PROPERTIES OF LIVER MITOCHONDRIA FROM IRON-LOADED RATS

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Summary: The increased iron content in livers from iron-loaded rats is almost exclusively confined to the mitochondria. The ten- to twenty-fold higher level of nonheme iron in such mitochondria decreases the respiratory control with pyruvate-malate, but not with 3-hydroxybutyrate or succinate as substrates, and has no effect on the capacity for phosphorylation and substrate oxidation. Iron loaded mitochondria have a malondialdehyde level which is about three times higher than that of control mitochondria, even after repeated washings with bovine serum albumin and EDTA. This is suggestive of an on-going process of lipid oxidation presumably catalyzed by the accumulated iron. Differences between the present *in vivo* data and *in vitro* results obtained by others are discussed.

INTRODUCTION

Aberrant function of vital organs is a well-recognized consequence of excessive tissue iron content. Information on the metabolic consequences of iron overload is, however, still scanty. The liver is a major organ of iron deposition; liver iron is increased in idiopathic (familial) hemochromatosis (1), porphyria cutanea tarda (2), states of high dietary iron intake (3), repeated transfusions (4), congenital atransferrinemia (5,6), and also in anemias with aberrant heme biosynthesis (7). In the latter disorder nonheme iron in erythroid cells is located mainly in the cytosol in the form of ferritin aggregates, but also in the mitochondria (8).

Little is known about the subcellular distribution of readily available iron in the liver of humans or experimental animals. Iron depletion and resupplementation neither alters the concentration of cytochromes b_5 and P-450

Abbreviations used: EDTA, ethylenediamine tetraacetate; MDA, malondialdehyde; RCA, respiratory control ratio (see Text).

in rat liver, nor changes the ability of these heme proteins to respond to induction with phenobarbital (9). *In vitro* experiments with rat liver mitochondria led to recognition of one energy-dependent and one energy-independent uptake mechanism for iron (III) chelates (10-16). Mitochondria contain the major enzymes of the heme biosynthesis pathway (17) and can, therefore, be expected to play a key role in the regulation of iron metabolism. Information is lacking as to how iron and heme pass the mitochondrial membranes *in vivo*, which carrier substances partake in their intracellular distribution and how large the readily available iron pool is, i.e., iron not bound to ferritin or hemosiderin. To our knowledge, the effect of alterations of the steady state concentration of iron on the tissue levels of heme and on the metabolic integrity of mitochondria has not been studied in *in vivo* experiments. The present communication reports preliminary data on iron content, metabolic performance and membrane stability of hepatic mitochondria obtained from iron-loaded and dietary anemic rats.

MATERIALS AND METHODS

Male Wistar rats were used. Control rats (300-400 gm) were fed Purina Rat Chow. Iron overload was achieved by injecting intraperitoneally 50 mg iron in the form of an iron-dextran complex (Imferon) three times over a six-day period. The rats were sacrificed three days after the last injection. Rats (300 gm maximum) were rendered anemic on a complete diet with low iron content (Taklad Mills, Wisc., 7 ppm iron) from the post-weanling stage until 4 to 6 months of age. At this time, the average hematocrit was 20%.

Rat liver mitochondria were prepared according to ref. 18. Studies of respiratory control and oxidative phosphorylation were performed at 30° with a Clark-type electrode according to ref. 19. Malondialdehyde was determined according to ref. 20. Other experimental details are given in the legend to the Tables.

ADP, Nargarse and bovine serum albumin (A Grade) were purchased from P-L Biochemicals, Enzyme Development Corp., and Calbiochem, respectively. Other

reagents were of the highest commercially available purity. Rutamycin (Oligomycin D) was a gift from Eli Lilly and Co.

RESULTS

Iron Content. Liver tissue and liver mitochondria from iron-loaded animals are dark red-brown in comparison to those obtained from normal and anemic rats. The iron analyses shown in Table I demonstrate a hepatic iron content increasing from anemic (67%) to normal (100%) to iron-loaded rats (158%). Strikingly, the iron content of liver mitochondria differs by more than one order of magnitude between mitochondria from normal or anemic rats, and mitochondria from iron-loaded rats. The results suggest that essentially all the iron taken up by the liver is concentrated in the mitochondria (Table I, last column).

Mitochondrial Function. The extent of respiratory control and the efficiency of oxidative phosphorylation are sensitive indicators of the functional integrity of mitochondria (23). Therefore, we have examined mitochondria from normal, iron-loaded and anemic rats with respect to these parameters. The data are shown in Table II. The respiratory control is measured by the respiratory control ratio (RCR), i.e., the rates of respiration in the presence and absence of ADP (state 3 rate and state 4 rate, respectively) (19). A similar parameter is the ratio of state 3 rate : state 4 rate in the presence of oligomycin, a specific inhibitor of mitochondrial oxidative phosphorylation. Ideally, both of these parameters should be identical. However, in most preparations of mitochondria, some endogenous energy dissipation processes take place which utilize ATP and produce small amounts of ADP. The ADP increases the apparent state 4 rate and lowers the RCR. The presence of oligomycin prevents the stimulatory effect of ADP on state 4 rates and the ensuing decrease of the RCR.

The data in Table II, columns 3 and 4 show no significant difference for the control ratios using succinate and 3-hydroxybutyrate as substrates. In contrast, the control of pyruvate-malate oxidation decreases in the order: normal > anemic > iron-loaded mitochondria. Especially significant is the difference between normal and iron-loaded mitochondria: the RCR value decreases from 5.04

TABLE I

Iron Content of Liver and of Liver Mitochondria from Normal, Iron-loaded and Anemic Rats

Type[1]	Liver[2]		Mitochondria (ng atoms Fe/mg protein)[3]		
	μg Fe / g wet weight	ng atoms / g wet weight	observed	calc. for even distribution[4]	calc. assuming all extra iron in mito.[4]
N	24 ± 13 (100%)	429	3.1 ± 0.9 (100%)	2.2	2.2
L	38 ± 16 (158%)	679	32.1 ± 12.1[5] (1050%)	3.4	27.2
A	16 ± 4 (67%)	286	2.4 ± 0.8 (80%)	1.4	1.4

1 - N, L, A: mitochondria from normal, iron-loaded and anemic rats, respectively.
2 - Determined by dry-ashing and colorimetry according to Beinert as described in ref. 21. Average of three determinations ± experimental standard deviation.
3 - Determined by wet-ashing and colorimetry according to Beinert as described in ref. 21. Average of four determinations ± experimental standard deviation.
4 - Based on a yield of about 10 mg mitochondrial protein/g liver wet weight, and on a ratio of mitochondrial wet weight/mitochondrial protein of approx. 5 (calculated from data given in ref. 22).
5.- $P < 0.01$ when compared to corresponding values obtained with mitochondria from normal rats.

TABLE II

Control and Efficiency in Oxidative Phosphorylation Catalyzed by Mitochondria from Normal, Iron-loaded and Anemic Rats

Substrate[1]	Type[2]	Respiratory control[3,4]	$\frac{\text{Rate (ADP)}}{\text{Rate (oligomycin)}}$[4,5]	ADP/O ratio[4]
3-hydroxybutyrate	N	4.66 ± 1.19	10.3 ± 3.9	2.16 ± 0.25
	L	4.10 ± 1.76	10.1 ± 2.9	2.14 ± 0.28
	A	4.19 ± 1.06	9.0 ± 1.3	2.16 ± 0.27
pyruvate & malate	N	5.04 ± 1.22	10.5 ± 2.9	2.17 ± 0.25
	L	3.21 ± 0.35[6]	6.0 ± 0.2[6]	2.03 ± 0.10
	A	3.99 ± 1.28	8.3 ± 3.4	2.10 ± 0.21
succinate	N	4.36 ± 0.28	6.0 ± 0.6	1.59 ± 0.17
	L	4.28 ± 0.84	5.4 ± 0.9	1.51 ± 0.20
	A	4.19 ± 0.66	6.0 ± 0.7	1.53 ± 0.17

1 - 3-hydroxybutyrate: 20 mM; pyruvate and malate: 10 mM each; succinate: 3 mM; other components in the assay medium as given in Fig. 1 of ref. 24.
2 - Designations as in Table I.
3 - Ratio of rates of oxygen uptake in the presence of ADP and in its absence. For experimental details see ref. 24.
4 - Average of four determinations ± experimental standard deviation.
5 - Ratio of rates of oxygen uptake in the presence of ADP, and in the presence of oligomycin (2-4 μg/mg mitochondrial protein) without ADP.
6 - $p < 0.05$, when compared to corresponding values obtained with mitochondria from normal rats.

to 3.21, which means that the degree of control was lowered by 45%*. A similar decrease by 47% is observed in the values in column 4. However, no significant differences were detected in the phosphorylation efficiencies (column 5) or in the rates of substrate oxidation (not shown). Thus, iron-load *in vivo* affects only certain control parameters of oxidative phosphorylation but not the rates of oxidation and phosphorylation themselves.

Membrane Stability. Peroxidation of microsomal (25) and mitochondrial (26,27) phospholipids is catalyzed by iron salts, nonheme iron and cytochromes. One of the products is malondialdehyde which is derived mainly from highly unsaturated fatty acids such as arachidonic acid and docosahexaenoic acid (28,29) which are constituents of phospholipids. Phospholipids are integral parts of the mitochondrial membranes, and degradation of the former inflicts damage on the latter (30). Since respiratory control is particularly dependent on the intactness of the mitochondrial inner membrane, and has been partially lost in iron-loaded mitochondria with at least one substrate, we have tested for the occurrence of MDA as an indication for membrane damage caused by lipid oxidation. Indeed, mitochondria from iron-loaded rats contain approximately three times more MDA (0.35 nmole/mg protein) than normal and anemic mitochondria (0.10 to 0.12 nmole/mg protein). These data are especially significant in view of the repeated washings carried out in the presence of bovine serum albumin during mitochondrial preparation.

DISCUSSION

In response to iron-load *in vivo* accomplished by intraperitoneal injections of iron (III) dextran, rat liver mitochondria accumulate an enormous amount of iron, apparently without severe metabolic consequences. In normal mitochondria, we found a total of 3.1 ng atoms Fe/mg protein (Table I), about 30% of which is heme iron (31). The content of nonheme iron in liver mitochondria from iron-loaded rats is increased from approximately 2 to 31 ng atoms Fe/mg protein. For

* $(3.21 - 1)/(5.04 - 1) = 0.55$

perspective, this number may be compared with the mitochondrial concentration of phospholipids (∿ 200 nmoles/mg protein*) or proteins (∿ 25 nmoles/mg protein**). On a molar basis, the amount of excess iron is thus comparable, in the order of magnitude, to that of the main constituents of the mitochondrial membrane.

It is surprising that iron-loading does not cause more extensive changes in oxidative phosphorylation. We tested two substrates (pyruvate-malate and 3-hydroxybutyrate) which energize phosphorylation sites I, II, and III, and one substrate (succinate) which energizes only sites II and III (33). Only the response to pyruvate-malate in iron-loaded mitochondria differed significantly from that of normal animals. This suggests that neither of the three phosphorylation sites is damaged. The basic mechanism of respiratory control is unknown. Nevertheless, there are several possibilities which could contribute to a preferential effect of iron-loading on the control of malate utilization. 1) Malate dehydrogenase is a water-soluble matrix enzyme, whereas 3-hydroxybutyrate and succinate dehydrogenase are membrane bound enzymes (17). Soluble forms of accumulated iron may therefore interfere selectively with malate dehydrogenase. 2) Iron may be chelated more readily by malate and its product, oxaloacetate, than by succinate or 3-hydroxybutyrate (34). Iron-substrate complexes may influence the respiratory control. 3) The excessive iron in mitochondria of iron-loaded rats may alter the steady state ratio of NADH/NAD, which may decrease the degree of respiratory control. 4) The excess iron may lead to membrane damage (as indicated by the increased production of MDA) which in turn may affect the activities of the Krebs cycle enzymes, including malate dehydrogenase, more than the membrane bound enzymes. Anomalies in the fluxes of substrates, products, and cations can be envisioned.

Unexpectedly, MDA was found in iron-loaded mitochondria after procedures of preparation that include two washings with buffer containing bovine serum

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* Based on a phospholipid content of about 0.18 mg/mg protein (32) and an average molecular weight of 800.

** Based on an average molecular weight of mitochondrial proteins of about 40,000.

albumin. This finding as well as the fact that MDA is freely membrane-permeable (35) and oxidizable by rat liver mitochondria (36) strongly suggests that the MDA is of mitochondrial and not microsomal origin, and that MDA formation occurs even in isolated mitochondria.

Iron uptake by isolated rat liver mitochondria has been carefully studied by Romslo and by Flatmark (10-16). It is useful to compare their results obtained *in vitro* with our results *in vivo*. These authors find two modes of iron uptake by isolated rat liver mitochondria: a high-affinity, energy-dependent accumulation of up to 6 ng atoms Fe/mg protein, and a low-affinity, energy-independent accumulation about 25 ng atoms Fe/mg protein. The energy-dependent pathway appears to be similar but is not identical with the energy-dependent calcium accumulation (11,16). Iron-sucrose complex increases state 3 and state 4 rates, more so with succinate than with pyruvate-malate (15). Most of the iron taken up by the energy-dependent mechanism is found in the matrix in a soluble form, whereas the energy-independent pathway leads to deposition of iron preferentially on the outer and inner membranes (12). The authors assume that iron accumulated inside the mitochondria is mainly in its electrochemically divalent state (15,16). No lipid peroxide formation was detected (15), although Hunter (37) observed lipid oxidation in mitochondria upon exposure to divalent iron.

Our results *in vivo* differ in several respects from the results of these authors. The iron content of iron-loaded mitochondria is present in such a form that two washings with bovine serum albumin and EDTA will not remove it. This suggests the iron to be bound inside the mitochondria, to an extent which is five times higher than the maximal high-affinity binding found *in vitro* (13). It should also be noted that in our experiments, iron is given intraperitoneally as an iron-dextran complex. The intracellular iron is probably bound to naturally occurring carriers, and accumulates in liver mitochondria in the presence of substrates. In contrast, iron uptake by mitochondria *in vitro* is inhibited by the presence of substrate because of the chelating properties of the latter (14). Whereas iron-load *in vitro* affects mostly succinate and to a minor degree

pyruvate-malate oxidation, the findings in the *in vivo* situation are *vice versa*. Furthermore, in contrast to *in vitro* experiments (15), we find significant amounts of MDA indicative of a considerable degree of mitochondrial lipid peroxidation.

The study of liver mitochondria from iron-overloaded rats appears promising in several respects. One can: 1) study the relationship between iron levels and the activities of ALA-synthetase and ferrochelatase; 2) follow the transport of iron from the inside to the outside of the mitochondria; 3) evaluate *in vitro* the treatment of conditions leading to mitochondrial iron-load *in vivo*.

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