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INHIBITION OF Ca^{2+} SEQUESTRATION IN FOETAL LIVER MICROSOMES BY CARBON TETRACHLORIDE AND BROMOTRICHLOROMETHANE

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Abstract—The purpose of the present work is to establish to what extent the calcium uptake of foetal liver microsomes can be modified, as in the adult, by classical hepatotoxins. The administration of liver toxins ($BrCCl_3$, CCl_4) to the pregnant rat or their addition to foetal and maternal liver microsome preparations causes a decrease in the level of cytochrome P-450 and a drop in the calcium storage capacity of microsomes. Lipid peroxidation of membrane phospholipids is observed in the mother but not in the foetus. On the 20th day of gestation, the foetal liver shows cytochrome P-450 dependent metabolic activity and constitutes a good model illustrating the hypothesis of calcium pump inhibition by $\cdot CCl_3$ radicals without lipoperoxidation.

In the foetus, the conditions favouring liver damage on treatment of the pregnant female with hepatotoxins are still poorly understood. The level of hepatic cytochrome P-450, the terminal oxidase of the mixed-function oxidase system, can only be measured on the very last days of gestation and increases steadily after birth [1]. This can explain the resistance of the new-born, compared to the adult, towards liver damage induced by CCl_4 [2]. Furthermore, during gestation the metabolism of xenobiotics by the maternal liver is also decreased. This is mainly due to the drop in the level of hepatic cytochrome P-450 [3–5].

From these various observations it can be seen that foetal protection is difficult to define. However, the presence of foetal liver damage caused by the activity of transaminases or by histological changes [6–8] was observed after administration of hepatotoxins to the mother. In adult animals alteration of calcium metabolism seems to be one of the first consequences of poisoning by certain hepatotoxins such as organochlorinated compounds. Calcium is thus considered to be a potential mediator of cell damage which can lead to necrosis [9]. Several authors [9–12] have shown that on peroxidation of microsome membrane phospholipids—observed after poisoning with CCl_4 or $BrCCl_3$—the calcium uptake capacity of the liver microsomes is reduced.

In the foetus the calcium sequestration capacity of the hepatic microsomes is high [13]. The purpose of the present study was therefore to see to what extent this activity can be modified, as in the adult, by the action of classical hepatotoxins. Such a modification would support the hypothesis of a cytochrome P-450-dependent metabolic capacity and could constitute indirect proof for the activation of the xenobiotics by the foetal liver.

MATERIALS AND METHODS

Animals and materials.

The animals used in this study were nulliparous pregnant female Sprague–Dawley rats (20th day of gestation) weighing 200–220 g at the date of mating, and new-born rats (5 days old). Insemination was detected by examining vaginal smears the morning after contact with a male. The finding of sperm was considered evidence of insemination and that day was recorded as day 1. The animals were placed in rooms having a constant temperature of 23 ± 1°, 50% humidity, and with automatically regulated lighting (light 07.00 h–19.00 h). They were given a standard feed and tap water *ad libitum*.

NADP, ATP, isocitrate and isocitrate dehydrogenase (E.C. 1.1.1.4.2) were obtained from the Sigma Chemical Co., St Louis, MO. $^{45}Ca^{2+}$ was supplied as aqueous $^{45}CaCl_2$ by the I.R.E. (Fleurus, Belgium).

Experimental procedures.

Pregnant females and new-born rats were decapitated. The foetuses were removed *in utero* and decapitated. Two grams of maternal liver or the same weight of foetal or new-born liver pooled (obtained from a single litter) was homogenized with 20 ml of ice-cold 3 mM EDTA, 154 mM KCl, at pH 7.4. The microsomal fraction was prepared [10], and calcium sequestration capacity was assayed [14].

In vivo administration. Five females received an intragastric dose of 2.5 ml/kg CCl_4 or $BrCCl_3$ undiluted 1 hr before killing.

In vitro assay. The experimental procedure involved initial incubation of the microsomal fraction (37° for 30 min) from untreated rats (pregnant females, foetuses and new-born) with the concentrations of $BrCCl_3$ or CCl_4, given in Fig. 1, in conditions suitable for organochloride metabolism [10]. Then aliquots of incubation medium were used for malondialdehyde (MDA) determination [15] and for a second 30 min incubation in order to determine the calcium sequestration capacity.

Other procedures

Protein was determined according to Lowry *et al.*

Table 1. Effects of chlorinated hydrocarbons on liver microsomes during pregnancy

		Control	$BrCCl_3$	CCl_4
Calcium Sequestration (nmoles/mg prot. per 30 min)	Mother	59 ± 4.8	7 ± 0.5*	18 ± 2.5*
	Foetus	258 ± 13	151 ± 29†	203 ± 17‡
Cytochrome P-450 (nmoles/mg prot.)	Mother	0.535 ± 0.03	0.193 ± 0.018*	0.385 ± 0.042‡
	Foetus	0.086 ± 0.012	<0.02	0.051 ± 0.011‡
Conjugated dienes absorbance of extracted lipid (1 mg/ml of cyclohexane) at 235 nm	Mother	0.297 ± 0.042	0.770 ± 0.17‡	0.530 ± 0.075‡
	Foetus	0.384 ± 0.024	0.389 ± 0.051	0.335 ± 0.059

Calcium uptake, cytochrome P-450 and appearance of conjugated dienes in liver microsomes of mother and 20-day foetuses. Pregnant rats fasted overnight were given 2.5 ml/kg either saline (control) or undiluted organochlorides ($BrCCl_3$, CCl_4) by gastric intubation, and killed 1 hr after. Values are expressed as mean ± SEM of 5 animals. * Significantly different from control ($P < 0.001$). † Significantly different from control ($P < 0.01$). ‡ Significantly different from control ($P < 0.05$).

[16]. Lipid peroxidation *in vivo* was determined as conjugated dienes at 235 nm as described by Rao and Recknagel [17]. The cytochrome P-450 content was measured as described by Omura and Sato [18].

RESULTS

The results obtained on oral administration of the two organochlorides to rats on the 20th day of gestation are summarized in Table 1. It can be noted that the calcium uptake capacity of the hepatic microsomes of the mother is inhibited by 64% on CCl_4 administration and by 88% with $BrCCl_3$. For the foetal hepatic microsomes this capacity is inhibited by 21% on administration to the mother of CCl_4 and by 41% on administration of $BrCCl_3$ under the same conditions. The level of cytochrome P-450 of the maternal microsomes was strongly decreased (29% with CCl_4 and 64% with $BrCCl_3$). For the foetal microsome the level of cytochrome P-450 is already very low in the controls and it can be considered that it is reduced to zero on $BrCCl_3$ treatment as it is no longer measurable with the technique used. At the same time, the degree of peroxidation brought about by these compounds was evaluated. In the mother, the level of conjugated dienes rose by 78% on CCl_4 treatment and by 159% with $BrCCl_3$. In the foetus, no signs of peroxidation were detected (no change in the level of conjugated dienes after treatment).

Figure 1 represents the level of malondialdehyde, the calcium sequestration capacity as percentage of control, and the percentage inhibition of cytochrome P-450, respectively, against the concentration of $BrCCl_3$ or CCl_4 used *in vitro*. In the microsomes of both the new-born and the mother the level of malondialdehyde increases progressively with $BrCCl_3$, whereas in the foetus there is no change. Note that in spite of the absence of peroxidation in the foetal microsomes, the calcium sequestration capacity was inhibited by 76% at a concentration of 0.08 μl $BrCCl_3$/ml. The level of cytochrome P-450 in the maternal and new-born microsomes was greatly reduced with the higher concentrations of $BrCCl_3$ used. In the foetus, it was not possible to measure the level of cytochrome P-450 owing to the very small amount of material available.

Note that at the concentrations used, CCl_4 causes the same modifications as $BrCCl_3$, but at a lesser intensity.

In order to check the role of cytochrome P-450 in the organochloride metabolism *in vitro* tests were performed by incubating maternal or foetal liver microsomes with CCl_4 in the presence or absence of carbon monoxide (CO). It can be seen that the significant inhibition by CCl_4 of the calcium uptake capacity of the maternal and foetal microsomes (comparison between control and CCl_4 groups) is no longer significant when the microsomes are previously treated with CO (CO control to CO + CCl_4 comparison). In the mother the production of MDA caused by the CCl_4 is greatly reduced in the presence of CO; in the foetus the results confirm the absence of peroxidation in the presence of CCl_4 (see Fig. 1).

DISCUSSION

The results concerning the level of cytochrome P-450 in the hepatic microsomes of the mother (25% lower than in non-pregnant adults of the same age) are in agreement with generally accepted data [3, 4]. In relation to adult rats [11] the calcium sequestration capacity of the pregnant rat was practically the same, but in the foetus on the 20th day of gestation, the capacity was 4 times greater and it was still twice as high as in the new-born rat (5 days old) [13].

In spite of the lower level of cytochrome P-450 the maternal liver is able to catalyse reductive carbon halogen bond cleavage of $BrCCl_3$ and CCl_4. This is seen through the inhibition of the maternal

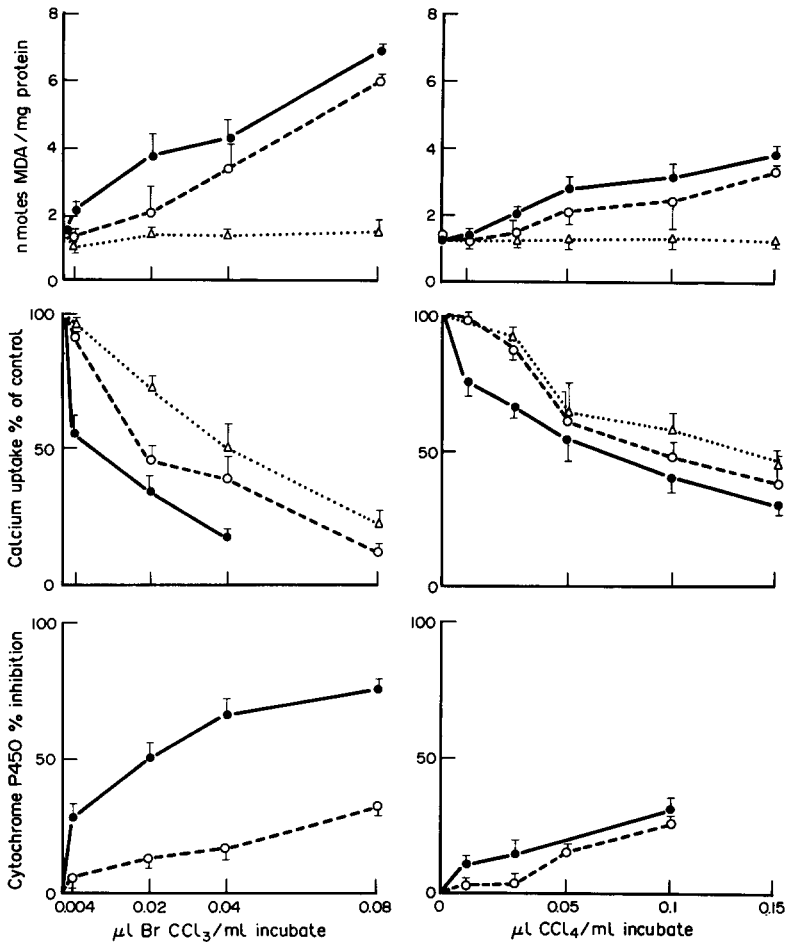

Fig. 1. Effect of increasing concentrations of $BrCCl_3$ (left) and CCl_4 (right) on malondialdehyde production, calcium uptake, cytochrome P-450 content of maternal—●, foetal—△, and new-born —○ hepatic microsomes. Analysis and assays were as described in materials and methods. Values given are means ± SEM of liver preparations from 5 animals.

microsome calcium sequestration capacity. The inhibition results from the effects of the · CCl_3 radical which can become both covalently bound to the constituents of the microsomes and act as initiator of membrane phospholipid peroxidation [12]. Although the two halogenated derivatives were administered at the same dose, the inhibition observed with $BrCCl_3$ was almost total whereas with CCl_4 the calcium sequestration capacity was decreased by 70%. The difference corresponds to the relative hepatotoxic potential of the two compounds [19]. Similar results were obtained on measuring the calcium sequestration capacity by foetal liver microsomes, but the inhibition caused by the two organochlorides was much less.

Parallel to the calcium pump inhibition, a decrease was seen in the level of cytochrome P-450, accompanied in the mother by production of conjugated dienes (lipid peroxidation indicators), whereas in the foetus no signs of lipid peroxidation were seen.

With the *in vivo* experiments, it is not possible to determine in which compartment, maternal or foetal, the active metabolite responsible for the inhibition of the foetal microsome calcium pump is produced. However, the migration of · CCl_3 radical produced in the maternal liver across the placenta to the foetal liver is improbable. To explain the observed effects it can be imagined that the intact haloalkane crosses the placenta to be then activated by the foetal liver: this seems to be confirmed by the *in vitro* inhibition of Ca^{2+} uptake and cytochrome P-450. In our experimental conditions the increasing concentrations of the two halogenated derivatives cause proportional inhibition of the calcium sequestration capacity in the hepatic microsomal fraction of the mother, the foetus and the new-born. In the same conditions the pretreatment of the microsomes with CO prevents the calcium pump inhibition showing the role of cytochrome P-450 in CCl_4 activation. In opposition to other results [20] the CCl_4-induced MDA production in the maternal microsomes is lowered in the presence of CO.

In the foetal microsomes the inhibition of calcium sequestration with CCl_4 and $BrCCl_3$ is not paralleled by the formation of malondialdehyde; this confirms

Table 2. Rat liver microsomal metabolism of CCl_4 in the presence and absence of carbon monoxide: effects of CCl_4 (0.1 μl/ml) on MDA production and Ca^{2+} sequestration capacity

	Conditions	MDA (nmoles/mg prot.)	Calcium sequestration (nmoles/mg prot. per 30 min)
Mother	Control	1.01 ± 0.073	51.8 ± 6.94
	+ CCl_4	3.39 ± 0.41*	22.5 ± 4.54†
	Control + CO	1.22 ± 0.16	48.4 ± 9.14
	+ CCl_4 + CO	1.93 ± 0.32	39 ± 7.11
Foetus	Control	1.49 ± 0.24	193 ± 17
	+ CCl_4	1.51 ± 0.21	127 ± 16‡
	Control + CO	1.45 ± 0.20	179 ± 20
	+ CCl_4 + CO	1.45 ± 0.18	162 ± 18

An aliquot of maternal or foetal liver microsomal fraction were bubbled with CO for 4 min. These preparations were incubated for 30 min at 37° in the absence (control and control + CO) or in the presence of 0.1 μl/ml of CCl_4 (CCl_4 and CCl_4 + CO). Then the level of MDA and Ca^{2+} sequestration capacity were determined as indicated in Materials and Methods. Values given are means ± SEM of liver preparations from 6 animals. * Significantly different from the corresponding control ($P < 0.001$). † Significantly different from the corresponding control ($P < 0.01$). ‡ Significantly different from the corresponding control ($P < 0.05$).

the absence of peroxidation observed *in vivo*. However, in a previous work [13] we showed that the phospholipids of foetal liver microsomes can be peroxidized by an Fe^{2+}/NADPH system, like newborn microsomes [21]. According to our previous results [13] a MDA production of 3.9 nmoles/mg protein was obtained with 0.6 μM of $FeSO_4$ giving a 40% inhibition of calcium uptake.

This work brings a new element to the relative contribution of covalent binding of $\cdot CCl_3$ and of lipid peroxidation to the inhibition of the calcium pump. In the adult the two mechanisms coexist and the importance of each can be investigated using agents blocking lipid peroxidation [12]. The foetal liver microsomes therefore appear to give the first illustration of Ca^{2+} pump inhibition by haloalkanes without peroxidation. Also the production of $\cdot CCl_3$ radicals by the microsomal foetal liver mixed-function oxidase system by the 20th day of gestation is quite probable. In effect, the level of cytochrome P-450 starts to rise from this date [1] and, as in the adult, it is destroyed *in vivo* by the hepatotoxins. The prevention of calcium pump inhibition by CO shows that the cytochrome P-450 in foetal liver is metabolically active. The disturbance of hepatocellular Ca^{2+} homeostasis may be a link between the metabolism of CCl_4 and the pathological phenomena described in the liver of foetuses whose mothers received CCl_4 [6–8].

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