Iron overload has been associated with damage of the liver and other organs of patients with primary or secondary increased iron load. In order to study the effect of iron overload on the pathophysiology of kidney lysosomes, experimentally induced iron overload models were employed. Iron overload was achieved through intraperitoneal injections of Fe-dextran (Imferon) in male rats, at different final iron concentrations (825 and 1650 mg/kg, single and double dose groups respectively). Controls were injected with dextran following a similar protocol. The animals were killed at different time points after the last injection. Subcellular fractionation studies of kidney homogenates were carried out by differential centrifugation and density gradient centrifugation. The kidney iron load was increased with both doses. Iron appeared to accumulate mainly in the lysosomes, bringing about distinct changes in the behaviour of the organelles as judged by subcellular fractionation studies. Lysosomes became more fragile and showed increased density. The extent of the above changes seemed to correlate with the extent and duration of iron accumulation and could be reversed when the iron load was reduced. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Iron overload; Kidney lysosome; Lysosomal enzyme

1. Introduction

Iron overload has been shown to result in several structural and functional changes in various tissues of patients with primary or secondary increased iron load [1–3]. Lysosomes appear to be a target of iron toxicity and they are believed to play a major role in the pathogenesis of tissue injury [4–9].

Considerable evidence suggests that iron can exert renal toxicity and it has been implicated in the pathogenesis of acute as well as chronic renal diseases. The kidney dysfunction in the latter has been related to the iron-laden lysosomes of the proximal tubular cells [10–14].

Recently a statistically significant increase in the urinary levels of the lysosomal enzyme β-hexosaminidase (β-Hex) (EC 3.2.1.52) has been reported in β-Thalassaemia major patients with increased iron load. Interestingly, the urinary levels of another lysosomal enzyme, α-mannosidase (EC 3.2.1.24), remained normal in the above patients [15].

The aim of the present study was to investigate, in experimentally induced iron overload models, the effect of iron overload on renal lysosomes, the behaviour of lysosomal enzymes as well as the possibility of reversing any injury caused by iron load.
2. Materials and methods

2.1. Fe-dextran injections

Iron overload was achieved in male Wistar rats, weighing about 100 g, through intraperitoneal injection of Fe-dextran (Imferon).

Fe-dextran was given in 11 doses (three doses per week) of 75 mg Fe/kg each (single dose regime) and of 150 mg Fe/kg each (double dose regime). The animals were sacrificed 1 week (group F-1), 2 weeks (group F-2) or 5 weeks (group F-5) after the last injection. Each group consisted of five animals. Control rats were injected with dextran following a similar protocol (groups: D-1, D-2, D-5).

Spleen, liver and kidney were removed and homogenised in an isotonic solution of sucrose (0.25 M) at a final concentration of 5% (w/v). The whole procedure was carried out at 4°C and total iron was assayed in their homogenates. Sections of the three tissues were histochemically stained for haemosiderin (Perl’s stain) according to the method of Lillie and Fullmer [16]. Histochemical stain was performed in eight animals.

2.2. Subcellular fractionation studies

Subcellular fractionation studies were carried out in 5% (w/v) whole kidney homogenate in sucrose 0.25 M, at 4°C, and the pellets collected were resuspended in known volumes of sucrose 0.25 M.

In particular, differential centrifugation studies were carried out according to the method of Shibko and Tappel [17]. Five fractions were obtained containing cell debris and unbroken cells (fraction A), lysosomes-mitochondria (fraction B), mitochondria-microsomes (fraction C), microsomes (fraction D) and cytosol (fraction E). They were characterised by assaying the marker enzymes: acid α-mannosidase, β-Hex, β-galactosidase (EC 3.2.1.23) for lysosomes, cytochrome-c-oxidase (EC 1.9.3.1) for mitochondria, glucose-6-phosphatase (EC 3.1.3.9) for microsomes and lactate dehydrogenase (LDH) (EC 1.1.1.27) for cytosol.

Total protein and total iron were determined both in the whole homogenate and in the fractions obtained.

Total enzyme recoveries ranged from 85 to 101% in all groups studied.

For the Percoll density gradient studies the method of Harikumar and Reeves [18] was used with slight modifications. Thus, the Percoll density gradient was prepared by spinning the gradient mixture (pH 7.0) at 48000×g for 75 min. The mitochondrial-lysosomal fraction was layered on this gradient and after centrifugation at 48000×g for 60 min, 10 fractions were collected, from the lightest (fraction 1) to the densest (fraction 10) fraction. In all fractions lysosomal and mitochondrial marker enzymes were assayed.

2.3. Marker enzymes – total iron and protein assays

Lysosomal enzymes were assayed using the respective fluorescent substrates [19–21].

Glucose-6-phosphatase was assayed by the method of Hers et al. [22] while phosphorus was determined by the method of King and Wooton [23].

LDH and cytochrome-c-oxidase were assayed by the methods of Plummer and Wilkinson [24] and Appelmans et al. [25] respectively.

Total protein was determined by the method of Lowry et al. [26] and total iron by using the Ferkit of bioMérieux (France).

2.4. Hypotonic lysis of lysosomal-mitochondrial fraction

The lysosomal-mitochondrial pellets collected by the method of Shibko and Tappel [17] were resuspended in known volumes of hypotonic sucrose solution 0.085% (w/v). They were subsequently incubated at 37°C for 1 h and then spun at 100000×g for 1 h, at 4°C, to give a supernatant and a pellet. Lysosomal enzymes and total iron were assayed in both fractions.

2.5. Statistical analysis

Three-way analysis of variance was initially used for each of the dependent variables.

The three independent factors were: time, with three levels (1, 2, 5 weeks); Fe administration, with two levels (yes, no); Fe dose, with two levels (single, double).
Since interactions of dose with both time and Fe administration were found to be statistically significant, the data for each dose were subsequently analysed separately by two-way analysis of variance (independent factors: time and Fe administration).

3. Results and discussion

Iron overload has been associated with injury of the liver and other organs in patients with primary or secondary increased iron load [1–3].

Histochemical and subcellular fractionation studies of the liver of such patients as well as animals with experimentally induced iron overload have shown that iron is accumulated in lysosomes as ferritin and haemosiderin [5,27].

Enhanced fragility of hepatic lysosomes, possibly due to the intralysosomal accumulation of iron compounds and/or the lipid peroxidation of lysosomal membranes by OH radicals, a product of the iron-catalysed Haber–Weiss reaction, has been found in experimentally induced iron overload [28,29]. Increases in density [4–6], pH [8] and size as well as changes in the morphology of lysosomes were also observed [6,8,9]. Taken together the above results indicate that hepatic lysosomes are a target of iron toxicity.

Functional renal abnormalities related to tissue deposition of iron have been found in adult patients with β-Thalassaemia/βE disease [30] and increased urinary β-Hex levels have been observed in patients with β-Thalassaemia major with increased iron load [15]. However, the effect of increased iron load on the kidney lysosomes has not been studied as extensively as in the case of the liver.

The aim of the present study was to investigate the effect of different iron loads on kidney lysosomes in an experimentally induced iron overload model.

As judged by total iron quantitation and histochemical staining, iron administration resulted in an increase of kidney iron load and haemosiderin respectively, which was, however, lower than the increase observed in liver and spleen (Tables 1 and 2).

One week after the last injection and irrespective of the administered iron dose, the accumulation of haemosiderin was the same in all tissues studied. It remained unchanged in the double dose groups, whereas a slight reduction was observed in the single dose groups, 2 and 5 weeks after the last injection (Table 1).

The increase of kidney iron load was the same in both dose regimes with a tendency to decrease with time in the single dose regime. In liver and spleen the increase of iron concentration remained more or less constant over the period studied in both the single

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<td>Histochemical stain for haemosiderin in tissues of animals injected with iron (single and double dose regime), 1, 2 and 5 weeks after the last injection (F-1, F-2, F-5)</td>
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<td>Single dose</td>
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<td>Total iron concentration (ng/mg of homogenate protein) in kidney, spleen and liver of animals injected with iron (single or double dose regime) 1, 2 and 5 weeks after the last injection (F-1, F-2, F-5) and the respective controls (D-1, D-2, D-5)</td>
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and double dose regimes, but it was more pronounced in the latter (Table 2).

Statistical analysis of the results in the single dose regime showed a statistically significant interaction of iron and time on the total iron concentration of the kidney ($P = 0.021$). Furthermore, a statistically significant effect of iron on the total iron concentration of the kidney ($P < 0.001$) in the double dose regime as well as on the total iron concentration of the spleen and the liver in both dose regimes (single dose: spleen $P < 0.001$, liver $P < 0.001$; double dose: spleen $P < 0.001$, liver $P < 0.001$) was also found.

The above differences observed in the tissues studied could reflect the different ability of iron uptake by these tissues, which could be related to the different cell types they contain, since the number of transferrin receptors is not the same in all cell types [6,31].

The presence of the iron did not affect the intralysosomal distribution of the lysosomal enzymes ac-
According to the results of the hypotonic lysis of mitochondrial-lysosomal pellets. Thus β-Hex, α-mannosidase and iron cosedimented in the membrane fraction (82, 79, 80% of the total recovered activity respectively), whereas β-galactosidase was recovered in the supernatant (81% of the total recovered activity).

Iron administration resulted in small but consistent changes in the distribution of lysosomal enzymes, the profile of which was similar to that of iron at all time points studied, indicating that iron was mainly accumulated in the lysosomes. Thus, irrespective of the administered dose, an increase of β-Hex, α-mannosidase and total iron was observed in fraction D, whereas an increase of β-galactosidase was observed in fraction E. The observed increase showed a tendency to normalisation 5 weeks after the last injection only in the single dose regime (Figs. 1–4).

Statistically significant additive effects of both iron...
and time on the percentage recovered activity of α-mannosidase in fraction D (single dose: iron $P < 0.001$, time $P < 0.001$; double dose: iron $P < 0.001$, time $P = 0.008$) and of β-galactosidase in fraction E (single dose: iron $P = 0.022$, time $P = 0.004$; double dose: iron $P < 0.001$, time $P = 0.014$) as well as on the percentage recovered concentration of iron in fraction D (single dose: iron $P < 0.001$, time $P = 0.002$) were found. A statistically significant effect of iron on the percentage recovered activity of β-Hex in fraction D (single and double dose: $P < 0.001$) and on the percentage recovered concentration of iron in the same fraction (double dose: $P < 0.001$) was also observed.

In contrast to the single dose regime, where the distribution profile of the other parameters did not change in a statistically significant way, in the double dose and at all time points a reduction of all the parameters studied was observed in fraction A, except for cytochrome-c-oxidase which showed an increase.

In particular, a statistically significant effect of iron
on the percentage recovered activity of β-Hex
\( (P < 0.001) \), α-mannosidase \( (P = 0.001) \), β-galactosidase \( (P < 0.001) \), LDH \( (P = 0.044) \), glucose 6-phosphatase \( (P = 0.002) \), cytochrome-c-oxidase \( (P = 0.009) \) and on the percentage recovered concentration of protein \( (P = 0.042) \) and iron \( (P < 0.001) \) in fraction A was found.

The above changes could be the result of increased fragility of iron-loaded lysosomes and/or membrane disruption.

Increasing the administered iron dose had a longer lasting effect as the iron load was not reduced over the period studied, remaining with haemosiderin at the same level and affecting the whole cell. Thus, although in the single dose regime the distribution of the lysosomal enzymes tended to normalise 3 weeks after the decrease of iron load, in the double dose regime it remained unchanged at all time points studied. The observation that the reversal of the effect of iron was not immediate indicated that the

Fig. 4. Distribution profile of the iron percentage recovered concentration in the subcellular fractionation studies (mean ± S.D.).
The effect of iron is not purely a mechanical one but it also involves chemical modifications of the constituents of the lysosomal membrane such as lipid peroxidation.

In accordance with previous observations regarding liver lysosomes [4–6], iron administration in the present study brought about an increase in the density of kidney lysosomes.

The distribution of the enzymes in the 10 fractions collected after Percoll gradient centrifugation is presented in Figs. 5–7.

A shift of the distribution of lysosomal enzymes to higher density fractions occurred at all time points studied in the double dose but only 1 week after the last injection in the single dose regime.

In the single dose regime a statistically significant effect of iron on the percentage recovered activity of β-Hex ($P = 0.012$), α-mannosidase ($P = 0.005$) and β-
galactosidase \( (P = 0.004) \) in fraction 6 and a statistically significant interaction of iron and time on the percentage recovered activity of \( \beta \)-Hex \( (P = 0.015) \), \( \alpha \)-mannosidase \( (P = 0.048) \) and \( \beta \)-galactosidase \( (P = 0.031) \) in fraction 7 was found.

In the double dose regime statistically significant additive effects of both iron and time on the percentage recovered activity of \( \beta \)-Hex \( (P = 0.033, \) time \( P = 0.008) \) and of \( \beta \)-galactosidase \( (P = 0.027, \) time \( P = 0.002) \) in fraction 6, as well as of \( \beta \)-Hex both in fraction 7 \( (P = 0.025, \) time \( P = 0.030) \) and in fraction 8 \( (\text{iron } P < 0.001, \text{time } P = 0.026) \) was observed. Furthermore a significant interaction of iron and time with the percentage recovered activity of \( \alpha \)-mannosidase and \( \beta \)-galactosidase in fraction 7 \( (\alpha \)-mannosidase: \( P = 0.003 \), \( \beta \)-galactosidase: \( P = 0.003) \), fraction 8 \( (\alpha \)-mannosidase: \( P = 0.008, \beta \)-galactosidase: \( P = 0.001) \) and of all three enzymes in fraction 10 \( (\alpha \)-mannosidase: \( P = 0.002, \beta \)-galactosidase \( P < 0.001, \beta \)-Hex: \( P < 0.001) \) was also found in the double dose regime.

The observed increase in the density of the lyso-

Fig. 6. Distribution profile of the \( \alpha \)-mannosidase percentage recovered activity in Percoll density gradient studies (mean \( \pm \) S.D.).
somes was related to the accumulation of iron and haemosiderin. Thus, in the single dose regime the shift of the lysosomes to higher densities in Percoll density gradients returned to normal 2 and 5 weeks after the last injection when the iron and haemosiderin contents were also reduced, whereas in the double dose regime the iron effect was more pronounced, so that lysosomes shifted to even higher densities and remained so, over the period studied.

In conclusion then, iron overload leads to intralysosomal storage of iron in the kidney, which brings about distinct changes in the behaviour of the organelles in subcellular fractionation studies. Lysosomes become more fragile and show increased density. The extent of the above changes seems to correlate with the extent and duration of iron accumulation and could be reversed when the iron load is reduced, an observation that may be of importance in the treat-
ment of thalassaemic patients and in the understanding of the pathogenesis of the renal involvement in this disorder.

References