Ferritin-dependent radical generation in rat liver homogenates

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ABSTRACT

The hypothesis of this study was that mammalian ferritin (FER) has the ability of releasing Fe in the tissue to catalyze the generation of free radicals, such as ascorbyl ($A^{\bullet}$) and hydroxyl radical ($^{\bullet}OH$), that might lead to the damage of FER itself. The rat liver homogenates exhibited an electron paramagnetic resonance (EPR) signal with the spectral features (a_H = 1.88 G, g = 2.0054) of $A^{\bullet}$. The addition to the reaction medium of isolated rat liver FER increased by 3-fold the EPR signal, as compared to the recorded value in its absence. Isolated microsomes from rat liver incubated during 10 min showed a signal with the spectral features (a_H = 15 G, g = 2.0062) of $^{\bullet}OH$. The addition of FER in the presence of either ethylenediamine-tetraacetic acid (EDTA) or adenosine-5'-triphosphate (ATP) significantly increased the recorded spectra. The labile Fe pool (LIP) in the homogenate was assessed by EPR. The rat liver homogenates exhibited an EPR signal with the spectral features (g = 4.3) of the Fe^{2+} and was significantly increased by the addition of FER (3-fold). The oxidation profile of the isolated FER from rat liver was analyzed after incubation with 10 mM ascorbate (AH^-). A drastic increase in the width of the band suggested alterations to the protein structure. The FER content of tryptophan (Trp) and thiols was significantly lower when the incubation was performed in the presence of AH^- as compared to the recorded effect in its absence. The data in tissue homogenates presented here showed that radical generation is associated to FER Fe release, and moreover that the FER protein itself was affected during this process.

1. INTRODUCTION

The amount of Fe within the cell is carefully regulated in order to provide an adequate level of micronutrient while preventing its accumulation and toxicity. Fe is transported and stored in specific proteins (transferrin, lactoferrin, and ferritin (FER)) (Galatro et al., 2007). Fe is sequestered in FER, the main intracellular Fe storage protein. However, there are indications that FERs may have other functions in addition to the well assessed role in storing intracellular Fe. FER is a nanobox protein designed to contain and maintain in solution up to four thousands Fe atoms, which otherwise would aggregate in toxic precipitates (Arosio and Levi, 2002). FER is mostly cytosolic but it is also found in mammalian mitochondria and nuclei (Arosio et al., 2008). Higher eukaryotes often have two major FER genes that encode subunits with different properties, named H (heavy) and L (light) and co-assemble to form heteropolymers (Arosio et al., 2008). FER has evolved a molecular design that limits the Fe chemistry within its interior, avoiding nonspecific Fe oxidation and hydrolysis reactions from occurring within the cytosol of the cell. In this way, other proteins and nucleic acids are protected from the toxic effects of labile Fe. However, previous data from *in vitro* chemical studies indicate that fully Fe-loaded proteins can release some Fe (Arouma and Halliwell, 1987), and O`Connell and Peters (1987) reported that Fe-storage proteins release Fe to a range of chelators and reducing agents and that the released Fe promoted both hydroxyl radical ($^{\bullet}OH$) formation in the presence of hydrogen peroxide, and lipid peroxidation in liposomes. Moreover, since FER Fe is reduced effectively by reductants with redox potentials more negative than about -200 mV, superoxide anion (O_2^-) (redox potential -300 mV) has the potential to reduce Fe from FER in a hydrofobic environment. Data from Puntarulo and Cederbaum (1993) suggested a role for microsomal cytochrome P_{450} on the release of catalytic active Fe from FER, and the induction of cytochrome P_{450} isozymes increased FER-dependent dye oxidation and chemiluminescence (Puntarulo and Cederbaum, 1996). On the other hand, FER can also stimulate the inactivation of enzymes, such as microsomal glucose-6-phosphatase, by a reaction which appeared to reflect FER stimulated lipid peroxidation resulting in cellular damage (Puntarulo and Cederbaum, 1994).

The cytosolic Fe pool (LIP) is a transitory, catalytically active compartment that has been implicated in cell Fe homeostasis and in metal-induced cytotoxicity. It has been postulated that whereas FER levels provide an index of long-term or cumulative Fe loading, LIP measurements provide an “instantaneous” parameter of Fe availability

within hepatocytes (Zanninelli et al., 2002). Moreover, this intracellular pool of low molecular weight Fe compounds acts as an intermediate between extracellular Fe and a wide variety of intracellular processes, and it is in equilibrium with Fe in storage and Fe in enzymes (Jacobs, 1977). Transition metals catalyze the formation of oxygen radicals by the Fe-catalyzed Haber-Weiss reactions (Haber and Weiss, 1934). Grady et al. (1989) and Van Eden and Aust (2001) showed the production of $^{\bullet}OH$ during the oxidative deposition of Fe into horse spleen FER. Hydroxyl radical (E_0 = 2.31 V) is a potent oxidant that reacts at diffusion limited rates (10^8-10^9 $M^{-1}s^{-1}$) with almost every type of molecule found in living cells including sugars, amino acids, nucleic acids, lipids and organic acids (Van Eden and Aust, 2001). No reports of direct experimental evidence concerning FER-dependent $^{\bullet}OH$ generation by Fe released from FER in tissues are available.

Non-enzymatic antioxidants are the primary protectants against oxidative damage (Hubel et al., 1997). Ascorbate (AH^-) is found in rat liver tissue at a concentration of 0.6 mM (Skrzydlewska and Farbiszewski, 1997), and during its antioxidant action undergoes two consecutive one electron oxidations to dehydroascorbic acid (DHA) with intermediate formation of the ascorbyl radical ($A^{\bullet}$) (Hubel et al., 1997). $A^{\bullet}$ has a relatively long lifetime (approximately 50 s) compared with other free radicals (Buettner, 1993), and it is easily detectable by electronic paramagnetic resonance (EPR) even at room temperature in aqueous solution. It is well known that transition metals, such as Fe, catalyze the oxidation of AH^- *in vitro* (Buettner, 1990), leading to the generation of $A^{\bullet}$. Moreover, *in vivo* studies showed that the $A^{\bullet}$ radical content increased significantly in plasma of Fe-overloaded rats after Fe-dextran administration (Galleano et al., 2002).

The hypothesis of this study was that mammalian FER has the ability of releasing Fe in the tissue to catalyze the generation of free radicals, such as $A^{\bullet}$ and $^{\bullet}OH$, that might be a clue factor leading to the damage of FER itself that could have metabolic relevance. The content of radicals and the LIP was assessed by EPR in liver homogenates added with rat liver FER. The damage to FER was analyzed as the oxidation of the protein, the decrease in the content of tryptophan (Trp), the formation of bi-tyrosines and the decrease in the content of thiols in the isolated FER during the release of Fe.

2. Materials and Methods

Experimental preparations

Male Wistar rats, weighing 170-200 g, were starved overnight. Rat livers were excised and homogenized for 30 s in a blender with 40 mM potassium phosphate buffer (pH 7.4). Liver microsomes were isolated as previously described (Klein et al., 1983) by differential centrifugations, utilizing a buffer containing 0.25 M sucrose-0.01 M Tris, pH 7.4. The microsomes were washed with 125 mM KCl, suspended in 125 mM KCl, and stored at -70º C.

All reagents were of the highest grade available. Chemicals: Ammonium sulfate and sodium chloride were purchased from Mallinckrodt (Paris, KY, USA). β-mercaptoethanol, potassium phosphate buffer (PBS) and potassium chloride were purchased from Merck (Darmstad, Germany). Western blotts reagents were obtained from Invitrogen, CA, USA. All other reagents were obtained from Sigma-Aldrich Chemical Co. The buffers and the water used to prepare all solutions were passed through columns containing Chelex-100 resin to remove metal contaminants.

Purification of rat liver FER

FER from rat liver was isolated according to Thomas et al. (1985) with modifications. The excised livers were homogenized in a blender in 2 volumes of extraction buffer (25 mM sodium acetate, 20 mM ethylenediamine-tetraacetic acid (EDTA), pH 4.8) at 4ºC. The homogenate was centrifuged for 20 min at 1,500*g* at 4ºC. The supernatant was filtered through 53 μm nylon filter and added to an equal volume of 50% (w/v) saturated ammonium sulfate with continuous stirring for 15 min, and allowed to settle overnight. The pellet was dissolved in 25 mM sodium acetate buffer with 20 mM EDTA, pH 4.8, and centrifuged at 100,000*g* for 1 h 30 min. The pellet was suspended in PBS (0.02 M phosphate buffer, pH 7.4, containing 0.1 M sodium chloride), and the non-solubilized material was removed by centrifugation at 10,000*g* for 1 h. The supernatant was centrifuged again at 100,000*g* for 90 min. The pellet containing the FER was resuspended in 20 mM PBS buffer (pH 7.4) with 0.1 M NaCl, and the supernatant was then loaded on a Sephacryl S-300 column (1.6 cm x 35 cm), and equilibrated in the same buffer. Fractions were collected and proteins and Fe concentrations were assessed. The Fe-rich fractions were pooled and then concentrated through filters with 30,000 nominal molecular weight limit (Centricon YM30). The FER pellets were dissolved in 40 mM potassium phosphate buffer, 0.15 M NaCl, pH 7.4

and stored a 4ºC until use. Protein content in the sample was measured according to Bradford (1976) using bovine albumin (Sigma) as standard. Total Fe content was determined spectrophotometrically after reduction with thioglycolic acid measuring the absorbance at 535 nm in the presence of bathophenanthroline (Brumby and Massey, 1967). FER purity was verified by an SDS-page (14% (w/v) total acrylamide gel) and ran at room temperature under conditions of constant electrophoretic voltage (150 V) for 60 min. Prior to use, isolated FER samples were incubated on ice in the presence of 10 mM EDTA for 60 min and passed through a Sephadex G-25 column equilibrated with 0.3 M NaCl (pH 7.0) to remove loosely associated Fe (Saito et al., 1985). The Fe content of FER was usually about 2300 atoms per FER shell equivalent to 5 μmol non-heme Fe per mg protein.

Rate of release of Fe from FER

Fe release from purified FER was measured spectrophotometrically using the ferrous chelator ferrozine as a chromophore by measuring the absorbance at 562 nm (ε = 27.9 mM^{-1} cm^{-1}) (Hynes and Coinceanainn, 2002). The reaction mixture (1 ml final volume) contained 100 μg FER, 60 mM Hepes buffer (pH 7.0), 500 μM ferrozine, and reactions were initiated by the addition of AH^- at the indicated concentration. The increase in absorbance at 562 nm was continuously monitored using a Beckman DU Series 7000 diode array spectrophotometer during 20 min at 37ºC.

Determination of $A^\bullet$ content in rat liver homogenates by EPR

Rat liver homogenates were incubated for 1 min under the conditions indicated in each assay, then an equal volume of dimethyl sulphoxide (DMSO) was added, and the sample was immediately transferred to a Pasteur pipette for EPR detection. $A^\bullet$ spectra were measured at room temperature on a Brucker ECS 106 EPR, equipped with a ER 4102ST cavity, operating at the following conditions: microwave power, 20 mW; microwave frequency, 9.75 GHz; modulation amplitude, 1 G; centerfield at 3487 G; time constant, 163.84 ms; sweep width, 15 G; and modulation frequency, 50 kHz. The quantification was performed using an aqueous solution of 2,2,6,6-Tetramethyl piperidine-N-oxyl (TEMPO) introduce into the same sample cell. EPR spectra for both, sample and TEMPO solutions, were recorded at exactly the same spectrometer settings

and the first derivative of the EPR spectra were double integrated to obtain the area intensity, calculating the concentration of A$^{\bullet}$ according to Kotake et al. (1996).

Determination of $^{\bullet}$OH generation by EPR

Isolated microsomes (0.5 mg protein/ml) were incubated in the presence of 100 mM 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 0.5 mM azide, and 40 mM potassium phosphate (pH 7.4). Reactions were started by the addition of 0.5 mM NADPH and the samples were immediately transferred to a Pasteur pipette for direct observation of the reaction in a Brucker ECS 106 EPR spectrometer operating at 9.75 GHz. The EPR spectra were recorded at room temperature as a function of time employing the instrumental settings as follows: microwave power, 20 mW; modulation amplitude, 0.475 G; time constant, 327.68 ms; scan time, 2684 s; and modulation frequency, 50 kHz. The cursor was fixed at 3478 G. The increasing height of the peak was recorded during 8 at 16 min to assess the rate of generation of $^{\bullet}$OH.

Determination of LIP in rat liver homogenates

The LIP was determined by EPR at 77K, according to Woodmansee and Imlay (2002) with modifications. Rat liver samples were homogenized in 10 mM Tris-HCl buffer, 120 mM KCl (pH 7.4), and 1 mM deferoxamine (DF). Samples were incubated at room temperature for 10 min, and then frozen with liquid nitrogen in a syringe. EPR spectra were recorded under the following experimental conditions: 9.75 GHz, microwave frequency; 20 mW, microwave power; 50 kHz, modulation frequency; 4.759 G, modulation amplitude, 1600 G, centered field; 81.92 ms, time constant, and 800 G, sweep width.

Determination of the content of carbonyl groups in proteins

Carbonyl groups in proteins were derivatized as described by Levine et al. (1994). Samples were mixed with an equal volume of SDS (12% w/v) and then with 2 volumes of 20 mM dinitrophenylhydrazine dissolved in 10% (v/v) trifluoracetic acid. This mixture was incubated for 25 min at room temperature, and the reaction was stopped by adding 1.5 sample volumes of 2 M Tris-HCl/30% (v/v) glycerol. Proteins (0.3 μg per well) were loaded in 12% (w/v) acrylamide concentration mini-gels and ran at room temperature under conditions of constant electrophoretic voltage (150 V) for 1

h. For western blotting, proteins were electro-transferred to nitrocellulose membranes at 120 V for 1 h. Blots were blocked with 5% (w/v) non-fat dry milk dissolved in PBS-T (10 mM potassium phosphate buffer pH 7.4, 150 mM NaCl, 0.1% (v/v) Tween 20), incubated overnight with primary antibody dissolved in blocking buffer (1/100), and washed several times with PBS-T. For carbonyl groups detection, the primary antibody was rabbit anti-DNP (Zymed, USA). Blots were then incubated for 2 h with the secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase) prepared 1/10,000 in PBS-T with 1% (w/v) non-fat dry milk; washed several times with PBS-T and developed with a chemiluminescence detection kit (Bio-Rad, USA).

Fluorescence spectroscopy

Fluorescence spectroscopy was used to monitor for a reduction of intrinsic fluorescence of tryptophan (Trp) content in FER upon Fe release (λ_{ex}=285 nm, λ_{em}= 345 nm). FER (20 μg/ml ≈ 0.05 μM) was incubated in 50 mM Hepes buffer (pH 7.0), and AH^- was supplemented to the incubation medium at 37ºC at the indicated concentration in each assay. The appearance of bi-tyrosine fluorescence (λ_{ex} = 325 nm, λ_{em}= 405 nm) was also evaluated.

Total thiol content

FER (50 μM) was incubated in the presence of 3.2 mM β-mercaptoethanol for 30 min at room temperature in 0.15 M sodium chloride (pH = 7) and dialyzed for 48 h against the indicated buffer. The resulting solution was assayed for total thiol content according to Sedlak and Lindsay (1968), employing 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and measuring absorbance at 412 nm. Glutathione was used as standard.

Statistical analyses

All results are from experiments carried out in duplicate and replicated with at least three different preparations. Where indicated, values refer to mean ± S.E.M. Statistical tests were carried out using Statview for Windows, ANOVA, SAS Institute Inc., version 5.0.

3. Results

FER (0.3 mg/g Fresh Weight (FW)) was isolated from rat liver and showed an amino acid composition (Rousseau et al., 2008), and a profile on a SDS gel (Fig. 1A) compatible with the features of previously described commercially L-, H- and (L+H)-chains of horse liver FER, and with the theoretical values in the literature (Takafuji et al., 2002).

The *in vitro* spontaneous release of Fe from the isolated rat liver FER was not detected, however upon the supplementation of the reaction medium with AH^- the amount of Fe released from FER was significantly increased upon a 20 min period (Fig. 1B). Moreover, Fe release depended on AH^- addition to the reaction medium in a concentration dependent manner (Fig. 1B Insert). Since Fe released from FER could catalyzed $A^\bullet$ generation in the tissue, $A^\bullet$ content in rat liver homogenates was assessed by EPR. The rat liver homogenates exhibited an EPR signal with the spectral features (a_H = 1.88 G, g = 2.0054) of $A^\bullet$ (Fig. 2A). The addition to the reaction medium of FER increased the $A^\bullet$-dependent EPR signal by 3-fold as compared to the recorded value in the absence of exogenously added FER (Fig. 2A and B). The addition to the incubation medium with Fe-EDTA, included as a positive control since it is an active catalyst of $A^\bullet$ generation, drastically increased the signal by 4 to 5-fold, meanwhile the supplementation of the reaction medium with either Fe-citrate, or Fe-desferrioxamine (Fe-DF) did not affect the endogenous signal of the homogenate (Fig. 2B), showing the critical role of the nature of the Fe chelator in terms of the metal catalytic ability, as it was previously suggested (Puntarulo and Cederbaum, 1988). Thus, the effect on the content of $A^\bullet$ in the liver homogenates exposed to several Fe chelators was tested, both in the presence and the absence of FER. Supplementation of the rat liver homogenates with NADPH, DF or citrate did not affect $A^\bullet$ content, neither in the presence nor in the absence of added FER (Table 1). The lack of effect of NADPH addition suggested that the cellular endogenous reducing power is not deficient even in the presence of exogenous added FER. The lack of effect of DF or citrate addition is consistent with the reported effect of these Fe-complexes, as shown in Fig. 2B. Data on Table 1 shows that in the absence of exogenous FER the addition of EDTA to the reaction medium affected the $A^\bullet$ content in the homogenates. In the presence of added FER, $A^\bullet$ content was significantly higher than in its absence, but EDTA addition did not show any significant effect over FER supplementation. This lack of effect recorded after EDTA addition in

the presence of FER, could be due to the fact that the endogenous cellular amount of AH^- would act as a limiting factor for $A^\bullet$ generation.

It has been previously reported that also $^\bullet OH$ generation could be significantly increased by Fe, and this effect strongly depends on the nature of the Fe chelator (Puntarulo and Cederbaum, 1988). Isolated microsomes from rat liver were incubated during 10 min as indicated in Material and Methods section, and a signal with the spectral features (a_H = 15 G, g = 2.0062) of $^\bullet OH$ was recorded (Fig. 3). The addition of Fe-EDTA, as a positive control, increased the recorded EPR signal of $^\bullet OH$ (Fig. 3). Even though the addition of isolated rat liver FER to the homogenate did not increase significantly the $^\bullet OH$ generation rate, the supplementation with either EDTA or ATP in the presence of added FER increased $^\bullet OH$ production rate by 13 and 85.9%, respectively, as compared to the measured values in the absence of the chelator (Table 2). The addition of the reaction medium with DF did not significantly affect the $^\bullet OH$ generation rate neither in the absence of added FER nor in its presence (Table 2).

From these data it can be postulated that the labile Fe pool (LIP) in the homogenate might be increased by the addition of FER. The accessibility of cellular Fe to chelators, such as DF, is commonly used as the criterion of 'lability'. The LIP (Fe^{2+}+Fe^{3+}) in the homogenate was assessed by EPR in the presence of DF, since its favors Fe reduction and chelation as Fe^{2+}. The rat liver homogenates exhibited an EPR signal with the spectral features (g = 4.3) of the Fe^{2+}, and it was drastically increased (40-fold) by the supplementation with 50 μM Fe-EDTA (Fig. 4A), tested as a positive control. The EPR-signal was significantly increased by the addition of 10 μg/ml FER (3-fold), and the effect was not further modified by NADPH supplementation to favor metabolic activity of the tissue (Fig. 4B).

Data in Fig. 5A shows comparatively the western blott profile of carbonyl content resulting from the oxidation of FER isolated from rat liver, before and after incubation with 10 mM AH^-. A drastic increase in the width of the band after incubation with AH^-, suggested alterations to the protein structure. In this regard, the presence of bi-tyrosines in the isolated FER was slightly increased, and total thiols content in the FER was significantly decreased after incubation in the presence of AH^-, as compared to the recorded content after incubation in its absence (Fig. 5 B). Moreover, the FER content of Trp, assessed as the intrinsic fluorescence, was significantly lower when the

incubation was performed in the presence of AH^-, as compared to the recorded content after the same incubation period in its absence (Fig. 5 B).

4. Discussion

Although it is well known that the Fe from FER is recycled and readily available for cellular needs, less is known on the physiological mechanisms of its release. Two mechanisms have been proposed for the release of Fe from FER. Fe could be either released upon the degradation of the FER molecule or released from the intact FER molecule (Aisen, 1991; Deiss, 1983). However, the relative importance of Fe release *in vivo* by the one or the other of these mechanisms is not known. It was suggested that the release of Fe after proteolytic degradation of the protein could be studied only in complex artificial conditions (Koorts and Viljoen, 2007). *In vitro* studies showed that the Fe of the FER core is stable in the absence of reducing agents, does not exchange among molecules and can be released only slowly by strong Fe^{3+} chelators, such as DF (Chasteen, 1998; O`Connell and Peters, 1987). Moreover, it was suggested that O_2^- (Puntarulo and Cederbaum, 1996), and various molecules with a low oxidation potential (Double et al., 1998) might reduce FER Fe and induce its release even in the absence of protein degradation. This mechanism would produce $^{\bullet}OH$ (Arouma and Halliwell, 1987) and may be involved in both physiological responses and pathological conditions. The data presented here showed that the addition of FER to rat liver homogenates has the ability of generating not only $^{\bullet}OH$ but other physiologically relevant reactive species, such as $A^{\bullet}$ radicals, by increasing the LIP in the medium. Thus, even though AH^- is a powerful antioxidant at the cellular level, and FER was long considered as a *safe* way to store Fe, the combination of both components in the same cellular compartment could result in cellular injury. The evidence presented here should be carefully considered when AH^- is used with therapeutic purposes if Fe metabolism is affected, such as in Fe overload, since it could lead to cellular damage instead of exerting protection against oxidative stress, in agreement with Neinhuis et al. (1980).

Zanninelli et al. (2002) reported that LIP levels in liver rat hepatocytes were of approximately 0.2 μM and raised by 1.8-fold following Fe loading, and changes were accompanied by raises by 19-fold in FER-levels, suggesting that LIP measurements provide an “instantaneous” parameter of Fe availability within hepatocytes. The *in vitro* model provided here affords experimental evidence that shows that the supplementation with isolated FER to rat liver homogenates, resulted not only in the generation of active free radicals but also to a significant increase in the amount of Fe available for catalyzing the free radical-generating reactions or for physiologically important

purposes. However, this release of Fe from FER that increases the LIP also leads to damaging effects to the protein itself. Early studies with liposomes by O`Connell and Peters (1987) proposed that FER protein is modified during Fe release both by free radical cleavage and addition reactions with aldehyde products of lipid peroxidation. The results presented here showed that after exposure of the rat liver homogenate to AH^- in the presence of excess of FER, the FER migration in the western blott was abnormal (smeared) appearing as a heterogenous population, and the content of carbonyl groups in the FER as index of oxidation of the protein, was significantly higher than in the absence of AH^-. This observation is consistent with previous observations by Van Eden and Aust (2001) when studying in a chemical system apoFER incorporation of Fe. Moreover, the results shown here indicate that Trp content of the protein was also decreased, suggesting a deterioration of the FER structure that could lead to protein degradation. Also, it was suggested that the release of Fe from the intact FER molecule was sensitive to changes in conserved amino acids near the outside of the FER channels which are likely to be involved in regulating the localised unfolding of the protein shell in order to open the channels and release the reduced Fe (Jin et al., 2001). This seems as an important observation since it could represent that Fe release from FER could be increased by protein degradation. Thus, the physiological mechanism for increasing Fe availability from FER could involve both aspects previously proposed as the required pathways for Fe release. On the other hand, hemosiderin has long been postulated as being derived from FER. Hemosiderin is thought to be formed from the aggregation and the partial degradation of the protein shell by lysosomal enzymes (Grady et al., 1989). From the relationship between FER and hemosiderin it was concluded that hemosiderin peptides are derived from FER peptides and that free radical catalyzed reactions are likely involved in the transformation (O`Connell and Peters, 1987). Furthermore, studies of the amino acid sequence of both FER and hemosiderin showed that hemosiderin has a relatively low abundance of thiol and aromatic residues, residues that are likely to react with $^{\bullet}OH$ (O`Connell and Peters, 1987). The data presented here showed that, under the conditions tested here, not only these residues were affected by the treatment, but protein primary structure started to be affected since Trp content was decreased. The results of the present study lend support to this hypothesis. Clearly, radicals are produced during Fe release from FER and these radicals coupled with the presence of Fe^{2+} within the environment of the shell of the FER, may result in the oxidation and

fragmentation of the nearby amino acids. However, it is unclear to what extent radical production in the cell contributes to hemosiderin formation since its depends on Fe^{2+} and O_2 concentration and their flux in the environment of the FER. However, under pathological conditions FER Fe core solubilization and recycling involving the production of pro-oxidant intermediates could be a key factor contributing to make FER Fe potentially toxic. In this regard, it was showed that oxidative damage (Arosio et al., 2008), and the expression of L-FER pathogenic mutants linked to neuroferritinopathy increase the proteasoma FER degradation pathway (Cozzi et al., 2006), confirming that multiple routes of FER degradation can occur through different mechanisms which can vary in different cellular conditions.

Taken as whole, the data presented here are experimental evidence, employing a biological medium (rat liver homogenate), that showed that radical generation is associated to FER Fe release, and moreover that the protein itself was affected during this process. However, it is critical in order to get an integrative picture of the cellular situation, to take into account the central role of FER as a cytoprotective protein, since its main function is to serve as a Fe storage depot, as supported by several *in vivo* experiments with cells and animals (Galatro et al., 2007). Thus, the observations reported here may be relevant to understand FER Fe release and to further analyze secondary effects linked to several pathologies involving Fe metabolism where FER ability to safely store Fe could be overwelhemed. FER remains a central protein of Fe homeostasis and crucial aspects of its biological functions need to be clarified to get new insights on many disorders including neurogeneration and senescence.

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6. REFERENCES

Aisen, P., 1991. Ferritin receptors and the role of ferritin in iron transport. Targeted Diag. Ther. 4, 339-354.

Arosio, P., Ingrassia, R., Cavadini, P., 2008. Ferritins: A family of molecules for iron storage, antioxidation and more. Biochim. Biophys. Acta. doi: 10.1016/j.bbagen.2008.09.004.

Arosio, P., Levi, S., 2002. Ferritin, iron homeostasis, and oxidative damage. Free Radic. Biol. Med. 33, 457-463.

Arouma, O.I., Halliwell, B., 1987. Superoxide-dependent and ascorbate-dependent formation of hydroxyl radicals from hydrogen peroxide in the presence of iron. Are lactoferrin and transferrin promoters of hydroxyl radical generation? Biochem. J. 241, 273-278.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Brumby, P.E., Massey, V., 1967. Determination of nonheme iron, total iron and cooper. Meth. Enzymol. 10, 463-474.

Buettner, G.R., Chamulitrat, W., 1990. The catalytic activity of iron in synovial fluid as monitored by the ascorbate free radical. Free Radic. Biol. Med. 8, 55-56.

Buettner, G.R., Jurkiewicz, B.A., 1993. Ascorbate free radical as a marker of oxidative stress: an EPR study. Free Radic. Biol. Med. 14, 49-55.

Chasteen, N.D., 1998. Ferritin. Uptake, storage, and release of iron. Met. Ions Biol. Syst. 35, 479-514.

Cozzi, A., Santambrogio, P., Corsi, B., Campanella, A., Arosio, P., Levi, S., 2006. Characterization of the l-ferritin variant 460InsA responsible of a hereditary ferritinopathy disorder. Neurobiol. Dis. 23, 644-652.

Deiss, A., 1983. Iron metabolism in reticuloendothelial cells. Semin. Hematol. 20, 81-90.

Double, K.L., Maywald, M., Schmittel, M., Riederer, P., Gerlach, M., 1998. In vitro studies of ferritin iron release and neurotoxicity. J. Neurochem. 70, 2492-2499.

Galatro, A., Rousseau, I., Puntarulo, S., 2007. Ferrritin role in iron toxicity in animals and plants. Res. Trends Curr. Topics Toxicol. 4, 65-76.

Galleano, M., Aimo, L., Puntarulo, S., 2002. Ascorbyl radical/ascorbate ratio in plasma from iron overloaded rats as oxidative stress indicator. Toxicol. Lett. 133, 193-201.

Grady, J.K., Chen, Y., Chasteen, N.D., Harris, D.C., 1989. Hydroxyl radical production during oxidative deposition of iron in ferritin. J. Biol. Chem. 264, 20224-20229.

Haber, F., Weiss J., 1934. The catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. Ser. A. 147, 332-333.

Hubel, C.A., Kagan, V.E., Kisin, E.R., McLaughlin, M.K., Roberts, J.M., 1997. Increased ascorbate radical formation and ascorbate depletion in plasma from women with preeclampsia: implications for oxidative stress. Free Radic. Biol. Med. 23, 597-609.

Hynes, M.J., Coinceanainn, M.O., 2002. Investigation of the release of iron from ferritin by naturally occurring antioxidants. J. Inorg. Biochem. 90, 18-21.

Jacobs, A., 1977. Low molecular weight intracellular iron transport compounds. Blood 50, 433-439.

Jin, W., Takagi, H., Pancorbo, B., Theil, E.C., 2001. "Opening" the ferritin pore for iron release by mutation of conserved amino acids at interhelix and loop sites. Biochemistry 40, 7525-7532.

Klein, S.M., Cohen, G., Lieber, C.S., Cederbaum, A.I., 1983. Increased microsomal oxidation of hydroxyl radical scavenging agents and ethanol after chronic consumption of ethanol. Arch. Biochem. Biophys. 223, 425-432.

Koorts, A.M., Viljoen, M., 2007. Ferritin and ferritin isoforms I: Structure-function relationships, synthesis, degradation and secretion. Arch. Physiol. Biochem. 113, 30-54.

Kotake, Y., Tanigawa, T., Tanigawa, M., Ueno, I., Allen, D.R., Lai, C.S., 1996. Continuous monitoring of cellular nitric oxide generation by spin trapping with an iron-dithiocarbamate complex. Biochim. Biophys. Acta 1289, 362-368.

Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. Meth. Enzimol. 233, 346-357.

Nienhuis, A.W., Griffith, P., Strawczynski, H., Henry, W., Borer, J., Leon, M., Anderson, W.F., 1980. Evaluation of cardiac function in patients with thalassemia major. Ann. N. Y. Acad. Sci. 344, 384-396.

O`Connell, M.J., Peters, Y.J., 1987. Ferritin and haemosiderin in free radical generation, lipid peroxidation and protein damage. Chem. Phys. Lipids 45, 241-249.

Puntarulo, S., Cederbaum, A.I., 1988. Comparison of the ability of ferric complexes to catalyze microsomal chemiluminescence, lipid peroxidation, and hydroxyl radical generation. Arch. Biochem. Biophys. 264, 482-491.

Puntarulo, S., Cederbaum, A.I., 1993. Stimulation of microsomal chemiluminescence by ferritin. Biochim. Biophys. Acta 1157, 1-8.

Puntarulo, S., Cederbaum, A.I., 1994. Ferritin-dependent inactivation of microsomal glucose-6-phosphatase. Biochim. Biophys. Acta 1200, 41-47.

Puntarulo, S., Cederbaum, A.I., 1996. Role of cytochrome P_{450} in the stimulation of microsomal production of reactive oxygen species by ferritin. Biochim. Biophys. Acta 1289, 238-246.

Rousseau, I., Robello, E., Galatro A., Puntarulo, S., 2008. Ferritin effect on ascorbyl radical content in biological systems, in: Alvarez, S., Evelson, P. (Eds), Free Radical Pathophysiology. Transworld Research Network, Kerala, India, pp.73-84.

Saito, M., Thomas, C.E., Aust, S.D., 1985. Paraquat and ferritin-dependent lipid peroxidation. Free Radic. Biol. Med. 1, 179-185.

Sedlak, J., Lindsay, R.H., 1968. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. Anal. Biochem. 25, 192-205.

Skrzydlewska, E., Farbiszewski, R., 1997. Liver and serum antioxidant status after methanol intoxication in rats. Acta Biochim. Pol. 44, 139-145.

Takafuji, V.A., Sharova, L.V.Crisman, M.V., Howard R.D., Nucleotide sequence. Submitted (May - 2002) to the EMBL/GenBank/DDBJ databases.

Thomas, C.E., Morehouse, L.A., Aust, S.D., 1985. Ferritin and superoxide-dependent lipid peroxidation. J. Biol. Chem. 260, 3275-3280.

Van Eden, M., Aust, S.D., 2001. The consequences of hydroxyl radical formation on the stoichiometry and kinetics of ferrous iron oxidation by human apoferritin. Free Radic. Biol. Med. 31, 1007-1017.

Woodmansee, A.N., Imlay, J.A., 2002. Quantitation of intracellular free iron by electron paramagnetic resonance spectroscopy. Meth. Enzymol. 349, 3-9.

Zanninelli, G., Loréal, O., Brissot, P., Konijn, A.M., Slotki, I.N., Hider, R.C., Ioav Cabantchik, Z., 2002. The labile iron pool of hepatocytes in chronic and acute iron overload and chelator-induced iron deprivation. J. Hepatol. 36, 39-46.

LEGENDS TO FIGURES

Figure 1: **A.** Characterization of isolated FER. SDS gel was performed as indicated in Materials and Methods section. Line 1 shows the molecular weight standards, line 2 commercially available FER from horse spleen, and line 3 isolated FER from rat liver. **B**. Fe released from FER as a function of time. The Fe released from FER in the absence (●) or presence of 1 (○), 3 (▼) and 5 (∇) mM AH^- over a 20 min period. **Insert**: Rate of Fe release from FER as a function of AH^- concentration.

Figure 2: $A^\bullet$ radical generated by rat liver homogenates. **A**. Typical EPR spectra of the $A^\bullet$ radical. (a) Spectra of computer simulated EPR spectra using the parameters: a_H = 1.88 G, g = 2.0054; (b) spectra in the presence of Fe-EDTA (1:2) 50 μM; (c) typical EPR spectra of rat liver homogenates; (d) in the presence of FER (10 μg/ml) and (e) in the absence of homogenates. **B**. Quantification of the spectra of the $A^\bullet$ radical in the presence of Fe complexes.
FW: stands for Fresh Weight. Results are from experiments carried out in duplicate and replicated with three different preparations.
*significantly different from $A^\bullet$ content in rat liver homogenates ($p \leq 0.01$, ANOVA).

Figure 3: $^\bullet OH$ radical generated by rat liver microsomes. Typical EPR spectra of the $^\bullet OH$ radical. (a) Spectra of computer simulated EPR spectra using the parameters: a_H = 15 G, g = 2.0062; (b) typical EPR spectra of rat liver microsomes incubated as indicated in Materials and Methods section during 10 min; (c) in the presence of 10 μg/ml FER; (d) in the presence of 10 μg/ml FER and 1 mM EDTA; (e) in the absence of microsomes.
Results are from experiments carried out in duplicate and replicated with four different preparations.

Figure 4: LIP content in the homogenate. **A**. (a) Typical spectra of rat liver homogenates with the spectral features (g = 4.3) of the Fe^{2+}; (b) spectra in the presence of 10 μg/ml FER; (c) spectra in the absence of homogenates, and (d) spectra of 50 μM Fe-EDTA, please note that the scale for this spectrum is 10-times smaller that the used

in the rest of the spectra. **B**. Quantification of the LIP. Effect of FER addition. NADPH (100 μM) was added where indicated.

Results are from experiments carried out in duplicate and replicated with three different preparations.

*significantly different from values in rat liver homogenates ($p \leq 0.01$, ANOVA).

Figure 5: Effect of AH^- exposure to FER. **A**. Western blott profile showing the oxidation of the FER protein isolated from rat liver after incubation in the absence and the presence of 10 mM AH^-. **B**. Effect to the FER protein (100 μg) of exposure to 10 mM AH^- on Trp (▨), bi-tyrosine (▩) and total thiol (□) content. ΔF stands for the difference in fluorescence between the values at 0 and 10 min of incubation. The incubation was performed either in the presence or the absence of AH^-. The ΔF represents a decrease for the Trp content and an increase for the bi-tyrosine content. Total thiol content is shown as the difference between the values at 0 and 10 min of incubation. The value of this difference is shown both in the presence or the absence of AH^- during the incubation period. The bars represent decreases in total thiol content after the incubation procedure under each condition.

Results are from experiments carried out in duplicate and replicated with three different preparations.

*significantly different from values obtained when incubations were performed in the absence of AH^- ($p \leq 0.01$, ANOVA).

Table 1

Effect of the addition of Fe chelators on the A• in rat liver homogenates supplemented with isolated rat liver FER.

	A• content (pmol/mg FW)	
	- FER	+ FER
rat liver homogenates	2.2 ± 0.3	6.6 ± 0.8*
+ 100 μM NADPH	2.0 ± 0.1	7.1 ± 0.9*
+ 100 μM EDTA	3.8 ± 0.4**	8.3 ± 1.1*
+ 1 mM DF	1.2 ± 0.1	6.1 ± 0.3*
+ 50 μM citrate	2.8 ± 0.1	7.4 ± 0.4*

*significantly different from A•content in rat liver homogenates in the absence of FER under the same experimental conditions ($p \leq 0.01$, ANOVA). 10 μg/ml of FER were exogenously added.

**significantly different from A• content in rat liver homogenates in the absence of FER without any other addition ($p \leq 0.01$, ANOVA).

FW: stands for Fresh Weight

Table 2

Effect of the addition of Fe chelators on the •OH generation by rat liver microsomes supplemented with isolated rat liver FER.

	•OH generation rate (pmol/s)	
	- FER	+ FER
rat liver microsomes	74 ± 10	92 ± 10
+ 1 mM ATP	128 ± 10**	238 ± 10*,***
+ 1 mM EDTA	293 ± 10**	330 ± 10*,***
+ 1 mM DF	55 ± 10	74 ± 10

*significantly different from •OH generation rate by rat liver microsomes in the absence of FER under the same experimental conditions ($p \leq 0.01$, ANOVA). 10 μg/ml of FER were exogenously added.

**significantly different from •OH generation rate by rat liver microsomes in the absence of FER and any other addition ($p \leq 0.01$, ANOVA).

***significantly different from •OH generation rate by rat liver microsomes in the presence of FER and no other addition ($p \leq 0.01$, ANOVA).