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Influence of parenteral iron preparations on non-transferrin bound iron uptake, the iron regulatory protein and the expression of ferritin and the divalent metal transporter DMT-1 in HepG2 human hepatoma cells

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Abstract

It is widely assumed that standard parenteral iron preparations are degraded in the reticuloendothelial cells and that the iron is subsequently incorporated into transferrin. Hepatocytes or other epithelial cells have been considered as not affected. We show that this picture should be carefully reconsidered. By using the human hepatoma cell line HepG2 we showed that the parenteral iron preparations ferric saccharate and ferric gluconate donated iron to the cells as efficiently as low molecular weight iron and stimulated non-transferrin bound iron uptake. This led to inactivation of the iron regulatory protein 1 and to an increase in the expression of ferritin and of the divalent metal transporter (DMT-1). Ferric dextran was only a weak stimulator of ferritin and DMT-1 expression. The observed changes in iron metabolism occurred at concentrations of parenteral iron that can also be found in the plasma of patients after i.v. infusion. We conclude that parenteral iron also influences the iron metabolism of non-reticuloendothelial cells like HepG2 cells. Further the increase in the expression of the transporter DMT-1 in HepG2 cells after iron treatment is in contrast to the regulation in the duodenum and may be involved in the upregulated uptake of potentially toxic non-transferrin bound iron from the circulation to store it in the non-toxic form of ferritin.

Keywords: Parenteral iron; Hepatoma cells; Non-transferrin bound iron; Ferrous iron transporter; Labile iron pool; Ferritin

1. Introduction

Parenteral iron therapy is widely used for the treatment of anemias not treatable by oral iron. Most prominent among these are hemodialysis and chronic renal failure [1–3], but it is also used to treat anemia in some forms of arthritis and other diseases [4–6]. The iron preparations currently in clinical use are all complexes of ferric iron with carbohydrate-like molecules, which are themselves polymeric, such as dextran, or form a polynuclear chelate with the metal ions, such as sucrose (yielding ferric saccharate) or gluconate [7].

All these forms of parenteral iron are considered as principally safe and non-toxic under the conditions used for clinical application [8–10]. Nevertheless, some forms of intravenous iron preparations, particularly the dextran moiety of ferric dextran, can induce anaphylactic reactions [11]. The non-toxicity of the other preparations is not generally accepted either [12–14]. There is therefore a certain controversy, particularly in the case of renal failure and hemodialysis, how to balance iron and erythropoietin, and whether to apply iron in frequent small doses or at higher amounts per injection, but with lower frequency, to spare the patient time and the general side-effects of the injections [15–17].

The general pathway of these preparations is to be first taken up by the cells of the reticulo–endothelial system,

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Abbreviations: IRP, iron regulatory protein; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; DTPA, diethylene triamine pentaacetate; IRE, iron regulatory element; NTBI, non-transferrin-bound iron; UTR, untranslated terminal region; LIP, labile iron pool; FAC, ferric ammonium citrate; DFO, desferrioxamine; DMT, divalent metal transporter; Nramp, natural resistance associated macrophage protein; SDS, sodium dodecylsulfate.

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where they are degraded and the iron subsequently bound to transferrin for delivery to the erythropoietic system [7,18,19]. However, some parenteral iron is also found in other tissues, particularly in the liver. It is considered safe as long as it is not degraded, but this has never really been proven. Initiation of endothelial dysfunction by parenteral iron has recently been demonstrated [20]. The intracellular metabolism of these polymers is largely unknown, and especially the consequences of long-term treatment are not really clarified and correlated to the applied doses.

In this study, we show that intravenous iron leads to deactivation of the IRP1 [21,22] and stimulation of non-transferrin bound iron uptake, a property frequently observed for labile iron [23,24] as well as the biosynthesis of ferritin and the iron transporter DMT-1/Nramp2.

2. Materials and methods

2.1. Cell cultures

The HepG2 cells (a gift of the Institute for Cancer Research, University of Vienna, Austria) were grown in DMEM with 10% heat inactivated fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine under standard tissue culture conditions and plated in 6-well plates at a density of 4.5×10^5 cells/well for 2 days. For the experiments cells were first washed twice with serum-free DMEM and then, if not indicated otherwise, depleted of iron by treatment with 50 μM DFO overnight in serum-free DMEM. Parenteral iron is for treatment of iron deficiency, which was therefore simulated by iron depletion of the cells.

2.2. Iron preparations

The preparations for testing were the ferric saccharate mixtures Ferrum Vitis (Neopharma), Venofer (Vifor), the ferric gluconate Ferrlecit (Rhone-Poulenc Rorer, A. Nattermann & Cie) and the ferric dextran INFeD (Schein Pharamceuticals). For the study, they were supplied by Dr. P. Lehmann (Roche Diagnostics).

2.3. Iron loading of the cells

HepG2 cells were incubated with FAC as positive control for low molecular weight iron, with 0.1 mg/mL diferric transferrin, or with the parenteral iron preparations, all diluted in serum-free DMEM. The uptake of diferric transferrin via the transferrin-receptor and endocytosis is strictly controlled and cannot lead to iron overload and was therefore used as control for cells with normal iron status. At the end of the various treatments cell viability was determined by means of trypan blue exclusion and LDH release [25].

2.4. RNA band-shift assay

The levels of IRP1 were detected with a probe for the IRE kindly supplied by Dr. Ernst Müllner (Department of Medical Biochemistry, Vienna). The ^{32}P-signal was detected by autoradiography. To estimate the total amount of IRP1, 2 mM mercaptoethanol were added to reactivate the IRE-binding capacity of IRP1 [26,27].

2.5. Immunoblotting of DMT-1/Nramp2 and ferritin

Expression of both DMT-1 isoforms in HepG2-cells was detected by western blotting. As control for the effects of iron treatment ferritin expression was assayed by the same method. After iron treatment and extensive washings the cells were lysed with cell culture lysis reagent (Promega), transferred to a microcentrifuge tube and treated for 15 sec with a Branson ultrasonicator. After centrifugation (6000 *g*, 10 min, 4°), aliquots of the supernatant (cell lysate) were stored at −80° until use. Fifty micrograms of proteins were separated on 8% SDS–polyacrylamide gel electrophoresis under non-reducing conditions using Prosieve 50 Gel solution (BMA, BioWhittaker) and Tris/Tricine-electrode buffer (0.1 M Tris, 0.1 M Tricine, 0.1% SDS) and electroblotted onto nitrocellulose membranes. After blocking with 2% dry milk (Maresi), 0.05% Tween 20 in PBS, pH 7.4, the blots were incubated with primary antibody in incubation buffer (PBS, pH 7.4, 0.1% dry milk, 0.05% Tween 20) overnight at 4°. Primary antibodies were either rabbit–anti-human ferritin (from DAKO) (1:500) or rabbit–anti-human DMT-1 antibody (a gift from Dr. Guenter Weiss, Department of Internal Medicine, Innsbruck, Austria), (1:250). After washings the blots were incubated with secondary goat–anti-rabbit-HRP antibody (from DAKO), (1:7500) for 45 min at room temperature, and detection was performed using the SuperSignal reagent (Pierce) and the FluoroS-MultiImager from Bio-Rad.

2.6. Uptake of NTBI

Uptake of NTBI was assayed with the ferric-DTPA method described earlier [28]. Though DTPA is a non-physiological chelator, it offers the possibility to measure true NTBI-uptake. When citrate, the most probable natural chelator of NTBI is used, the iron may bind to transferrin endogenously produced by the hepatocytes [29]. For the uptake studies $^{55}FeCl_3$ in 0.5 M HCl (obtained from New England Nuclear) was mixed with DTPA to obtain a molar ration of Fe:DTPA of 1:4 and diluted in 37° prewarmed hepatocyte incubation buffer (68.4 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 0.6 mM $MgCl_2$, 1.1 mM KH_2PO_4, 0.7 mM Na_2SO_4, 30.2 mM Hepes, 30.1 mM TES, 36.3 mM Tricine, 40 mM NaOH, pH 7.4) and immediately used for the uptake experiments. After removal of surface bound iron by incubation with 50 μM DTPA in DMEM for 5 min and two more washings with DMEM alone, the cells

were incubated for the indicated times (0–20 min) with 0.5 μM ^{55}Fe-DTPA at 37° in hepatocyte incubation buffer. The uptake was terminated by removal of the uptake solution. To remove extracellular iron the cells were incubated for 5 min in ice-cold washing buffer (142 mM NaCl, 6.7 mM KCl, 1.2 mM $CaCl_2$, 10 mM Hepes, 5 mM NaOH, pH 7.4) containing 50 μM DTPA and two more washings with washing buffer alone. The cells were lysed with 1 mL/well of 0.5 M KOH with 0.1% Triton X-100 overnight and neutralized with 1 M HCl. The radioactivity was counted in a Packard Liquid Scintillation counter. The kinetic data were normalized for the amount of protein present in each sample.

3. Results

3.1. Changes in the activity of IRP1 in HepG2-cells by iron preparations

To get the IRP in its iron-depleted active form, HepG2 cells were first depleted overnight with 50 μM DFO in DMEM. After removal of DFO by extensive washing, incubation of the cells with 300 μM polymeric iron or FAC for 3 hr resulted in a slight depression of the RNA-binding activity of IRP1, but not of its total amount, by ferric saccharate and gluconate (Fig. 1). In this case, FAC was much more effective, whereas ferric dextran had very little effect.

3.2. Influence of loading with iron on the expression of the iron transporter DMT-1 and ferritin

The DMT-1 iron transporter is responsible for the duodenal absorption of iron as well as for the passage of transferrin-iron from the endosomal compartment to the cytoplasm [30–32]. In rat livers, an up-regulation of this protein in iron overload has been recently demonstrated [32], and reflects the role of the liver as an iron scavenger. When HepG2 cells were first iron-depleted by incubation with 50 μM DFO, extensively washed and loaded with one of the four parenteral iron preparations or with FAC (300 μM iron), the observed down-regulation after iron depletion was reversed after 24 hr treatment with FAC as well as with ferric gluconate and ferric saccharate, whereas the reversal needed 48 hr with ferric dextran (Fig. 2).

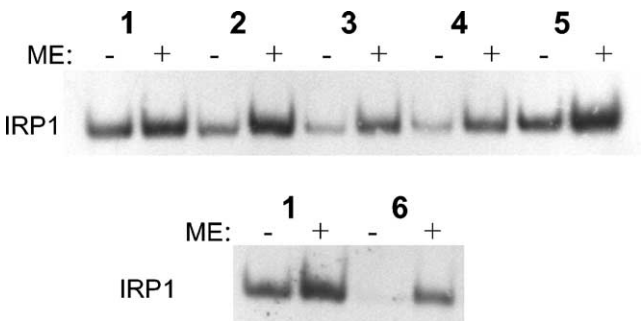

Fig. 1. Dependence of the inactivation of the RNA-binding of IRP1 on the incubation with iron. HepG2 cells were first depleted overnight by treatment with 50 μM DFO to render IRP1 in its highly active iron depleted form. After washings the cells were incubated for 3 hr at 37° either with control medium, containing 0.1 mg/mL holotransferrin (negative control) (1) or with one of the four iron preparations at an iron concentration of 300 μM. Ferrlecit (2), Ferrum Vitis (3), Venofer (4), INFeD (5). FAC at the same iron concentration was used as positive control (6). The IRE-binding activity of IRP1 was determined by the RNA-bandshift assay. The total amount of IRP1 was obtained by addition of mercaptoethanol (+ME) to the cell lysates. Data are representative of one out of four independent assays.

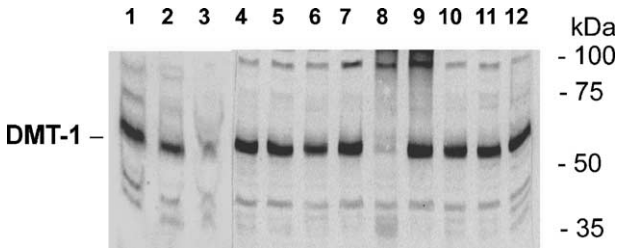

Fig. 2. Expression of the DMT-1 iron transporter in HepG2 cells after depletion with DFO and loading with different forms of iron. HepG2-cells grown in DMEM containing 10% FCS (lane 1) were first depleted of iron by incubation with 50 μM DFO (24 hr, lane 2; 48 hr, lane 3), and then, after extensive washings, reincubated for the indicated times with one of the four iron preparations (300 μM iron): Venofer (24 hr, lane 4; 48 hr, lane 5); Ferrum Vitis (24 hr, lane 6; 48 hr, lane 7); INFeD (24 hr, lane 8; 48 hr, lane 9), Ferrlecit (24 hr, lane 10; 48 hr, lane 11). FAC at the same iron concentration was used as positive control (24 hr, lane 12). Cell lysates (50 μg protein/lane) were electrophoresed on 8% SDS–polyacrylamide gels under non-reducing conditions using a Tris/Tricine-electrophoresis buffer. DMT1 was detected by western blotting using a chemiluminescence protocol (see Section 2).

Loading the cells with iron in any of the used forms leads to an increase in ferritin expression. This is also true for ferric dextran, despite the much lower iron loading found after treatment of the cells with this preparation (Fig. 3). In all cases the increase in ferritin expression is time and concentration dependent and needs the highest concentrations and longest incubation times (48 hr) with ferric dextran, whereas ferric gluconate is most effective. With Ferrlecit a slight increase in ferritin expression can be observed even after short time incubation (8 hr) with lowest concentration tested (25 μM). Ferric sucrose (Venofer) is less effective than ferric gluconate but much more effective than ferric dextran in increasing ferritin expression (not shown). The levels of ferritin parallel the degree of inactivation of the IRP, which leads to increased translation of the protein because of its iron-mediated release from the mRNA [26].

3.3. Stimulation of uptake of NTBI after loading with iron preparations

The basic uptake rate of HepG2-cells for NTBI is almost zero. Whereas ferric dextran was absolutely ineffective, ferric saccharate as well as ferric gluconate were similar to

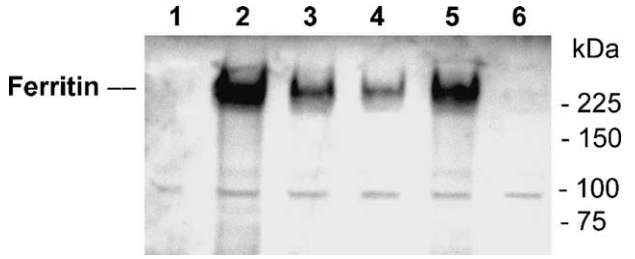

Fig. 3. Ferritin levels in HepG2 cells after loading with different forms of iron. The cells were first depleted of iron by treatment with 50 μM DFO for 48 hr (lane 1) and, after washings, incubated for 48 hr with either FAC (300 μM iron) as positive control (lane 2) or with one of the parenteral iron preparations at the same iron concentration. Venofer (lane 3), INFeD (lane 4), Ferrlecit (lane 5). Incubation with 0.1 mg/mL diferric transferrin, the physiologic iron donor, was used as control for normal iron status of the cells (lane 6). At the end of the incubations, the cells were lysed as described in Section 2. Fifty micrograms protein of the cell lysates were loaded in each lane and electrophoresed on 8% SDS–polyacrylamide gels under non-reducing conditions using a Tris/Tricine-electrophoresis buffer. Ferritin was detected by western blotting using a chemiluminescence protocol (see Section 2). One typical ferritin–protein blot out of four independent experiments is shown.

FAC in their ability to stimulate NTBI-uptake after loading with an equivalent of 300 μM iron for 3 hr (Fig. 4). The effect was dependent on the concentration of iron and was detectable at 70–80 μM iron with all effective iron forms (not shown). The low stimulation of NTBI-uptake from ferric dextran was not caused by cell damage, as assessed by means of trypan blue exclusion and LDH-release (not shown). Very similar data could also be obtained with rat hepatocytes in short-time culture (not shown).

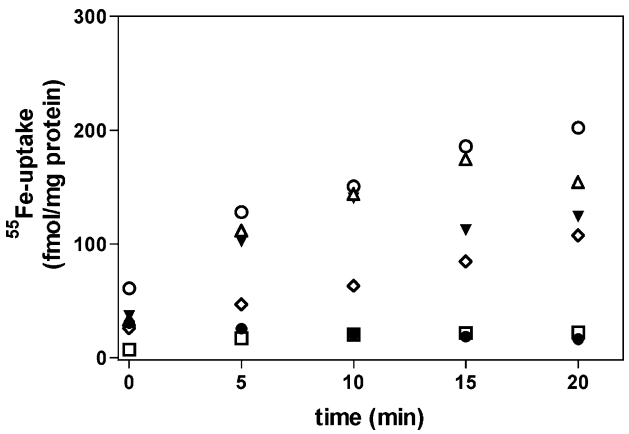

Fig. 4. Stimulation of NTBI-uptake in HepG2 cells by iron preparations. The cells were first depleted of chelatable iron by treatment with 50 μM DFO overnight. Loading with iron was then carried out for 3 hr at 37° with the iron preparations (300 μM iron) or as positive control with FAC at the same concentration. After removal of excess iron the uptake of NTBI was assayed with ^{55}Fe-DTPA as described previously [28] (see Section 2). (●), Control (loading of the cells with 0.1 mg/mL diferric transferrin); (○), FAC (positive control); (◇), Ferrlecit; (△), Ferrum Vitis; (▼), Venofer; (□), INFeD. Uptake was normalized according to the protein concentration. Data represent one typical experiment from a set of three independent uptake experiments which yielded essentially the same results. Shown are the means of triplicates only, the error bars are smaller than the symbols.

4. Discussion

A number of pharmacokinetic studies have been published for all iron preparations in clinical use, but relatively little is known about the biochemistry underlying its therapeutic efficacy. It is generally accepted that the cells of the reticuloendothelial system degrade the polymers and deliver the iron to plasma transferrin which then supplies the erythropoietic cells with iron [7,18,19].

It is assumed that epithelial cells like hepatocytes are very little affected by the iron because it enters them only very sluggishly, if at all, and the slow degradation within the cell enables to store it safely without any cellular damage. According to the data shown in this study, these assumptions must be reconsidered with care, especially when the safe single dose for injection of iron is determined.

Ferric saccharate as well as ferric gluconate, much less ferric dextran, added iron to the intracellular LIP in HepG2 cells. This was indirectly demonstrated by two characteristic effects: it deactivated RNA-binding ability of the IRP1 [21,22] when offered to the cells at concentrations above 50 μM, and it increased the uptake rate for non-transferrin bound iron. This is typical for labile iron and is considered to be a means of protection from the ill effects of free iron [23,24]. Intracellular labile iron is less dangerous than extracellular free iron because it can be stored away in ferritin. In both respects, ferric saccharate and ferric gluconate were completely equal to FAC in their efficacy.

The exact mechanism of NTBI-uptake is not known, but it is quite possible that the endosomal iron transporter DMT-1/Nramp2 may be overexpressed under the conditions of iron overload [32] and relocated to the plasma membrane. Two transcriptional variants are known: one contains IRE-elements in the 3′-UTR, which renders the mRNA subject to a control mechanism identical to that of the transferrin receptor. This form is expressed in the duodenal villi and is down-regulated by iron, respectively up-regulated in iron deficiency. The other form does not contain any IREs, and nothing is known about regulatory connections between the iron content of the cells and its expression [33]. This second form is expressed in most other tissues and may respond in the opposite direction by a mechanism which is unknown at the moment, as reported for the situation of iron overload in the rat. This same response was observed in this study in HepG2 cells after acute *in vitro* loading with iron.

The two protein isoforms differ in the N-terminal region. The antibodies used in this work could detect the whole amount of DMT-1 protein, since they were directed against a C-terminal peptide, which is identical in both isoforms, and thus cannot differentiate between them. This is subject to further study.

The cells responded to the increase in intracellular iron by synthesizing more ferritin. The elevated LIP resulting from iron loading is therefore probably short-lived, but it does appear and represents a possible danger. Intracellular

labile iron may initiate oxidative cell damage even when present for short times [34,35]. Toxicity may also result from the other changes in iron metabolism shown in this study: from stimulated NTBI-uptake which will further increase the intracellular LIP before it can be stored away in ferritin, by deactivation of IRP which of course results in protection by increased ferritin synthesis, but which also results in a decreased uptake from transferrin-bound iron via receptor mediated endocytosis, due to decreased transferrin receptor expression at the same time. In patients this could result in higher transferrin saturation in the serum and therefore reduced iron-binding capacity of transferrin which could stimulate the occurrence of non-transferrin bound iron in the plasma. Also the increased DMT-1 expression should be considered, thought the exact role of DMT-1 in iron metabolism of the liver (and other non-intestinal cells) is not known at the moment.

Preliminary experiments yielded similar results in other cells types (e.g. fibroblasts and HeLa cells, not shown). Thus, since the half-life of intravenous iron is several hours [17], untoward iron loading of non-target tissues by intravenous iron cannot be excluded.

Acknowledgments

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