Journal of Bioenergetics and Biomembranes, Vol. 21, No. 4, 1989

Lipid Composition and Fluidity of Liver Plasma Membranes from Rats with Chronic Dietary Iron Overload

A. Pietrangelo,[1] A. Tripodi,[2] N. Carulli,[2] A. Tomasi,[3] D. Ceccarelli,[3] E. Ventura,[1] and A. Masini[3]

Received January 20, 1989; revised March 13, 1989

Abstract

Liver plasma membranes isolated from rats with chronic dietary iron overload showed a large modification of their phospholipid fatty acid composition. Specifically, a significant decrease in polyunsaturated fatty acids and a parallel increase in saturated fatty acids was observed. This pattern was consistent with the *in vivo* occurrence of lipoperoxidative reactions in the liver plasma membranes. However, neither change in the cholesterol/phospholipid molar ratio nor in the lipid/protein ratio was detected. Direct measurement of the plasma membrane fluidity state by electron spin resonance spectrometry did not reveal any difference between control and iron-treated rats. These findings indicate that chronic dietary iron overload can induce lipid peroxidation of rat liver plasma membranes, but this event does not bring about modification in the physical state of the membrane.

Key Words: Iron overload; phospholipid fatty acids; cholesterol; electron spin resonance; plasma membrane; rat liver.

Introduction

Iron overload in the liver has been associated with hepatic injury, fibrosis, and cirrhosis (Powell *et al.*, 1980). It has been proposed that the pathogenesis of liver damage is related to membrane lipid peroxidative events initiated by iron which could lead to structural and/or functional damages of subcellular structures and finally to cell death (Tribble *et al.*, 1987).

[1]Clinica Medica III, Università di Modena, 41100 Modena, Italy.
[2]Patologia Medica, Università di Modena, 41100 Modena, Italy.
[3]Patologia Generale, Università di Modena, 41100 Modena, Italy.

0145-479X/89/0800-0527$06.00/0

Experimental evidence for the *in vivo* occurrence of lipid peroxidation in mitochondria, microsomes, and lysosomes from rats made siderotic by different models of chronic iron overload has been reported (Bacon *et al.*, 1983; Masini *et al.*, 1984a, 1984b, 1986; Peters *et al.*, 1985; Bacon *et al.*, 1985). However, in these studies, a direct correlation between lipid peroxidation and cell damage did not appear (Tribble *et al.*, 1987; Poli *et al.*, 1987).

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 (Curtis *et al.*, 1984). 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 have been shown to be influenced by molecular order or fluidity (Schachter, 1984). The analysis of these physicochemical parameters may therefore give a better insight into the mechanism of iron hepatotoxicity.

In view of these considerations, due to the crucial role of plasma membrane integrity in the cytotoxic process (Farber and El-Mofty, 1975), the present research was aimed at investigating the phospholipid fatty acid composition and the fluidity state of liver plasma membranes from rats with chronic dietary iron overload, an experimental model which closely resembles human primary iron overload conditions (Bacon *et al.*, 1983).

Materials and Methods

Female albino rats (150–180 g body weight) were fed a diet supplemented with 2.5% (w/w) carbonyl iron over a 9-week period. At the end of iron treatment rats were killed by decapitation, and the liver was quickly removed, weighed, and processed for plasma membrane separation as specified below. A small liver sample was used for histologic and iron atomic absorption evaluation.

Liver plasma membranes (LPMs) were prepared following the method of Boyer *et al.* (1983). Proteins were assessed by Lowry's method (1952). Membrane purification was evaluated by the enrichment of the activities of various marker enzymes between homogenate and LPM preparations: the activity of Na^+, K^+ ATPase, and Mg^{2+} ATPase was 11-fold, and that of 5′-nucleotidase 8-fold, higher in membrane than in homogenate.

Lipid Analysis

LPM lipids were extracted with 2 : 1 (v/v) chloroform : methanol (Folch *et al.*, 1957). 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, Merk) 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 the individual fatty acids, they were converted into methyl esters by a 2 M solution of potassium hydroxide in methanol and extracted with petroleum ether. Methyl esters were then analyzed on a Varian 3400 gas chromatograph equipped with a 30-feet capillary column cross-linked SE 54. The detector was set up at 230°C, the injection part at 230°C, and the oven temperature programmed from 150 to 210°C at a heating rate of 5°C/min. Peaks were identified by comparison with standards of methyl esters of fatty acids (Supelco). They were also singularly analyzed by a Hewlett Packard GC/MS spectrometer. Total phospholipids were determined by the Bartlett method (1959), and total cholesterol by an enzymatic method (Boehringer, Mannheim, FRG).

Spin Labeling

The spin label 5-doxylstearic acid (Sigma) was evaporated from chloroform solution to form a thin film in a small glass vial. The LPM preparation (3 mg protein/ml) was added to the vial and gently stirred for 5 min and incubated overnight at 4°C. The labeling was kept constant at 20 nmol spin label/mg protein, and the probe/phospholipid molar ratio was 0.05. 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 G, microwave power 10 mM, scan width 50 G. Order parameters were calculated using the method of Hayes Griffith and Jost (1976) and Lange *et al.* (1985).

The isolation, lipid extraction, and evaluation of physicochemical parameters of LPMs were performed in the presence of 0.01% butylated hydroxytoluene.

Results and Discussion

The iron content of the liver of rats fed a diet supplemented with 2.5% (wt/wt) carbonyl iron increased from 181 $\pm$ 18 μg/g wet tissue in control animals to 1605 $\pm$ 52 μg/g wet tissue in iron-treated animals after 9 weeks of treatment. At this level of iron overload, in the same experimental model, the occurrence of lipid peroxidative reactions in the mitochondrial membranes

Table I. Effect of Chronic Dietary Iron Overload on the Phospholipid Fatty Acid Composition of Rat Liver Plasma Membranes

Fatty acids	Control	Iron-treated
16:0	29.04 ± 0.15	33.60 ± 1.42[a]
18:0	27.84 ± 2.43	39.62 ± 1.10[b]
18:1	9.85 ± 0.47	5.37 ± 0.59[b]
18:2	12.44 ± 0.72	9.48 ± 0.53[a]
20:4	12.15 ± 0.26	9.36 ± 0.43[a]
20:6	9.18 ± 2.59	2.55 ± 0.97[c]
Double-bond index[d]	1.36 ± 0.05	0.77 ± 0.04[c]

Each value is given as a percentage of total fatty acids and represents the mean ± SD for five control and five iron-treated rats. Statistical analyses were performed by using Student's *t* test. Significantly different from control: *a*, $p < 0.02$; *b*, $p < 0.01$; *c*, $p < 0.001$.
[d]Double-bond index is the sum of the fraction of each fatty acid times the number of double bonds in that acid.

was detected by the formation of conjugated dienes, whereas no lipid peroxidation was revealed in the microsomal membranes (Bacon *et al.*, 1983; Masini *et al.*, 1986). A change in the fatty acid composition of lysosomal membranes, consistent with the occurrence of iron-catalyzed lipid peroxidation, was also observed at a similar degree of hepatic iron overload, but in a quite different experimental model (i.e., intraperitoneal injection of iron-nitrilotriacetate) (Peters *et al.*, 1985).

In our study, the analysis of the phospholipid fatty acid composition of LPMs revealed a large modification in the iron-treated rats. Indeed, as can be seen from Table I, a significant decrease in the polyunsaturated fatty acids and a concomitant increase in the saturated fatty acids was observed after iron treatment. In particular, the fatty acid profile of LPMs showed a dramatic decrease in the proportion of docosahexaenoic acid (−72%) and a marked increase in that of stearic acid (+42%). In accordance with these events, the double-bond index significantly fell in LPMs of iron-treated animals. These results are therefore consistent with the *in vivo* induction by iron of lipoperoxidative processes in the plasma membranes. So far, a similar modification in the phospholipid fatty acid composition of LPMs was reported only after the *in vitro* induction of lipid peroxidation by addition of ADP–Fe^{3+} complex to LPM preparations (Ungemach, 1985). Moreover, in this study no data were presented on the physical state of the membrane under these experimental conditions. Indeed, a change in the fatty acid composition of LPMs could lead to a modification in membrane fluidity. Other factors, however, along with the fatty acid composition, may be responsible for changes in the physical state of the membrane, the most important including: (a) the cholesterol/phospholipid ratio; (b) the lipid/protein ratio; and (c) the lecitin/sphingomyelin ratio (Shinitzky and Henkart, 1979; Bartoz, 1981;

Table II. Effect of Chronic Dietary Iron Overload on the Cholesterol and Phospholipid Content of Rat Liver Plasma Membranes[a]

	Control	Iron-treated	Significance
Total cholesterol (μg/mg of protein)	96.8 ± 3.4	98.2 ± 2.6	NS
Total phospholipids (μg/mg of protein)	282.3 ± 9.6	276.2 ± 6.8	NS
Cholesterol/phospholipid molar ratio	0.620 ± 0.05	0.643 ± 0.04	NS

[a]Cholesterol and phospholipids were determined as described in Materials and Methods. Results are expressed as mean ± SD of three experiments. All other conditions as in Table I. NS, not significant.

Yamada and Lieber, 1984). Table II shows that the total cholesterol and phospholipid content of LPMs as well as their cholesterol/phospholipid molar ratio were unmodified by iron treatment. Also the lipid/protein ratio was not significantly influenced under the present experimental conditions. In fact, the iron treatment did not even appreciably modify the LPM protein recovery: 0.85 ± 0.1 in control vs. 0.80 ± 0.2 μg/g wet tissue in iron-loaded rats.

To directly assess the effect of iron intoxication on the membrane molecular order, we measured the membrane fluidity state by incorporating a spin-labeled fatty acid (5-doxylstearic acid) and determining the temperature dependence of the so-called order parameter S (Hayes Griffith and Jost, 1976; Lange *et al.*, 1985). In our experimental model the iron treatment performed *in vivo* did not influence the order parameter of the plasma membrane. In fact, Fig. 1 shows that the temperature-dependent decrease in order parameter S denoted the same increase in plasma membrane fluidity in the control and in iron-treated rats.

In conclusion, these results indicate that during chronic dietary iron overload the enhancement of lipid peroxidation of liver plasma membranes, as indicated by a specific modification in their phospholipid fatty acid composition, is not associated with membrane fluidity changes, an event which could lead to membrane dysfunction and cell damage. This finding may indicate that lipid peroxidative reactions induced by chronic dietary iron overload are not able *per se* to cause a direct damage to LPMs, so adding substantial weight to the current view that lipid peroxidation appears to be more important in triggering and/or amplifying other damage events than directly causing injury (Poli *et al.*, 1987; Tribble *et al.*, 1987).

On the other hand, another possible explanation is that the extent of lipid peroxidation in LPMs from siderotic rats remained, *in vivo*, below a

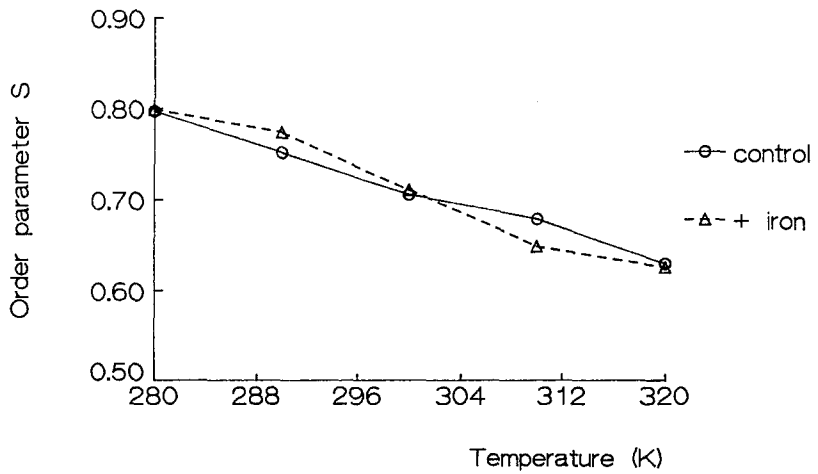

Fig. 1. Effect of chronic dietary iron overload on the rat plasma membrane fluidity. Temperature-dependent plasma membrane fluidity was measured as described in Materials and Methods. Data represent the mean for four different experiments.

critical value. In fact, Debretsov *et al.* (1987), studying the effect of the *in vitro* lipid peroxidation, 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 came to the same conclusion studying different liver membrane fractions (Richter, 1987; Griffith-Green *et al.*, 1988). The finding that the content of hepatic GSH was unmodified at this level of iron intoxication (unpublished observation), so indicating that cellular defense mechanisms were not compromised, may also support the above proposal.

Acknowledgments

This work was supported by a grant of the Consiglio Nazionale delle Ricerche, contract 87.015 48.04, and by a grant from M.P.I. 40% "Patologia Radicali Liberi."

References

Bacon, B. R., Tavill, A. S., Brittenham, G. M., Park, C. H., and Recknagel, R. O. (1983) *J. Clin. Invest.* **71**, 429–439.

Bacon, B. R., Park, C. H., Brittenham, G. M., O'Neil, R., and Tavill, A. S. (1985) *Hepatology* **5**, 789–797.

Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468.

Bartoz, G. (1981) *Biochim. Biophys. Acta* **664**, 69–73.

Boyer, J. L., Allen, R. M., and Ng, O. C. (1983) *Hepatology* **3**, 18–28.

Curtis, M. T., Gilfor, D., and Farber, J. (1984) *Arch. Biochem. Biophys.* **235**, 644–649.
Dobretsov, G. E., Borschevsckaya, T. A., Petrov, V. A., and Vladimirov, Yu. A. (1977) *FEBS Lett.* **84**, 125–128.
Farber, J. L., and El-Mofty, B. K. (1975) *Am. J. Pathol.* **81**, 237–250.
Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) *J. Biol. Chem.* **226**, 497–509.
Griffith-Green, E. A., Zaleska, M. M., and Erecinskca, M. (1988) *Biochem. Pharmacol.* **37**, 3071–3077.
Hayes Griffith, O., and Jost, P. C. (1976) In *Spin Labeling, Theory and Applications* (Berliner, L. J., ed.), Academic Press, New York, pp. 454–523.
Lange, A., Marsh, D., Wassmer, K-H., Meier, P., and Kothe, G. (1985) *Biochemistry* **24**, 4383–4392.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1952) *J. Biol. Chem.* **193**, 265–275.
Masini, A., Ceccarelli-Stanzani, D., Trenti, T., and Ventura, E. (1984a) *Biochim. Biophys. Acta* **802**, 253–258.
Masini, A., Ceccarelli-Stanzani, D., Trenti, T., Rocchi, E., and Ventura, E. (1984b) *Biochem. Biophys. Res. Commun.* **118**, 356–363.
Masini, A., Trenti, T., Ceccarelli-Stanzani, D., and Muscatello, U. (1986) *Ann. N.Y. Acad. Sci.* **488**, 517–519.
Peters, T. J., O'Connel, M. J., and Ward, R. J. (1985) In *Free Radicals in Liver Injury* (Poli, G., Cheeseman, K. M., Dianzani, M. U., and Slater, T. F., eds.), IRL Press, Oxford, pp. 107–115.
Poli, G., Albano, E., and Dianzani, M. U. (1987) *Chem. Phys. Lipids* **45**, 117–142.
Powell, L. W., Bassett, M. L., and Halliday, J. W. (1980) *Gastroenterology* **78**, 374–381.
Richter, C. (1987) *Chem. Phys. Lipids* **44**, 175–189.
Schachter, D. (1984) *Hepatology* **4**, 140–151.
Shinitzky, M., and Henkart, P. (1979) *Int. Rev. Cytol.* **60**, 121–147.
Tribble, D. L., Aw, T. Y., and Jones, D. P. (1987) *Hepatology* **7**, 377–387.
Ungemach, F. R. (1985) In *Free Radicals in Liver Injury* (Poli, G., Cheeseman, K. M., Dianzani, M. U., and Slater, T. F., eds.), IRL Press, Oxford, pp. 127–134.
Yamada, S., and Lieber, Ch. S. (1984) *J. Clin. Invest.* **74**, 2285–2289.