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THE ROLE OF PENTACHLOROPHENOL IN CAUSING MITOCHONDRIAL DERANGEMENT IN HEXACHLOROBENZENE INDUCED EXPERIMENTAL PORPHYRIA

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Abstract—Hexachlorobenzene feeding to rats for 60 days to induce experimental porphyria resulted in partial and constant uncoupling of oxidative phosphorylation of liver mitochondria from the early phase (i.e. 20 days) of treatment. Direct experimental evidence has been presented that this uncoupling is completely due to the action of pentachlorophenol endogenously formed by metabolism of hexachlorobenzene. The complete restoration of membrane potential by albumin under these conditions indicates that no irreversible damage occurs in the mitochondrial membrane. No appreciable correlation between concentrations of pentachlorophenol and the degree of porphyria has been observed.

The experimental hepatic porphyria induced in animals by hexachlorobenzene (HCB) closely resembles human chronic hepatic porphyria, i.e. porphryia cutanea tarda (PCT) [1, 2] and it is used as a good model for this pathological state [2, 3]. Accumulation of uroporphyrins and heptacarboxy-porphyrins under these experimental conditions is related to a decrease in the activity of hepatic uroporphyrinogen decarboxylase [2]. The porphyrogenic effect of HCB, however, is not due to a direct interaction with heme synthesis, but, rather, a metabolite or a reactive intermediate formed by metabolism of HCB is suspected to be responsible for the disturbance of this metabolic pathway [2]. In this regard, attention has been paid to the structural and functional properties of mitochondria in rats subjected to HCB treatment. Anomalies in oxidative phosphorylation have been reported to occur [4, 5]. The enhancement of either mitochondrial porphyrins or fatty acids was proposed as a possible cause of this functional disturbance [4, 5]. More recently, it has been suggested that pentachlorophenol (PCP), a well known uncoupler of oxidative phosphorylation in mitochondria [7, 8], endogenously formed by metabolism of HCB [2, 9], may play a primary role in disturbing the oxidative phosphorylation mechanism [10]. The possibility of changes in mitochondrial membrane permeability induced by HCB has also been considered [6].

It seems therefore of interest to estimate directly the amount of HCB and of endogenous PCP linked to the mitochondrial fraction isolated from rats treated for different periods with HCB. The degree of correlation between the concentration of PCP, the level of porphyrins and the extent of mitochondrial derangement, as a function of treatment time, may serve to clarify the nature of the mitochondrial abnormality in the HCB experimentally induced porphyria.

MATERIALS AND METHODS

Female Wistar albino rats (160–200 g body wt) were divided into 2 groups. The first group was given 0.2% HCB in food *ad libitum* for 60 days. A control group was maintained without treatment. Animals of each group were killed by decapitation on days 20, 40, 60 after an overnight starvation, during which urine and faeces were collected. The livers were immediately used for the preparation of mitochondria in 0.25 M sucrose, according to a standard procedure [11].

Porphyrins were measured according to Gaetani *et al.* [12] by high performance liquid chromatography (HPLC) on a Varian Aerograph 8500 high-pressure liquid chromatograph equipped with a Varian detector (Fluorichrom).

The oxygen uptake was assayed with a Clark oxygen electrode at 25° in the final volume of 3 ml. The incubation medium for assaying both respiratory and electrochemical parameters had the following composition: 100 mM NaCl; 10 mM $MgCl_2$; 10 mM Tris–HCl buffer (pH 7.4); 10 mM Na, K-phosphate buffer (pH 7.4) and 1.6 mM Na-pyruvate plus 0.4 mM L-malate as the substrate. The respiratory states were those defined by Chance and Williams [13] on the basis of the factors limiting the respiratory rate.

The transmembrane potential ($\Delta\psi$) was measured at 25° in a final volume of 1.5 ml by monitoring with a tetraphenylphosphonium selective electrode the movements of tetraphenylphosphonium across the membrane, as in [14, 15]. An inner mitochondrial volume of 1.1 μl/mg protein was assumed.

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‖ Abbreviations used: HCB, hexachlorobenzene; PCT, porphyria cutanea tarda; PCP, pentachlorophenol.

The mitochondrial protein concentration, as determined by the biuret method, ranged between 3 and 3.5 mg protein/ml.

HCB and PCP were measured by gas liquid chromatography (GLC). Samples for gas chromatographic analyses were extracated in toluene. Calibration curves and retention time were determined using pure PCP and pure HCB dissolved in toluene. Gas–liquid chromatographic analyses were carried out on a Varian 3600 gas–liquid chromatograph, fitted with an electron capture detector and using a DA 1240 column (Supelco, Inc., U.S.A.); detector and injecting temperature, 250°; oven temperature, 200°. Pure PCP and HCB were obtained from Fluka (Switzerland) and Serva (Germany) respectively.

When data are expressed as mean ± S.E. their significance was assessed by the variance analysis and the difference among media by the Student's *t*-test.

RESULTS

The changes in the content of porphyrins in urine, liver and liver mitochondria as a function of time of treatment with HCB are reported in Table 1. It appears that HCB feeding results in a consistent and progressive increase in porphyrin level. In the early period of treatment, i.e. 20 days, urinary porphyrins are not significantly enhanced. In contrast, hepatic and mitochondrial porphyrin level is largely increased since this time.

The efficiency of oxidative phosphorylation was parallely tested. Table 2 shows that HCB treatment leads to partial uncoupling, whose extent is nearly constant through the whole period of intoxication. The presence of albumin, a known scavenger of uncoupling agents, fully restores the phosphorylative efficiency at all stages of HCB feeding. Essentially similar results were obtained when succinate was used as the substrate (not shown).

The concentration of HCB and PCP in liver and liver mitochondria, as a function of time of intoxication, is reported in Table 3. It appears that PCP is present both in liver homogenate and in mitochondrial fraction from the early period of treatment; the mitochondrial concentration of this compound is of the order of 0.3–0.4 nmoles/mg protein and it remains nearly at a steady-state value through the whole treatment period. A similar pattern is presented by the PCP content in the hepatic tissue. It is interesting to note that the level of HCB associated to mitochondria reaches a threshold value at 20 days

Table 1. The effect of hexachlorobenzene feeding of rats on the concentration of porphyrins in urine, liver and liver mitochondria

Animals	Period on HCB diet (days)	Porphyrins		
		Urine* (μg/l)	Liver† (μg/g)	Mitochondria‡ (μg/mg protein)
HCB-treated	0	23 ± 2	0.3 ± 0.1	0
	20	21 ± 3§	10 ± 3‖	0.03 ± 0.01¶
	40	500 ± 90	109 ± 38	0.9 ± 0.2
	60	740 ± 160	182 ± 14	2.3 ± 0.4

Porphyrin analyses were performed by high performance liquid chromatography (HPLC). Porphyrins represent the sum of uroporphyrins and heptacarboxyporphyrins. Mean values of 6–8 animals are given ± S.E.

* $P < 0.005$; † $P < 0.001$; ‡ $P < 0.005$.

§ NS; ‖ $P < 0.05$; ¶ $P < 0.005$, in respect to zero time.

Table 2. The effect of hexachlorobenzene feeding of rats on oxidative phosphorylation in liver mitochondria

Animals	Period on HCB diet (days)	− BSA		+ BSA	
		RCI	ADP/O	RCI	ADP/O
HCB-treated	0	3.98	2.52	4.10	2.50
	20	2.18	2.08	3.80	2.38
	40	2.03	1.98	3.83	2.30
	60	1.90	1.76	3.83	2.36

Mitochondria were incubated for 2 min under State 4 conditions (standard medium plus substrate) with pyruvate as substrate, followed by the addition of 0.33 mM ADP to induce the activated respiratory rate, or State 3. The respiratory control index (RCI) is the ratio of the respiration measured in State 3 to that in State 4. When present, 0.1% bovine serum albumin fatty acid free (BSA), was added to the incubation medium before addition of mitochondrial suspension. The data are from one of four identical experiments in which the results were within 5% of each other.

Table 3. HCB and PCP concentration in liver and liver mitochondria as a function of treatment time of rats fed with HCB

Animals	Period on HCB diet (days)	Liver		Mitochondria	
		HCB* (nmoles/g)	PCP† (nmoles/g)	HCB‡ (nmoles/mg protein)	PCP§ (nmoles/mg protein)
HCB-treated	0	0	0	0	0
	20	740 ± 140	76 ± 7	3.9 ± 1.0	0.35 ± 0.04
	40	950 ± 280	88 ± 5	4.3 ± 0.8	0.41 ± 0.09
	60	1360 ± 130	92 ± 16	4.4 ± 0.3	0.44 ± 0.06

HCB and PCP concentrations were measured by gas liquid chromatography (GLC). All other conditions as in Table 1.
* $P < 0.0001$; † NS; ‡ NS; § NS.

Table 4. A comparison between the degree of uncoupling caused by PCP endogenously formed *in vivo* and that by PCP externally added *in vitro*

Animals	State 4 respiration (ng atom O/ min. mg protein)	RCI	ADP/O	$\Delta\psi$ (mV) − BSA	$\Delta\psi$ (mV) + BSA
Control	12.4	4.00	2.52	178	184
HCB-treated	21.8	1.88	1.80	156	174
Control + 0.25 μM PCP	14.1	3.52	2.20	168	180
Control + 1.0 μM PCP	22.0	2.00	1.86	158	176
Control + 2.5 μM PCP	33.1	1.42	1.40	130	164

When indicated, either 0.1% bovine serum albumin (BSA) or PCP were added to the incubation medium before the addition of mitochondrial suspension. The membrane potential ($\Delta\psi$) was measured under State 4 conditions with pyruvate as substrate, in the presence of 20 μM tetraphenylphosphonium chloride. All other conditions as in Table 2.

and then it remains nearly constant, whereas a significant increase is detected in the hepatic tissue during the course of intoxication.

The effect of external PCP, added to control mitochondria at various concentrations, on the respiratory parameters and the transmembrane potential is presented in Table 4. It can be seen that the addition of external PCP, at a concentration similar to that actually found *in vivo*, to control mitochondria elicits an impairment of the respiratory control index and of the ADP/O ratio to a value exactly comparable to mitochondria from HCB fed rats. By analogy, control mitochondria, in the presence of this concentration of external PCP, on addition of substrate, develop a membrane potential of about 158 mV, a value very similar to that from intoxicated animals, but consistently lower than that of tightly coupled mitochondria under State 4 conditions, i.e. of about 180 mV [14]. It is worthy to note that albumin fully restores the membrane potential of mitochondria either when PCP is endogenously formed *in vivo* or when it is added *in vitro*. Addition of 10 μM HCB to control mitochondria does not appreciably modify any of the parameters examined (not shown).

DISCUSSION

The results of the present work lead to the conclusion that the partial and constant uncoupling of oxidative phosphorylation exhibited by rat liver mitochondria during the whole period of HCB treatment here investigated is almost completely due to the action of endogenous PCP. Direct experimental evidence has been presented that this metabolite of HCB is present since the early phase of intoxication at a steady-state concentration which completely accounts for the inhibition of ATP synthesis, impairment of RCI and fall in membrane potential. In fact, it has been shown that PCP externally added at a concentration equal to that actually found in the mitochondrial fraction from HCB treated animals caused disturbances in control mitochondria closely resembling those observed in intoxicated rats. All these findings strongly support a previous suggestion that endogenous PCP plays a primary role in causing alterations of oxidative phosphorylation in liver mitochondria from HCB treated rats [10]. These results give also experimental support to a previous hypothesis that PCP acts *in vivo* as it does *in vitro* [16]. Two other possibilities were envisaged to account for the decreased capacity for oxidative phosphorylation in this experimental model: an enhanced level of porphyrins [5] and an increased amount of fatty acids [4]. However, the results here reported indicate that these two factors may contribute to a little extent to this mitochondrial derangement.

Another aspect which merits consideration concerns the mechanism of induction of porphyria by HCB. In a previous report it was shown that there is no correlation between derangement of oxidative

phosphorylation and urinary and hepatic porphyrin content [10]. This observation has been here confirmed (see Table 2). A comparison between the pattern of endogenous PCP concentration and of porphyrin level during the time course of intoxication (see Table 1 and Table 3) makes it reasonable to conclude that PCP is not directly involved in the development of porphyria, in agreement with the conclusion by Koss *et al.* [17]; if PCP was in fact primarily involved in the development of porphyria, a better correlation would be expected between concentration of PCP and urinary and hepatic porphyrins as a function of intoxication time.

Since mitochondria are able to bind PCP extensively [18], the fact that the amount of endogenous PCP found in the mitochondrial fraction is nearly at a constant value during the whole period of HCB feeding, may indicate that there is a steady-state in the production and elimination processes of this metabolite.

As to the problem concerning changes and/or damages in the membranes in the liver of the experimental animals fed with porphyrogenic doses of HCB [4, 6], the present results clearly demonstrate that no irreversible damage occurs in the mitochondrial membrane under the experimental conditions here used, since the membrane potential is not irreversibly depressed, as indicated by the restitutive effect on it by albumin.

The presence of consistent amount of this inhibitor of the mitochondria energy transducing process, here reported, should be taken into account in future studies *in vivo* of mitochondrial pathology in the experimental model.

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