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BIOTRANSFORMATION OF BENZO[a]PYRENE AND 7-ETHOXYRESORUFIN AND HEME-STAINING PROTEINS IN MICROSOMES FROM HUMAN FETAL LIVER AND PLACENTA

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Abstract—Microsomal proteins from human fetal livers and mid-gestational and term placentas were stained for heme in an attempt to detect multiple forms of cytochrome P-450. In fetal liver microsomes five protein bands staining for heme in the mol. wt region 46,000–60,000 were found. In the placentas two bands were seen in the region 46,000–52,000. Fetal liver and placental microsomes were assayed for metabolism of benzo[*a*]pyrene (B[a]P) and 7-ethoxyresorufin (7-EOR). The B[a]P metabolites were separated using high performance liquid chromatography. Following incubations with fetal liver microsomes, in general only phenols were detectable, while after incubations with mid-gestational as well as term placentas from smoking women, the 9,10-, 4,5- and 7,8-dihydrodiols were also formed. No quinones were detected. Placental microsomes from non-smoking women did not catalyse the formation of B[a]P metabolites. The 7-EOR *O*-de-ethylase activity was in the same range (2–5 pmol/min · mg microsomal protein) in the fetal livers as in the mid-gestational placentas. The activities were somewhat higher in the placentas originating from smokers. No correlation between enzymatic activities *in vitro* and intensity of any specific protein band was observed for the fetal livers or placentas studied.

The human fetus has been shown to catalyse the oxidation of several drugs and steroid hormones from 10–12 weeks of gestation [1, 2]. The implications of this capacity of the fetus to metabolize endogenous as well as foreign compounds, with respect to toxicity, teratogenicity and transplacental carcinogenicity, are still a matter of discussion [3].

Involvement of cytochrome P-450-dependent enzymes in the biotransformation of various endogenous substrates, e.g. steroid hormones, has been well established in fetal liver as well as in placenta. Human fetal liver microsomes have been shown to carry out hydroxylations of, e.g. testosterone [1, 4], progesterone [5], androsterone [6–8], estrone [8] and pregnenolone [9] as well as hydroxylations of a variety of sulfoconjugated steroids [8]. Recent results also give evidence of the formation of catechol estrogens in fetal tissues, including fetal liver and placenta [10, 11].

Various tissues from the human fetus also have the capacity to metabolize a wide variety of xenobiotic substrates [2, 12–16] and to catalyze the formation of epoxides from endogenous [14] and exogenous [15, 16] substrates of the hepatic microsomal mono-oxygenase system. The placenta, on the other hand, has a somewhat more restricted substrate specificity towards endogenous [2, 17, 18] as well as exogenous substrates [19].

The inducing effect of smoking on the placental enzymes which metabolize polycyclic aromatic hydrocarbons has been shown by several authors [2, 20, 21] but it has not been clarified whether a similar effect is seen in the human fetal liver. Studies in rats, however, have demonstrated that 3-methylcholanthrene induces cytochrome P-450 in fetal liver during late gestation [22]. Nau *et al.* [23] have shown induction of drug metabolizing enzyme activities by phenobarbital in organ cultures from human fetal liver. On the other hand, it has so far not been possible to give positive evidence for transplacental induction of drug oxidizing enzymes in the human fetal liver. For adult liver, on the other hand, there is evidence for an inducing effect of cigarette smoking on antipyrene as well as benzo[*a*]pyrene metabolism [24, 25].

The question why there is such an early development of the drug metabolizing enzymes in the fetal liver in man, as compared to other species, remains unclear. Is, e.g. the capacity to metabolize steroids (and drugs) as early as during the mid-gestational phase physiologically important for normal fetal development? Does this capacity make the human fetus more vulnerable to environmental chemical exposure? Does it constitute a protective mechanism as well? The metabolising system may in fact have a dual role depending on whether the metabolic step represents a detoxification or a bioactivation reaction.

Our previous preliminary results [26] showing several heme positive protein band in human fetal liver microsomes prompted us to further investigate the nature of these bands in fetal livers and placentas and their relation to metabolic activities. In this study we have used SDS-polyacrylamide gel electrophoresis to detect protein bands staining for heme in the mol. wt region ranging from 46,000 to 60,000 in

human placental and fetal liver microsomes. To estimate cytochrome P-450 dependent enzyme activities we have used two enzyme assays, the *O*-deethylation of 7-EOR and the oxidation of B[a]P.

MATERIALS AND METHODS

Human fetal livers and corresponding placentas were obtained following legal abortions whereas term placentas were obtained after normal vaginal deliveries. The legal abortions were performed via hysterotomy or by administration of ethacridine (Rivanol®) or prostaglandins. All abortions were performed for sociomedical reasons. The gestational age of the fetuses varied between 15 and 27 weeks and the placentas were obtained from pregnancies between week 10 of gestation and term. Health status, smoking history and pharmacotherapy of the women were recorded (Table 1). This information was obtained from the medical records as well as from postpartum interviews. Two livers from adults were also used in some analyses (Table 1 and Fig. 3).

Tissues were immediately frozen at −70°. No significant loss of activity was observed in tissue stored for up to 1 year. Some fetal livers and corresponding placentas were frozen at −20° and assayed within 24 hr. No differences were observed between results obtained with tissues frozen at −70° or at −20°, respectively.

Microsomes were isolated according to standard procedures and used during the same day. The microsomal metabolism of 7-EOR [27] and B[a]P was studied [28]. The microsomal protein concentration used in the 7-EOR assay was 1 mg/ml. The reaction was allowed to proceed for 10 min. The incubation with B[a]P were performed with a protein concentration of 2.5 mg/ml. B[a]P (100 nmoles) and 1×10^6 dpm [^{3}H] B[a]P were added to each incubation mixture. The incubations were terminated after 20 min by the addition of 1 ml acetone. The B[a]P metabolites were separated using high performance liquid chromatography according to Holder *et al.* [28]. SDS-polyacrylamide gel electrophoresis of microsomes was performed according to Laemmli [29]. Gels were stained for heme with 3,3′, 5,5′-tetramethylbenzidine (TMBZ)–H_2O_2 as described by Thomas *et al.* [30], as well as for protein with Coomassie Brilliant Blue. When gels were stained for heme the β-mercaptoethanol was excluded from the samples. Densitometric tracings of gels stained for heme were recored at 600 nm whereas gels stained for protein were scanned using a wavelength of 550 nm.

RESULTS

Metabolism of 7-EOR

Five pairs of fetal livers and corresponding placentas with gestational ages varying between 18 and

Table 1. Clinical data of the patients

Patient No.	Gestational age of fetus (weeks)	Cigarettes smoked per day	Drugs
1	18	30–40	0
2	24	10–20	0
3	24	20	Carisoprodol
4	10	4	0
5	21	0	0
6	23	0	0
7	16†	10	0
8	18	20	0
9	15	0	0
10	15	0	0
11	19	0	0
12	21‡	0	0
13	22	—§	—§
14	22	0	Paracetamol, terbutalin, salbutamol
15	25	0	0
16	27÷	0	0
Adult livers			Alcohol
17 Man, 22 years		—§	Phenobarbital
18 Woman, 59 years		—§	Pentobarbital
Term placentas			
19 (n = 5)		2–30	0
20 (n = 5)		0	0

* From patients Nos. 1–3 and 5–6 both fetal livers and placentas were studied while only fetal livers were studied from patients 7–16. No. 4 represents a patient where only the placenta has been studied.

† Abortion was performed by administration of ethacridine (Rivanol®).

‡ Abortion was performed by administration of prostaglandins.

§ Unknown.

Table 2. *In vitro* metabolism of benzo[*a*]pyrene and 7-ethoxyresorufin in human fetal liver and placental microsomes*

Fetus/placenta No.		Gestational age	pmol B[a]P metabolites formed/min·mg protein		B[a]P dihydrodiols† (% of total metabolites)		pmol 7-EOR/min × mg protein	
			Fetal liver	Placenta	Fetal liver	Placenta	Fetal liver	Placenta
1	Smokers	18	19	4	n.d.‡	39	2	2
2		24	10	4	n.d.	38	5	2
3		24	12	10	n.d.	36	2	5
4		10	—	≤0.5	—	—	—	≤0.5
5	Non-smokers	21	8	≤0.5	n.d.	—	3	1
6		23	3	≤0.5	n.d.	—	3	≤0.5
Term placentas								
Smokers ($n = 5$)§		—	—	14(4–20)	—	47(45–50)	—	7(2–13)
Non-smokers ($n = 5$)§		—	—	≤0.5	—	—	—	1(1–2)

* The data represent the mean of duplicate analyses.
† Represents the sum of the 9,10-, 4,5- and 7,8-dihydrodiols.
‡ n.d., Not detectable.
§ Means and ranges are presented.

24 weeks had 7-EOR *O*-de-ethylase activities within the same range. The activity in the fetal liver microsomes ranged between 2 and 5 pmol/min·mg and there was no indication of any correlation with maternal smoking habits. The three mid-gestational placentas originating from smokers showed activities between 2 and 5 pmol/min·mg while the activities in the two placentas from non-smokers were lower, ≤0.5 and 1 pmol/min·mg protein, respectively. One placenta from early gestation (10 weeks) did not metabolize 7-EOR to any detectable extent (≤0.5 pmol/min·mg) although it originated from a smoker. Finally, the 10 term placentas showed *O*-de-ethylase activities between 1 and 13 pmol/min·mg microsomal protein. The average *O*-de-ethylase activity was seven-fold higher in placentas from smokers than in placentas from non-smokers (Table 2).

Metabolism of B[a]P

Analysis of the B[a]P metabolites formed by fetal liver microsomes supported earlier results [3], indicating the formation of mainly phenolic metabolites (Table 2). No dihydrodiols could be detected. Somewhat smaller quantities of phenols were formed in incubations with fetal livers originating from non-smoking women than with fetal livers from smoking women. As, however, the amount of metabolites formed was very low, the analysis so far should be considered as mainly qualitative. The mid-gestational and term placentas from smokers catalysed B[a]P to hydrodiols (9,10-, 4,5- and 7,8-dihydrodiols) and phenolic metabolites (9-OH and 3-OH phenols). No quinones could be detected. Placental microsomes from non-smokers and the placenta from week 10 of gestation originating from a smoker did not catalyse the oxidation of B[a]P.

The rate of formation of total metabolites varied between 4 and 10 pmol/min·mg when mid-gestational placentas were used with 36 and 39% being retrieved as dihydrodiols (Table 2). The corresponding values for term placentas varied between 4 and 10 pmol/min·mg, the dihydrodiols making up to about 45–50 % of the total amount of metabolites.

SDS-polyacrylamide gel electrophoresis

When staining placental microsomes for heme, at least two protein bands, with apparent mol. wts of 46,000 and 52,000 (Fig. 1) were clearly visible. The possible existence of other protein bands staining for

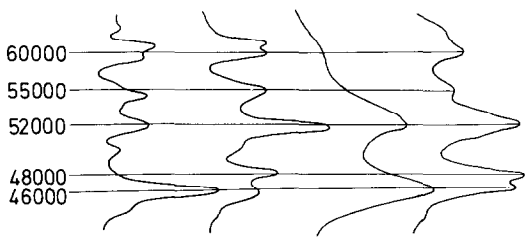

Fig. 1. Densitometric tracings of SDS-polyacrylamide gels. Microsomes stained for peroxidase activity (a and b) and for protein (c and d). (a) and (c) fetal liver microsomes. (b) and (c) placental microsomes. The gestational age of the fetus was 24 weeks (No. 2, see Table 1).

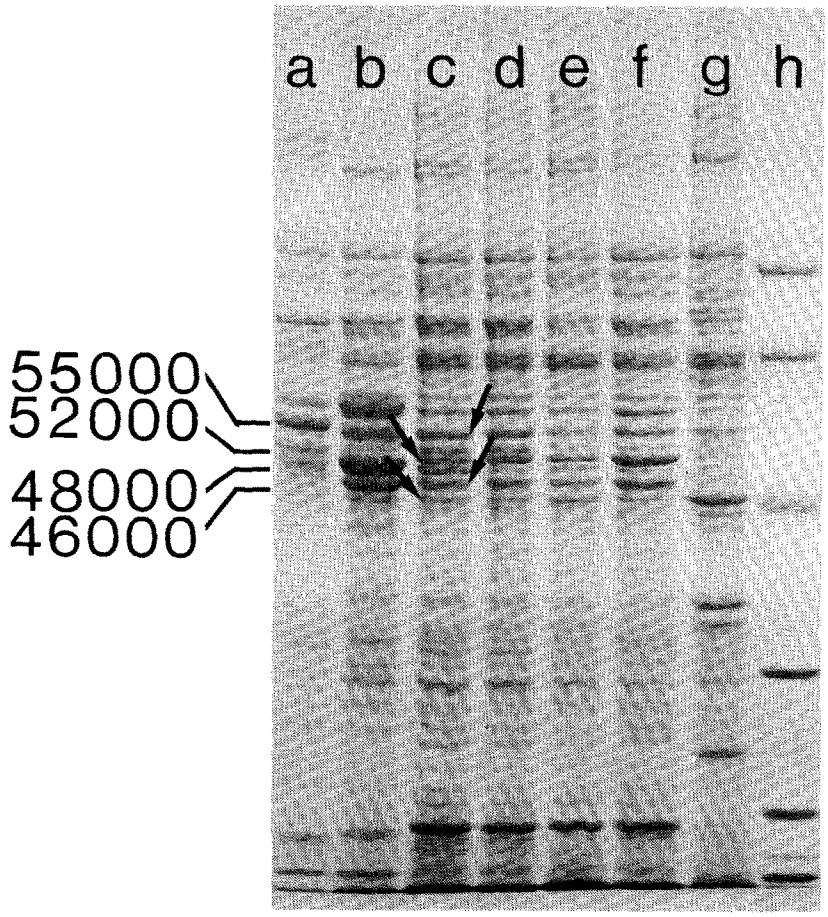

Fig. 2. SDS-gel electrophoresis of microsomes from adult and fetal liver and one corresponding placenta. For comparison microsomes from rat liver can also be seen. The gel was stained for protein with Coomassie Brilliant Blue. The proteins in the liver microsomes tentatively identified as heme proteins are indicated. (a) β-Naphthoflavone induced rat liver; (b) adult liver (No. 18, see Table 1); (c) fetal liver (21 weeks, No. 7, see Table 1); (d) fetal liver 21 weeks, No. 12, see Table 1); (e) fetal liver (27 weeks, No. 16, see Table 1); (f) fetal liver; and (g) placenta (24 weeks, No. 3, see Table 1); (h) the mol. wts of the standard proteins are 94,000, 68,000, 45,000, 30,000 and 21,000.

heme could, however, not be excluded. When gels were stained for protein with Coomassie Brilliant Blue, no differences in the intensities of the different placental microsomal protein bands were observed with respect to gestational age (10 weeks to term) or maternal smoking habits.

In the 15 fetal and two adult livers studied five heme positive protein bands were detected. The apparent mol. wts were 46,000, 48,000, 52,000, 55,000 and 60,000 (Figs. 1 and 2). The heme positive protein band in the region of 60,000 probably originated from catalase as the subunits of most mammalian catalases have mol. wts of approximately 60,000 [31]. This protein band was excluded when estimating the total amount of heme positive proteins by densitometry.

When analysing the ontogenesis of the four proteins with mol. wts of 46,000, 48,000, 52,000 and 55,000, we found an age dependency for the 52,000 protein band (Fig. 3). The relative intensity of this protein band seemed to increase with gestational

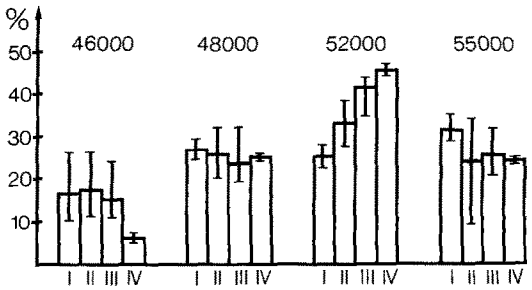

Fig. 3. Per cent of the total amount of microsomal heme proteins with a mol. wt between 46,000 and 55,000. Means are illustrated for four different groups of livers. The quantitations are based on results obtained from gels stained for protein with Coomassie Brilliant Blue. (I) Fetal livers, gestational age 15–16 weeks ($n = 3$). (II) Fetal livers, gestational age 18–21 weeks ($n = 5$). (III) Fetal livers, gestational age 22–27 weeks ($n = 7$). (IV) Adult livers ($n = 2$). See also Table 1. The range of the observations are illustrated by vertical bars.

age. It was not possible to correlate the relative amounts of the protein bands to any environmental factor.

DISCUSSION

Human fetal liver and placental (term and mid-gestational) microsomes have the capacity to metabolize 7-EOR as well as B[a]P. Smoking apparently induced the placental metabolism of both substrates excpet in one case (gestational age 10 weeks). These findings support earlier reports concerning metabolism in term placenta [19, 32–35] as well as results indicating maturation of the placental enzymes metabolizing xenobiotics during the second trimester [35].

Although maternal smoking seems to be a prerequisite for the formation of B[a]P metabolites in the placenta it was not possible to correlate the total amount of metabolites formed to the amount of cigarettes smoked. This might be due to difficulties in obtaining correct information about smoking habits and inhalation technique, as well as to the limited amount of placentas studied.

The fetal livers originating from smokers showed a somewhat higher enzyme activity towards B[a]P than the livers originating from non-smokers. For this reason the possibility of transplacental induction must be taken into consideration. The number of investigated liver samples was, however, limited and a larger number of observations is needed before any final conclusions can be drawn on the issue of transplacental effects of enzyme inducers in cigarette smoke. It should be noted that even if we were not able to show any formation of dihydrodiols in the incubations with fetal liver microsomes, Pelkonen [36] have reported formation of diols in incubations with fetal liver homogenate.

The difficulties in verifying the hypothesis of transplacental induction of drug-oxidizing enzymes have been discussed by Pelkonen *et al.* [37]. An attempt was made to induce drug metabolism in human fetal liver and placenta by the administration of phenobarbital to mothers prior to abortion. The data of these authors did not give any final evidence for the occurrence of transplacental induction but in two extreme cases where one of the mothers took denatured alcohols, technical solvents and drugs, and the other used excessive amounts of tranquilizers, the case of transplacental induction seemed highly probable.

A hypothesis has been presented that fetal tissues might be refractory to enzyme induction during fetal life, due to hormonal influence. The hormones suggested as inhibitors of enzyme induction are progesterone [38] and growth hormone [39]. On the other hand, Atlas *et al.* [40] have provided evidence for temporal control of fetal cytochrome P-450 structural gene products, by studying the effects of induction, with 3-methylcholanthrene, on different mono-oxygenase activities in rabbits from 5 days before birth to adult life.

When staining SDS-polyacrylamide gels for peroxidase activity the possible existence of multiple forms of cytochrome P-450 was indicated in placental as well as in fetal and adult liver microsomes. Only two heme positive protein bands could be seen in the placental microsomes, as has already been suggested [20], while in the fetal and adult livers at least five heme positive protein bands were observed. A multiplicity of P-450s in human adult liver has been reported by several investigators [41–43]. In these studies the mol. wts of the partially purified cytochrome P-450s were estimated to be 45,000 [43], 53,000 and 55,500 [41]. The study of Leboeuf *et al.* [42] indicates a heme staining band with a mol. wt of 50,000, which is possibly also a cytochrome P-450 isoenzyme. Our results concerning the mol. wts of the different microsomal heme staining proteins differ slightly from the results referred to above but might be due to methodological differences.

The densitometric tracings of placental microsomes stained for protein did not show any significant differences between the placentas investigated. These findings are consistent with those of Pelkonen *et al.* [20], who failed to demonstrate any differences in relative amounts of microsomal proteins originating from different placentas in which the aryl hydrocarbon hydroxylase activities varied 50-fold.

In the fetal livers studied interindividual differences in the enzyme activities as well as in the relative amounts of the different heme positive protein bands were observed. The relative amounts of the heme positive protein band corresponding to a mol. wt of 52,000 seemed to increase with gestational age. Constitutional or hormonal factors might explain the interindividual differences observed, as no correlation to maternal drug intake or smoking habits could be seen.

It is reasonable to assume that a major biological role of the cytochrome P-450 dependent enzymes in fetal liver and placenta is to metabolize steroid hormones which are present in large amounts in the feto-placental unit. The capacity of placenta, fetal liver and other fetal tissues to transform not only steroids but also various drugs does, however, also constitute a potential risk to the fetus in terms of toxicity, teratogenicity and transplacental carcinogenicity. In the discussion of these risks to the fetus, other steps in the process of bioactivation and detoxification must be considered. Since fetal tissues are able to oxidize various compounds into reactive intermediaries including epoxides, the further metabolism of such metabolites by epoxide hydrolase and glutathione *S*-transferase(s) are important steps in the detoxification pathway. This is illustrated by the work of Pacifici and Rane [44, 45] which has demonstrated the presence of styrene oxide metabolizing enzymes in various fetal tissues. Thus, e.g. the fetal liver epoxide hydrolase activity was higher than in the placenta. Because of the widely different sizes of these organs their relative contribution to the detoxification process *in vivo* is not necessarily reflected by the specific activities in sub-cellular preparations.

Various tissues in the fetus also contain measurable activities of glutathione *S*-transferase. The activity of glutathione *S*-transferase found in mid-gestational placentas was about two-fold higher than in term placentas [45]. As placental microsomes are able to form dihydrodiols of B[a]P during mid-gestation this high activity of glutathione *S*-transferase

may be of importance for the placental detoxification of epoxides generated in the maternal organism.

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