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Effects of pregnancy and protein-energy malnutrition on monooxygenase *O*-dealkylation activity in rat liver microsomes

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Abstract

Xenobiotic metabolism is influenced by a variety of physiological and environmental factors including pregnancy and nutritional status of the individual. Pregnancy has generally been reported to cause a depression of hepatic monooxygenase activities. Low-protein diets and protein-energy malnutrition have also been associated with a reduced activity of monooxygenases in nonpregnant animals. We investigated the combined effects of pregnancy and protein-energy malnutrition on liver monooxygenase *O*-dealkylation activity. On pregnancy day 0 rats were assigned at random to a group fed *ad libitum* (well-nourished, WN) or to a malnourished group (MN) which received half of the WN food intake (12 g/day). WN and MN rats were killed on days 0 (nonpregnant), 11 or 20 of pregnancy and ethoxy- (EROD), methoxy- (MROD) and penthoxy- (PROD) resorufin *O*-dealkylation activities were measured in liver microsomes. Only minor changes in enzyme activities were observed on pregnancy day 11, but a clear-cut reduction of monooxygenase activities (pmol resorufin min^{-1} mg $protein^{-1}$) was noted near term (day 0 *vs* 20, means ± SD, Student *t*-test, $P<0.05$) in WN (EROD: 78.9 ± 15.1 *vs* 54.6 ± 10.2; MROD: 67.8 ± 10.0 *vs* 40.9 ± 7.2; PROD: 6.6 ± 0.9 *vs* 4.3 ± 0.8) and in MN (EROD: 89.2 ± 23.9 *vs* 46.9 ± 15.0; MROD: 66.8 ± 13.8 *vs* 27.9 ± 4.4; PROD: 6.3 ± 1.0 *vs* 4.1 ± 0.6) dams. On pregnancy day 20 MROD was lower in MN than in WN dams. Malnutrition did not increase the pregnancy-induced reduction of EROD and PROD activities. Thus, the present results suggest that the activities of liver monooxygenases are reduced in near-term pregnancy and that protein-energy malnutrition does not alter EROD or PROD in pregnant rats.

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- Undernutrition
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- Biotransformation

Xenobiotic metabolism is known to be influenced by a variety of endogenous and environmental factors such as diseases (e.g., diabetes, infections, inflammation, hepatoma, cirrhosis), age, gender, diet, nutritional status, genetic background, stress, pregnancy, diurnal and seasonal cycles, and exposure to other chemical agents (1,2). The toxic effects of foreign chemicals may result either from the parent compound itself or from its reactive metabolites (i.e., metabolic activation). Since toxicity depends on the balance between activation and detoxication reactions, biotransformation-modifying factors

may either enhance or reduce toxicity by decreasing/increasing the rate of detoxication or, alternatively, by increasing/decreasing the rate of formation of reactive metabolites (2). In principle, any phase I or phase II biotransformation enzyme can form proximate (intermediate) or ultimate (reactive species) toxic metabolites that bind to macromolecules and DNA. Phase I oxidation enzymes, however, are the enzymes most frequently involved in the metabolic activation of toxicants. Isoenzymes of the CYP1A subfamily (1A1, 1A2), for example, are considered as one of the most important groups of monooxygenases that form reactive metabolites from polycyclic aromatic hydrocarbons, aromatic and heterocyclic amines, azobenzene derivatives and planar polyhalogenated biphenyls (2). CYP2B subfamily monooxygenases, on the other hand, are involved in the detoxication of barbiturates, phenytoin, DDT, and other chemicals, and also take part in the metabolic activation of several hepatotoxic, mutagenic and teratogenic substances (e.g., bromobenzene, cyclophosphamide) (2).

Pregnancy has been reported to cause a reduction in the activity of several hepatic monooxygenases and changes in the concentrations - as measured by spectral determinations - of hepatic cytochrome P450s in the rat (3-5). The liver content of cytochrome P450s has been shown to be decreased when it is expressed per g of tissue or per mg of microsomal protein (3-5), and either to remain unchanged (4), or to be increased when it is calculated on a whole organ basis (6). Moreover, it has also been shown that the induction of microsomal enzymes by phenobarbital (a CYP2B inducer) but not the induction by 3-methylcholanthrene (a CYP1A inducer) is impaired in pregnant rats (6,7).

The effects of malnutrition (e.g., low protein diets, fasting or restricted feeding) on the metabolism of xenobiotics have been investigated in male and nonpregnant female rats by several research groups (8-11). Despite the marked differences in experimental design, these studies have generally found that protein deficiency and protein-energy malnutrition (i.e., restricted feeding) cause a depression of hepatic monooxygenase activities, thereby slowing the rate of xenobiotic biotransformation (8,10,11).

Protein-energy malnutrition is still endemic in large areas of the developing world where pregnant malnourished women are often exposed to drugs and environmental chemicals. Despite the importance of this exposure, to our knowledge, no study has been performed on the combined effects of pregnancy and malnutrition on the activity of enzymes involved in the biotransformation of xenobiotics.

The present study was designed to investigate the combined effects of pregnancy and protein-energy malnutrition on the activities of CYP1A and CYP2B monooxygenases in the rat liver.

The study was conducted on male and nulliparous female Wistar rats from the FIOCRUZ Central Animal House breeding stock, with a body weight range of 250-320 g and 202-240 g, respectively. The rats were housed individually in standard plastic cages on white pine shavings bedding, given tap water *ad libitum* and fed a standard pelleted diet (Nuvital®, Nuvilab Ltd., Curitiba, PR, Brazil). Temperature (23 ± 1°C), humidity (approximately 70%) and photoperiod (lights on from 10:00 to 22:00 h) were controlled in the animal's room. Mating was performed by transferring two females to the cage of one male of known fertility for 2 h (8:00 to 10:00 h) and copulation was confirmed by vaginal smears. The day on which spermatozoa were found in the vaginal smear was designated as day '0' of pregnancy. On pregnancy day '0' rats were assigned at random to a well-nourished (WN) group or to a malnourished group (MN). Females of the WN group received food *ad libitum* throughout pregnancy, whereas females of the MN group were given only 12 g of food per day, i.e., approximately half of the daily food

intake by the WN group. Nonpregnant malnourished females were killed 20 days after the beginning of the food restriction regimen.

Food was removed from all cages 16 h previous to sacrifice by decapitation. Immediately after sacrifice livers were quickly removed, weighed, frozen and stored at -80°C. Liver microsomes were prepared as follows. Hepatic tissue was homogenized with a motor-driven Potter-Elvejhem homogenizer and the homogenate was centrifuged at 9000 *g* for 20 min. The supernatant was then centrifuged at 100,000 *g* for 60 min. The pellet obtained by ultracentrifugation was suspended in buffer (0.1 M Tris-HCl, 0.15 M KCl, pH 7.4) and centrifuged again at 100,000 *g* for 60 min. The pellet of the second ultracentrifugation was suspended in a phosphate buffer solution (0.1 M, pH 7.4) containing glycerol (20% v/v) and EDTA (1 mM) and stored in liquid nitrogen until use. Protein concentration in the microsomal fraction was determined by a colorimetric method using Coomassie brilliant blue G dye (Bradford Reagent, Sigma Chemical Co., St. Louis, MO, USA) and bovine serum albumin (Sigma) as the standard. Ethoxyresorufin-*O*-deethylase (EROD), methoxyresorufin-*O*-demethylase (MROD) and pentoxyresorufin-*O*-depentylase (PROD) activities were determined essentially as reported by Burke et al. (12), except for the use of an NADPH regenerating system which consisted of 0.25 mM ß-NADP, 2.5 mM $MgCl_2$, 5 mM glycose-6-phosphate and 0.5 units of glucose-6-phosphate-dehydrogenase per ml of incubation mixture. Resorufin, pentoxy-, ethoxy- and methoxyresorufin were also purchased from Sigma. Reactions were initiated by the addition of the regenerating system and were carried out in quartz cuvettes at 37°C. The reaction rate was measured by the increasing fluorescence caused by the accumulation of resorufin. The spectrofluorimeter (Shimadzu RF-5000) settings were as follows: excitation wavelength 550 nm and emission wavelength 582 nm, with a 5-nm band slit width.

As expected, pregnancy and protein-energy malnutrition affected both liver and maternal body weights. Food restriction to approximately 50% of *ad libitum* food intake drastically reduced weight gain from day 0 to day 20 of pregnancy (Table 1). Moreover, pregnancy-induced liver enlargement was much smaller in MN females (23% heavier on day 20) than in WN dams (93% heavier on day 20) (Table 1). The liver to maternal body weight ratio was also altered by pregnancy and protein-energy malnutrition. While the relative liver weight (%) increased from 2.5 ± 0.2 (day 0) to 3.4 ± 0.3 (day 20) in WN females, it increased from 2.7 ± 0.3 to 3.0 ± 0.3 in MN females (Table 1). Therefore, food restriction appeared to hamper the pregnancy-induced liver enlargement in rats. Liver enlargement associated with pregnancy was first reported by Bokelmann and Scheringer (13) and seems to be due to a transient swelling of parenchymal cells (13,14). The concentrations of liver microsomal proteins were reduced in WN dams on days 11 and 20 compared to pregnancy day 0 (nonpregnant females) (Table 1). On the other hand, no significant difference among groups was detected with regard to the total amount of microsomal protein per liver (Table 1). Thus, the reductions in microsomal protein concentration observed in WN rats were apparently due to an increase in liver size (hypertrophy) unaccompanied by a comparable increase in the total content of microsomal proteins. In MN rats, whose livers showed only minor variations in size, microsomal protein levels seemed to have remained fairly constant throughout pregnancy (Table 1).

Data about the activities of liver monooxygenases in WN and MN pregnant rats are shown in Table 2. Since pregnancy and malnutrition affected both liver and body weights, enzyme activities (MROD, EROD and PROD) were also reported on a whole liver

Table 1 - Effect of pregnancy and malnutrition on rat liver microsomal protein.

Data are reported as means ± SD. [+]Well-nourished rats were fed *ad libitum*. Malnourished rats received only 12 g of food per day from pregnancy day 0 on. Nonpregnant malnourished females were on a food restriction regimen for 20 days before sacrifice. [+]Weight on the day of sacrifice. [++]Pregnant females: weight on the day of sacrifice - weight on pregnancy day 0. Nonpregnant females: weight on the day of sacrifice - weight 20 days before sacrifice. Weight gain and relative liver weight were analyzed by the Kruskal-Wallis test followed by the Mann-Whitney U-test. All other parameters were evaluated by one-way ANOVA followed by the Student *t*-test. [a]P≤0.05 compared to the respective WN or MN nonpregnant group; [b]P≤0.05 compared to the WN group on the same pregnancy day.

Groups[+]	N	Body weight[+] (g)	Weight gain[++] (g)	Liver weight (g)	Liver weight/ maternal weight (%)	Concentration of microsomal protein (mg/g liver)	Amount of microsomal protein per liver (mg)
Nonpregnant females							
Well-nourished	6	232.7 ± 7.7	3.8 ± 4.3	5.8 ± 0.5	2.5 ± 0.2	24.4 ± 1.9	142.4 ± 20.1
Malnourished	8	220.9 ± 11.2	-18.3 ± 8.0[b]	6.0 ± 0.7	2.7 ± 0.3	23.7 ± 3.0	142.9 ± 11.8
Pregnant females							
Day 11							
Well-nourished	5	239.3 ± 34.1	36.7 ± 10.5[a]	7.4 ± 1.3	3.2 ± 0.4[a]	17.7 ± 2.3[a]	132.6 ± 35.4
Malnourished	7	207.2 ± 20.8	-1.3 ± 4.9[a b]	6.6 ± 0.7	3.2 ± 0.2[a]	22.7 ± 1.3	148.6 ± 16.8
Day 20							
Well-nourished	6	333.0 ± 16.5[a]	107.2 ± 8.6[a]	11.2 ± 1.3[a]	3.4 ± 0.3[a]	15.7 ± 3.8[a]	174.0 ± 41.8
Malnourished	5	243.7 ± 3.5[ab]	29.0 ± 14.9[ab]	7.4 ± 0.7[ab]	3.0 ± 0.3	23.8 ± 4.1[b]	176.0 ± 36.6
Analysis of variance		P<0.001	P<0.001	P<0.001	P = 0.003	P<0.001	P = 0.12

Table 2 - Ethoxy- (EROD), methoxy- (MROD) and pentoxy- (PROD) resorufin *O*-dealkylase activities in liver microsomes.

Enzyme activity (amount of resorufin produced per minute) is reported per mg of microsomal protein, on a whole liver basis and per g of body weight. Data are reported as means ± SD. [+]Well-nourished rats were fed *ad libitum*. Malnourished rats received only 12 g of food per day from pregnancy day 0 on. Nonpregnant malnourished females were on a food restriction regimen for 20 days before sacrifice. Data were analyzed by one-way ANOVA followed by the Student *t*-test. [a]P≤0.05 compared to the respective WN or MN nonpregnant group; [b]P≤0.05 compared to the WN group on the same pregnancy day.

	Rate of *O*-dealkylation								
Groups[+]	MROD (pmol resorufin min^{-1} mg protein^{-1})	EROD	PROD	MROD (nmol resorufin min^{-1} liver^{-1})	EROD	PROD	MROD (pmol resorufin min^{-1} g body weight^{-1})	EROD	PROD
Nonpregnant females									
Well-nourished	67.8 ± 10.0	78.9 ± 15.1	6.6 ± 0.9	9.7 ± 2.4	11.2 ± 2.6	0.9 ± 0.2	42.0 ± 10.6	48.6 ± 12.9	4.1 ± 1.1
Malnourished	66.8 ± 13.8	89.2 ± 23.9	6.3 ± 1.0	9.5 ± 2.5	12.6 ± 3.9	0.9 ± 0.2	43.3 ± 12.8	57.7 ± 19.2	4.1 ± 1.0
Pregnant females									
Day 11									
Well-nourished	56.4 ± 7.5	73.2 ± 14.7	6.0 ± 0.9	7.0 ± 1.6	9.9 ± 5.1	0.8 ± 0.4	30.2 ± 4.6	42.0 ± 17.2	3.6 ± 1.1
Malnourished	59.2 ± 10.4	48.4 ± 16.7[a]	5.7 ± 0.6	8.8 ± 2.0	7.1 ± 2.4[a]	0.8 ± 0.1	42.3 ± 6.3[b]	30.5 ± 3.9[a]	4.1 ± 0.5
Day 20									
Well-nourished	40.9 ± 7.2[a]	54.6 ± 10.2[a]	4.3 ± 0.8[a]	7.0 ± 1.4[a]	9.2 ± 1.4	0.7 ± 0.1	20.9 ± 4.1[a]	27.6 ± 3.4[a]	2.2 ± 0.4[a]
Malnourished	27.9 ± 4.4[ab]	46.9 ± 15.0[a]	4.1 ± 0.6[a]	4.8 ± 0.9[a]	8.3 ± 3.1[a]	0.7 ± 0.1	19.9 ± 3.4[a]	34.1 ± 12.7[a]	2.9 ± 0.3[ab]
Analysis of variance	P<0.001	P<0.001	P<0.001	P = 0.002	P = 0.053	P = 0.317	P<0.001	P = 0.006	P<0.001

basis and per g of body weight.

Pregnancy-induced reduction of monooxygenase activity was evident when activities were expressed per mg of microsomal protein. In mid-pregnancy (day 11) the EROD, MROD and PROD activities of WN and MN dams were only slightly lower than those of nonpregnant females (day 0). Later in pregnancy (day 20), however, the activities of the three monooxygenases were markedly reduced both in WN and MN rats. It is interesting to note that, in most instances, reductions were considerably less pronounced when activities were calculated on a whole liver basis (Table 2). For example, differences among treatment groups were not significant for PROD (ANOVA, $P = 0.317$), and were only marginally significant for EROD ($P = 0.053$) when activities were expressed on a whole liver basis. Although the difference was nonsignificant, the amount of microsomal proteins per liver appeared to be approximately 20% higher in rats pregnant for 20 days compared to nonpregnant females (Table 1). Thus, the apparent reductions in EROD, MROD and PROD activity when activity was expressed per mg of microsomal protein may have been due, at least in part, to a higher content of microsomal proteins other than these monooxygenases in the liver of near-term pregnant females.

Owing to the maternal weight gain from days 0 to 20 of pregnancy, EROD, MROD and PROD activities, expressed per unit of body weight, were all markedly decreased near term (day 20). Since PROD is a marker for CYP2B isoenzymes which play an important role in the metabolism of barbiturates (2), this finding is consistent with literature data showing that hexobarbital sleeping time was considerably longer in full-term pregnant rats compared to nonpregnant rats (4,6). However, when hexobarbital was administered to full-term pregnant rats at doses based on their nonpregnant weights, duration of sleeping time remained almost unchanged (4,6). The absence of effects of pregnancy on sleeping time when hexobarbital doses were based on nonpregnant weights is consistent with results from this study showing that PROD activity expressed on a whole liver basis did not differ among treatment groups.

Neale and Parke (4) reported that 4-hydroxylation of biphenyl and cytochrome P450 content, when expressed per unit of liver weight, were both decreased by 25-30% in pregnant rats. On the other hand, no change was found when the same parameters were calculated on a whole organ basis, a result considered to indicate that liver capacity to metabolize xenobiotic compounds was not altered in pregnant animals (4). At variance with the foregoing interpretation, most authors have found a depression of monooxygenase activities, expressed per mg of hepatic microsomal protein, during pregnancy (3,14).

Our results with EROD, MROD and PROD are consistent with previously published data showing that pregnancy depresses microsomal oxidative reactions such as aniline hydroxylation, ethylmorphine *N*-demethylation, benzopyrene hydroxylation, 3-hydroxylation of coumarin, 16α-hydroxylation of progesterone and others (3,14,15).

It should be pointed out that the extent to which pregnancy reduces enzyme activity apparently varies from monooxygenase to monooxygenase. Borlakoglu et al. (14), for instance, found that, while activities of EROD and aminopyrine *N*-demethylase in rat hepatic microsomes were clearly reduced from pregnancy day 10 to 20, activities of 4-nitroanisole-*O*-demethylase and aniline hydroxylase were only slightly decreased, and dimethylnitrosamine *N*-demethylase and aldrin epoxydase activities remained unchanged.

The *O*-dealkylation of pentoxyresorufin has been widely used as a selective marker for CYP2B isoenzymes, while the *O*-dealkylation of ethoxy and methoxy resorufin has been considered to be a marker of CYP1A

isoforms in rat liver microsomes (12). According to this interpretation, the present findings would also suggest that CYP2B and CYP1A monooxygenase activity is depressed during near-term pregnancy. Data recently provided by Burke et al. (16) confirmed that PROD is a rather selective probe for CYP2B1 in phenobarbital-induced microsomes and that EROD and MROD are selective probes for CYP1A1 and 1A2 in 3-methylcholanthrene-induced microsomes. Nonetheless, these authors also showed that, in non-induced microsomes, isoforms other than CYP2B and CYP1A also contribute to the *O*-dealkylation of pentoxy-, ethoxy- and methoxyresorufin, thereby implying that this selectivity is partially lost in untreated rats (16).

The mechanism by which pregnancy causes a depression of certain P450-catalyzed reactions is still far from being entirely understood. The reduction of monooxygenase activities has been attributed to the pronounced hormonal changes that occur during pregnancy, in particular to the higher plasma levels of progesterone and its metabolites. Data showing that progesterone and its metabolites cause *in vitro* inhibition of P450-catalyzed reactions (17) apparently support this interpretation. Results from *in vivo* studies, however, do not seem to be consistent with this hypothesis. It was reported, for instance, that continuous exposure of nonpregnant rats to high doses of progesterone in the diet causes liver hypertrophy and the induction of hepatic monooxygenases such as ethylmorphine, aminopyrine and benzphetamine *N*-demethylases (18). It has also been suggested that reduction of phase I and phase II metabolism during pregnancy may result from alterations in the microsomal phospholipid environment, i.e., from decreases in the ratio of phosphatidylcholine to phosphatidylethanolamine (15). Strobel et al. (19) reported that phosphatidylethanolamine inhibited phosphatidylcholine-stimulated oxidation of benzphetamine, a finding that is consistent with the latter hypothesis.

No effect of malnutrition on liver monooxygenase activity was found in nonpregnant or in 11-day-pregnant females (Table 2). Except for a reduction of approximately 30% in MROD activity, no other effect of malnutrition on monooxygenase activities was noted on pregnancy day 20 (Table 2). Since the body weight of MN females on pregnancy day 20 was about 27% lower than that of WN dams (Table 1), no malnutrition-induced decrease of MROD was found when enzyme activities were expressed per unit of maternal body weight (Table 2).

Low-protein diets and protein-energy malnutrition (e.g., restricted feeding, starvation) have generally been found to decrease both phase I oxidative metabolism and phase II conjugation reactions in nonpregnant animals (8,10,11). Our data suggest that, at least as far as EROD and PROD are concerned, protein-energy malnutrition does not increase the reduction of enzyme activity caused by pregnancy itself. It should be pointed out that, as shown in Table 1, the food restriction regimen adopted in this study was severe enough to produce rather drastic changes in pregnancy weight gain as well as in maternal liver weight.

In conclusion, the present results indicate that EROD, MROD and PROD monooxygenase activities in rat liver microsomes are depressed during late pregnancy. It was also noted that these effects of pregnancy were less pronounced when monooxygenase activities were calculated on a whole liver basis. Additionally, it was found that, except for a lower activity of MROD on day 20, protein-energy malnutrition did not intensify pregnancy-induced inhibition of oxidative metabolism.

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