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LIPID COMPOSITION AND FLUIDITY OF LIVER MITOCHONDRIA, MICROSOMES AND PLASMA MEMBRANE OF RATS WITH CHRONIC DIETARY IRON OVERLOAD

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Abstract—The effect of chronic dietary iron overload on the lipid composition and physical state of rat liver mitochondria, microsomes and plasma membranes was investigated. After 9 weeks of iron treatment, a significant decrease of polyunsaturated and a parallel increase of saturated fatty acids was observed in mitochondrial and plasma membrane phospholipids. By contrast, no appreciable modification of the fatty acid composition of microsomal membranes was detected. The cholesterol/phospholipid molar ratio as well as the lipid/protein ratio, did not reveal any significant difference in any of the fractions studies. Finally, no change in the molecular order of the various membranes, as assessed by electron spin resonance spectrometry, was observed following iron intoxication. These data indicate that, although *in vivo* chronic hepatic iron overload induces a modification of fatty acid profile in cellular structures consistent with the *in vivo* occurrence of lipid peroxidation, these changes do not bring about appreciable modifications of other physico-chemical parameters relevant to membrane integrity and cell viability.

Iron overload in the liver has been associated with alteration in heme metabolism [1], as well as hepatic injury, fibrosis and cirrhosis [2]. The pathogenetic role of iron in these abnormal events has been recently defined in terms of iron-catalysed free radical-mediated lipid peroxidation. Iron can initiate lipid peroxidation either by acting as a catalyst in the formation of the highly reactive hydroxyl radical [3] or by complexing with oxygen directly to yield the reactive perferryl and ferryl ions [4].

In experimental models of chronic iron overload in rats, evidence for the occurrence of lipid peroxidation in mitochondria, microsomes and lysosomes, has been presented [5–10]. Moreover, functional anomalies were detected concomitantly with the enhancement of lipid peroxidative reactions in the mitochondrial membranes, such as disturbances in the oxidative metabolism [6–8], in the calcium transport [9] and in the process of porphyrin accumulation [11].

However, a direct involvement of lipid peroxidation in the pathogenesis of liver injury due to iron overload, does not appear from these studies [12, 13]. Particularly, the effect of *in vivo* chronic iron overload on the physico-chemical properties of liver membranes, has not been addressed so far. In fact, in the course of oxidative cell injury the membrane lipids represent a primary target of the peroxidative attack [13]. The peroxidative reactions may result in a change in the unsaturation/saturation ratio of membrane phospholipid fatty acids, in the chain length percentage distribution of the fatty acids and, possibly, in the induction of covalent cross-links between adjacent lipid radicals in the membrane [14]. Such alterations in the chemical nature of the fatty acids may engender physical changes in the molecular order of membranes. Various membrane functions such as selective permeability, enzyme activity and ion transport, had been shown to be influenced by molecular order or fluidity [15]. The analysis of these physico-chemical parameters may therefore give a better insight into the mechanism of iron hepatotoxicity.

In view of these considerations, we carried out a study on the membrane lipid composition (i.e. phospholipid fatty acid profile; cholesterol and phospholipid content) and the fluidity state of rat liver membranes in an experimental model of chronic dietary iron overload at three different cellular levels: mitochondria, microsomes and plasma membrane.

MATERIALS AND METHODS

Female albino rats (150–180 g body wt) were fed a standard diet (Dottori Piccioni, Gessate Milano, Italy) supplemented with 2.5% (w/w) carbonyl iron (Fluka, Buchs, Switzerland) over a 9 week period. Control rats, paired by sex, age and weight, were fed standard diet alone *ad lib*. At the end of iron treatment iron-treated and control rats were killed by decapitation, liver was quickly removed, weighed and processed for the separation of the various subcellular fractions, as specified below. A small liver sample was used for histologic and iron atomic absorption evaluation.

Mitochondria and endoplasmic reticulum vesicles

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were isolated from rat liver as described in Refs 16 and 17, respectively. Liver plasma membranes were prepared following the method of Boyer *et al.* [18]. Protein content was assessed by method of Lowry *et al.* [19].

Glucose-6-phosphatase activity, a microsomal marker enzyme, was assayed as in Ref. 20. The mitochondrial marker enzyme, succinate-dehydrogenase, was assayed as in Ref. 21. 5'-Nucleotidase was selected as a plasma membrane marker and measured as in Ref. 20.

As an index of cell damage, the levels of serum glutamate-pyruvate (SGPT), glutamate-oxaloacetate transaminases (SGOT), were measured (optimized UV enzymatic methods, C. Erba, Milano, Italy).

Hepatic reduced glutathione (GSH) was measured by high pressure liquid chromatography as specified in Ref. 22.

Lipid analysis. Membrane lipids of the various subcellular fractions were extracted with 2:1 (v/v) chloroform: methanol [23]. The organic phase was dried and phospholipid classes were separated by thin layer chromatography using a 0.25 mm thin layer plate (Silica Gel G. Merck, Darmstadt, F.R.G.), and *n*-hexane, diethyl ether and acetic acid (70/30/1, v/v/v) as solvent system. The phospholipids remaining at the origin were scraped and processed for fatty acid analysis as specified below. For the determination of individual fatty acids, they were converted into methyl esters in a 2 M solution of potassium hydroxide in methanol, and extracted with petroleum ether. Methyl esters were then analysed on a Varian 3400 gas chromatograph equipped with a 30 foot capillary column of crosslinked SE 54. The detector was set at 230°, the injection port at 230°, and the oven temperature was programmed from 150 to 210° at a heating rate of 5°/min. Peaks were identified by comparison with standards of methyl esters of fatty acids (Supelco). They were also individually analysed using a Hewlett Packard GC/MS spectrometer.

Total phospholipids were determined by the Bartlett method [24] and total cholesterol by an enzymatic method (Boehringer Mannheim, F.R.G.).

Spin labelling. The spin label 5-doxylstearic acid (Sigma Chemical Co., Poole, U.K.) was evaporated from chloroform solution to form a thin film in a small glass vial. Each subcellular preparation (3 mg protein/ml) was added to the vial and gently stirred for 5 min and incubated overnight at 4°. The labeling was kept constant at 20 nmol spin label/mg protein. An aliquot of spin-labeled membrane preparation was transferred to a capillary silica tube and inserted in the cavity of the electron spin resonance (ESR) spectrometer. Spectra were recorded using a Bruker ER 200 SRC spectrometer fitted with a variable temperature control.

The usual instrument settings were: modulation amplitude 1 Gauss, microwave power 10 mM, scan width 50 Gauss. Order parameters were calculated using the method of Hayes Griffith and Jost [25] and Lange *et al.* [26].

The isolation, lipid extraction and physico-chemical analyses of the various subcellular fractions were performed in the presence of 0.01% butylated hydroxytoluene (BHT).

RESULTS

Characteristics of rats after iron treatment

Feeding rats a diet supplemented with 2.5% (w/w) carbonyl iron for 9 weeks, did not result in a significant change in body weight (270 ± 20 g in iron-treated vs 255 ± 25 g in control rats). The hepatic iron concentration increased from 178 ± 8 μg/g wet tissue in control animals up to 1650 ± 26 μg/g wet tissue in iron-treated rats after 40 days and then remained nearly constant. No significant differences in the levels of serum enzymes related to cell integrity were found in iron-treated vs control rats: glutamate-oxaloacetate transaminase 72 ± 18 vs 60 ± 12; glutamate-pyruvate transaminase, 50 ± 10 vs 38 ± 15.

The hepatic level of reduced glutathione was unaffected by the iron treatment: 20.9 ± 1.8 nmol/mg protein in iron-treated vs 21.4 ± 2.1 nmol/mg protein in control rats.

Protein recovery enrichment of marker enzymes and purity of isolated fractions

The protein recovery was not influenced by the iron treatment. Also the relative specific activities of mitochondrial, microsomal and liver plasma membrane marker enzymes (succinate dehydrogenase, glucose-6-phosphatase and 5'-nucleotidase, respectively), which are measures of the enrichment of the enzymes in each subcellular fraction, were similar in iron-treated and in control groups (Table 1). The degree of contamination of each fraction by the others, as assessed by using the relative specific activity of each marker enzyme, did not differ between the two groups (data not shown).

Lipid composition of liver membranes

The phospholipid content of hepatic mitochondria, microsomes and plasma membrane are reported in Table 2. No significant changes in the phospholipid content of cellular subfractions were detected after iron intoxication.

Also the total cholesterol content of these membrane preparations did not appear to be modified by iron treatment (Table 2). The cholesterol-phospholipid molar ratio was unchanged as well. The fatty acid composition of mitochondria phospholipids presented a marked decrease of polyunsaturated fatty acids (Table 3). Specifically, the fatty acid profile of mitochondrial membranes showed a large decrease of arachidonate (−52%) and a parallel enhancement of the relative content of palmitic acid (+58%). Accordingly, the double bond index in the mitochondrial fraction fell (−35%) after iron intoxication. In the case of the microsomal fraction, no appreciable modifications in the fatty acid composition at the end of iron treatment were detected (Table 3).

As to the phospholipid fatty acid composition of liver plasma membrane, Table 3 shows a significant decrease of polyunsaturated fatty acids and a concomitant increase of saturated fatty acids. Both monoenoic and polyenoic fatty acids appeared to be similarly affected by iron treatment. Specifically, the fatty acid profile of liver plasma membrane showed a

Table 1. Relative specific activities of subcellular fractions marker enzymes in the liver from iron treated and control rats

Enzyme	Fraction	Specific activity ratio*	
		Control	Iron-treated
5'-Nucleotidase	Plasma membrane	11.9 ± 1.1	10.9 ± 1.3
Succinate dehydrogenase	Mitochondria	3.26 ± 0.5	2.96 ± 0.4
Glucose-6-phosphatase	Microsomes	7.34 ± 0.7	7.68 ± 0.8

Results are expressed as mean ± SD of at least three experiments.
* Enzyme specific activity ratios of each subcellular fraction/homogenate.

Table 2. Effect of chronic dietary iron overload on the cholesterol and lipid phosphorous content of rat liver membranes

	Mitochondria		Microsomes		Plasma membranes	
Cholesterol (μg/mg protein)	5.7 ± 0.6	5.5 ± 0.4	34.2 ± 4.4	32.1 ± 3.8	96.8 ± 3.4	98.2 ± 4.5
Phospholipid (μg/mg protein)	95.4 ± 9.6	100.3 ± 10.6	306.5 ± 18.2	266.7 ± 19.3	282.4 ± 10.3	276.6 ± 7.2
Molar ratio of cholesterol/phospholipid	0.108 ± 0.006	0.099 ± 0.005	0.202 ± 0.012	0.218 ± 0.017	0.620 ± 0.05	0.643 ± 0.03

Cholesterol and phospholipids were determined as described in Materials and Methods. Results are expressed as mean ± SD of three experiments. Statistical analysis was performed by using Student's *t*-test. Data were not significantly different.

Table 3. Effect of chronic dietary iron overload on the phospholipid fatty acid composition of rat liver membranes

Fatty acids	Mitochondria		Microsomes		Plasma membranes	
	Control	Iron-treated	Control	Iron-treated	Control	Iron-treated
16:0	23.08 ± 2.05	36.5 ± 1.38†	26.03 ± 3.8	26.56 ± 1.4	29.04 ± 0.15	33.60 ± 1.42*
18:0	31.37 ± 0.69	29.12 ± 0.16*	31.08 ± 0.3	31.60 ± 0.3	27.84 ± 2.43	39.62 ± 1.10†
18:1	3.91 ± 0.93	7.77 ± 0.40‡	5.69 ± 0.1	7.31 ± 0.2*	9.85 ± 0.47	5.37 ± 0.59†
18:2	18.70 ± 0.40	13.90 ± 0.77†	14.66 ± 0.5	14.13 ± 0.9	12.44 ± 0.72	9.48 ± 0.53*
20:4	18.13 ± 1.38	8.75 ± 1.56‡	17.45 ± 3.2	14.60 ± 0.8	12.15 ± 0.26	9.36 ± 0.43*
22:6	4.79 ± 1.40	4.43 ± 1.02	6.13 ± 0.5	6.35 ± 0.8	9.18 ± 2.59	2.55 ± 0.97†
Double bond index§	1.45 ± 0.03	0.94 ± 0.02†	1.40 ± 0.17	1.22 ± 0.07	1.36 ± 0.05	0.77 ± 0.04‡

Each value is given as percentage of total fatty acids and represents the mean ± SD for five control and five iron-treated rats. Statistical analysis was performed by using Student's *t*-test. Significantly different from control: * $P < 0.02$; † $P < 0.01$; ‡ $P < 0.001$).
§ Double bond index is the sum of the fraction of each fatty acid times the number of double bonds in that acid.

large decrease in the proportion of docosahexaenoic acid (−72%) and a marked increase in that of stearic acid (+42%). The double bond index was also significantly decreased in the liver plasma membrane fraction of the iron-treated rats (−43%).

Spin labelling

A convenient method to directly measure membrane fluidity is to incorporate a spin labelled fatty acid (5-doxyl stearic acid) and determine the temperature dependence of the so-called order parameter *S* [25, 26]. The order parameter continuously decreases as the temperature increases, showing that the fluidity of the lipid increases as the temperature increases.

In this experimental model, iron overload "*in vivo*" did not influence the order parameter *S* of mitochondria, microsomes or plasma membrane (Table 4).

DISCUSSION

The present results show that in an experimental model of chronic dietary iron overload the phospholipid fatty acid composition of rat liver mitochondria and plasma membrane was significantly modified, whereas no appreciable change was observed at the level of the endoplasmic reticulum membrane. The possibility that these changes could be the result of degradative processes occurring *in vitro* during the

Table 4. Effect of chronic dietary iron overload on the order parameter(s) of lipids of rat liver membranes

Fraction	*S* (Relative) (%)	
	Control	Iron-treated
Mitochondria	0.524 ± 0.01	0.527 ± 0.03
Microsomes	0.543 ± 0.03	0.537 ± 0.01
Plasma membranes	0.667 ± 0.01	0.685 ± 0.02

ESR measurements were performed at 310°K as described in Materials and Methods.

S, order parameter. Data represent the mean ± SD for three different experiments. Data were not significantly different.

fractionation and analytical procedures due to the presence of excess iron, is unlikely, since all these experimental manipulations were performed in the presence of an antioxidant (BHT). Therefore, it seems reasonable to consider this modification of fatty acid composition as representative of the original *in vivo* situation. The phospholipid fatty acid profile of mitochondrial membranes presented a marked decrease in polyunsaturated fatty acids with a concomitant relative increase in saturated fatty acids. In this context, we can speculate that the striking increase of oleic acid (+88%) may represent a compensatory response to the loss of polyunsaturated fatty acid [10, 27]. The overall modification in the fatty acid profile of mitochondrial membranes appears to be consistent with the *in vivo* occurrence of iron-catalysed free radical mediated lipid peroxidation [28, 29], in accordance with previous reports showing the *in vivo* occurrence of lipid peroxidation of liver mitochondria, as evaluated by the formation of conjugated dienes, in a very similar experimental model of hepatic iron overload [5, 6, 9]. Under the present experimental conditions, however, a similar change of phospholipid fatty acid composition could conceivably result from differences in membrane biosynthesis and/or degradation. This possibility can be reasonable excluded since direct measurement of conjugated dienes revealed the occurrence of lipid peroxidation in the mitochondrial fraction, whereas no peroxidative processes were detected in the microsomal one (A. Masini *et al.*, to be published elsewhere).

The results regarding the mitochondrial membrane phospholipids may be clouded by the fact that our mitochondrial preparation, obtained by differential centrifugation, has some lysosomal contamination [30]. As to this point, Peters *et al.* [10] found a significant reduction in the polyunsaturated fatty acid content of lysosomal membrane phospholipids isolated from rats made siderotic by i.p. injection of FeNTA. However, since the lipid content of mitochondria is about 100 times that of lysosomes [31], the possible contribution from lysosomal membrane lipids to the mitochondrial lipids would appear to be negligible.

No significant modification in the phospholipid fatty acid composition of microsomal membranes from iron-treated rats was observed. This agrees well with our measurement of conjugated dienes (see above) as well as previous observations [5, 32]. Indeed, in these studies, lipid peroxidation as well as functional abnormalities of microsomes isolated from iron-loaded rats were detected only at a hepatic iron concentration much higher than that achieved in the present study (i.e. 4000 μg/g wet tissue vs 1650 μg/g wet tissue).

The phospholipid fatty acid composition of liver plasma membrane from iron-treated rats showed a marked reduction in all the unsaturated fatty acids and a concomitant increase of saturated classes, suggesting that iron overload *in vivo* also results in lipid peroxidative reactions in this subcellular structure. A similar modification in the phospholipid fatty acid composition of liver plasma membrane has been reported after the *in vitro* induction of lipid peroxidation by addition of ADP–Fe^{3+} complex to liver plasma membrane preparations [33].

In previous studies, no data were presented on the effect of iron-catalysed lipid peroxidation on the physical state of membranes. Indeed, a change in the fatty acid composition of membrane lipids could bring about a modification in membrane fluidity. Other factors, however, along with the fatty acid composition, may be responsible for changes in the membrane physical state: the most important include the cholesterol/phospholipid molar ratio and the lipid/protein ratio [34–37]. The present data (Table 2) indicate that these parameters were unmodified by iron treatment. Finally, the direct measurement by electron spin resonance of the effect of iron intoxication on the membrane molecular order, did not reveal any difference between control and iron-treated rats in any of the subcellular structures examined.

Therefore, it seems reasonable to conclude that during chronic dietary iron overload and enhancement of lipid peroxidation in liver mitochondria and plasma membranes, as indicated by a specific modification in their phospholipid fatty acid composition, is not associated with membrane fluidity change, an event which could lead to membrane dysfunction and cell damage. The cell structural integrity at this degree of hepatic siderosis is further indicated by the observation that no release of intracellular enzymes occurred under these conditions.

A possible explanation for these findings is that the extent of *in vivo* lipid peroxidation in mitochondria and plasma membrane from siderotic rats remained below a critical value. In fact, Dobretsov *et al.* [38], studying the effect of the lipid peroxidation *in vitro* on the fluidity state of rat liver mitochondrial membranes, showed that when the extent of lipid peroxidation overwhelmed a threshold level, modifications in membrane viscosity occurred, which in turn brought about the loss of membrane functions. Other authors have come to the same conclusion using different liver membrane fractions [39]. The finding that the content of hepatic GSH was unmodified at this level of iron intoxication, indicating that cellular defence mechanisms were not compromised, may support the above proposal.

As to the pathogenetic mechanism of liver injury due to iron overload, we conclude that, in accordance with a current view [12, 13], lipid peroxidation appears to be more important in triggering and/

or amplifying other damaging events rather than directly causing cell injury. In fact a strict correlation was found between the induction of lipid peroxidative reactions in mitochondrial membrane and the occurrence of derangement in Ca^{2+} transport as well as anomalies in oxidative metabolism under conditions of *in vivo* iron overload [6–9]. Such impairment of mitochondrial functions may possibly constitute one of the causal factors for the onset of cell damage in experimental hepatic iron overload.

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