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Hydroxylation of 3,4-benzpyrene in human fetal tissue homogenates

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SEVERAL reports in the literature[1–3] have indicated that, during the early stages of gestation, the human fetal liver lacks the enzymes requisite to catalyze the hydroxylation of 3,4-benzpyrene (BP), an environmental carcinogen of considerable importance. In this communication we wish to report the detection of BP hydroxylation, catalyzed by an enzyme system referred to as aryl hydrocarbon hydroxylase or benzpyrene hydroxylase, in several organs of the human feto-placenta unit at 13–20 weeks of gestation. Activity also was detected in the fetal liver, adrenal gland, kidney and lung of a subhuman primate species at 65 days of gestation. Possible explanations for previous inability to detect this enzyme system in human fetal tissue also are presented.

Experimental

Human fetal and placental tissues were obtained after therapeutic abortions via the Central Embryology Laboratory, University Hospital, Seattle, Wash. In one case the tissues utilized were obtained after a spontaneous abortion, but fetal heartbeat could be detected briefly after delivery of the conceptus. Tissues from a single pigtail monkey (*Macaca nemestrina*) fetus were obtained from the Regional Primate Center via the Central Embryology Laboratory. Gestational ages were estimated from measurements of crown-rump length and foot length. Livers of adult male Sprague–Dawley rats (Simonsen Laboratories, Gilroy, Calif.) were employed for purposes of comparison.

Fetal tissues and rodent livers were homogenized in ice-cold isotonic solutions of potassium chloride in a Potter homogenizer with a plastic pestle. Placental tissues were homogenized in an identical solution in a Waring Blendor set at high speed for 1 min. In most cases, whole homogenates were utilized in the assay for benzpyrene hydroxylase activity. As positive controls, 9000 *g* supernatant fractions of rat liver homogenates were studied. In one experiment, the whole homogenate of adrenal gland was centrifuged at 1000 *g* for 20 min (2×10^4 *g*-min) in an IEC refrigerated centrifuge, and assays were performed on the whole homogenate, 1000 *g* supernatant and the 1000 *g* pellet. The pellet was reconstituted to the original volume of the whole homogenate with 1·15% KCl solution. In another experiment, whole homogenates of liver and adrenal gland were centrifuged at 600 *g* for 10 min (6×10^3 *g*-min) and the resultant supernatants were centrifuged at 10,000 *g* for 20 min (2×10^5 *g*-min). The 10,000 *g* supernatant was centrifuged at 104,000 *g* for 1 hr ($6{\cdot}24 \times 10^6$ *g*-min). Benzpyrene hydroxylase activities were determined in the whole homogenates, reconstituted pellets and 104,000 *g* supernatant fractions.

The rates of hydroxylation of BP in fetal and placental tissue homogenates and subfractions were determined by measuring the appearance of the fluorescent hydroxylated metabolites according to slight modifications of the method of Wattenberg *et al.*[4] as previously described.[5] Typical reaction mixtures consisted of 0·5 ml tissue homogenate or subfraction, 0·1 ml BP in acetone (10^{-4} M, final concentration), 0·2 ml NADPH in phosphate buffer ($1{\cdot}2 \times 10^{-3}$ M, final concentration), 0·5 ml glucose 6-phosphate in phosphate buffer ($7{\cdot}4 \times 10^{-3}$ M, final concentration) and sufficient 0·1 M potassium phosphate buffer (pH 7·35) to yield a total volume of 2·0 ml in the incubation flasks. In the assay of particulate fractions, 2 units of Torula yeast glucose 6-phosphate dehydrogenase (Sigma) also were added to the flasks to ensure saturating concentrations of NADPH. This mixture was incubated with shaking in a Dubnoff metabolic incubator (50–60 rpm) under a saturated oxygen gas phase at 37° for 1 hr, except where indicated. Flasks were incubated in duplicate. Control flasks for each tissue homogenate or homogenate subfraction were incubated under the same conditions but contained no NADPH or glucose 6-phosphate. For adrenal, liver, kidney and placental homogenates, product formation was linear with respect to time of incubation and tissue concentration. Lung and brain were not tested due to very low or negligible activity in those organs. Heated homogenates or subfractions (100°, 5 min) were completely inactive. Activities were expressed as millimicromoles of BP hydroxylated per gram of protein per hour. Quinine sulfate in 0·1 N sulfuric acid was employed as the standard, and specific activities were calculated on the basis of the observation that 0·036 nmole/ml in 1 N NaOH of an authentic 8-hydroxybenzpyrene standard emits the same fluorescence as 0·3 μg/ml of quinine sulfate in 0·1 N sulfuric acid at an excitation wavelength of 400 nm and an emission wavelength of 522 nm.[6] Protein concentrations were determined according to the method of Lowry *et al.*[7]

Results and discussion

Tissue homogenates from four human fetuses and one monkey fetus were analyzed for benzpyrene

hydroxylase activity and the results are presented in Table 1. In each case the adrenal gland exhibited the highest specific activity when expressed either as rate of metabolism per gram of protein or per gram of tissue (wet wt). Whole homogenates of kidneys exhibited surprisingly high specific activities on a per weight protein basis, but when expressed as per gram of tissue were lower than those observed in liver homogenates. The protein content of fetal kidney homogenates, however, was consistently very low in comparison to each of the other organs investigated. Whole homogenates of fetal livers exhibited significant benzpyrene hydroxylase activity in each of the five primates studied, but calculated specific activities were markedly lower than those observed in fetal adrenal gland homogenates. In none of the experiments could activity be detected in fetal brain homogenates, and only very low or negligible activities were observed in preparations of lung homogenates. Low but readily detectable levels of activity could be observed in placental homogenates from these early stages of gestation, in agreement with previously reported observations.[3]

Preliminary efforts also were made to determine why previous investigations had yielded negative data with respect to benzpyrene hydroxylase activity in human fetal liver preparations. The results reported in Table 1 represent enzymic activities in homogenates of very fresh tissues. Maximum time elapsing between delivery of the fetus and incubation of tissue preparations was 4 hr, with the exception of experiment 5 in which 8 hr elapsed between completion of the operation and incubation of the tissue homogenates. In that experiment, high specific activities were observed only in the adrenal gland, even though each of the tissues had been stored in the cold (0–4°) 4 hr prior to incubation. Homogenates assayed in experiment 1 subsequently were stored at −15° and again analyzed after 5 days. Specific activities for adrenal gland, liver and kidney homogenates decreased by 81, 44 and 98 per cent, respectively, during the 5-day storage period. Such observations indicated that the fetal enzyme systems were much more labile than that present in adult rat hepatic preparations.[3] Since other laboratories had reported the lack of detectable activities in microsomal fractions[1,2] and 9000 *g* supernatant fractions[3] of human fetal liver homogenates, we wished also to determine whether lack of detectable activity might be a function of the homogenate subfraction investigated. Experiments performed with liver and adrenal homogenates (Table 2) tended to suggest that activity was localized in mitochondrial and microsomal-rich subfractions of homogenates prepared from either organ. Little or no activity could be detected in the cell soluble fractions. Specific activities were usually as high (or higher) in whole homogenates as in the various particulate subfractions, but it has not yet been determined whether this observation was attributable to lability of the enzyme system or to a requirement for the presence of two or more subfractions for optimal activity. The results obtained, however, suggested that previous inability to detect benzpyrene hydroxylase activity in human fetal tissues was less likely to be a function of the homogenate subfraction under investigation than the lability of the enzyme system(s). It should be emphasized that these experiments allow only a preliminary indication of the subcellular distribution of the fetal enzyme(s), since rigorous methods for determination of intracellular distribution of enzymes in human fetal tissues at various gestational stages have not yet been reported. Electron microscopy, marker enzymes and chemical analyses will be necessary for such determinations.

Smoking of cigarettes is known to enhance markedly the rates of benzpyrene hydroxylation in human placentas delivered at term.[3,8,9] Activities in human adrenal homogenates from smokers were higher than those observed in corresponding preparations of non-smokers (approximately 3-fold). There appeared to be little correlation between smoking habits and hydroxylation activities observed in homogenates of the other human fetal organs (Table 1). It should be emphasized that these data were not sufficient to allow definitive correlations. Patient 1 had smoked (2 packs/week) prior to her pregnancy but had abstained totally during her pregnancy. This patient had at least some degree of toxaemia associated with each of six previous pregnancies, had coronary artery disease and had been on a normal regimen of phenylbutazone 6 weeks prior to her hysterectomy. Patient 2 had a spontaneous abortion at approximately 94 days of gestation, but fetal heartbeat was detected upon delivery. This patient had smoked heavily during her pregnancy (more than 2 packs/day). Unfortunately, only the adrenal gland was available for investigation of benzpyrene hydroxylase activity in this instance. Patient 3 had an uncomplicated pregnancy and did not smoke, but was on chronic diphenylhydantoin therapy for treatment of seizure disorders. The drug history of patient 4 (*Macaca nemestrina*) was exceedingly free of complications, although she had received four daily doses of 6-aminonicotinamide (6 mg/kg/day) on days 21–24 of her gestational period (the normal gestational period for this species is 178 days). As might be expected, patient 4 did not smoke. Patient 5 smoked approximately 10 cigarettes/day and also occasionally smoked marihuana. In addition, she had a history of considerable drug abuse including the self-administration of heroin, methamphetamine, barbiturates and many others. Her pregnancy, however, was uncomplicated and an apparently normal fetus was delivered. Thus, it is not possible at this time to draw conclusions regarding the effects of a drug-taking history on human fetal benzpyrene hydroxylase activity.

In summary, we have succeeded in detecting benzpyrene hydroxylase activity in homogenates of

TABLE 1. BENZPYRENE HYDROXYLASE ACTIVITIES IN WHOLE HOMOGENATES OF PRIMATE FETAL ORGANS

Patient	Species	Gestational age (days)	Cigarettes smoked (No./day)	Benzpyrene hydroxylase activity*					
				Adrenal	Liver	Kidney	Lung	Brain	Placenta
1	Human	107	None	1083 (133)	290 (26)	576 (9)	0† (0)	0 (0)	34 (3)
2	Human	94	40–50	3762 (271)					
3	Human	105	None	1077 (114)	331 (43)	529 (15)		0 (0)	
4	Monkey	65	None	4030 (485)	94 (21)	490 (12)	45 (5)	0 (0)	
5	Human	135	10–15	4896 (297)	75 (7)	13 (2)	0 (0)	0 (0)	39 (4)

* Numbers of the table represent millimicromoles 3,4-benzpyrene hydroxylated per hour per gram of protein. Numbers in parentheses represent millimicromoles hydroxylated per hour per gram of tissue (wet weight). For comparison, rat hepatic homogenates and 9000 *g* supernatant fractions yielded specific activities of approximately 5000–7000 and 8000–10,000 millimicromoles hydroxylated per hour per gram of protein respectively. Flasks incubated with adrenal, liver, kidney, lung, brain and placental homogenates contained 17, 167, 83, 167, 167 and 167 mg tissue (original wet weight) per flask.

† Blanks indicate that no determinations were made. Zero indicates that fluorescence detected in test samples was not significantly different from that of the corresponding blanks.

TABLE 2. BENZPYRENE HYDROXYLASE ACTIVITIES IN SUBFRACTIONS OF HUMAN FETAL LIVER AND ADRENAL GLAND HOMOGENATS;

Patient	Organ	Homogenate subfraction*	Specific activity†
2	Adrenal gland	Whole homogenate	3762
2	Adrenal gland	2×10^4 *g*-min pellet	2656
2	Adrenal gland	2×10^4 *g*-min supernatant	5534
5	Liver	Whole homogenate	75
5	Liver	6×10^3 *g*-min pellet	24
5	Liver	2×10^5 *g*-min pellet	87
5	Liver	$6{\cdot}24 \times 10^6$ *g*-min pellet	123
5	Liver	$6{\cdot}24 \times 10^6$ *g*-min supernatant	4
5	Adrenal gland	Whole homogenate	4896
5	Adrenal gland	6×10^3 *g*-min pellet	45
5	Adrenal gland	2×10^5 *g*-min pellet	398
5	Adrenal gland	$6{\cdot}24 \times 10^6$ *g*-min pellet	446
5	Adrenal gland	$6{\cdot}24 \times 10^6$ *g*-min supernatant	40

* Homogenate subfractions represent material obtained from successive centrifugations for each organ investigated (see Experimental). Measurements were made on subfractions reconstituted to the original volume of the whole homogenate.

† Numbers in the table represent millimicromoles 3,4-benzpyrene hydroxylated per gram of protein per hour.

human and monkey fetal adrenal glands, livers and kidneys. Specific activities approaching those observed in adult rat liver preparations were observed in adrenal gland homogenates. It is not possible at present to determine why previous investigations resulted in a lack of detectable activity in human fetal tissues *in vitro*, although several possible explanations can be given. These include; (1) insufficient incubation time to allow measurement of significant quantities of metabolite(s); (2) deterioration of enzymic activity between the time of delivery of the fetus and beginning of incubations; (3) insufficient concentrations of enzyme, substrate or cofactors; or (4) utilization of inappropriate incubation conditions. The activity of this enzyme system in fetal tissues may be a highly important determinant of human fetal pharmacology and toxicology.

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