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SHORT COMMUNICATIONS

Radical activation of carbon tetrachloride in foetal and maternal rat liver microsomes

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The initial event responsible for CCl_4 hepatotoxicity is now known to be the homolytic cleavage by the hepatic mixed-function oxidase system of the endoplasmic reticulum of a chlorine-carbon bond which produces trichloromethyl radical [1, 2]. Evidence of the production of this radical was given by spin-trapping on microsomal preparations either *in vitro* [3] or after *in vivo* administration of CCl_4 [4]. Formation of covalent binding of CCl_4 carbon with phospholipids and proteins in smooth endoplasmic reticulum [5] and peroxidation of the lipids in these membranes [6] are due to CCl_3 radical reactivity. These two phenomena coexist in adults and were related with the first cell disordering observed: perturbation of Ca^{2+} homeostasis due to inhibition of microsome ability to store calcium [7].

From the point of view of metabolism at microsomal level, the period of gestation is characterized by a decrease of the activity of enzymatic systems implicated [8–10]. In rat foetal liver, cytochrome P-450-dependent monooxygenase system being active in the very last days of gestation [11]. We showed that in mother and foetus, the liver microsome capacity to store Ca^{2+} was inhibited after oral administration of CCl_4 or $CBrCl_3$ into female rat on the twentieth day of pregnancy or after *in vitro* incubation of microsomes with haloalkanes in the presence of a NADPH generating system [12]. In these conditions maternal liver microsome membranes are peroxidated whereas we have never seen any lipoperoxidation in the foetus (no increase in MDA or conjugated dienes was observed). Therefore, foetal liver microsomes seem to give the first illustration of Ca^{2+} pump inhibition by haloalkanes without peroxidation [12]. In these conditions, inhibition observed should be attributed solely to trichloromethyl radical covalent binding in microsome membranes, which supposes the existence of a metabolic capacity responsible for CCl_4 radical activation.

The aim of this study is to show the existence of this covalent binding and of the radical derived from CCl_4. In order to confirm the predominant role played by the covalent binding in the inhibition of the calcium pump in the foetus, a correlation was made between the membrane modifications of liver microsomes induced by CCl_4 (lipoperoxidation, inhibition of cytochrome P-450 and of capacity to store calcium) and the covalent binding level of this molecule.

Materials and methods

Animals and materials. The animals used in this study were nulliparous pregnant female Sprague–Dawley rats (twentieth day of gestation) weighing 200–220 g at the date of mating. 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 hr–19.00 hr). They were given a standard feed and tap water *ad libitum*.

NADP, ATP, isocitric and isocitrate dehydrogenase (EC 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), $^{14}CCl_4$ was supplied by NEN (Boston, MA) and PBN (N tert. butyl-phenylnitrone) by Aldrich Chemical Co.

Microsome preparation. Twentieth day pregnant female rats were killed by decapitation. The foetuses were removed *in utero* and decapitated. Five grams of maternal liver or the same weight of foetal liver pooled (obtained from two litters) were homogenized with 50 ml of ice-cold 3 mM EDTA, 154 mM KCl at pH 7.4. The microsomal fraction was prepared [13] and the pellet was resuspended in cold Tris–maleate buffer (0.05 M Tris, 0.05 M maleate, 0.1 M KCl, pH 7.4).

Experimental procedures. The experimental procedure involved initial incubation at 37° of the foetal and maternal microsomal fraction (0.5 mg protein/ml and 1 mg protein/ml respectively) with 0.1 μl CCl_4/ml, in the presence of a NADPH generating system [13]. Microsome capacity to store calcium [14], level of cytochrome P-450 [15] and level of MDA produced [16] were measured at different times. Controls were performed without CCl_4.

Total binding of $^{14}CCl_4$ carbon to microsomes was determined in the same experimental condition of the foetal and maternal microsomes with $^{14}CCl_4$ (0.3 mCi/mmol). Control values were performed without NADPH generating system. After incubation, a sample of 3 ml was mixed with 60 μl of 1 M $CaCl_2$ to aggregate the microsomes [17] and was then filtered on cellulose acetate filters (Millipore). The filters were rinsed with buffer several times and then three times with methanol, 5 ml per wash. The filters with microsomes were immersed in liquid scintillation and monitored for radioactivity. This procedure measures all radioactivity bound to microsomal lipids and proteins [7].

Electron spin resonance studies (ESR). Maternal and foetal liver microsomes (2 mg protein/ml) were incubated in presence of NADPH generating system, 0.028 M PBN and 2.5 μl CCl_4/ml. Incubation time (30 min) and CCl_4 concentration were chosen after preliminary studies in order to obtain a good balance between the level of radicals produced by the microsomes and the efficiency of PBN in incubation medium. After incubation, the medium was extracted with chloroform (3/1, v/v). The chloroform phase was collected and concentrated by evaporation under nitrogen stream in order to obtain a PBN adduct concentration sufficient for its detection (for the foetus, chloroform was concentrated seven times more than for the mother). The detection of radical was performed in the chloroform layer by electron spin resonance with a Brucker 200 D spectrometer equipped with a double cavity. Samples were examined in the $g = 2$ G region. The conditions for spectrum recording were as follows: power 20 mW, modulation 16, scan range 100 G, room temperature.

Results

Effects observed on maternal and foetal liver microsomes after the incubation period with 0.1 μl/ml CCl_4 for various lengths of time are summarized in Fig. 1.

For the mother, MDA level increases very rapidly for the first 5 min, and then more slowly until it reaches 4.6 nmoles/mg prot. after 90 min incubation. For the foetus MDA level remains constant during whole experiment. Control values obtained without CCl_4 are represented as time 0.

Covalent binding of $^{14}CCl_4$ to maternal microsomal membranes very quickly reaches an almost maximum value which varies later. For the foetus, $^{14}CCl_4$ covalent binding

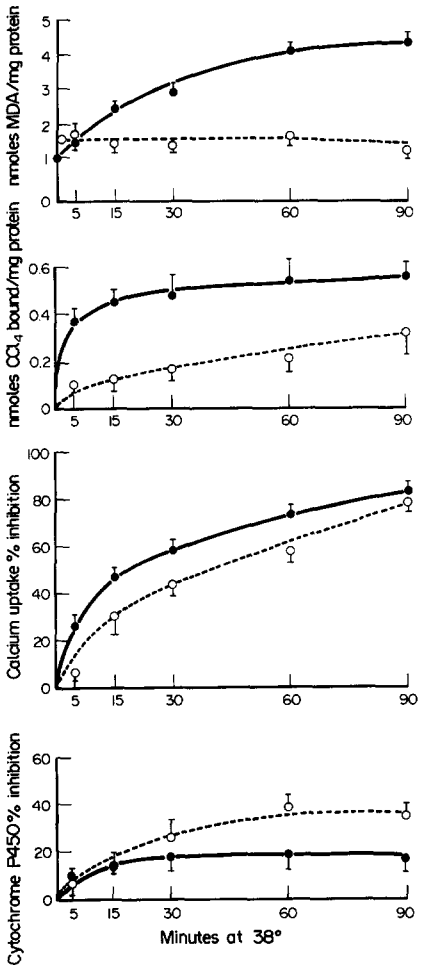

Fig. 1. Time course of the effect of carbon tetrachloride on MDA production, covalent binding, calcium uptake and cytochrome P-450 content of maternal (●——) and foetal (○– – –) hepatic microsomes. Analysis and assays were as described in Materials and Methods. Values given are means ± S.E.M. of liver preparations from 5 animals.

level in microsome membranes is low during the first 15 min, then increases linearly between 15 and 90 min. The control values are lower than 0.1 nmoles CCl_4 bond/mg protein at all the times.

The basic level of cytochrome P-450 of maternal and foetal liver microsomes are respectively 0.53 ± 0.08 and 0.08 ± 0.015 nmoles/mg prot. In our experimental conditions maternal cytochrome P-450 is weakly inhibited. For the foetus, cytochrome P-450 inhibition follows the same evolution as for the mother for the first 30 min, but reaches 40% after an hour.

The absolute values of calcium uptake in control microsomes (time 0 i.e. without CCl_4) were 240 ± 14 nmol/mg protein/30 min for the foetus and 55 ± 6.1 nmol/mg protein/30 min for maternal microsomes. Percentages were calculated in relation to their corresponding controls. The time course of calcium uptake in foetal and maternal liver microsomes are similar but the inhibition percentages are higher in the mother.

From experimental results we were able to calculate the correlation coefficients between the most important parameters in order to show their interdependence.

A good correlation (r = 0.98) exists between lipoperoxidation and inhibition of Ca^{2+} pump for maternal microsome membranes whereas there is no relation for the foetus (r = 0.15). Concerning the evolution of the capacity to store calcium in function of covalent binding, a good correlation between these two effects must be noticed for mother (r = 0.99) and foetus (r = 0.89). The percentage of mother cytochrome P-450 inhibition depends on covalent binding and MDA levels (respectively r = 0.85 and r = 0.85). On the contrary, a correlation between covalent binding (r = 0.71) and the percentage of cytochrome P-450 inhibition can be observed in the foetus but there is no relation between the latter and lipoperoxidation (r = 0.03).

Results on Table 1 confirm that for pregnant females, PBN is a good scavenger for radicals formed from CCl_4 during microsome metabolism (decrease of lipoperoxidation and protection of cytochrome P-450). Results were obtained using CCl_4 concentrations 25 times higher than for previous experiments (Fig. 1), which was a sufficient margin in order to insure a good PBN efficiency in our experimental conditions.

Figure 2 shows results concerning electron spin resonance studies after incubation of maternal and foetal microsomes with CCl_4 and results obtained after incubation with $CBrCl_3$, haloalkanes with a higher aptitude to produce CCl_3·. No signal was obtained when foetal and maternal microsomes were incubated in the presence of an NADPH generating system alone and in the presence of CCl_4 or $CBrCl_3$ without a NADPH generating system. In the presence of an NADPH generating system and PBN, both maternal and foetal microsomes give an ESR signal with CCl_4 as well as with $CBrCl_3$. The spectra consists of 6 lines corresponding to a triplet of doublets characteristic of PBN adduct. Nitrogen and β-hydrogen hyperfine splitting constants for both CCl_4 and $CBrCl_3$ signals were respectively $a'_N = 14.4 \pm 0.2$ G and $a'_H = 2.0 \pm 0.2$ G. In both, maternal and foetal, ESR signal obtained from $CBrCl_3$ was more intense than CCl_4 signal, all the more so as $CBrCl_3$ concentration was 2.5 times lower; experimental conditions were otherwise identical.

Discussion

Results show that foetal liver during the last days of pregnancy, as well as mother liver, can metabolize CCl_4 molecule into a free radical. The ESR spectra characteristics of these radicals are similar to the ones described for adult male rats [18, 19]. From the spectra obtained, their hyperfine splitting constants, and the absence of peroxidation radical reaction in foetal microsomes, we can assert that the radical showing after incubations with CCl_4 and $CBrCl_3$ is in fact the CCl_3·–PBN adduct radical. $CBrCl_3$ higher reactivity compared to CCl_4 [1, 20] is confirmed by the intensity of the signal obtained. The scavenger role of PBN [21] is confirmed in pregnant female microsomes (Table 1) by the reduction of the MDA level and the protection of cytochrome P-450.

It seems that in spite of a lower activity of cytochrome P-450 dependent system when compared to the male [8–10], the capacity to produce CCl_3· is maintained as confirmed by ESR. This radical may bind covalently to the membranes and is at the origin of the phospholipid peroxidation. The effect of CCl_4 on MDA production, covalent binding and calcium uptake in pregnant female hepatic microsomes are similar to those described for male rats [7, 22, 23]. Our results do show that there is a relation between degree of peroxidation, covalent binding, destruction of cytochrome P-450 and inhibition of capacity to store calcium.

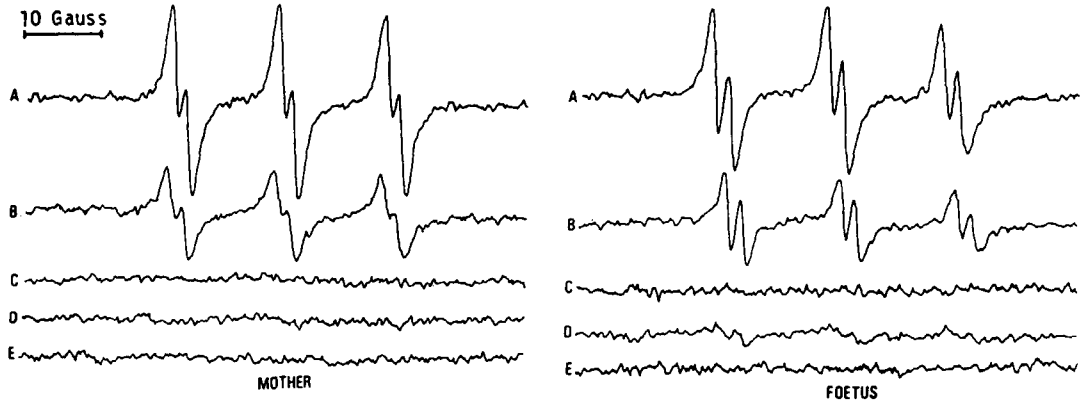

Fig. 2. Electron spin resonance spectra of PBN adducts in chloroform from maternal and foetal liver microsomes incubated in presence of: (A) 1 μl $CBrCl_3$/ml and the NADPH-generating system; (B) 2.5 μl CCl_4/ml and the NADPH-generating system; (C) 1 μl $CBrCl_3$/ml; (D) 2.5 μl CCl_4/ml; (E) the NADPH-generating system.

Table 1. Protecting effect of PBN upon CCl_4-induced lipid peroxidation and cytochrome P-450 inhibition.

	MDA (nmoles/mg prot.)	Cytochrome P-450 (nmoles/mg prot.)
Control	0.89 ± 0.26	0.714 ± 0.077
CCl_4	3.98 ± 0.87	0.320 ± 0.033
Control + PBN	1.20 ± 0.21	0.760 ± 0.076
CCl_4 + PBN	1.60 ± 0.43	0.562 ± 0.067

Maternal liver microsomes (with NADPH-generating system) were incubated for 30 min at 37° in the absence [Control and Control + PBN (0.028 M)] or in the presence of 2.5 μl CCl_4/ml [CCl_4 and CCl_4 + PBN (0.028 M)]. The level of MDA and cytochrome P-450 were determined as indicated in material and methods. Values given are means ± SEM of 3 assays.

As far as the foetal liver is concerned there is a relation between these parameters, except for lipoperoxidation. The inhibition of the foetal microsomal ability to store calcium is a sensitive and early biological test of liver disturbance due to production of reactive metabolites by haloalkanes. However, the evolution of the microsomal ability to store calcium during the perinatal period has not often been studied [24, 25]. For the foetus, absence of microsome membrane lipoperoxidation observed during 30 min incubation of these microsomes with various doses of CCl_4 [12] was confirmed. Our results showed that the decrease in cytochrome P-450 level and calcium pump inhibition are mainly due to covalent binding consecutive to the radical activation shown by ESR.

In the adult covalent binding and lipoperoxidation coexist simultaneously. The importance of the first mechanism can be shown only after inhibition of lipoperoxidation using either incubation in anaerobiosis [26, 27] or agents blocking peroxidative processes [7, 28]. From our results we can say that the evolution of both phenomena during the perinatal period seems to be a good model in order to define the predominance either of lipoperoxidation or of covalent binding in the occurance of liver necroses observed mainly in adults after intake of CCl_4, and which is still subject to controversy [29–32].

Although it is not very well defined, it would seem that CCl_4 perinatal liver toxicity is a consequence of the radical activation and covalent binding observed. Some authors [33] noted either little or no histological alterations after carbon tetrachloride poisoning. Others [34] gave a description of the variable alterations with a few cases of centrolobular necroses. Moreover, the level of serum transaminase increases, with more or less intensity, 48 hr after CCl_4 administration to the female on the 21st day of pregnancy.

Other experimental results would therefore be needed in order to draw a parallel between these toxic manifestations and the appearance of a foetal liver ability to activate CCl_4.

In summary, results obtained with the spin trapping method showed the ability of foetal liver microsomes at the end of pregnancy, similarly to adult microsomes, to produce $CCl_3\cdot$ radical from CCl_4. This radical has a covalent binding to the microsomal membranes and causes the destruction of cytochrome P-450 as well as the inhibition of one of the main microsomal activities, the ability to store Ca^{2+}. Contrary to adults, the production of $CCl_3\cdot$ radicals in the foetus does not provoke a membrane phospholipid peroxidation. This difference shows the importance of parallel studies of covalent binding and lipoperoxidation both in mother and foetus, in view of evaluating their respective roles in the occurance of liver necroses observed after administration of CCl_4.

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Protein binding of nomifensine and its three main metabolites

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The binding of drugs to plasma proteins can have a profound effect on their pharmacokinetics [1, 2]. A large inter-individual variation in plasma protein concentrations may cause different clinical responses when highly bound agents are used [3–5]. In recent years it has been recognized that in addition to albumin, α_1-acid glycoprotein (α_1-AG) also plays a significant role in the binding of basic drugs.

Nomifensine is a psychotropic agent having antidepressive properties. It has three main metabolites, 4-hydroxynomifensine (M_1), 4-hydroxy-3-methoxynomifensine (M_2) and 3-hydroxy-4-methoxynomifensine (M_3) [6] (Fig. 1). Nomifensine is slightly basic, so binding to α_1-AG could be assumed. Plasma protein binding of nomifensine has been reported to be about 60% [7]. The binding of nomifensine to different components of plasma, as albumin or α_1-acid glycoprotein, has not been published. There is no information of the binding of nomifensine metabolites to plasma proteins.

In the present study the binding of nomifensine and its main metabolites to whole plasma protein, albumin and α_1-acid glycoprotein was studied. Protein binding was evaluated over a wide concentration range. The effect of protein concentrations was also evaluated.

Materials and methods

Materials. Nomifensine maleate as a pure substance and in gelatine capsules (Nomival®) were obtained from Leiras Pharmaceuticals (Turku, Finland). 4-hydroxy-, 4-hydroxy-3-methoxy- and 3-hydroxy-4-methoxynomifensine were gifts from Hoechst AG (Frankfurt am Main, F.R.G.). Human serum albumin (Fatty acid free) and α_1-acid glycoprotein (orosomucoid, human) were purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade. Fresh human plasma of a healthy subject was obtained from Finnish Red Cross.