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The role of endocytic pathways in cellular uptake of plasma non-transferrin iron

Running title: Mechanism of iron overload by native plasma NTBI

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Abstract

Background. In transfusional siderosis, plasma transferrin’s iron binding capacity is often surpassed, with concomitant generation of non-transferrin-bound iron. Although implicated in tissue siderosis, non-transferrin-bound iron modes of cell ingress remain undefined, largely because of its variable composition and association with macromolecules. Using fluorescent tracing of labile iron in endosomal vesicles and cytosol, we examined the hypothesis that non-transferrin-bound iron fractions detected in iron overloaded patients enter cells via bulk endocytosis.

Design and methods. Fluorescence microscopy and flow cytometry served as analytical tools for tracing non-transferrin-bound iron entry into endosomes with the redox-reactive macromolecular probe Oxyburst-Green and into the cytosol with cell-laden calcein green and calcein blue. Non-transferrin-bound iron-containing media were from sera of polytransfused thalassemia major patients and model iron substances detected in thalassemia major sera; cell models were cultured macrophages, and cardiac myoblasts and myocytes.

Results. Exposure of cells to ferric citrate together with albumin, or to non-transferrin-bound iron-containing sera from thalassemia major patients caused an increase in labile iron content of endosomes and cytosol in macrophages and cardiac cells. This increase was more striking in macrophages, but in both cell types was largely reduced by co-exposure to non-transferrin-bound iron-containing media with non-penetrating iron chelators or apo-transferrin, or by treatment with inhibitors of endocytosis. Endosomal iron accumulation traced with calcein-green was proportional to input non-transferrin-bound iron levels ($r^2=0.61$) and also preventable by pre-chelation.

Conclusions. Our studies indicate that macromolecule-associated non-transferrin-bound iron can initially gain access into various cells via endocytic pathways, followed by iron translocation to the cytosol. Endocytic uptake of plasma non-transferrin-bound iron is a possible mechanism that can contribute to iron loading of cell types engaged in bulk/adsorptive endocytosis, highlighting the importance of its prevention by iron chelation.
Introduction

The occurrence of iron forms in the plasma that are not tightly bound to transferrin has been described for various iron overload disorders. These forms, commonly referred to as non-transferrin bound iron or NTBI, appear in two pathological scenarios: a. when plasma transferrin binding capacity for iron is exceeded due to an outpouring of iron into plasma or b. when the iron transferred to plasma fails to be catalytically incorporated into transferrin due to aceruloplasminemia or atransferrinemia. Persistently high plasma NTBI levels can lead to uncontrolled ingress of labile iron into cells and ensuing tissue damage in organs such as liver, endocrine glands and heart over time. These properties have led NTBI to be considered an indicator of impending tissue iron overload and a target of chelation therapy. However, the identification of membrane-permeant iron species in plasma NTBI and their routes of ingress into particular cells have not been established, largely because of the heterogenic nature of NTBI itself and its variable composition in different pathologies. In systemic iron overload, NTBI has been claimed to be associated with various plasma components such as organic acids like citrate, phosphates and proteins. The results of such associations are heterogeneous mixtures of chemical composition that varies with the source and flux of incoming iron, the previous treatment of patients by chelation or phlebotomy or conversely, varies with the frequency of blood transfusions or parenteral iron administration.

Previous attempts to experimentally define the pathways of NTBI entry into various cell types have comprised: a. the application of iron(III) complexed to various organic acids as model permeant substrates; b. the supplementation of agents capable of reducing ferric complexes so as to maintain it in the ferrous state, which is presumed to be the only ionic permeant form of the metal and c. the avoidance of incorporating proteins in the transport assays, as those might reduce the chemical activity of iron or its salts and thereby prevent their cell uptake. This contrasts with the fact that in thalassemic patients most of the plasma NTBI is excluded by size filtration and that its chemical determination necessitates harsh extraction measures. This would indicate that in thalassemic plasma in general and, as we found in this study in plasma from non-chelated patients, the chemically active forms of NTBI consist of high-molecular weight complexes, which may be protein-associated. We predict that for macromolecular forms to gain access to cells, they would need to be transferred initially as fluid cargo or membrane adsorbed species that are taken up by endocytosis and subsequently by transmembrane mechanisms into the cytosol. With the view of assessing the initial steps of ingress of plasma NTBI into model cells, we used as NTBI substrate, the original sera from thalassemia major (thalassemia major) patients who were only sporadically treated with iron chelators, so that levels of Fe-chelates in circulation...
are negligible. As model NTBI forms, we used whole human serum or only its albumin (HSA) fraction supplemented with iron-citrate or other iron sources. The ingress of iron into cell compartments was followed by time-lapse fluorescence imaging tracing in two formats: 1. in endosomes by following the rise in fluorescence of Oxyburst-green, an endosomal macromolecular ROS indicator, and particularly its sensitivity to iron chelators; 2. in cytosol, by following the quenching of the fluorescent metalosensors calcein-green (CALG) or calcein-blue (CALB) and the reversal by permeating iron chelators as iron identification tools. We used both confocal and epifluorescence microscopy to image cell fluorescence and when applicable also flow cytometry on suspended cells. We used cell lines of macrophages, endocrine glands and heart, as those are major cells types that accumulate iron in polytransfused patients. Specific blockers of endocytosis were used as experimental tools for probing the involvement of endocytic steps in NTBI cell loading and iron chelators for associating fluorescence changes with labile iron.

**Design and methods**

**Materials:** Oxyburst-green (OBG =albumin conjugated dichlorodihydrofluorescein), calcein green (CALG= 3,3‘-bis[N,N-bis(Carboxymethyl) aminomethyl] fluorescein), calcein blue (CALB= methylumbellifereone-8-methyleneiminodiacetic acid) and their respective acetomethoxy (AM) precursors CALG-AM and CALB-AM were from Molecular Probes (Invitrogen Corp., Carlsbad, CA); SIH (salicyl isonicotinoyl hydrazine) was a gift from Dr P. Ponka (Montreal), the iron-saccharate polymer Venofer™ was from Vifor International. St Gallen, Switzerland. F-DFO (N-(fluorescein-5-thiocarbamoyl) desferrioxamine from Molecular Probes (Eugene, OR, USA), Deferrioxamine (DFO) was from Novartis-Pharma (Basel). Hydroxyethyl-starch-DFO, M.W. ~ 10 kDa (HES-DFO) was from Biomedical Frontiers Inc, Minneapolis MN. Apo-transferrin (aTf) was from Kamada Ltd. (Kibbutz Kama, Israel). Verapamil, Nifedipine, Rhodamine Dextran (R-D) (MW 10 kDa), Wortmannin, W7(N-(6-Aminohexyl)-5-chloro1-naphthalenesulfonamide hydrochloride), Chloroquine, nocodazole (N) and ML9 (M), cytocholasin D (cyD) and human serum albumin (Fraction V) (HSA) were from Sigma Chemical Co. (St Louis, MO).

**Cell culture:** RAW264.7 mouse macrophage cells and the RAW264.7 macrophage cell lines stably transfected with a functional NRAMP1 (RAW 37) or NRAMP1 antisense variant (RAW 21) provided generously by Dr. H. Barton, University of Southampton, U.K were grown in 5% CO₂ Dulbecco’s modified Eagle’s (DMEM) medium supplemented with 10% fetal calf serum, 4.5g/l D-glucose, glutamine and antibiotics (Biological Industries, Kibbutz Bet Haemek, Israel). For experimentation, cells were plated onto 12-well plates, or onto microscopic slides glued onto perforated 3-cm diameter tissue culture plates. The plates were analyzed
by confocal microscopy with a FV-1000 confocal microscope (Olympus, Japan) equipped with an IX81 inverted objective and placed in a thermostated CO₂ environmental chamber, or by a Nikon TE 2000 microscope equipped with optigrid and autofocus systems and a Hamamatsu Orca-Era CCD camera. The system was operated with a Velocity 4 operating system (Improvision, Coventry, UK) that was used for both image data acquisition and analysis, aided also with NIH Image J program.¹³,¹⁴

RAW cells and H9c2 cultured cardiomyoblasts were grown as previously described.¹³,¹⁴ Rat INS1 cells clone 832/13 (generously provided by Dr. H.E. Hohmeier, Duke University, NC, USA) derived from rat pancreas were stably transfected with the human proinsulin gene. The cells were grown in RPMI containing 10% fetal bovine serum, 10mM Hepes, 1mM Sodium Pyruvate and 50 µM β-Mercaptoethanol. Flow cytometric analysis was performed with an automated Eclipse instrument (iCyt, Champaign, IL, USA) on H9c2 cardiomyoblasts after trypsinization, as described elsewhere for other cells.¹³⁻¹⁵ We also used cultured HL-1 cardiac muscle cells derived from the AT-1 mouse atrial cardiomyocyte tumor lineage which continuously divide and spontaneously contract while maintaining a differentiated cardiac phenotype (generously provided by Dr. W.C. Claycomb, LSU, New Orleans, USA).¹⁶

**Measurement of DCI (Directly chelatable Iron) in serum samples.**

The concentration of DCI in the sera of patients was determined as described previously.¹⁷,¹⁸ The assay is based on the binding of NTBI in serum to fluorescein-DFO (Fl-DFO), causing quenching of its fluorescence. Briefly, each serum sample is measured under two separate conditions: A, with Fl-DFO only, and B, as in condition A, but in the presence of a large excess of non-fluorescent DFO. This ensures that the change in fluorescence is due to the binding of iron to Fl-DFO rather than due to other, unknown factors in the sample. The concentration of DCI is calculated using iron calibration solutions, from the difference between fluorescence under conditions A and B divided by the maximal fluorescence of the sample (under condition B). As there is a strong positive correlation between DCI and NTBI,¹⁷,¹⁸ sera were defined as NTBI-positive or negative if their DCI values were above or below 0.4 µM respectively. The fact that inclusion of nitrilotriacetic acid in the DCI assay did not reveal additional DFO chelatable material was taken as indication that the DCI values represented most of the NTBI fraction.¹⁸

**Human sera samples.** The sera samples used in this study were primarily from thalassemia major (thalassemia major) adolescents (age 14-21) living in the Gaza area, who were regularly transfused but only sporadically chelated and did not undergo any chelation for at least 6 months prior to the present study. The study, approved by the Helsinki ethics committee of the European Hospital in Gaza, was conducted on patients who provided
written informed consent. All sera were initially tested for iron related parameters serum ferritin and transferrin saturation and for non-transferrin bound iron (NTBI) measured as directly chelatable iron (DCI).\textsuperscript{18} Sera with DCI >1.2 µM were used as source of high NTBI (hNTBI) and those with values <0.4 µM as low NTBI (lNTBI). Model NTBI-containing sera were prepared by supplementing 20 µM ferric citrate to human sera that had originally lower than 70% transferrin saturation and thereby attained NTBI levels of 10-15 µM (measured as DCI, see below). All human sera were applied to cells in culture at 30% concentrations in DMEM media.

\textit{Measurement of labile iron in endosomes.} Cells were incubated for up to 90 min in growth media containing the Oxyburst green probe (Oxyburst-green) (40 µg/ml) and either 30% human serum (with or without NTBI) or human serum albumin (40 µM) supplemented (or not) with 30 µM ferric citrate. After the incubation, cells were washed and bathed in DMEM-HEPES medium and subsequently reacted with H\textsubscript{2}O\textsubscript{2} (50 µM) for 10 min at 37°C.

\textit{Treatment of cells with different iron containing media.} RAW264.7 cells were perfused with DMEM-HEPES (20 mM pH 7.4) medium containing either 30% human sera, or human serum albumin (50 µM) or iron-saccharate (Venofer) (500 µM), generally for up to 3 hours at 37°C and subsequently washed with DMEM-HEPES alone. In some studies, CALG (5-30 µM) was added in order to trace iron within cells by addition of the permeant iron chelator SIH (50 µM), which reveals all Fe quenched complexes. The sera used were from: (a) normal individuals (N); (b) from thalassemia patients (thalassemia major) with high NTBI (hNTBI) and (c) from patients with low NTBI (lNTBI) but rendered hNTBI by incubating them with 10 µM ferric citrate (FC).

\textit{Measurement of cytosolic CALG or CALB fluorescence in cells exposed to NTBI-containing media}
Cells which were pre-exposed (up to 3 hrs) to medium containing 30% sera with high NTBI (hNTBI) or low NTBI (lNTBI), or human serum albumin (HSA) supplemented or not with ferric citrate, were cytosolically loaded with CALG via 1 min incubation at 37°C with CALG-AM (1 µM) in DMEM-HEPES medium or with CALB via CALB-AM (10 µM at 37°C for 10 min). Cells were subsequently washed with HEPES-buffered saline (HBS= 130 mM NaCl, 20 mM Hepes, pH 7.4) and bathed at 37°C in DMEM-HEPES containing 0.5 mM probenecid (to minimize probe leakage).\textsuperscript{13,19,20} For assessing fluorescence properties the cells were analyzed either microscopically or by flow cytometry (following release by trypsinization). Epi-fluorescence microscopy analysis of CALG was done using EXC: 488nm and Em: 520 nm and for CALB Exc: 390 nm and Em: 430 nm.
Results

NTBI uptake into cells as revealed with cytosolic iron markers
The mechanism of plasma NTBI uptake by cells depends both on the chemical nature of the substrate, the composition of the medium and the cell in question. The canonical mode of assessing transport of metals or molecules into cells is by tracing substrate ingress into the cytosol. For tracing ingress of labile iron into the cytosol we used CALG, a green fluorescent metal sensor that is loaded into cells via its CALG-AM precursor.\textsuperscript{13,20} The probe undergoes swift and stoichiometric quenching by interacting with labile iron and recovers its fluorescence when challenged with iron chelators. Using CALG-laden RAW macrophages and fluorescence microscopy live imaging, we found that ferric compounds like ferric citrate, considered a major component of NTBI in iron overloaded sera,\textsuperscript{11,12} failed to evoke changes in cytosol fluorescence over a period of 90 minutes, unless the medium was supplemented with human serum albumin (HSA) (Fig. 1 shows data for only two time points: 0 and 40 min). The degree of CALG quenching in the cytosol that resulted from exposure of cells to various types of iron-containing media is given as bar graph in Fig. 1 (lower right panel), indicating several features: 1. The change in fluorescence evoked by ferric citrate applied together with HSA was iron related, as addition of the impermeant iron chelator hydroxyethyl-starch-DFO to the medium, essentially abrogated the change. Similarly, HSA alone led to a lower but significant cytosolic quenching that was related to contaminating iron, as hydroxyethyl-starch-DFO also abrogated it; 2. The observation that quenching evoked by both ferric citrate + HSA and HSA alone was markedly reduced when cells were pretreated with blockers of endocytosis, nocodazole + ML9 or cytocholasin D, strongly suggested a cellular endocytic route in the process of iron delivery to cytosol from a surrogate NTBI source. The enhancing effect of HSA on endocytic uptake of iron can be interpreted in terms of the observations of Evans et al,\textsuperscript{12} showing that while ferric citrate consists of low-molecular weight forms (< 30 kD) in the absence of HSA, it acquires high-molecular weight properties (> 30 kD) immediately after addition of HSA.

NTBI uptake into cells as revealed with endosomal iron markers
In order to assess if iron derived from native plasma/serum NTBI was initially taken up into the endosomal compartment, we supplemented NTBI-containing media with the albumin-tagged Oxyburst-green probe which is endocytosed and whose ability to fluoresce upon addition of H\textsubscript{2}O\textsubscript{2} can be attributed to labile iron due to its sensitivity to iron chelators. RAW cells exposed to media containing Oxyburst-green showed a punctuated fluorescence when the medium contained ferric citrate + HSA but not ferric citrate alone. Pictures of cells exposed only to either ferric citrate or serum or HSA were very similar and are indicated in the figure as control. Importantly, the endosome associated fluorescence is attributable to
endosomal labile iron, as addition of a permeant chelator (SIH) after exposure to iron-containing media, but prior to hydrogen peroxide, abrogated the rise in fluorescence. More importantly, Oxyburst-green supplemented to sera from thalassemia major patients showed similar properties as those of HSA + ferric citrate while hydroxyethyl-starch-DFO or apo-transferrin abrogated the fluorescence changes. All this information links labile iron to the fluorescence changes occurring within endosomes and traces its origin to the NTBI-containing medium, either associated with native thalassemia major serum or with the artificially formulated NTBI in the form of HSA + ferric citrate.

A similar approach of monitoring iron ingress into cytosol following exposure to NTBI-containing medium was applied to H9c2 cardiomyoblasts and to HL-1 contractile cardiomyocytes. The cardiac cells are markedly less active in endocytosis than the RAW macrophages and accordingly, the follow up of NTBI uptake by cardiomyoblasts and cardiomyocytes demanded extended exposure of cells to NTBI-containing media so as to enable fluorescence detection of iron-dependent signals. NTBI-evoked rise in fluorescence was observed a. in endosomes using Oxyburst-green in conjunction with fluorescence microscopy (Supplement: Figs. S1 and S2) and b. in cytosol using CALG in conjunction with fluorescence microscopy (Supplement Fig. 3) or flow cytometry following release of attached cells by trypsinization (Supplement Fig. S4). The analysis revealed qualitatively similar features of NTBI uptake into endosomes (Supplement Figs. S1 & S2) and to cytosol (Supplement Figs. S3 & S4) of cardiomyoblasts and cardiomyocytes as compared to RAW macrophage cells (Figs. 1 & 2), particularly their susceptibility to inhibitors of endocytosis and/or chelating agents.

**NTBI uptake into cells as revealed with a fluorescent membrane impermeant iron marker**

The implication of the above results is that initial stages of cellular uptake of NTBI occur via endocytosis of whole complexes of iron together with its presumed ligands (citrate and albumin). In order to assess this possibility by an alternative means, we sought to generate a complex simulating NTBI by use of an iron-responsive fluorescent probe with iron-binding moieties similar to those of citrate. In this approach, ferric citrate is replaced with ferric-calcein green (CALG-Fe), a fluorescence-quenched NTBI surrogate whose metal-quenched fluorescence can be revealed in cell compartments by addition of permeant chelators such as SIH. We initially explored the possibility that CALG added to sera might show different fluorescence responses depending on the presence or absence of NTBI. We used for that purpose two different cell lines representative of transfusional siderosis, macrophages and cardiomyoblasts and assessed uptake of the fluorescent (partially quenched by the
complexed iron) NTBI simulator CALG-Fe into the endosomal compartment (Fig. 3). Macrophages showed prominent acquisition of endosomal fluorescence following exposure to NTBI-containing media supplemented with CALG, possibly related to their high constitutive endocytic activity. A similar property was observed in macrophages when using polymeric forms of NTBI such as the iron-saccharate Venofer (Sohn et al unpublished observations) that is administered iv in various treatment regimens of iron deficiencies.

In both, macrophages and cardiomyoblasts, we found that the endosomal fluorescence rose following addition of the permeant chelator SIH, indicating the presence of chelatable iron. Moreover, similarly to RAW cells exposed to serum NTBI-containing medium and probed with Oxyburst-green, the H9c2 cardiomyoblasts also showed uptake of NTBI into a cell compartment shared with the fluid-endocytosis marker rhodamine-dextran (R-D).

To confirm that the endosome-associated fluorescence originating from CALG-Fe, as observed in HSA-containing medium (Fig. 3), is also demonstrable with genuine sera from thalassemia major patients containing NTBI, we incubated RAW cells with NTBI-positive sera from thalassemia major patients supplemented with: a. the red fluorescent R-D as marker of fluid endocytosis and b. CALG as in situ green fluorescent marker of native plasma NTBI (Fig. 4).

We noted that both markers applied separately or together yielded similar cell patterns of green and of red fluorescence associated with the endosomal compartment, as highlighted in Fig. 4 (right column). When both probes were applied on the same cultures, the calculated degree of probe co-localization (Fig. 4, bottom panel) was 70-75% indicating similar uptake features of both markers. As both cell fluorescence signals were: a. markedly intensified when the thalassemia major sera used were from patients with high NTBI (hNTBI) and b. green but not red fluorescence was reduced when sera were pretreated with DFO (Sohn and Cabantchik, unpublished observations), we also deduced that sera from thalassemia major patients has most likely components that might promote endocytosis beyond what is observed with HSA alone.

**NTBI uptake and the status of iron in thalassemia major sera as assessed with CALG.**

Initial studies on the interaction of CALG with iron overloaded sera revealed that a substantial fraction of the fluorescent probe CALG becomes adsorbed to serum components (Supplement: Fig. S4). Incubation of 6 different thalassemia major sera with 20 μM CALG followed by ultrafiltration on 30 kD cutoff filters showed that 69% of CALG fluorescence was associated with the filter-retained, high molecular weight fraction, compared to 7% in the absence of serum. In addition, when CALG was added to thalassemia major sera it also underwent fluorescence quenching (and dequenching following addition of excess chelators such as SIH), in a manner roughly proportional to the NTBI content of the sera (Sohn et al,
unpublished observations). Thus, CALG appears to bind labile iron in thalassemia major sera, similarly to labile iron in solution and concomitantly becomes associated with serum proteins, similarly to ferric citrate. Ultrafiltration experiments of CALG incubated with purified human serum albumin showed similar CALG-binding properties as whole serum (Supplement: Fig. S4), indicating that albumin is the probable CALG-binding serum component. These observations, together with the fact that most of the NTBI fraction was not filterable (with or without CALG), led us to the assumption that exposure of cells to sera from thalassemia major patients doped with CALG might provide some clues as to the fate of NTBI uptake into cells.

We found that endocytic CALG uptake into cells was saturable and did not increase further at concentrations ≥ 10 µM probe for a particular hNTBI serum (Fig. 5A). However, we noticed (Fig. 3A inset) that the fraction revealed by SIH, namely CALG-Fe, was essentially independent of CALG concentration used in the range 5-50 µM.

In order to assess to what extent CALG fluorescence associated with endosomes depended on plasma NTBI, we compared the fluorescence intensity of cells exposed to sera with high NTBI and low NTBI following their pre-treatment with deferrioxamine (DFO) or its macromolecular conjugate hydroxyethyl-starch-DFO. As shown in Fig. 5B, pretreatment with these impermeant chelators markedly (about 50%) reduced CALG uptake and virtually eliminated the NTBI component revealed with SIH. We interpret these observations to indicate that the fluorescence associated with cells originated from both protein-associated CALG (DFO-insensitive fluorescence uptake) as well as CALG-Fe complexes formed in hNTBI thalassemia major serum that were taken up by cells in the 3 h incubation period. Conceivably, some of the CALG-Fe complexes dissociated in the endosomal compartment, so that SIH revealed only a residual fraction of CALG-Fe.

Finally, as CALG-Fe complexes are essentially dissociable, we considered the possibility that during the incubation of cells with CALG-supplemented thalassemia major sera, a fraction of the endosomal CALG-Fe undergoes dissociation followed by Fe translocation into the cytosol via NRAMP1 and/or 2 (DMT1) the two principal iron transporters in endocytic vesicles. That possibility was examined in two sublines of RAW cells over-expressing functional NRAMP1 (RAW 37) or non-functional anti-sense NRAMP1 (RAW 21). RAW 37 cells exposed to high NTBI sera showed higher levels of endosomal CALG than either WT or RAW 21 cells following 3 hs incubation with CALG supplemented thalassemia major sera (Fig. 6A). However, the fluorescence increase exerted by SIH, which reveals endosomal CALG-Fe complexes, showed higher values in WT and RAW 21 relative to RAW 37 cells. We tentatively attribute that to the egress of Fe dissociated from endosomal CALG-Fe complexes into the cytosol of NRAMP1-positive subline 37. As shown in Fig 5B (kinetics) and Fig. 6 (C & D, images), there was a time-dependent increase in endosomal CALG
fluorescence concomitant with a time dependent decrease in cytosol CALB fluorescence, indicating translocation of Fe from endosomes into cytosol, particularly in the NRAMP1 expressing subline 37.

**Discussion**

The appearance of plasma NTBI in systemic iron overload caused by iron hyperabsorption or blood hypertransfusions is a phenomenon that has pathophysiological, diagnostic and therapeutic implications.\(^3,22\) The pathological accumulation of iron in multiple tissues observed in iron overload conditions is thought to be caused by excessive influx of iron into plasma as well as unbalanced erythrophagocytosis by reticuloendothelial cells in spleen and liver that lead to persistently high circulating NTBI levels, which are responsible for the systemic iron dispersal. The hypothesis that tissue iron overload is caused by NTBI is largely based on the assumption that NTBI is transported into cells randomly by unregulated mechanisms, presumably via non-specific divalent cation transporters\(^23,24\) or calcium channels\(^25-27\) subsequent to extracellular reduction of iron(III) to iron(II) by a cell-surface iron reductase such as Dcytb. Chronic exposure of cells *in vitro* to artificial iron complexes that presumably mimic NTBI (usually ferric citrate)\(^10-12,31,32\) has been shown to generate cellular iron overload as indicated by increased ferritin levels, ROS generation, protein and DNA oxidation and other indicators of cell damage.\(^28-30\) However, the relevance of such models to cell iron overload *in vivo* is open to question because of various factors associated with the presence of NTBI in plasma: a. the association/complexation of NTBI with various acids and plasma proteins,\(^31\) b. the changes in chemical composition of NTBI with changing iron concentrations\(^15\) and plasma oxidation\(^28,32\) and c. the changes in NTBI properties due to fluctuations in plasma component composition. Adsorption of NTBI to macromolecular plasma components such as albumin,\(^31,32\) may restrict its access to iron reductases and divalent ion transporters at the cell surface due to steric hindrance. Moreover, in thalassemia or other iron overload disorders, it is not only difficult to experimentally simulate NTBI *per se*, but also to reproduce the possible chemical modifications of plasma components that become exposed to oxidative stresses due to a rise in labile iron and depletion of antioxidants.\(^28,32\) Despite the above limitations, attempts have been made to assess NTBI transport by using NTBI-simulating complexes of radiolabelled iron in protein free settings and by artificial inclusion of reductants in order to render the iron transportable by various voltage activatable Ca channels\(^25,27\) or by a putative Zn transporter.\(^23,24\) The pathophysiological significance of such approaches is still arguable and likewise the effects of Ca-channel blockers on iron-associated cardiac damage.\(^27\)
Considering the complex nature of plasma NTBI, we opted for using native NTBI containing sera from hypertransfused thalassemia major patients in conjunction with two major strategies for tracing iron ingress into cell compartments. One was based on the Oxyburst-green probe that is taken up from medium into the endosomal compartment and which fluoresces when activated by hydrogen peroxide in a metal-dependent/chelator-sensitive manner (Fig. 2 and supplement Figs S1 &S2). The other strategy monitored labile iron ingress into the cytosolic CALG-laden or CALB-laden compartment by following metal evoked quenching of fluorescence (Figs. 1&6 and supplement Figs. S3&S4). Proofs that both strategies monitored processes that involved endocytosis of NTBI leaned on the effects of inhibitors of endocytosis and on those of impermeant iron chelators such as hydroxyethyl-starch-DFO (Figs1&5 and supplement FigsS1&S2), whereas proof that the species monitored in endosomes or cytosol was labile iron leaned on the action of permeant iron chelators such as SIH or deferiprone (Figs 3-6). In addition to thalassemia major sera containing NTBI, we also used ferric-citrate, an accepted component of plasma NTBI, either by itself or supplemented to human sera or human serum albumin (Figs1&2 and Supplement Figs. S1-S3). While cell exposure to ferric-citrate alone at concentrations measured in thalassemia major sera failed to evoke significant iron-associated changes in endosomal or cytosolic iron pools, its addition together with human sera or serum albumin (HSA) renders it demonstrably accessible to cells: first by undergoing endocytosis and subsequently by releasing labile iron in endosomes and translocating it to the cytosol, in the case of RAW cells, via NRAMP1 (Fig. 6). That the processes monitored by endosomal Oxyburst-green or cytosolic CALG were associated with NTBI and not with TBI was deduced from the fact that addition of apotransferrin to media containing NTBI, native or artificial, abrogated the processes similarly to hydroxyethyl-starch-DFO.

We also found that the proposed steps of NTBI uptake into RAW cells were largely recapitulated with thalassemia major sera probed with CALG, that reversibly binds iron, including components of plasma NTBI and is endocytosed commensurately with NTBI levels (Fig. 3). However, a key question is to what extent CALG added to sera reports NTBI ingress into cells rather than promotes NTBI ingress by binding to plasma NTBI, whether present as low-molecular weight complexes or bound to plasma proteins. Since endocytic uptake of CALG-Fe and thalassemia major sera with CALG is inhibited by impermeant iron chelators while the uptake of rhodamine-dextran is unaffected by the same chelators, it may be concluded that CALG is a reporter rather than a promoter of endocytosis of NTBI. This is further supported by our observation that cellular uptake of CALG-Fe was negligible in the absence of serum or serum albumin. Moreover, not only CALG-Fe, but ferric-citrate in the presence of albumin, and NTBI in thalassemia major sera also exist in macromolecular
forms\textsuperscript{12} that could be taken up by cells by a similar bulk mechanism of endocytosis, whether adsorptive or pinocytic, which would be in line with the same mechanistic concept.

Taken together, the data presented indicate that a major component of plasma NTBI ingress into cells is associated with bulk mechanisms of endocytosis that prevail in cells of various organs.\textsuperscript{34-38} The proposed mechanism explains how NTBI species bound to plasma components gain access to particular cells, but does not exclude others which comprise some plasma NTBI forms transportable by resident membrane transporters or channels. Endocytic uptake of NTBI, which is demonstrated primarily in macrophages, also operates, though to a lesser extent, in other cells such as cardiomyoblasts and cardiomyocytes (Supplement Fig. S1-S4 and Fig. 4) and insulinoma cells (Glickstein and Cabantchik, unpublished observations). The relative contribution of NTBI endocytosis to iron accumulation in the above organs will depend on both, their endocytic/pinocytic activities, and on the levels of NTBI in plasma that undergoes significant modifications in chronic iron overload. Macropinocytic uptake of a variety of macromolecules has only recently become recognized as a regulated pathway with features that distinguish it from clathrin-dependent endocytic processes, such as receptor-mediated endocytosis.\textsuperscript{34} Numerous stimuli regulate macropinocytosis, among which the best defined are growth factor signalling and surface binding of intracellular pathogens. Pinocytic activity is particularly high in macrophages, epithelial and endothelial cells and cells of the immune system.\textsuperscript{34} However it also prevails, though at relatively lower levels, in various cell types and tissues, including those susceptible to iron overload such as the heart\textsuperscript{35-37} and endocrine glands.\textsuperscript{38} A surprising observation in this study was the enhanced endocytosis of rhodamine-dextran in the presence of sera containing NTBI (Fig. 4). Although the biochemical basis for this effect is still unclear, it could comprise a key component in the mechanism of tissue iron loading in iron overloaded thalassemia major patients. As the uptake of rhodamine-dextran added to thalassemia major plasma is not affected by DFO, the contribution of NTBI per se to endocytosis is likely to involve pre-oxidized plasma components, for which there is ample evidence in hypertransfused patients with inadequate chelation treatment.\textsuperscript{26} Furthermore, albumin, especially in its oxidized form has been shown to avidly bind iron\textsuperscript{32} and has been suggested to be a possible plasma carrier of NTBI.\textsuperscript{32} Considering that the half-life of oxidized human albumin in mice is reduced by almost 50\%, mainly due to liver clearance,\textsuperscript{28} it is conceivable that oxidized albumin or other plasma components are preferentially endocytosed by macrophages. In fact, macrophages are pivotal for recycling of iron extracted from senescent erythrocytes and the control of systemic iron levels.\textsuperscript{33} These properties have implications for the pathophysiology and treatment of iron overload and highlight the need for eliminating NTBI from the circulation by chelation as a means of reducing plasma protein oxidation and ensuing tissue iron overload. However, further studies
are also required in order to assess whether a similar mode of NTBI ingress by endocytosis prevails in other disorders of systemic iron overload like hemochromatosis or transfusional siderosis like MDS and SCD.

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AUTHORSHIP AND DISCLOSURES
Yang-Sung Sohn: design and performance of experiments, analysis and MS writing. No coi to declare. William Breuer: design and performance of experiments, analysis and MS writing. No coi to declare. Hussam Ghoti: design of clinical aspects and patient care and data collection and preparation. No coi to declare. Eliezer Rachmilewitz: design of clinical aspects and patient care and data collection and preparation. No coi to declare. Samah Attar: design of clinical aspects and patient care and data collection and preparation. No coi to declare. Guenter Weiss: design of experimental studies and data analysis and preparation No coi to declare and Z. Ioav Cabantchik: design and performance of the study, analysis and MS writing. Received honoraria from Novartis ESB and consulting fees from Coremedix Inc and Chiasma, Ltd (none related to the present work)
References


LEGENDS TO FIGURES

Fig 1. Uptake of ferric citrate into the cytosol of RAW cells. Effect of human serum albumin, inhibitors of endocytosis and impermeant iron chelators

RAW cells labeled in the cytosol with CALG via its AM precursor were followed for up to 1 hour by epifluorescence microscopy while perfused in DMEM-HEPES supplemented with ferric citrate only (Fe:citrate10: 30 µM) (FC), or together with 50 µM human serum albumin (FC+HSA) and the indicated substances: Nocodazole (N=30 µM), ML-9 (M=100 µM) (FC+HSA+N+M), cytochalasin D (cyD= 100 µM) (FC+HSA+CyD), and hydroxyethyl-starch-DFO (50µM) (FC+HSA+HES-DFO). Systems without added ferric citrate were HSA alone (50 µM) (HSA), HSA with Nocodazole (N=30 µM) and ML-9 (M=100 µM) (HSA+N+M) and HSA with hydroxyethyl-starch-DFO (50µM) (HSA+HES-DFO). All incubations and perfusions were carried out at 37°C. The pictures depict snapshots of the same field taken at 0 and 40min, and the graph depicts the mean fluorescence quenching (FQ) of each field (analyzed with NIH Image J (National Institutes of Health, Bethesda, MD) normalized to the initial fluorescence intensity (control cells).

Fig 2. Uptake of NTBI into endosomes of RAW cells as revealed with an endosomal ROS probe

RAW cells were incubated for 2-3 h in DMEM medium containing Oxyburst-green (OBG) (400 µg/ml) with 10 µM ferric citrate (FC), or 50 µM human serum albumin + 10 µM ferric citrate (FC+HSA), or 30% thalassemia major sera (TM sera), or HSA (50 µM) + 10 µM FC supplemented with either hydroxyethyl-starch-DFO (50 µM) (HSA+FC+HES-DFO) or the permeable chelator SIH (50 µM) (HSA+FC+SIH), or TM sera with hydroxyethyl-starch-DFO (50 µM) (TM sera+HES-DFO). After washing of cells, the fluorescence of Oxyburst-green was monitored before and 10 min after addition of H₂O₂ (50 µM). We used for that purpose live epi-fluorescence microscopy adapted for pseudo-confocal imaging with an Optigrid system. The pictures shown in the upper panel are snapshot images taken after addition of H₂O₂ and the bar graph in the lower panel represents the mean fluorescence intensities of Oxyburst-green in endosomes of 4 cells per field (±SEM) for one (out of three) representative experiments. The bar graph includes data obtained with TM sera + apo-transferrin (TM sera+aTf). Data are given in terms of arbitrary fluorescence units obtained by image analysis with Image J.

Fig 3. Uptake of NTBI into the endosomal compartment of various cell types

RAW, macrophages (left panels) and H9c2 cardiomyoblast cells (middle panels) were loaded with calcein-green-iron complexes (20µM) (CALG-Fe) for 3 hrs and washed.
extensively. SIH (50 µM) was added and the fluorescence in the same fields was inspected 10' later (lower left and lower middle panels). H9c2 cardiomyoblasts were exposed for 18 hrs to Oxyburst-green (400 µg/ml) + Rhodamine-dextran (R-D) (30µM) in growth medium supplemented with NTBI in the form of 30µM ferric citrate + HSA (50 µM) (RD+OBG+NTBI) (upper right panel) and after washing they were challenged with H2O2 (50 µM) (lower right panel) as described in Fig. 2 for RAW cells. Colocalization of Oxyburst-green and Rhodamine dextran appears as yellow spots.

Fig 4. Endocytosis of CALG and rhodamine-dextran by RAW cells in media containing sera from thalassemia major patients. RAW cells were incubated for 3 h with thalassemia major sera containing low and high NTBI (lNTBI or hNTBI) as indicated) and both CALG (10µM) and rhodamine-dextran (R-D) (30µM). After washing of cells and treatment with SIH 50 µM, the fluorescence of CALG and R-D was monitored by live epi-fluorescence microscopy equipped with an Optigrid system. The fluorescence intensity of CALG and R-D obtained from 5 different cell areas and the mean values obtained from 3 independent experiments are depicted in the body of the figure. Colocalization of merged images of CALG and R-D (shown in the 3rd row of images) done with the Volocity program was 73-75%.

Fig 5. CALG uptake into RAW cells exposed to thalassemia major sera is dependent on concentration of CALG and NTBI. (A). RAW cells were incubated for 3 h in DMEM medium containing 30% high-NTBI thalassemia major serum supplemented with the indicated concentrations of CALG and the mean fluorescence intensity measured in cells was plotted against CALG concentration (circles). The fluorescence intensity, ΔF, following addition of SIH (squares) was plotted against CALG (inset). (B). The uptake of CALG was measured in RAW cells exposed for 3h to 30% thalassemia major sera with low NTBI (INTBI) and high NTBI (hNTBI), as indicated, which were supplemented with 20 µM CALG without and with 50 µM DFO or hydroxyethyl-starch-DFO (HES-DFO). Fluorescence measurements were done before and after addition of SIH (ΔF corresponds to ΔSIH) and the mean cell CALG fluorescence of 4 cells per field (±SD, from 3 independent experiments) was calculated with Image J program. (C). Correlation between endosomal CALG-Fe acquired by RAW cells and NTBI levels detected in thalassemia major sera. RAW cells exposed to sera from thalassemia major patients (n=20) were examined microscopically after 3 h incubation before and after addition of SIH, as described in Figure 1(where ΔF corresponds essentially to ΔSIH). The mean cell CALG fluorescence (± std. dev.) associated with the endosomal compartment was calculated from 4 different cells in a given field. The increment in fluorescence following SIH addition (ΔF + SIH) was plotted against
serum DCI (in µM; a measure of serum NTBI, see Methods) and analyzed by linear regression analysis (slope 132 ± 30 fluorescence units per µM Fe; R²=0.61).

Fig 6. NTBI uptake and iron distribution between endosomes and cytosol in RAW cells expressing different levels of NRAMP-1.

(A) Endosomal levels of CALG in WT RAW cells and 2 RAW stable mutant cells overexpressing or underexpressing NRAMP1. WT RAW and the sublines 37 (NRAMP-1 overexpressing) and 21 (NRAMP-1 underexpressing) were incubated for 3h with thalassemia major sera containing high NTBI (hNTBI) in the presence of CALG (50µM) and washed. Cell CALG fluorescence intensity of 4 cells per field (±SD, from 3 independent experiments) was recorded before and 10 min after addition of the permeant SIH (50 µM) (in order to reveal the quenched CALG-Fe complexes formed by interaction of CALG with sera-containing NTBI). Data are given in terms of arbitrary fluorescence units.

(B) Fluorescence changes in endosomal CALG and cytosolic CALB in RAW 37 and 21 sublines following incubation with high NTBI sera. Cells of the RAW 21 and 37 lines (denoted as 21 hNTBI-diamonds and 37 hNTBI-squares) were exposed for 2 h to 30% hNTBI thalassemia major sera supplemented with CALG (50 µM) and subsequently loaded for 10 min with CALB-AM (10 µM). Changes in cytosolic CALB (filled symbols) and endosomal CALG (empty symbols) fluorescence were monitored with time after loading of the probes and are given relative to time 0 (beginning of measurements) for each subline and for each fluorescent probe. The images represent snapshot images taken in the same field at zero and 60 min time points for subline 37: (C) for CALG and (D) for CALB.
fig 2

![Bar chart showing O2G fluorescence (u.a.)](image)

- FC
- HSA+FC
- TM sera
- HSA+FC+HES-DFO
- HSA+FC+SIH
- TM sera + HES-DFO

+H2O2

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Supplement: legends to supplementary figures S1-S5.

Fig. S1. NTBI uptake into H9c2 cardiomyoblasts as revealed with the ROS probe Oxyburst-green (OBG)
H9c2 cells were incubated for 18 hr in DMEM medium containing Oxyburst-green (400 µg/ml) and HSA (50 µM) supplemented with 20 µM ferric citrate (FC) in the presence and absence of either hydroxyethyl-starch-DFO (HES-DFO, 50 µM) or aTf (25uM). After washing of cells, the fluorescence of Oxyburst-green was monitored before and 10 min after addition of H2O2 (50 µM) using live epi-fluorescence microscopy adapted for pseudo-confocal imaging with an Optigrid system. The pictures shown in the upper half are snapshot images taken after addition of H2O2 and the bar graph in the lower half represents the mean fluorescence intensities of Oxyburst-green (OBG in arbitrary units a.u.) in endosomes of 5 cells per field (±SEM) for one (out of three) representative experiments. Data are given in terms of arbitrary fluorescence units obtained by image analysis with Image J.

Fig. S2. NTBI uptake into HL-1 cardiomyocytes as revealed with the ROS probe Oxyburst-green (OBG)
HL-1 cells were incubated overnight in HL-1 growth medium16 containing Oxyburst-green (400 µg/ml) and HSA (50 µM) supplemented with 20 µM ferric citrate (FC) in the presence and absence of either hydroxyethyl-starch-DFO (HES-DFO, 50 µM). After washing of cells, the OBG fluorescence was monitored by confocal microscopy. The pictures shown on the upper half are snapshot images taken 10 min after addition of H2O2 (50 µM) and the bar graph on the lower half represents the mean fluorescence intensities (in arbitrary units a.u.) of 5 cells per field (±SEM) for one (out of three) representative experiments. Data are given in terms of arbitrary fluorescence units (a.u.) obtained by image analysis with Image J.

Fig. S3. Uptake of NTBI into the cytosol of HL-1 cardiomyocytes. Effect of human serum albumin and inhibitors of endocytosis
HL-1 cells labeled in the cytosol with CALG via its acetomethoxy precursor CALG-AM as described in methods were followed for up to 1 hour by epifluorescence microscopy while perfused in DMEM-HEPES supplemented with ferric citrate (Fe:citrate 10: 30 µM) or without it (control) and the indicated substances: HSA (50 µM), cytochalasin D (CyD= 100 µM). All incubations and perfusions were carried out at 37°C. The pictures depict snapshots of the same field taken at 0 at 40min, and the graph depicts the mean change in fluorescence (∆F) between 0 and 40 min of each field (analyzed with NIH Image J [National Institutes of Health, Bethesda, MD])

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Fig. S4. **Effect of cytoskeleton inhibitors and apo-transferrin on the uptake of NTBI into the cytosol of H9c2 cells.**

Cultured rat cardiomyocytes (H9c2 line) were cultured for 1 hr in medium containing 30% human serum (control serum, empty bars) or the same serum supplemented with 20 µM ferric citrate to generate 8.5 µM NTBI as measured by the DCI assay (DCI(+) serum, filled bars). In systems with cytoskeleton inhibitors, the cells were pre-incubated in serum-free medium for 15 min with nocodazole (20 µM) and ML-9 (100 µM) (N + M) prior to the addition of sera to a final concentration of 30%. In systems with apo-transferrin (aTf), sera were pre-mixed with 2 mg/ml apo-transferrin for 15 min prior to addition to cells. Immediately following incubation with sera, the cells were loaded with 0.125 µM CALG-AM for 10 min, suspended by trypsinization and mean cell fluorescence without and with 0.4 mM deferiprone (L1) was determined by flow cytometry. Data are presented as mean change in fluorescence (ΔMFI) after addition of the chelator L1 (mean ± SD) (n=4).

Fig. S5. **Binding of CALG to high molecular weight components in thalassemia major sera.**

CALG (20 µM) was incubated with HBS buffer only, or with 6 different thalassemia major sera with DCI values in the range 0–6.1 µM, or with HBS containing 20 mg/ml HSA for 1.5 hrs at room temperature, then the samples were diluted 1:5 in HBS and passed through a 30 kD cutoff filter. The fluorescence of the retentate (high MW) and filtrate (low MW) was measured in the presence of 100 µM DFO to obtain the (mean ± SD) (n=3) relative CALG concentrations in both fractions.
Sohn et al. Fig S2

OBG loaded HL-1 cells

FC

FC-HSA

HSA

FC+HSA+HES-DFO

OBG mean fluorescence

FC

FC-HSA

HSA

FC+HSA+HES-DFO