5. Decrease of $BPS\text{-}Fe^{2+}$ complex formation by delay of BPS addition. Cells were loaded with the indicated concentration of DHA as described in the legend to Fig. 2, washed, and incubated at 37° in KRH that contained 5 mM D-glucose, 40 μM ferric citrate, and either no BPS (■) or 80 μM BPS (●). After 30 min, 80 μM BPS was added to the BPS-free samples, and the incubation was continued for another 5 min before determination of $BPS\text{-}Fe^{2+}$ complex formation. Data are shown as means ± SEM from 3 experiments.

the curve (Fig. 4B). Apparent Michaelis–Menten parameters for these data were: control, K_m = 18 ± 5 μM, V_{max} = 38 ± 4 pmol · $(10^6\ cells)^{-1}$ · min^{-1}, no residual; and DHA-loaded, K_m = 20 ± 5 μM, V_{max} = 61 ± 7 pmol · $(10^6\ cells)^{-1}$ · min^{-1}, no residual. Failure to have BPS present during the entire incubation with ferric citrate lowered the amount of $BPS\text{-}Fe^{2+}$ complex formed, regardless of whether the cells had been pre-loaded with DHA (Fig. 5). This finding could indicate oxidation of ferrous iron, or more likely, based on the ability of the cells to take up ^{55}Fe (Fig. 1), it reflects uptake of ferrous iron by the cells during the period when BPS was not present. Together, these results suggest that extracellular reduction of ferric citrate is saturable, that DHA loading increases the maximal rate achieved, and that BPS detects ferrous iron that can be taken up by the cells.

The effects of DHA loading on $BPS\text{-}Fe^{2+}$ complex formation contrast with the ability of the cells to reduce extracellular ferricyanide. As shown in Table 1, U-937 cells reduced ferricyanide at a slightly greater basal rate than was

TABLE 1. Reduction of ferric iron and ferricyanide by U-937 cells

Treatment	$BPS\text{-}Fe^{2+}$ formation	Ferricyanide reduction
	pmol · $(10^6\ cells)^{-1}$ · min^{-1}	
Control	13 ± 2*	23 ± 5
DHA	26 ± 3	92 ± 10†
DHA + AO	16 ± 1*	94 ± 9†

Cells (4 × 10^6/mL) were incubated for 15 min at 37° in the presence or absence of 200 μM DHA in KRH buffer, washed 3 times by centrifugation, and suspended to the same cell concentration for assay of $BPS\text{-}Fe^{2+}$ complex formation or ferricyanide reduction. Incubations were carried out for an additional 30 min at 37° in KRH that contained 5 mM D-glucose, and, where indicated, 4 U/mL ascorbate oxidase (AO). Data are shown as means ± SEM from 6 experiments for $BPS\text{-}Fe^{2+}$ complex formation in the presence of 40 μM ferric citrate and 80 μM BPS, and 4 experiments for ferricyanide reduction.

*$P < 0.05$ compared to incubation with DHA alone.

†$P < 0.05$ compared to the control incubation.

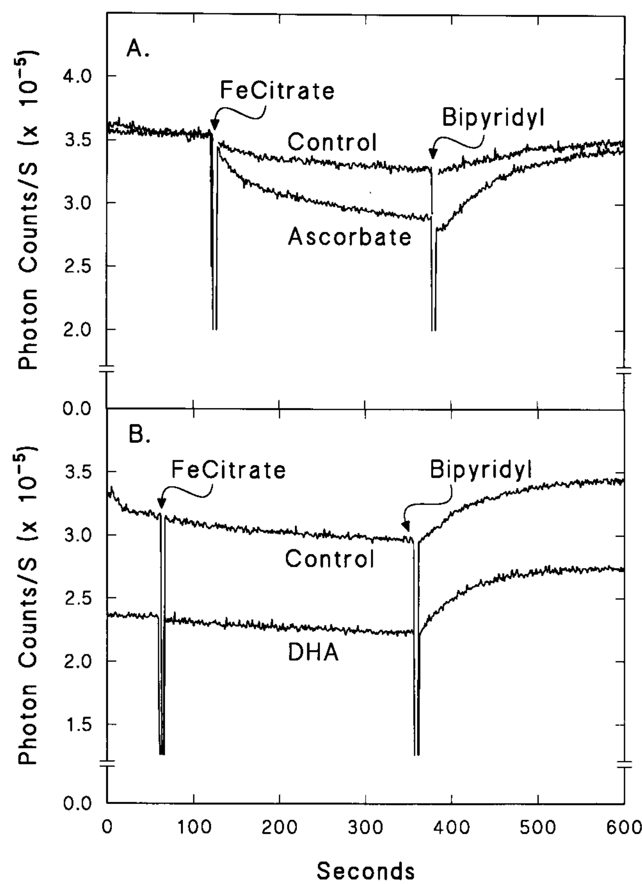

FIG. 6. Effects of ascorbate and DHA on intracellular calcein fluorescence. Cells that had been loaded with the acetoxymethyl ester of calcein were incubated as described in Materials and Methods. Tracings are shown from an experiment in which samples were incubated for 10 min in the dark at 37° without additions, or with either 200 μM ascorbate (Panel A) or 200 μM DHA plus 4 U/mL ascorbate oxidase (Panel B). Additions of 50 μM ferric citrate and 50 μM 2,2′-bipyridyl were made where noted. Data from a representative experiment of four that were performed are normalized to the fluorescence of each sample just before the addition of ferric citrate.

observed for BPS-Fe^{2+} complex formation. In contrast to the doubling of BPS-Fe^{2+} complex formation, ferricyanide reduction was stimulated 4-fold by DHA pre-loading of the cells. As expected from the studies in Figs. 1 and 2, ascorbate oxidase completely blocked the DHA-dependent stimulation of the BPS-Fe^{2+} complex formation. However, it had no effect on the ability of the cells to reduce extracellular ferricyanide. This suggests that ferrous iron generation and ferricyanide reduction occur by different mechanisms.

Although intracellular ascorbate does not affect the extracellular reduction and uptake of ferric iron, it could affect the redox status of intracellular iron, especially during uptake. Therefore, the effects of extra- and intracellular ascorbate on intracellular iron during its uptake were evaluated (Fig. 6). The labile pool of intracellular iron was assessed using calcein, which has been shown to lose fluorescence intensity on chelation of intracellular ferrous iron [21–23]. The fluorescence of cells that had been loaded with calcein-AM was followed to establish a steady-state level of intracellular fluorescence. Baseline fluorescence levels varied with different samples, probably due to differences in cell stirring or in the position of the cuvette in the chamber. Neither ascorbate nor DHA had consistent effects on the baseline level or slope before ferric citrate addition. Upon addition of ferric citrate, a curvilinear decrease in calcein fluorescence was observed, which was reversed when the cell-penetrant chelator of ferrous iron, 2,2′-bipyridyl, was added. As is evident in Fig. 6, the extent of reversal by 2,2′-bipyridyl was variable, but usually approximated the initial baseline. When 200 μM ascorbate was added to the cells for 10 min before the addition of ferric citrate, the initial decrease in calcein fluorescence following the addition of ferric citrate was more rapid than in the control sample (Fig. 6A). Further, addition of 2,2′-bipyridyl reversed the decrease in fluorescence in both control and ascorbate-treated cells (Fig. 6A). Exposure of cells to 200 μM DHA in the presence of ascorbate oxidase, to increase only the intracellular ascorbate content, resulted in fluorescence changes that were no different than control (Fig. 6B). Although the ascorbate content of the cells was not measured in these experiments, consideration of the 15-min time course of Fig. 3A suggests that the intracellular ascorbate concentration at the end of this 14–16 min incubation with 200 μM DHA would be in the range of 4–6 mM. Whereas extracellular ascorbate increased the cell content of ferrous iron that could be chelated by calcein and 2,2′-bipyridyl, an increase in intracellular ascorbate in response to DHA loading had no effect on the amount of intracellular ferrous iron that could be detected by this method.

DISCUSSION

Despite the fact that intracellular ascorbate enhances ferricyanide reduction in U-937 cells, the ascorbate content of the cells had little effect on uptake of ^{55}Fe from ferric citrate, on extracellular reduction of ferric citrate, or on the intracellular labile pool of ferrous iron. Nevertheless, cellular release of the reduced form of the vitamin enhanced both ferric iron reduction, as determined by BPS-Fe^{2+} complex formation, and cellular uptake of ^{55}Fe. The ^{55}Fe uptake results in suspension-cultured U-937 cells were similar to those observed previously by Han *et al.* [6] in Caco-2 cells, a cultured cell model for intestinal iron uptake. Under the conditions used in the present studies, the measured extracellular ascorbate concentrations were less than 1 μM in the face of intracellular concentrations that were as high as 6 mM. The addition of much higher concentrations of ascorbate directly to cells resulted in no further stimulation of ^{55}Fe uptake (Fig. 1), which may indicate that iron transport rather than reduction limits the uptake process under these conditions.

Since the plasma ascorbate concentration is 20–60 μM in unsupplemented individuals [24], there appears to be

more than enough ascorbate to reduce any ferric iron to which it has access. As pointed out by Buettner and Jurkiewicz [25], the combination of ascorbate and transition metals is to be avoided, since the ferrous iron that results from ascorbate-dependent reduction of ferric iron can react with molecular oxygen to produce superoxide, H_2O_2, and eventually the damaging hydroxyl radical. On the other hand, it has been shown recently that even in iron-overloaded plasma that contains appreciable amounts of free iron, ascorbate continues to act as an antioxidant and protects against lipid peroxidation until its concentration falls to less than 10% of that present initially [26]. Moreover, if such potentially toxic ferric iron reduction occurs in the presence of cells, the resulting ferrous iron will be taken up quickly by the cells and removed from potential redox cycling with molecular oxygen and ascorbate. In this manner, ascorbate released locally from cells could remove extracellular free iron by facilitating its cellular uptake.

The present results suggest that the cell-surface ferric iron reductase activity presumed to be responsible for reduction of extracellular iron is distinct from the ubiquitous trans-plasma membrane ferricyanide reductase activity. Although it is clear that ferricyanide inhibits radioactive iron uptake [6, 8], such inhibition could occur either by competition for reducing substrate at the cell-surface ferric iron reductase or by depletion of intracellular reducing equivalents. Support for the latter mechanism derives from studies in which ferricyanide was found to cause NADH depletion in HeLa cells [11] and ascorbate depletion in human erythrocytes [17]. The ability of extracellular ascorbate oxidase to block ferric citrate reduction by DHA-loaded U-937 cells, but not ferricyanide reduction, provides support for the notion that the two reductase activities have different mechanisms. That is, ferric iron reduction requires direct reaction with ascorbate, whereas ferricyanide reduction can use electrons derived from intracellular ascorbate or NADH.

As in human erythrocytes [14, 15], when ascorbate is present within U-937 cells, their ability to reduce ferricyanide increases 3- to 4-fold over basal. Monocytes freshly prepared from blood have ascorbate concentrations of about 6 mM [27], which is in the range achieved by DHA loading in this study (Fig. 3A). Therefore, ascorbate may be the major natural substrate for the transmembrane ferricyanide reductase activity in these cells, as well as in human erythrocytes [15]. The authors of a recent study suggest further, on the basis of differential sensitivity to inhibition by *p*-chloromercuribenzene sulfonic acid, that basal and ascorbate-dependent ferricyanide reductase activities are mediated by different enzymes [28]. Our results do not rule out the possibility that an ascorbate-independent ferricyanide reductase activity contributes to extracellular ferric iron reduction. They do indicate that reduction of extracellular iron by ascorbate is direct and not mediated by trans-plasma membrane electron transfer.

It has been shown that the intracellular pool of labile iron measured by chelation with calcein is increased by treatment of K562 cells with hydroperoxides or with β-mercaptoethanol [22]. Further, the presence of an oxidant stress induced by *tert*-butyl hydroperoxide during treatment of K562 cells with transferrin increases both the labile intracellular iron pool and the susceptibility of the cells to lipid peroxidative damage [29]. Ascorbate can reduce ferric iron bound to the iron storage protein ferritin [30], and this reduction has been associated *in vitro* with lipid peroxidation in LDL [31]. These considerations suggest that intracellular ascorbate could generate damaging oxygen free radical species by redox cycling catalyzed by iron. On the other hand, there was no indication from the present studies that increased amounts of intracellular ascorbate in DHA-loaded cells affected the labile pool of intracellular iron that can be chelated by calcein or bipyridyl. Although extracellular ascorbate increased this pool by enhancing iron uptake from ferric citrate, a similar effect was not observed for intracellular ascorbate. This suggests that, at least in U-937 cells, ascorbate does not reduce ferric iron in amounts that can be detected by the calcein method. Even in erythrocytes, which contain substantial amounts of iron in hemoglobin, we found no evidence for generation of intracellular H_2O_2 by increases in intracellular ascorbate following DHA loading [32]. In those studies, extracellular ascorbate did enhance H_2O_2 generation that could be detected as catalase inhibition in the presence of 3-amino-1,2,4-triazole. However, the latter effect was shown to be due to interaction of ascorbate with traces of Fe^{3+} in the medium. It may be that ascorbate has little access to the labile pool of intracellular iron, or that most iron in U-937 cells is already in the reduced form. For other cells that contain substantial amounts of ferritin, this may not be the case.

In conclusion, extracellular ascorbate enhances uptake of ferric iron by directly reducing it to ferrous iron. Intracellular ascorbate, although a substrate for a trans-plasma membrane ferricyanide reductase activity, does not provide electrons for reduction of extracellular ferric iron.

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