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THE EFFECT OF IRON OVERLOAD ON THE MITOCHONDRIAL PORPHYRIN LEVEL IN THE HEXACHLOROBENZENE INDUCED EXPERIMENTAL PORPHYRIA

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SUMMARY: Liver mitochondria isolated from rats treated with hexachlorobenzene plus iron, present a lower content of total porphyrin in respect to that of mitochondria from rats fed hexachlorobenzene alone.The *in vitro* mitochondrial porphyrin accumulation processes have been studied in mitochondria from iron loaded rats. It has been found that under these conditions the active porphyrin uptake process, which is driven by the K^+ transmembrane gradient, is maximally inhibited in the presence of pentachlorophenol at a concentration similar to that found *in vivo* in the hexachlorobenzene experimental porphyria. By contrast the same degree of inhibition is presented by control mitochondria only in the presence of pentachlorophenol plus valinomycin, a condition which collapses the transmembrane K^+ gradient. A strict correlation between porphyrin uptake and K^+ concentration has been found in control as well as in iron treated mitochondria. A possible involvement of peroxidative reactions in the mitochondrial membranes has been proposed as a cause of the changes in the permeability properties of the mitochondrial membranes in the experimental chronic hepatic porphyria under conditions of iron overload.

Hexachlorobenzene (HCB)-induced porphyria in man closely resembles naturally occurring porphyria cutanea tarda. both clinically and biochemically (1). It has been established that feeding HCB to rats leads to a marked decrease in liver uroporphyrinogendecarboxylase activity (2), which accounts for the hepatic accumulation and urinary excretion of higher carboxylated porphyrins. Hepatic iron overload is a feature of porphyria cutanea tarda (3) and depletion of the body's iron stores by repeated phlebotomy has become standard terapy in this condition (4). Similarly, prior treatment of animals with iron accelerates the onset of HCB-induced porphyria (5). However, the precise biochemical mechanism underlying the synergistic action of iron in the experimental model as well as in the human pathological condition has not been fully clarified (6). Given the

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central role plaied by mitochondria in heme metabolism, studies have been performed on the functional properties of liver mitochondria isolated from rats subjected either to HCB treatment alone or to HCB in combination with iron overload (HCB-Fe) (7-9). It has been shown that the partial and reversible uncoupling exhibited by liver mitochondria under these experimental conditions is due to the presence of pentachlorophenol (PCP), an *in vivo* metabolite of HCB (10-11). It has also been confirmed that iron exherts a synergistic action on the porphyrogenic effect of HCB, resulting either in an increased excretion of foecal and urinary porphyrins (12) or in an augmented amount of porphyrins in the hepatic tissue (9,13). In contrast, it has been found that the level of hepatic mitochondrial porphyrins appeared to be decreased in the case of HCB plus Fe treatment in respect to HCB alone (7-8). Experimental evidence has been presented that the *in vitro* porphyrin uptake by liver mitochondria takes place in part by an energy independent mechanism and in part by a mechanism which depends on the transmembrane K^+ gradient and metabolic energy (14-16).

In the present research the process of porphyrins accumulation by liver mitochondria isolated from rats made siderotic by dietary supplementation with carbonyl iron has been investigated with a view of giving an insight into the role of iron overload in decreasing the mitochondrial porphyrin level in the HCB-induced experimental porphyria.

MATERIALS AND METHODS

Female Wistar albino rats (150-180g body wt) were divided in four groups. The first group was given 0.2% (wt/wt) hexachlorobenzene in food ad libitum for 60 days. Another group was made siderotic by dietary supplementation with 2.5% (wt/wt) carbonyl iron for the same period. The third group received both these treatment contemporary. A control group was maintained without treatment. Animals from each group were killed by decapitation on day 60 after an overnight starvation period. Liver mitochondria were prepared in 0.25 M sucrose according to a standard procedure (17).

Total porphyrins of the hepatic tissue and of the mitochondrial fraction were measured according to Gaetani et al.(18) by high performance liquid chromatography (HPLC) on a Varian Aerograph 8500 high-pressure liquid chromatograph equipped with a Varian fluorimetric detector (Fluorichrom).

The accumulation of protoporphyrin IX by liver mitochondria was determined as described in Ref. 16. Mitochondria (2 mg/ml) were incubated in a standard medium at 25°C. The reaction was initiated by the addition of 9 uM protoporphyrin IX. 2 min later 0.5 ml samples were withdrawn and centrifuged for 1 min in an Eppendorf bench centrifuge (Mod. 5114 S). Porphyrins uptake was determined fluorimetrically by measuring the decrease in the amount of porphyrins in the supernatant (405 nm excitation and 605 nm emission).

The concentration of K^+ in the mitochondrial fraction was determined as follows: mitochondria (2 mg/ml) were incubated in a standard medium at 25°C. After 3 min 0.5 ml of mitochondrial suspensions was removed and centrifuged for 1 min in an Eppendorf centrifuge. The pellet was washed twice with cold standard medium without K^+ and then dispersed by extensive vortexing in 1% Na-cholate containing 1 mM EDTA. The K^+ content was then determined by atomic absorption.

The composition of the standard medium was as follows: 0.25 M sucrose, 10 mM Hepes buffer (pH 7.4) and 10 mM KCL.

Hexachlorobenzene was from Merck, Munchen (F.R.G.)

Carbonyl iron was obtained from Fluka, Buchs (Switzerland).

RESULTS AND DISCUSSION

The content of total porphyrins in the liver and in the mitochondrial fraction from rats treated for 60 days with either HCB, iron (Fe) or HCB plus Fe is reported in Table I. It appears that feeding HCB to rats leads to a large increase in the porphyrin level of the hepatic tissue. The combined treatment of HCB with iron induces a further, although slight, increase in this level. It is noteworthy that the amount of porphyrin in the mitochondrial fraction does not follow this pattern, the mitochondrial porphyrin content being lower of about 25% in the case of rats treated with HCB plus Fe in respect to those with HCB alone. All these data are in agreement with our previous reports (8,9) as well as with results from other Authors (7,13).

TABLE I. Porphyrin level in the hepatic tissue and in the mitochondrial fraction of rats treated for 60 days with either hexachlorobenzene (HCB), hexachlorobenzene plus iron (HCB + Fe) or iron (Fe)

Animals	Liver (μg/g)	Mitochondria (μg/mg protein)
Control	0.3 ± 0.1	0.03 ± 0.01
Fe	0.3 ± 0.1	0.02 ± 0.01
HCB	131 ± 29	3.18 ± 0.5
HCB + Fe	152 ± 36	2.39 ± 0.4

Porphyrin analyses were performed by high performance liquid chromatography (HPLC) as described in the Methods. Porphyrins represent the sum of uroporphyrin and hepta carboxyporphyrin. Mean values of 4-6 animals ± S.D. are given.

Table II. Protoporphyrin IX uptake by liver mitochondria isolated either from control or Fe-treated rats

Additions	Porphyrin uptake (nmol/mg protein)	
	Control	Fe-treated
None	3.34 ± 0.24	3.16 ± 0.09
PCP (1.5 μM)	2.24 ± 0.13	1.55 ± 0.11
Valinomycin (0.5 μg/mg)	3.20 ± 0.27	2.99 ± 0.10
Valinomycin + PCP	1.60 ± 0.18	1.62 ± 0.13

Mitochondria (2mg/ml) were incubated as described in the Methods for 1 min. Porphyrin was then added and the reaction terminated at 2 min. Porphyrin uptake was determined fluorimetrically as described in the Methods. Mean values of 4 different experiments ± S.D. are given.

A possible explanation to this observation is that the processes of mitochondrial porphyrin accumulation may be affected by iron. It is well established that *in vitro* liver mitochondria accumulate porphyrins by two different mechanisms, a passive binding and an active process driven by the transmembrane K^+ gradient. The experiments depicted below were carried out to test whether an excess of iron may interfere with these processes. Table II shows that the addition of PCP, at a concentration similar to that found in liver mitochondria isolated from HCB fed rats (10), impairs the energy dependent accumulation of protoporphyrin IX by control mitochondria. When valinomycin is also present, a condition which collapses the transmembrane K^+ gradient, this uptake is further decreased in agreement with Romslo et al (14-16). It appears from the same Table that mitochondria isolated from iron loaded rats present the minimum active uptake in the presence of PCP alone. The contemporary addition of valinomycin does not further modify this process. Similar results were obtained with uroporphyrins (not shown).

In order to assess whether the inhibitory action of iron on the energy dependent mitochondrial porphyrin uptake may result from a perturbation of the

TABLE III. Potassium content of liver mitochondria isolated either from control or Fe-treated rats

Additions	K^+ concentration (nmol/mg protein)	
	Control	Fe-treated
None	98 ± 15	74 ± 15
PCP (1.5 μM)	59 ± 16	39 ± 12
Valinomycin (0.5 μg/mg)	93 ± 17	70 ± 10
Valinomycin + PCP	44 ± 15	41 ± 15

Mitochondria (2 mg/ml) were incubated as described in the Methods. The K^+ concentration was determined by atomic absorption. Mean values of 4 different experiments ± S.D. are given.

mitochondrial K^+ level, the concentration of K^+ was parallely measured under the same experimental conditions. Table III shows that the concentration of mitochondrial K^+ exhibits a minimum value when PCP is added in combination with valinomycin to control mitochondria. By contrast mitochondria from iron loaded rats present the lowest level in the presence of PCP alone. As to this point it has to be noted that the *in vivo* induction of lipid peroxidation of the mitochondrial membranes was found to be associated with this degree of mitochondrial iron overload (7,19,20). Lipid peroxidation may thus constitute a plausible explanation for this event. Indeed, lipoperoxidative reactions may modify the permeability properties of the mitochondrial membranes. As to this aspect it has been recently shown that the *in vitro* induction of mitochondrial lipid peroxidation with ferrous iron leads to a drop of the membrane potential and K^+ efflux (21). A similar realationship between a lack of antioxidant capability, a fall in membrane potential and a decrease in the mitochondrial K^+ concentration was also observed in mitochondria from rats intraperitoneally injected with iron(III)-gluconate complex (22).

From Table II and III it may be derived a strict correlation between the active accumulation of protoporphyrin IX and the endogenous level of mitochondrial K^+ in the control as well as in the iron loaded mitochondria.

The results here presented demonstrate that the permeability properties of the inner mitochondrial membrane are modified by the presence of an excess of iron, so that the *in vitro* energy dependent process of porphyrin uptake by mitochondria is severely impaired under conditions which are very similar to those occurring *in vivo* in the HCB experimental model. Indeed, the maximum inhibition to the energy dependent porphyrin accumulation has been observed by adding the same PCP concentration which was detected *in vivo* in liver mitochondria from HCB treated rats. The above considerations and the observation that iron overload leads to a stimulation of δ-aminolaevulinate synthase (23) may thus reasonably explain the finding that the combined treatment of HCB with Fe results in higher level of tissutal porphyrin and lower level of mitochondrial porphyrin in respect to the treatment with HCB alone. The finding that iron loaded mitochondria on addition of PCP accumulate approx 30% less porphyrins than control, a value very similar to the per cent difference measured in the mitochondrial porphyrin level between HCB plus Fe and HCB treated rats, may give experimental support to the above proposal.

The present results may give a better insight into the biochemical mechanism by which excess iron exacerbates the condition of experimental chronic hepatic porphyria

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REFERENCES

1. De Matteis, F., Prior, R.E. and Rimington, C. (1961) Nature 191, 363-366.
2. Elder, G.H., Evans, J.O. and Matlin, S.A. (1976) Clin. Sci. Molec. Med. 51, 71-80.
3. Uys, C.J. and Eales, L.(1963) S. Afr. J. Lab. Clin. Med. 9,190-196.
4. Epstein, J.H. and Redeker, A.G. (1968) New Engl. J. Med. 279, 1301-1304.
5. Taljaard, J.J.F., Shanley, B.C., Deppe, W.M. and Joubert, S.M. (1972) Brit. J. Hemat. 23, 513-519.
6. De Matteis, F. and Stonard, M.(1977) Semin. Hematol. 14, 187-192.

7. Hanstein, W.G., Heitmann, T.D., Sandy, A., Biesterfeldt, H.L., Ljem, H.H. and Muller-Eberhard, U. (1981) Biochim. Biophys. Acta 678, 293-299.
8. Masini, A., Ceccarelli-Stanzani, D., Trenti, T., Rocchi, E. and Ventura, E. (1984) Biochem. Biophys. Res. Commun. 118, 356-363.
9. Masini, A., Ceccarelli-Stanzani D., Trenti, T. and Ventura, E. (1984) Biochim. Biophys. Acta 802, 253-258.
10. Masini, A., Ceccarelli-Stanzani, D., Tomasi, A., Trenti, T. and Ventura, E. (1985) Biochem. Pharmacol. 34, 1171-1174.
11. Trenti, T., Ventura, E., Ceccarelli, D. and Masini, A. (1986) in: Hexachlorobenzene (Morris, C.R. and Cabral, J.R.P. eds.) pp. 457-459, IARC Scientific Publications, Oxford University Press, Oxford.
12. Blekkenhorst, G.H., Day, R.S. and Eales, L. (1980) Int. J. Biochem. 12, 1013-1017.
13. Smith, A.G., Stewart, F.P. and Francis, J.E. (1986) in: Hexachlorobenzene (Morris, C.R. and Cabral, J.R.P. eds.) pp. 433-439, IARC Scientific Publications, Oxford University Press, Oxford.
14. Koller, M.E. and Romslo, I. (1978) Biochim. Biophys. Acta 503, 238-250.
15. Koller, M.E. (1979) FEBS Lett. 100, 47-51.
16. Koller, M.E. and Romslo, I. (1980) Biochem. J. 188, 329-335.
17. Masini, A., Ceccarelli-Stanzani, C. and Muscatello, U. (1983) Biochim. Biochim. Acta 724, 251-257.
18. Gaetani, E. Laureri, C.F., Vitto, M., Rocchi, E.,Gibertini, P., Farina, F., Cassanelli, M. and Ventura, E. (1982) J. Chromatogr. 231, 425-432.
19. Masini, A., Trenti, T., Ceccarelli, D. and Muscatello, U. (1986) Ann. N.Y. Acad. Sci. 488, 517-519.
20. Bacon, B.R., Tavill, A.S., Brittenham, G.M., Park, C.M. and Recknagel, R.O. (1983) J. Clin. Invest. 71, 429-439.
21. Marshansky, V.N., Novgorodow, S.A. and Yaguzhinsky, L.S. (1983) FEBS Lett. 158, 27-30.
22. Masini, A, Trenti, T., Ventura, E., Ceccarelli-Stanzani, D. and Muscatello, U. (1984) Biochem. Biophys. Res. Commun. 124, 462-465.
23. De Matteis, F. and Sparks, R.G. (1973) FEBS Lett.29, 141-144.