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RELATIONSHIP BETWEEN FREE IRON LEVEL AND RAT LIVER MITOCHONDRIAL DYSFUNCTION IN EXPERIMENTAL DIETARY IRON OVERLOAD

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The concentration of total iron in the hepatic tissue and mitochondria from rats fed a 2.5 % carbonyl iron supplemented diet progressively increased up to 40 days, then reached nearly a steady-state. By contrast the level of free iron (desferrioxamine-chelatable) exhibited a transient but significant increase at 40 days of treatment, only in this period of treatment the induction of lipid peroxidation and the resulting mitochondrial abnormalities in calcium transport was observed too. The enhancement of the energy dissipating mitochondrial calcium cycling was found to be associated with a significant decrease of endogenous mitochondrial ATP content. As to the pathophysiological mechanism for hepatocellular injury in iron overload, these results indicated that the transit pool of free iron.may play a critical role in initiating organelle dysfunctions, at least in this experimental model of iron overload.

Clinical evidence for toxicity caused by excess iron has been provided by studies of patients with hereditary hemochromatosis, African iron overload and secondary hemochromatosis caused by β-thalassemia; therapeutic reduction of hepatic iron by either phlebotomy or chelation therapy has resulted in clinical improvement (1-3). However, the specific pathophysiological mechanisms for hepatocellular injury, fibrosis and cirrhosis in hepatic iron overload are poorly understood. Iron induced lipid peroxidation of membrane phospholipids associated with functional abnormalities in hepatic organelles (mitochondria and microsomes) seems to represent a potential unifying mechanism (4). In chronic dietary iron

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overload, a model which closely resembles hepatocellular iron deposition of human hereditary hemochromatosis (5), the presence of lipid peroxidation and organelle functional disturbances has been correlated with the hepatic total iron concentration, even though the form of intracellular iron responsible for initiating the observed abnormalities is unknown (6-9). Under normal conditions iron is transported and stored in specific proteins (transferrin, ferritin, lactoferrin and haem proteins) which prevent or minimize its reaction with reduced oxygen derivatives (10). However, under various pathological conditions associated with iron overload it has been postulated that there is an increase in the intracellular transit pool of iron (11,12). In fact, within cells a cytosolic pool of low molecular weight chelatable iron derivatives (LMWC-iron or free iron) exists which appears to be catalytically active in initiating free radical reactions and lipid peroxidation (13-15). Iron in these forms is chelatable by desferrioxamine (DFO), so the complex DFO-iron may be directly measured (16).

In the present research we have measured free iron concentration in the hepatic tissue and in mitochondria in an experimental model of chronic dietary iron overload to define a correlation between free iron levels and mitochondrial functional efficiency in this pathological state.

MATERIALS AND METHODS

Animals and diet. Female Wistar albino rats (100-120g) (Morini, Reggio Emilia, Italy) were made siderotic by feeding a standard diet purchased from Dr. Piccioni (Brescia, Italy) supplemented with 2.5% (w/w) carbonyl iron.

Preparation of mitochondrial fraction. Animals were killed by decapitation after an overnight starvation period. Liver mitochondria were prepared in 0.25 M sucrose according to a standard procedure (17). The cytosolic contamination in mitochondrial preparation did not exceed 2% of the total protein content, as assessed by recovery of marker enzymes (18). The protein content of the final mitochondrial suspension was determined by the biuret method with bovine serum albumin as the standard.

Total iron determination. Total liver and mitochondrial iron concentration was determined in acid extracts by an atomic absorption Perkin-Elmer spectrophotometer model 306 (8).

Free iron determination. Hepatic tissue and mitochondrial free iron concentration was measured as desferrioxamine-iron complex (FO) as previously described (16). Briefly, livers were homogenized (10% w/v) in 0.25 M sucrose buffered at pH 7.4 containing 25 μM desferrioxamine; the mitochondria were resuspended in the same medium (60-90 mg prot/ml) and lysed by freezing and thawing. Both liver homogenate and mitochondria were centrifuged at 100.000 x g for 30 min. The supernatant was ultrafiltered by membrane cones (Centriflo CF 25, Amicon). The FO content of the aproteic ultrafiltrates was measured by an HPLC method (19).

Lipid peroxidation. The amount of malondialdehyde (MDA) in the mitochondrial fraction was measured by the thiobarbituric acid method (20).

Adenine nucleotide determination. Adenine nucleotides were determined as follows: 1 ml of mitochondrial suspension (ca. 8 mg prot/ml) was treated with 0.2 M of $HClO_4$ and centrifuged. 20 μl of the neutralized acid-soluble fraction were used for the HPLC analysis by a liquid chromatografic system (Hewlett-Packard HP 1090) equipped with a photo-diode array detector. The column was a LC-18 Hypersil 5ODS 4.6 mm X 25 cm (Lab Service, BO, Italy). The separation was accomplished according to Stocchi et al. (21). ATP, ADP and

AMP were revealed at 260 nm against known quantities of external standards (75 - 200 pmol).

Mitochondrial membrane potential measurement. The transmembrane potential ($\Delta\Psi$) was measured at 25°C by a tetraphenylphosphonium chloride (TPP^+) selective electrode (22). Mitochondria (2 mg protein/ml) were incubated in a standard incubation medium of the following composition: 100 mM NaCl; 5 mM sodium-potassium phosphate buffer (pH 7.4); 10 mM Tris-HCl buffer (pH 7.4); 10 mM $MgCl_2$ plus 5 μM rotenone and 20 μM TPP^+. An inner mitochondrial volume of 1.1 μl/mg protein was assumed. The respiratory states were those defined by Chance and Williams (23) on the basis of the factors limiting the respiration.

RESULTS

Fig.1 shows that the total iron content of the hepatic tissue of rats fed a diet supplemented with carbonyl iron progressively increases from 9.9 ± 0.9 nmol/mg prot of control up to 84.8 ± 12 after 40 days of treatment and then remains nearly constant during a period of 90 days tested. The very same pattern is presented by the total iron concentration in the mitochondrial fraction (2.8 ± 0.8 in control and 21.9 ± 1.2 nmol/mg prot after 40 days of treatment). By contrast, the time course of free iron concentration in both the hepatic tissue and mitochondrial fraction exhibits a sudden increase around 40 days of iron feeding up to 887 ± 58 and 817 ± 42 pmol/mg prot, respectively; then free iron level rapidly decreases to the control values of 270 ± 35 and 290 ± 40 pmol/mg prot respectively.

Fig. 2 shows that MDA production significantly increases only in the mitochondrial membranes from rats fed iron for 40 days reaching a value of 240 ± 32 compared with 100 ± 26 pmol/mg prot of the control. It is noteworthy that very similar results were obtained when an antioxidant, such as BHT, was present during the fractionation procedure, suggesting that

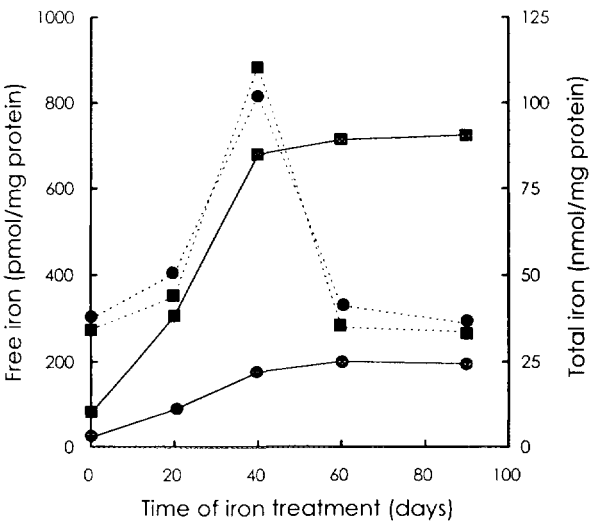

Fig. 1. Free iron (----) and total iron level (——) in the hepatic tissue (■) and in the mitochondrial fraction (●) were determined as described in the Methods. The data represent mean values of four separate experiments.

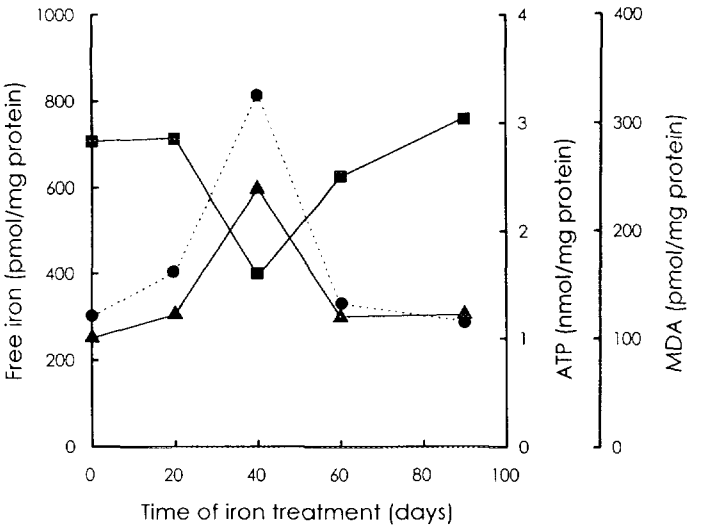

Fig. 2. Free iron (--●--), MDA (—▲—) and ATP (—■—) content in the mitochondrial fraction was determined as described in the Methods. The data represent mean values of four separate experiments.

the increased concentration of MDA reflected in vivo formation (not shown). The same figure also shows that the endogenous mitochondrial ATP content is significantly decreased by 44% only at this critical period of iron overload (1.58 ± 0.18 vs 2.83 ± 0.24 nmol/mg prot of the control). A 15% decrease in ADP level with no appreciable change in AMP concentration was observed too (not shown).

Fig. 3 presents the membrane potential pattern during a complete cycle of phosphorylation of control mitochondria as compared to that of 40 days iron treated rats. It appears that, at variance with control (Fig. 3A), the membrane potential of mitochondria from iron loaded rats does not maintain a steady state value after the phosphorylation of ADP is completed, but progressively decreases (Fig. 3B). Addition of EGTA, a specific Ca^{2+} chelator, of ruthenium red, an inhibitor of the electrophoretic Ca^{2+} uptake pathway, or of oligomycin, an inhibitor of ATP synthase, fully restores the membrane potential trace. On the contrary the addition of an antioxidant such as BHT, and of an iron chelator such as DFO, does not prevent the $\Delta\psi$ drop.

DISCUSSION

The present results demonstrate a direct correlation between the concentration of free iron and the induction of lipid peroxidation and the associated functional abnormalities in rat liver mitochondria in mild dietary iron overload.

The "in vivo" induction by iron of lipid peroxidation in rats loaded with iron by either parenteral administration of iron-chelates (5-7,24,25) or by dietary supplementation of

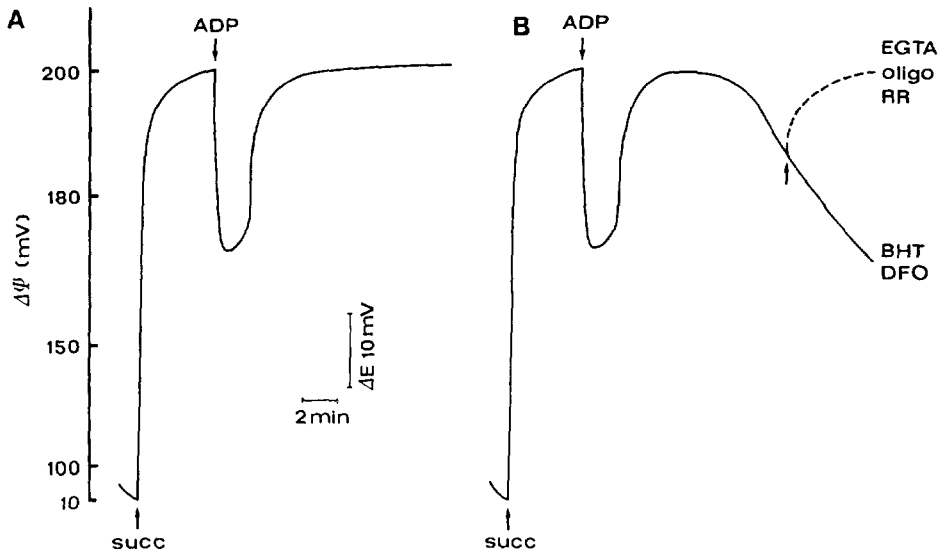

Fig. 3. Mitochondrial membrane potential measurements ($\Delta\psi$) were performed as described in the Methods. The arrows indicate the following additions: 2 mM succinate (succ); 0.25 mM ADP; 0.5 mM EGTA; 2 μg/mg protein oligomycin (oligo); 1 μM ruthenium red (RR); 30 μM butylated hydroxitoluene (BHT); 1 mg/mg protein desferrioxamine (DFO); ΔE, electrode potential. A, control mitochondria; B, mitochondria from 40 day iron treated rats.

elemental iron (5,6,8,9,26) has been demonstrated in whole animals (27), in the liver (26) and in subcellular organelles (5-7,9,10,24). No direct correlation may be derived from all these studies between the occurrence of lipid peroxidation and total iron level; in fact the critical iron concentrations for the occurrence of lipoperoxidative reactions were in a wide range of values from 1,000 to 5,000 μg/g wet weight. Since the form of intracellular iron responsible for initiating these processes remains unknown, various mechanisms of in vivo iron induced peroxidative reactions have been proposed; the most convincing involves an increase in the intracellular transit pool of free iron that under normal conditions represents from 3 to 10 % of total hepatic iron (10,28). In this vein, an association between iron content in the cytosol and chemiluminescence stimulation was found in a mild iron overload experimental model (25). Furthermore, ultrafiltrates from hepatic cytosol of dietary iron loaded rats were found to exhibit a greater prooxidant action than those from controls. The observation that DFO administration suppressed this prooxidant action indicated that its activity was iron dependent (13). Moreover an increase in non-haem iron observed in the hepatic and cerebellar cytosolic fraction of ethanol treated rats was reported to be associated with an enhancement in the content of free iron (28). An increased amount of this pool was also found to bring about the induction of lipid peroxidation in erythrocytes exposed to oxidizing agents (16,29). A correlation between the level of LMW-iron pool and MDA production was reported in an

experimental model of uroporphyria too (30). The transient increase in the concentration of free iron and MDA around 40 days of treatment, observed here under conditions of dietary mild iron overload (1,000 μg/g wet weight), gives direct experimental support to the proposal that iron overload results in an increase of the catalytically active cytosolic pool of free iron. It is conceivable that with progressive increase of iron deposition the capacity of the hepatocyte to maintain iron in storage forms is exceeded. Once iron has reached the hepatocyte, ferritin synthesis is stimulated (31), nevertheless the existing ferritin as well as the newly synthesized ferritin could not be able to completely accomodate the large excess of total iron which progressively accumulates up to 40 days (see Fig. 1). In the meantime the amount of free iron increases and enhances the generation of oxygen free radicals. As soon as total iron level reaches a steady state, the capability of iron withholding by specific proteins of the hepatocyte may be restored.

One aspect which deserves further consideration is that concerned with the time course of total iron and free iron concentration. The proportion of total iron in the hepatic tissue to that in the mitochondrial fraction appears to be constant during dietary overload tested at a value of about 3.5. This finding, in agreement with the view of the localization of iron bound to proteins, suggests a correlative rather than a specific change in iron content. Also the proportion between free iron concentration in the hepatic tissue and in the mitochondrial fraction remains constant but at a value of 1, a result which fits into the concept of free iron delocalization in the cell.

The in vivo induction by the transient increase of free iron of lipoperoxidative reactions in the mitochondrial membranes (see Fig. 2) is associated with impairment of the energy transducing capability of the inner membrane due to enhancement of the energy dissipating Ca^{2+} cycling process (see Fig. 3). The activation of a specific Ca^{2+} efflux from mitochondria via the oxidation of pyridine nucleotides due to the formation of lipid peroxidation products and their reduction by the glutathione enzyme cascade, previously reported in a very similar experimental model (8), may account for the enhancement of Ca^{2+} cycling. This energy wasting observed here in the absence of externally added Ca^{2+} may be responsible for the significant decrease in the endogenous mitochondrial ATP content (see Fig. 2).

The present results providing an explanation for the initiating form of iron which leads to organelle dysfunction and then to hepatocellular injury may give a clue for the therapeutic interventation in various disorders associated with excess tissue iron deposition.

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