BioMetals 1996, **9**, 98–103

'Free' iron, as detected by electron paramagnetic resonance spectroscopy, increases unequally in different tissues during dietary iron overload in the rat

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Received 8 April 1995; accepted for publication 24 July 1995

'Free' iron concentration, as determined by electron paramagnetic resonance (EPR) spectroscopy, and lipid peroxidation (LPO), as determined by thiobarbituric acid test, were assessed in the lung, heart, liver, spleen, brain and kidney of rats subjected to experimental iron overload. Two tests, Desferal- and NO-available iron, were used to measure 'free' iron and gave comparable results. The most pronounced accumulation of 'free' iron was observed in liver, kidney and spleen. Differences between control and iron loaded animals increased during the initial 90 days of treatment. Between 90 and 180 days 'free' iron concentration reached a steady state level, or even decreased, as in the case of liver. Lipid peroxidation level, measured in the organs of both treated and matched controls, did not give any significant difference during the initial 90 days of treatment. A significant augmentation was observed in liver, kidney, spleen and heart at 180 days. The results of the present research show that, under conditions of moderate siderosis, the occurrence of LPO is partially related to the level of 'free' iron.

Keywords: electron paramagnetic resonance spectroscopy, free iron, iron overload, lipid peroxidation

Introduction

Transition metal ions, in particular iron, are important mediators of oxidative damage in the organism (Halliwell & Gutteridge 1986, Halliwell & Gutteridge 1992, Lauffer 1992). A highly sophisticated system, present both in the intra- and extracellular compartment, usually precludes the possibility of potentially dangerous, metal-driven, oxidative reactions. Iron transport and storage proteins, like ferritin, transferrin and haemosiderin, efficiently control iron reactivity (Reif 1992). However, minute amounts of intracellular iron may exist in a form defined as 'free' (Halliwell *et al.* 1988, Kuzuya *et al.* 1990, Ferrali *et al.* 1993). Low molecular mass, non-transport (or storage) protein-bound iron complexes, sometimes defined also as desferrioxamine chelatable, are thought to represent an iron species catalytically active in initiating free radical reactions in a biological environment. In order to make iron available to the various cell compartments, the metal has to be released from transport and storage proteins, giving rise to a steady state concentration of 'free' iron within the cell. Any increase of this pool will cause a real danger for the cell, because of the destructive processes catalysed by iron, now 'free' to react.

'Free' iron is thought to increase within the cell either when released from ferritin or when excess iron saturates transport and storage systems. During experimental iron overload, total iron concentration in tissues increases (Bacon & Neill 1985, Iancu 1994). A correlation between the degree of siderosis and the extent of functional disorder has been described in a number of studies (Bacon *et al.* 1993, Pietrangelo *et al.* 1989, Whittaker *et al.* 1994). An increase in malondialdehyde and 4-hydroxynonenal protein adducts has been reported in the plasma and liver of rats with iron overload (Houglum *et al.* 1990, Whittaker *et al.* 1994). Increased hepatic chemiluminescence (Galleano & Puntarulo 1992) and conjugated dienes (Botti *et al.* 1989) in mild or moderate iron overload were also observed.

In a widely accepted view, at an early stage of iron overload, ferritin and other iron transport proteins are capable of preventing iron-mediated toxicity; while the oxidative damage and the alteration of the antioxidant status

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is observed after a long-term increase in iron concentration.

Nearly all the studies on iron overload-related effects have been carried out in the liver; few reports have taken in consideration different organs, like heart (Whittaker *et al.* 1994) and lung (Iancu *et al.* 1987, Sullivan *et al.* 1989). However, different tissues may well have different capacity to allocate iron under dietary load and different tissues may also have different sensitivity to iron.

The aim of this work was to study the time course of 'free' iron concentration in different rat tissues during experimental iron overload and to correlate it to a marker of lipid peroxidation (LPO). We chose to employ the method described by Kozlov *et al.* (1992), which utilizes electron paramagnetic resonance (EPR) spectroscopy in order to measure 'free' iron concentration and test LPO using the thiobarbituric acid as described by Mihara *et al.* (1980).

Materials and methods

Animals and diet

Female Wistar albino rats (100–120 g body weight) were purchased from Morini (Reggio Emilia, Italy). Rats were made siderotic by feeding a standard diet purchased from Piccioni (Brescia, Italy) supplemented with 2.5% (w/w) carbonyl iron, purchased from Fluka Chimica. Carbonyl iron is an extremely pure form of elemental iron in the form of microscophic spheres less than 5 μm in size.

Chemicals

Desferal (desferrioxamine) was obtained from Ciba-Geigy (Basel, Switzerland), 2-thiobarbituric acid (TBA) from Merck (Darmstadt, Germany), phosphoric acid, *n*-butanol, Tris and sodium chloride from BDH (Poole, UK).

Preparation of tissue homogenates

Lung, heart, liver, spleen, brain and kidney tissues were kept in 0.9% NaCl at 5°C. Tissues were washed thoroughly in order to remove blood and immediately frozen in liquid nitrogen, stored at −84°C and processed within 2 weeks. Tissues (1:2 w/v) were homogenized at 20°C in 10 mM Tris, 120 mM KCl, pH 7.4. Measurements were performed on the homogenates immediately after the preparation.

'Free' iron determination

'Free' iron concentration was measured by EPR spectroscopy as described in Kozlov *et al.* (1992) using two assays.

Nitrite assay (NO assay). Sodium nitrite (0.05 ml, 8.7 M) was added to 0.5 ml of homogenate. The mixture was incubated at 20°C for 10 min. Nitric oxide (NO), produced from the reduction of sodium nitrite, gives rise to a dinitrosyl iron complex with endogenous Fe^{2+}, characterized by an EPR absorption (Figure 1B) at $g = 2.03$ (Vanin 1967, Woolum

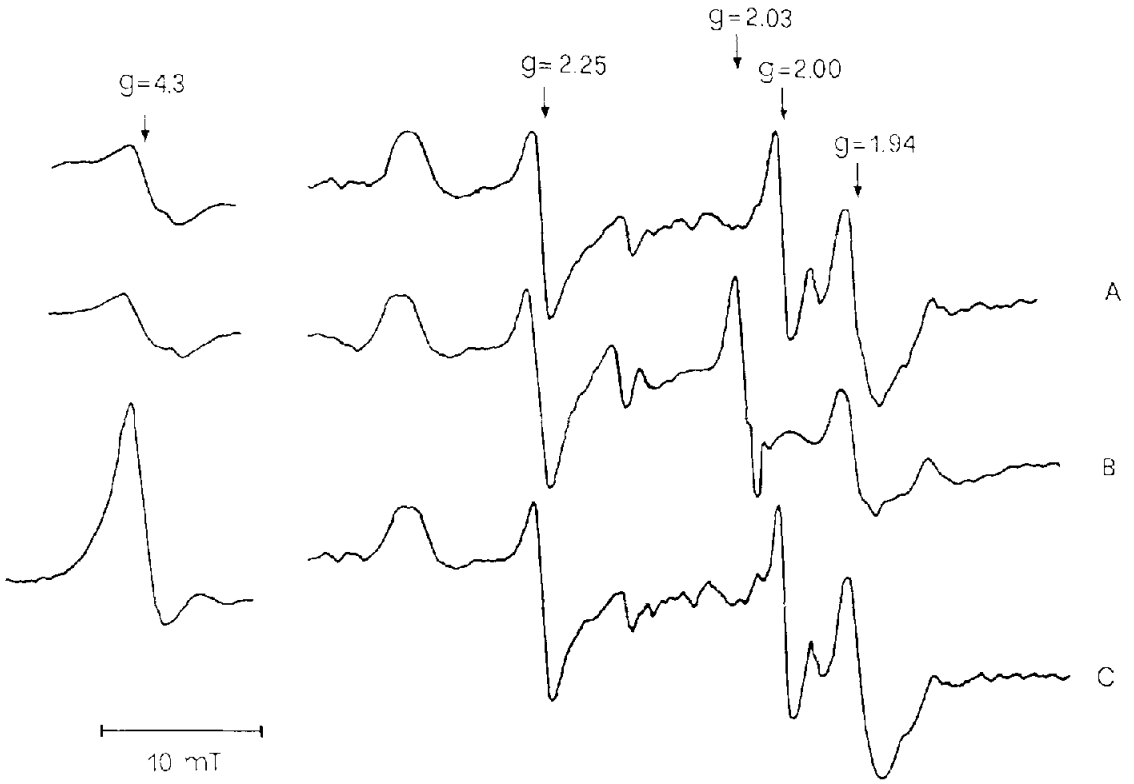

Figure 1. EPR spectra of 'free' iron in rat liver homogenate as detected by NO assay and DF assay. Spectrum A shows the EPR signals in control liver homogenate; spectrum B is from sodium nitrite treated liver homogenate (NO assay) and C from Desferal-treated (DF assay). The g = 2.03 signal (spectrum B) has been recorded at 0.125 × instrumental gain.

et al. 1968). The homogenate was placed in a cylindrical Teflon tube, frozen and stored in liquid nitrogen until use. EPR spectra were recorded on a Bruker 200 spectrometer at liquid nitrogen temperature under the following conditions: oscillator Klystron frequency 9.12 GHz, power 2.5 mW, modulation amplitude 0.25 mT. The intensity of the low field peak of $g=2.03$ signal was measured to estimate the concentration of nitrosyl-iron complexes.

Desferal assay (DF assay). The procedure was the same as described for the NO assay, but 0.05 ml of 10 mM Desferal was added instead of sodium nitrite. The intensity of the signal (Figure 1C) at $g=4.3$ was measured to estimate Desferal-iron concentration. The measurements were carried out at the following conditions: oscillator Klystron frequency 9.12 GHz, power 20 mW, modulation amplitude 2.0 mT. As a control for both the assays, an EPR spectrum of the same liver homogenate was recorded in the absence of Desferal and sodium nitrite (Figure 1A).

Calibration procedures. 'Free' iron concentration was calculated on a calibration plot obtained by adding incremental volumes of $FeSO_4 \cdot 7H_2O$ to tissue homogenate. Under these conditions, the relevant amount of reducing equivalents present in the homogenate maintains iron in the reduced form (Kozlov *et al.* 1992). The concentration of Fe^{2+} in the stock solution was measured using the *o*-phenanthroline assay (Vladimirov *et al.* 1990). The homogenate was then treated as described above and EPR spectra taken. Since no difference in the line-width of the spectra was observed under these experimental conditions, the calibration curve was built using the amplitude of the EPR signal and the concentration of added iron.

Thiobarbituric reactive substances (TBARS)

TBARS were determined as described (Mihara *et al.* 1980), with minor modifications (Kozlov *et al.* 1991): 0.25 ml of the tissue homogenate was mixed with 3.0 ml of 1.5% phosphoric acid and 1.0 ml of 0.5% TBA. Samples were incubated at 100°C for 45 min then cooled at room temperature and extracted in 4.0 ml *n*-butanol; the mixture was stirred and centrifuged at 1800 *g* for 10 min. The upper butanol fraction was collected and absorption spectra were recorded in the 514 554 region. The intensity of the 534 nm peak was measured with two base points at 514 and at 554 nm.

Statistics

Statistical significance was determined using Student's *t*-test for unpaired data.

Results

'Free' iron concentration and TBARS levels were measured in lung, heart, liver, spleen, brain and kidney at 0, 20, 40, 60, 90 and 180 days of iron-rich diet. In order to take into account possible variations of 'free' iron concentration in untreated animals, rats matching in age were kept on a control diet and sacrificed at the same experimental data point. Table 1 presents 'free' iron concentration observed in the different tissues of control rats. The DF assay gave a 'free' iron concentration lower than that observed with the NO assay, in agreement with previously described results (Kozlov *et al.* 1991). The relative distribution of iron within the tissues was, in any case, similar and independent of the test used.

Using the DF assay, 'free' iron concentration in tissues increased in the following order: lung $\leqslant$ brain < heart < spleen $\leqslant$ kidney < liver. In the case of the NO assay the observed order was: lung $\leqslant$ brain < spleen < kidney < heart < liver. The only difference in the ranking regarded the heart: third in the DF assay, fifth in the NO assay.

'Free' iron concentration, as measured by the NO assay during iron overload, is given in Figure 2 for liver and in Figure 3 for other tissues. Control values were measured at the same time on age-matched, control rats. The most remarkable increase in 'free' iron concentration observed in treated animals took place in the liver, where a significant difference was observed at 20 days of treatment. The increment continued during the whole experimental period up to a 10-fold increase at 90 days.

Approximately a 3-fold increase of 'free' iron level was observed at 60 and 90 days in the kidney and in the spleen. The lung, heart and brain of the same treated animals did not show any significant difference in 'free' iron concentration.

At 6 months of treatment, 'free' iron concentration in liver, kidney and spleen was inferior to the values observed at 90 days; however, significantly higher than in control animals.

The same measurements were carried out using the DF assay. Figures 2 and 4 summarize the results. 'Free' iron concentration in liver homogenate was consistently and significantly increased over the control value at any data point. A 20-fold increase was recorded in the liver at 90

Table 1. The mean values of 'free' iron concentrations in intact tissues of control rats, as estimated by the NO and DF assays (values are expressed in nmol Fe g^{-1} tissue)

	Brain	Lung	Heart	Spleen	Kidney	Liver
NO assay	4.98 ± 0.75^a	3.06 ± 0.33^a	36 ± 3.3^a	11.7 ± 1.59	26.91 ± 1.92^a	38.97 ± 5.07^a
DF assay	0.99 ± 1.08	0.78 ± 0.81	5.55 ± 1.29	11.73 ± 1.98	11.55 ± 2.13	18.63 ± 3.45

[a] $P < 0.05$, Student's *t*-test for unpaired data.

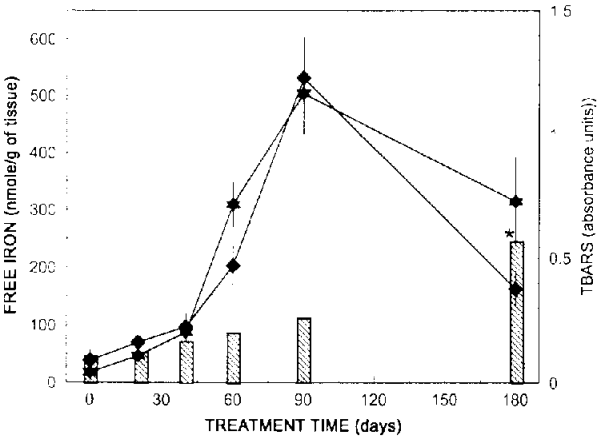

Figure 2. The time course of 'free' iron concentration and TBARS in liver tissue obtained from rats subjected to experimental iron overload. 'Free' iron concentration was measured by DF assay (◆) and NO assay (★) as described in Materials and methods. Empty bars indicate the amount of TBARS recorded at the same data point. $^{*}P \leq 0.05$ from the control value.

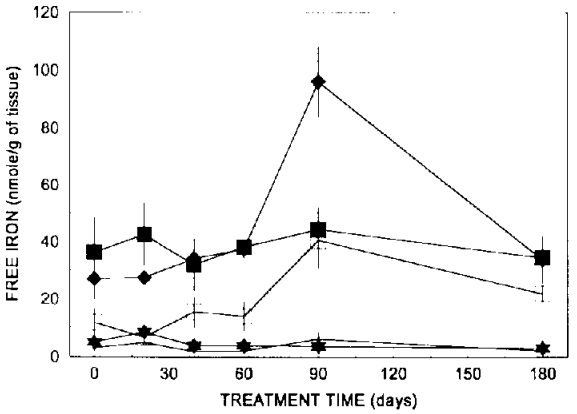

Figure 3. The time course of 'free' iron concentration in kidney (◆), heart (■), spleen (□), brain (★) and lung (*). 'Free' iron concentration was measured by the NO assay.

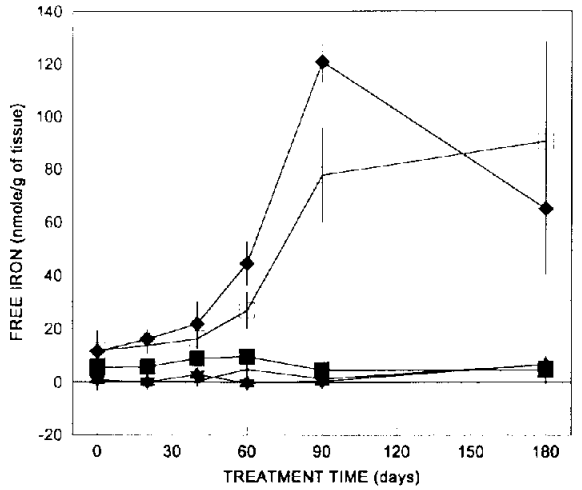

Figure 4. The time course of 'free' iron concentration in kidney (◆), heart (■), spleen (□), brain (★) and lung (*). 'Free' iron concentration was measured by the DF assay.

days; 'free' iron concentration increased in the kidney (6.5 times) and in the spleen (4.5 times). Different from the NO assay, the DF assay gave evidence of a significant iron increase also in the lung at 90 and 180 days of treatment. 'Free' iron concentration in the different tissues reached a steady state level at 90 days; afterward a certain decrease was observed in the case of the liver.

In short, tissues under iron-overload can be divided into three groups. (1) Liver, with a 10-fold increase in iron concentration; (2) kidney, spleen and lung, with a 4- to 7-fold increase; and (3) heart and brain, with no significant change in 'free' iron concentration.

TBARS, measured both in treated and matched controls, did not give any significant difference during the initial 90 days of treatment. Figure 2 reports the TBARS values observed in the liver during the entire experimental period, as compared to the variation of 'free' iron concentration. Table 2 reports the TBARS values observed in different tissues at 180 days, where a significant increase was observed for liver, kidney, spleen and heart. No significant difference was observed in the lung and brain at any time period.

Discussion

Two different tests, i.e. the NO and DF assay, have shown an increase in 'free' iron concentration in the liver, kidney and spleen of rats treated with an iron-rich diet. According to the DF assay only, a significant 'free' iron increase was observed also in the lung. Heart and brain 'free' iron concentration was not affected by the treatment.

It has been suggested that loosely bound non-haem iron ('free' iron) may initiate free radical processes, as ascorbate oxidation and LPO, and cause post-ischemic damages (Mergner *et al.* 1991). It has been proposed that the availability of catalytically active 'free' iron, rather than O_2^- or H_2O_2, is indeed the limiting factor in the development of the injury (Karwatowskaprokopczuk *et al.* 1992, Minetti *et al.* 1992, Minotti 1993).

An extensive literature indicates that Desferal-available iron can be considered as catalytically active in various experimental model systems. The addition of Desferal consistently inhibits free radical processes induced by the addition of exogenous iron, or following the release of 'free' iron from transport or storage proteins (Miller *et al.* 1992, Latour *et al.* 1992, Fantini & Yoshioka 1993).

In this study we have used two assays, the first assesses the Desferal available iron, the second utilizes sodium nitrite as a source of NO, which forms a dinitrosyl complex with catalytically active Fe^{2+}. We found statistically significant differences using the two methods when measuring 'free' iron in control tissues. This is explained by the extremely low concentration of 'free' iron present in the tissues and the detection of such low amount is at the lowest part of our titration curve, where the uncertainty is maximum (Table 1).

However, in treated rats when iron concentration increases (Figures 2–4), no significant differences between the two methods were noted, but in the myocardium; in fact,

Table 2. Effect of iron overload on TBARS concentration in different rat tissues after 180 days of treatment (results are expressed in absorbance units)

	Brain	Lung	Heart	Spleen	Kidney	Liver
Control	0.104 ± 0.006	0.045 ± 0.003	0.029 ± 0.004	0.050 ± 0.038	0.047 ± 0.013	0.101 ± 0.052
Iron	0.074 ± 0.006	0.060 ± 0.012	0.051 ± 0.011[a]	0.133 ± 0.024[a]	0.146 ± 0.042[a]	0.550 ± 0.185[a]

[a] $P < 0.05$. All other conditions as in Table 1.

myocardium ranked third in the DF assay and fifth in the NO assay (Table 1).

Equally, the more pronounced relative changes in iron concentration observed in the case of the DF assay between control and iron overload rats are related to the lower initial 'free' iron concentration observed in control tissues, than to a genuine increase observed in treated rats. In fact, the absolute amount of iron measured by both assays in treated animals is not significantly different (Figures 3 and 4)

Catalytically active iron ions induce LPO, and a strict correlation between iron concentration and LPO products accumulation, as measured by TBARS, has been demonstrated (Fantini & Yoshioka 1993, Linseman *et al.* 1993, Dabbagh *et al.* 1994). However, these studies report the correlation between total or non-heme iron and LPO, while the presence of catalytically active, low molecular weight iron is hypothesized.

In our model system at 20 days of treatment, the increase in 'free' iron in sensitive tissues was significant. At any data point betwen 20 and 90 days, 'free' iron concentration was significantly increased in the liver, kidney and spleen, but no significant increase in TBARS was observed in these tissues. A significant increase in TBARS was found in liver, kidney, spleen and heart only at 180 days, in spite of the fact that 'free' iron level reached a steady state level or even decreased, as in the liver, at this point.

A possible explanation to our finding may lay in the low level of reducing agents available in tissues during iron overload. 'Free' iron may well accumulate as Fe^{3+} low molecular weight complexes, which do not enter the redox cycle and do not activate LPO. Support to this point is given by experimental evidence indicating a relevant decrease in the liver content of NADH and NADPH (Galleano & Puntarulo 1994, Masini *et al.* 1989) and a partial depletion of ATP (Bacon *et al.* 1993, Ceccarelli *et al.* 1991) during experimental mild siderosis.

It has also to be taken in account that the TBA test estimates a steady state concentration of LPO products and TBARS are quickly eliminated *in vivo*. Under a continuous low-level oxidative stress that steady state concentration of TBARS may well be under the detection limit, differently from what has been observed under acute iron treatment. A further point supporting our finding lays in the reported increase of plasma antioxidant activity in animals during iron overload, surely an efficient protection against the provident 'free' iron mediated activity (Kawabata *et al.* 1989, Sullivan *et al.* 1989).

Thus, the absence of LPO products accumulation in the presence of high 'free' iron concentration is explained by the modification of various equilibria: (1) the balance between reducing and oxidizing agents, (2) the balance between production and metabolism of LPO products, and (3) the balance between pro-oxidant and antioxidant systems.

In conclusion, the results of the present research show for the first time that different tissues have different responses to iron overload. Under conditions of moderate siderosis, the occurrence of TBARS does not correlate with the maximum level of 'free' iron, but rather with the duration of the pathological condition. We suggest that alteration of cell metabolism and the exhaustion of antioxidant defence mechanisms may render the cell more vulnerable to the damaging action of 'free' iron over a long period of time.

Acknowledgements

A.K. thankfully acknowledges the support of the European Science Foundation, grant RF/93/35(T); the research was supported by a grant of the Italian National Research Council CNR, special project 'Ageing'.

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