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THE HETEROGENEITY OF ARYLHYDROCARBON HYDROXYLASE IN FETAL LIVER MICROSOMES OF RATS

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SUMMARY: The characteristic of arylhydrocarbon hydroxylase system in fetal liver microsomes of rat was investigated. NADH-synergistic effect on NADPH-dependent arylhydrocarbon hydroxylase was observed in fetal liver microsomes of rat but not in maternal liver microsomes. NADH-synergistic effect decreased in parallel with the decrease of the ratio of cytochrome b_5/cytochrome P-450 in liver microsomes. The cytochrome P-450 in arylhydrocarbon hydroxylase system in fetal liver microsomes of rat seemed to be different from that in offspring liver microsomes in respect of its dependency on cytochrome b_5 system for its maximum activity.

INTRODUCTION: This laboratory has been interested for some years in the comparative aspects of drug-metabolizing system of fetal liver and maternal liver microsomes of rat(1). Previous works in this(1,8) and other laboratories(2-6) have revealed some characteristics of the drug-metabolizing system in fetal liver microsomes of rat. These include differences in spectral and catalytic properties as well as inducibility by polychlorinated biphenyls(2,8), phenobarbital(3-4) and 3-methylcholanthrene(3-MC)(1,5-6). Arylhydrocarbon hydroxylase(AHH) is a typical example of monooxygenase specifically induced by administration of polycyclic hydrocarbons(5-7). The data obtained in the inductive effect of 3-MC on AHH in fetal liver microsomes of rats were similar to those obtained when male rats were pretreated with 3-MC(5). However, no further investigation of the characteristic of AHH system in fetal liver microsomes has been previously made. In this communication, we attempted to

Abbreviations used are; 3-MC, 3-methylcholanthrene, AHH, arylhydrocarbon hydroxylase, CO, carbon monoxide, b_5/P-450, cytochrome b_5/cytochrome P-450, PCMS, p-chloromercuribenzenesulfonic acid

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further study the properties of AHH system in fetal liver microsomes of rat in comparison with that in maternal liver microsomes.

MATERIALS AND METHODS: Animals and treatments Experimental animals used were Wistar strain rats weighing 250-350 g. Five females were mated with two males overnight. The next day was designated as day 0 of pregnancy and the birthday was designated as day 0 of age. 3-MC dissolved in olive oil(25 mg/2 ml/kg) was intraperitoneally administered.

Preparation of liver microsomes and assay of drug-metabolizing enzymes Liver microsomes from fetal, newborn, 3 day-old and maternal rats were prepared by the method as previously described(8). NADH-cytochrome b_5 reductase and NADPH-cytochrome c reductase determined as described by Takesue and Omura (9) and Williams and Kamin(10), respectively. AHH was assayed by the method of Nesnow et al.(11) using incubation time and protein concentration such reaction rates were linear with different preparation.

Preparation of microsomal fraction through Sepharose 2B column For the determinations of cytochromes, each microsomal fraction was prepared according to the method of Tangen et al(12). Contents of cytochrome b_5 and cytochrome P-450 were determined by the method of Omura and Sato(13). Protein content was determined by the method of Lowry et al.(15) using bovine serum albumin as the standard.

RESULTS AND DISCUSSIONS: The administration of 3-MC to pregnant rats increased AHH activities in both fetal and maternal liver microsomes of rat 21.4-fold and 14.4-fold, respectively(data not shown). Then, a comparison has now made to characterize AHH system in fetal liver microsomes of rat. As shown in Table 1, omission of NADP in the incubation mixture completely abol-

Table 1.
Characteristics of arylhydrocarbon hydroxylase in fetal and maternal liver microsomes of rats

Assays conditions	Arylhydrocarbon hydroxylase (nmoles/min/mg protein)			
	Maternal microsomes	%	Fetal microsomes	%
Complete system	1.217 ± 0.086	100	0.345 ± 0.010	100
- NADP	0	0	0	0
+ NADH(1.0 mM)	1.329 ± 0.100	109.2	0.518 ± 0.015**	150.1
+ CO (bubbled for 1 min)	0.630 ± 0.042**	51.9	0.062 ± 0.016**	18.1
+ Menadione(1.0 mM)	0	0	0	0

3-MC was administered to pregnant rats once daily on the 18th and the 19th day of pregnancy. Pregnant rats were sacrificed on the 21st day of pregnancy and livers were excised from fetal and maternal rats. The reaction mixture for AHH contained an NADPH-generating system consisted of 1.0 mM $NADP^+$, glucose-6-phosphate and glucose-6-phosphate dehydrogenase, 5 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM potassium phosphate buffer(pH:7.5) and 0.08 mM [^{14}C]benzo(a)-pyrene(58.5 mCi/mmole, The Radiochemical Center, Amersham, England) and total volume was adjusted to 1.0 ml. NADH in 0.02 ml or menadione in 0.002 ml of dimethylsulfoxide was added into 1.0 ml of incubation mixture.
Each value is mean ± S.E. Asterisks indicate a significant difference from control(** P<0.01).

ished AHH activities in fetal and maternal liver microsomes of rats as the presence of menadione which is an electron acceptor. The presence of carbon monoxide(CO) inhibited AHH activities in fetal and maternal liver microsomes of rat. The results suggested that AHH system in both fetal and maternal liver microsomes are mediated by NADPH-dependent monooxygenase. On the contrary, a striking difference was noted by the addition of NADH. The AHH activities in fetal liver microsomes were increased by the addition of NADH in an equimolar to that of NADPH, but no increase was observed in maternal liver microsomes of rats. Thus, we investigated the difference in NADH-synergistic effect on NADPH-dependent AHH activities in fetal and maternal liver microsomes. Correia and Mannering et al.(15) reported that NADH-synergism on NADPH-dependent drug-oxidation was seen in 3-MC-treated rat to lower degree comparing that in non-treated rat. In addition, Conney et al.(16) reported that NADH-synergism on AHH was not produced at a significant level. Based on early reports together with our finding, it is very interesting that NADH-synergistic effect on NADPH-dependent AHH was observed in fetal liver microsomes differing from that in maternal liver microsomes. In order to further examine the difference in NADH-synergism, we investigated the NADH-synergistic effect on NADPH-dependent AHH in microsomes from 3-MC-pretreated fetal, newborn, 3 day-old and maternal rats. As shown in Table 2, the degree of synergistic effect of NADH was the highest in fetal liver microsomes. The effect of NADH diminished in newborn rat liver microsomes immediately after birth and then the synergistic effect of NADH was not observed in 3 day-old as well as in maternal liver microsomes of rat. It is well known that cytochrome b_5 system participates in NADH-synergism and interpreted as evidence that second electron is transfered via cytochrome b_5 system to cytochrome P-450(16-18). However, little is known about the contents of cytochrome b_5 and its role and relationship to cytochrome P-450 in fetal liver microsomes of rat. One of the reasons is that contamination by hemoglobin makes it difficult to characterize cytochromes in fetal liver microsomes. Thus, we prepared liver microsomes by passing 10,000 x g supernatant fraction

Table 2.
Synergistic effect of NADH on NADPH-dependent arylhydrocarbon hydroxylase

Microsomes	Addition NADH (1.0 mM)	Arylhydrocarbon hydroxylase (nmoles/min/mg protein)	%
Fetal rat	−	0.346 ± 0.029	100
	+	0.533 ± 0.017**	154.0
Newborn rat	−	0.506 ± 0.007	100
	+	0.612 ± 0.034*	120.4
3 day-old rat	−	2.146 ± 0.041	100
	+	2.280 ± 0.111	106.3
Maternal rat	−	1.256 ± 0.075	100
	+	1.256 ± 0.086	100.1

Fetal and maternal livers were excised from the pregnant of the 21st day of pregnancy which were pretreated maternally with 3-MC once daily on the 18th and the 19th day of pregnancy. Newborn rat livers were excised from the newborn immediately after parturition which were pretreated maternally with 3-MC once daily on the 19th and the 20th day of pregnancy. 3 day-old rat livers were excised from the neonatal which were pretreated with 3-MC once daily on 0 and the 1st day of age. The reaction mixture for NADH-synergistic effect on AHH was the same component as described in the legend for Table 1. Each value is mean ± S.E. Asterisks indicate a significant difference from control(* $P<0.05$, ** $P<0.01$).

through Sepharose 2B column(12). The results are summarized in Table 3. Surprisingly, contents of cytochrome b_5 were larger than those of cytochrome P-450 and the ratio of cytochrome b_5/cytochrome P-450(b_5/P-450) was 1.95 in fetal liver microsomes. On the contrary, the ratio of b_5/P-450 decreased as a

Table 3.
Levels of cytochromes in rat liver microsomes as a function of age

Microsomes	Cytochromes (nmoles/mg protein)		Ratio: (b_5/P-450)
	b_5	P-450	
Fetal liver	0.352 ± 0.004	0.181 ± 0.012	1.95
Newborn rat	0.410 ± 0.001	0.411 ± 0.006	0.99
3 day-old rat	0.485 ± 0.007	1.418 ± 0.058	0.34
Maternal rat	0.302 ± 0.052	0.853 ± 0.042	0.35

Livers were excised from fetal, newborn, 3 day-old and maternal rats. The pretreatment with 3-MC was the same as the pretreatment described in the legend for Table 2. Each value is mean ± S.E.

function of age. As shown in Table 2 and 3, NADH-synergistic effect decreased in parallel with the decrease of ratio of b_5/P-450. In the next experiment, the effect of p-chloromercuribenzenesulfonic acid(PCMS) was examined to clarify that cytochrome b_5 system participates in NADH-synergism on NADPH-dependent AHH in fetal liver microsomes of rat(19). Table 4 showed that PCMS abolished NADH-synergistic effect in fetal liver microsomes(PCMS inhibited NADH-cytochrome b_5 reductase but not NADPH-cytochrome c reductase at the dose of 2.5 and 5.0 μM). This result promted us to suggest that AHH system in fetal liver microsomes requires cytochrome b_5 system for its maximum activity. Furthermore, inhibitions of NADPH-dependent AHH in addition of NADH in an equimolar to NADPH were examined. 7,8-Benzoflavone(2×10^{-4}M) inhibited AHH completely, SKF 525-A(1×10^{-4}M) inhibited AHH by 64.3 % and in the presence of 0.5 mM CN^-, AHH was not inhibited at all. These results indi-

Table 4.
Effect of p-chloromercuribenzenesulfonic acid on NADH-cytochrome b_5 reductase, NADPH-cytochrome c reductase and NADH-synergistic effect on NADPH-arylhydrocarbon hydroxylase

Addition PCMS (μM)	NADH-cytochrome b_5 reductase (nmoles/min/mg protein)	%	NADPH-cytochrome c reductase (nmoles/min/mg protein)	%
–	0.820 ± 0.029	(100)	26.12 ± 0.78	(100)
2.5	0.402 ± 0.043**	(51.2)	25.25 ± 0.09	(96.8)
5.0	0.244 ± 0.018**	(29.7)	25.53 ± 0.09	(97.8)

Addition NADH, (1.0 mM)	PCMS (μM)	Arylhydrocarbon hydroxylase (nmoles/min/mg protein)	%
–	–	0.351 ± 0.010	(100)
+	–	0.559 ± 0.007**	(159.3)
+	2.5	0.453 ± 0.001**	(129.5)
+	5.0	0.376 ± 0.007	(107.0)

3-MC was administered to pregnant rats once daily on the 18th and the 19th day of pregnancy. Pregnant rats were sacrificed on the 21st day of pregnancy and livers from fetal rats were excised. The reaction mixture for AHH was the same as described in the legend for Table 1. The reaction mixture for cytochrome c reductase activities contained microsomes, 8 μM cytochrome c and 0.1 M NADPH in 2.0 ml of 0.1 M potassium phosphate buffer(pH:7.5). The reaction mixture for NADH-cytochrome b_5 reductase activities contained microsomes, 5 μM cytochrome b_5 and 0.1 M NADH in 0.1 M potassium phosphate buffer(pH:7.5). PCMS in 0.05 ml of distilled water was added to the reaction mixture. Each value is mean ± S.E. Asterisks indicate a significant difference from control(** $P<0.01$).

cate that cytochrome P-450 is the terminal enzyme which catalyzes NADPH-dependent AHH in addition of NADH in an equimolar to NADPH in fetal liver microsomes of rat. The results hitherto, lead us to conclude that cytochrome b_5 of which contents are larger than those of cytochrome P-450 participates in NADH-synergism on NADPH-dependent AHH and cytochrome P-450 in AHH system in fetal liver microsomes seems to be different from that in offspring liver microsomes of rat in respect of its dependency on cytochrome b_5 system for its maximum activity. However, the decisive proofs will be presented only after the purification of each component of AHH system and we will present it in the near future.

REFERENCES

1. Mizokami,K., Inoue,K., Sunouchi,M., Fujimori,K., Takanak,A. and Omori,Y. (1982) Biochem. Biophys. Res. Commun. 107, 6-11
2. Alvares,A.P. and Kappas,A. (1975) FEBS Lett. 53, 205-210
3. Guenthner,T.M. and Mannering,G.J. (1977) Biochem. Pharmacol. 26, 567-575
4. Cresteil,Th., Flinois,J.P., Pfister,A. and Leroux,J.P. (1979) Biochem. Pharmacol. 28, 2057-2063
5. Welch,R.M., Gommi,B., Alvares,A.P. and Conney,A.H. (1972) Cancer Res. 32, 973-978
6. Conney,A.H., Miller,E.C. and Miller,J.A. (1957) J. Biol. Chem. 228, 753-766
7. DePierre,J.W., Moron,M.S., Johannessen,K.A.M. and Ernster,L. (1975) Anal. Biochem. 63, 470-484
8. Inoue,K., Takanaka,A., Mizokami,K., Fujimori,K., Sunouchi,M. and Omor,Y. (1981) Toxicol. Appl. Pharmacol. 59, 540-547
9. Takesue,S. and Omura,T. (1970) J. Biochem. 67, 259-266
10. Williams,C.H.,Jr. and Kamin,H. (1962) J. Biol. Chem. 237, 587-595
11. Nesnow,S., William,E.F. and Jefcoate,C.R. (1977) Anal. Biochem. 80, 258-266
12. Tangen,O., Johanessen,J. and Orrenius,S. (1973) Anal. Biochem. 54, 597-603
13. Omura,T. and Sato,R. (1964) J. Biol. Chem. 239, 2370-2378
14. Lowry,O.H., Rosenbrough,N.J., Farr,A.L. and Randall,R.J. (1951) J. Biol. Chem. 193, 265-275
15. Correia,M.A. and Mannering,G.J. (1973) Mol. Pharmacol. 9, 470-485
16. Katata,S., Sugiyama,T., Seki,K., Tarui,S., Okamoto,M. and Yamano,T. (1982) J. Biochem. 92, 305-311
17. Sugiyama,T., Miki,N. and Yamano,T. (1979) Biochem. Biophys. Res. Commun. 90, 715-720
18. Noshiro,M. and Omura,T. (1978) J. Biochem. 83, 61-77
19. Oshino,N., Imai,Y. and Sato,R. (1971) J. Biochem. 69, 155-167