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STRUCTURAL AND FUNCTIONAL PROPERTIES OF RAT LIVER MITOCHONDRIA IN HEXACHLOROBENZENE INDUCED EXPERIMENTAL PORPHYRIA

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SUMMARY: A possible link between changes in iron and porphyrin content in liver mitochondria, from rats treated with either hexachlorobenzene, iron, or hexachlorobenzene plus iron, as a function of treatment time and their structural-functional properties, has been investigated. Normal oxidative phosphorylation in mitochondria from rats treated with iron has been shown. By contrast a significant and constant uncoupling of the phosphorylative process, fully reversed by albumin, in mitochondria from rats treated with hexachlorobenzene and hexachlorobenzene plus iron has been presented. A possible involvement of pentachlorophenol in causing these abnormalities has been proposed.

Polyhalogenated aromatic hydrocarbons and particularly hexachlorobenzene (HCB), have been largely used to induce animal symptomatic porphyria, which biochemically closely resembles porphyria cutanea tarda (PCT) (1). Hepatic siderosis, which is consistently detected in PCT (2), can also be induced in animals by injecting iron complexes, either on their own or in addition to HCB treatment. In spite of a large number of studies, the mechanism of the porphyrogenic action of HCB and the role that iron plays in this process are still poorly understood (1,3,4).

Given the primary role played by mitochondria in heme metabolism, studies performed on mitochondria isolated from animals subjected to porphyrogenic treatment have revealed various modifications of their structural and functional properties (5,6). However, no definitive and complete evidence has emerged to clarify the mechanism underlying these modifications.

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The present research is aimed at elucidating the possible link between mitochondrial abnormalities and the pathological state of HCB induced experimental porphyria.

MATERIALS AND METHODS

Female Wistar albino rats (160-200 g body wt.) were divided into 4 groups. The first group was given 0.2% HCB in food ad libitum for 60 days. Another group was made siderotic by injecting intraperitoneally 12.5 mg iron as iron-sucrose complex (Ferlixit) 6 times during the 60 days period. The third group received both these treatments contemporary. A control group was maintained without treatment. Six animals for each group were killed by decapitation on days 20, 40 and 60 after an overnight starvation period, during which urine and faeces were collected. The livers were pooled within a group and used for the preparation of mitochondria in 0.25 M sucrose, according to a standard procedure (7).

Porphyrins and iron content analyses were performed on mitochondria previously broken by osmotic shock and freezing-thawing procedures (8). Iron was determined by an atomic absorption Perkin-Elmer spectrophotometer mod. 306. Porphyrins were measured according to Gaetani et al. (9) by high performance liquid chromatography (HPLC) on a Varian Aerograph 8500 high-pressure liquid chromatograph equipped with a Varian fluorimetric detector (Fluorichrom).

The oxygen uptake was assayed with a Clark oxygen electrode at 25 C in a final volume of 3 ml. The incubation medium had the following composition: 100 mM NaCl; 10 mM $MgCl_2$; 10 mM Tris-HCl buffer (pH 7.4); 10 mM NaK-phosphate buffer (pH 7.4) and 1.6 mM Na-pyruvate plus 0.4 mM L-malate or, where indicated, 2 mM Na-succinate as the substrate. The mitochondrial concentration, as determined by the biuret method, ranged between 3-3.5 mg protein/ml.

Ferlixit was obtained from Natterman and Cie. GMBH, Köln, (Germany). HCB was from Merck, München (Germany).

RESULTS

The changes in the content of porphyrins and iron in liver mitochondria isolated from rats treated with either HCB, iron (Fe) or HCB plus Fe, as a function of time of treatment, are reported in Table 1. It appears from the Table that HCB treatment results in a consistent increase in the mitochondrial porphyrins level since the early period. At this time, no appreciable variations in the urine and faeces level of porphyrins are observable (not shown) . HCB administration is also paralleled by a consistent increase in the iron content of mitochondria, whereas a combined treatment of HCB and Fe leads to a substantially lower content of porphyrins and iron than single treatments. These results are

TABLE 1. Iron and total porphyrins content of liver mitochondria from rats treated for different periods with either HCB, Fe, or HCB plus Fe.

Treatment	Total porphyrins (μg/mg of protein)			Iron (ng atoms/mg of protein)		
	[a]20 days	40 days	60 days	20 days	40 days	60 days
None	0.008	0.007	0.008	3.58	4.18	5.01
Fe	0.003	0.006	0.008	38.70	48.20	101.00
HCB	0.035	1.107	3.185	6.63	12.03	20.04
HCB+Fe	0.024	0.377	2.388	23.30	36.70	50.70

Iron and total porphyrins content of rat liver mitochondria were determined as described in the Methods. Total porphyrins represent the sum of uroporphyrins and heptacarboxyporphyrins.
[a]Days of treatment.

in agreement with previous findings referred to only one time of treatment, i.e. 60 days (6).

The efficiency of oxidative phosphorylation process was also tested. It appears from Table 2 that treatment of rats with Fe does not appreciably modify the properties of liver mitochondria. By contrast treatment with either HCB alone or with HCB plus Fe leads to substantial modifications since the early period of tre-

TABLE 2. Effect of different periods of treatment of rats with either HCB, Fe, or HCB plus Fe on oxidative phosphorylation in liver mitochondria.

Substrate	Treatment	RCI			ADP/O		
		[a]20 days	40 days	60 days	20 days	40 days	60 days
pyruvate + malate	None	3.78	3.90	3.56	2.58	2.48	2.43
	Fe	4.42	4.03	3.76	2.70	2.55	2.38
	HCB	2.18	2.03	1.83	2.08	1.98	1.66
	HCB+Fe	1.65	1.76	1.97	1.88	1.90	1.87
Succinate	None	5.12	4.70	4.80	1.86	1.83	1.75
	Fe	5.08	4.91	4.66	1.90	1.82	1.78
	HCB	2.74	2.42	2.32	1.55	1.54	1.36
	HCB+Fe	1.81	1.80	1.84	1.40	1.41	1.38

Mitochondria were incubated for 2 min in state 4, as described in the Methods, before addition of 0.33 mM ADP. The respiratory control index (RCI) is the ratio of the respiration rate measured in state 3 to that in state 4.
[a]Days of treatment.

atment. The extent of the impairment of respiratory parameters does not reflect the behaviour of porphyrins and iron levels as a function of treatment time, that is, it reamains nearly constant.

As the impairment of respiratory control essentially represents an increased state 4 respiration rate, a careful examination of this process was made. In this regard, it has been recently shown, by the use of oligomicin (10,11), that a significant part of respiration in this state (i.e. about 50%) is coupled to ATP synthesis. Therefore, the use of this antibiotic proves to be a valuable tool for obtaining more detailed information on the control of state 4 respiration: it can thus be assessed whether this enhanced oxygen uptake is due to a stimulation of oxidative phosphorylation or to the occurrence of other energy -dissipating processes (12). Table 3 shows that state 4 respiration of mitochondria from Fe treated rats is inhibited up to at least 50% by oligomycin, a value very similar to that observed in control mitochondria. By contrast, the lesser extent of inhibition by oligomycin of respiration of mitochondria from either HCB or HCB plus Fe treated rats, indicates that a larger part of respiration is not coupled to phosphorylation. The effect of albumin,a known scavanger of uncoupling agents, further confirms this observation.

Table 4 shows the effect of albumin on the oxidative phosphorylation after 60 days treatment. The presence of albumin fully

TABLE 3. Effect of oligomycin on state 4 respiration of liver mitochondria from either HCB, Fe, or HCB plus Fe treated rats.

Treatment	Respiratory rate (ngatoms $O.min^{-1}.mg\ protein^{-1}$)	% Inhibition by oligomycin	
		-BSA	+BSA
None	12.8	50	52
Fe	12.5	48	50
HCB	22.6	28	46
HCB+Fe	21.8	26	44

Mitochondria were incubated, as described in the Methods, for 2 min in state 4 before addition of oligomycin (1 µg/mg protein). When present, 0.1% bovine serum albumin, fatty acid free, was added to the incubation medium before addition of mitochondrial suspension. 60 days treated rats were used for the preparations of mitochondria.

TABLE 4. Effect of albumin on oxidative phosphorylation of liver mitochondria from either HCB, Fe, or HCB plus Fe treated rats.

Treatment	State 4 rate	RCI	ADP/O
None	11.6	4.10	2.50
Fe	10.7	4.50	2.44
HCB	10.8	3.56	2.46
HCB+Fe	9.6	3.76	2.39

Mitochondria were incubated as in Table 2. 0.1% albumin was present in all the incubations before addition of mitochondrial suspensions.

restores both respiratory control index and ADP/O ratio of mitochondria from either HCB or HCB plus Fe treated rats, whereas it does not appreciably affect the functional parameters of mitochondria from Fe treated rats. It must be stressed that mitochondria from rats subjected to all the different treatments, do not oxidize externally added NADH, thus indicating that the inner membrane is structurally and functionally unaltered (13).

Table 5 shows that albumin, if present at low concentrations, fails to restore normal state 4 respiration in mitochondria from HCB treated rats. Furthermore,when the metabolic medium contains both ATP and CoA, thus stimulating mitochondrial re-acylating sys-

TABLE 5. Effect of various concentrations of albumin on state 4 respiration of liver mitochondria from HCB treated rats.

Additions to system	State 4 rate	
	Control	HCB
None	12.60	21.95
Albumin (0.1 mg/ml)	10.32	17.83
" (0.2 mg/ml)	=	17.03
" (0.6 mg/ml)	=	10.56
" (1.0 mg/ml)	10.02	10.03
ATP (1mM)+CoA (0.2 mM)	10.82	18.25

Mitochondria from either control or HCB treated rats were incubated in state 4 as described in the Methods. Albumin and ATP plus CoA, when present, were added to the incubation medium before addition of mitochondrial suspension.60 days treated rats were used for the preparation of mitochondria.

tem, very low decrease in the oxygen uptake is detectable.Similar effects were observed on the phosphorylation efficiency (not shown).

DISCUSSION

The results here presented lead to the following conclusions: a) HCB and HCB plus Fe treatments uncouple mitochondrial oxidative phosphorylation at the same extent through the whole treatment period; b) no irreversible damages are observable under these experimental conditions; c) iron treatment does not appreciably modify mitochondrial functional properties.

Many causes may be envisaged to account for the anomalies of respiration in this pathological state. A loose coupling (i.e.an increased state 4 respiration but normal P/O ratios) of mitochondrial respiration, only with NAD linked substrates, was reported to occurr in mitochondria from rats made porphyric with allylisopropylacetamide (5); δ-aminolevulinic acid was indicated as responsible of these abnormalities (5). A similar loose coupling of respiration was observed in mitochondria which had accumulated in vitro an amount of porphyrins at least 2 or 3 fold higher than those here measured (15). This present work has shown however that the extent of impairment of oxidative phosphorylation does not parallel the progressive enhancement with time of mitochondrial porphyrin content and urinary δ-aminolevulinic acid level (unpublished observations). Furthermore, the modifications here observed in the phosphorylative process do not resemble a loose coupling condition but rather indicate an uncoupled state.

It is a general accepted view that lipid peroxidation can disturb mitochondrial functions. PCT is accompanied by epatic siderosis and iron, in various form, is known to catalyse this process (16,17). By the observation that HCB plus Fe treatment resulted in mitochondrial damage beyond reversible uncoupling, lipid peroxidation was indicated as the possible cause (6,14). However no direct correlation between the extent of lipid peroxidation and impairment of mitochondrial metabolic activity was presented to support this suggestion (6). The present results do not reveal

any modification in mitochondrial functions in concomitance to a progressive increase in iron content during the treatment period.

Fatty acids and chloro-phenolic metabolites of HCB seem to be the most plausible candidates to account for the uncoupling of oxidative phosphorylation here observed. In fact,fatty acids might accumulate as a consequence of phospholipases activation. On the other hand,pharmacokynetical studies have shown that, during administration of HCB to rats, pentachlorophenol,a well known uncoupler of oxidative phosphorylation both in vitro (18) and in vivo (19) is the major metabolite recovered in tissues (1). The results reported in Table 5 exclude that fatty acids may be to a large extent responsible for this uncoupled condition: in fact, concentrations of albumin that remove fatty acids but not PCP (20) fail to restore normal oxidative phosphorylation. Furthermore, this uncoupling is not substantially reversed also when the mitochondrial re-acylating system is stimulated by the addition of ATP and CoA.

The present results and the above considerations indicate that mitochondrial derangement in HCB induced experimental porphyria is to a large extent due to the action of phenolic metabolite of HCB rather than to disturbances of porphyrin metabolism. The presence of this metabolite may account not only for the uncoupling of phosphorylation observed when HCB is administered but also for the lower content of Fe measured in the case of combined HCB plus Fe treatment, when compared to treatment with Fe alone; since there is evidence that the energy-dependent uptake of Fe by mitochondria is largely reduced in the presence of uncouplers (21).

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