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INDUCTION OF CYTOCHROME P-450c IN HEMATOPOIETIC CELLS OF FETAL LIVER

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The transplacental inductive effect of β-naphthoflavone (βNF) on cytochrome P-450 isozymes was studied in separate hematopoietic and hepatocyte cells from fetal rat liver. Two fractions of dispersed fetal liver cells were isolated by Ficoll-Paque centrifugation and shown by histologic examination to be enriched in erythroblasts and hepatocytes, respectively. βNF treatment increased ethoxyresorufin-O-deethylase activity 250-fold in both erythroblast and hepatocyte cell fractions. Polyacrylamide gel electrophoresis and immunostaining techniques showed the induction of cytochrome P-450c, but not P-450d, in erythroblast and hepatocyte fractions.

Developmental studies of liver monooxygenase enzymes have shown that cytochrome P-450s are present at very low concentrations in fetal liver and increase after birth (1-4). While PAH are potent inducers of cytochrome P-450c[1] in fetal liver from several species, induced levels of fetal activities were much lower than those induced in neonatal or maternal tissues (1-4). A developmental lag in the inducibility of P-450d, relative to the inducibility of P-450c, has been reported in several species (1,3,4,7). Studies to date have not considered that the fetal liver is a major hematopoietic organ in which immature nucleated erythroid cells represent

[1]Cytochrome P-450c isolated from 3MC treated rats is identical with cytochromes P-448_{MC} (5) and presumably βNF_{β} (6). P-450d is identical with P-448_{HCB} (5) and presumably ISF_{G} (6). Analogous forms in mouse are P_1-450 and P_3-450 (4,7), and in rabbit LM_6 and LM_4 (1).

Abbreviations: βNF, 5,6-benzoflavone; 3MC, 20-methylcholanthrene; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis; PAH, polyaromatic hydrocarbons.

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about 70% of the liver population at gestation day 17 (8). Hematopoietic cells still comprise 40% of the liver population during the first four days after birth (9). The non-hepatic nature of the fetal liver makes difficult the interpretation of cytochrome P-450 induction data. Whereas P-450c is induced in both hepatic and extrahepatic tissues of the adult rat, P-450d is totally refractive to induction in extrahepatic tissues (10). The present study describes a cell separation procedure for investigation of the inductive effects of βNF on the separate populations of hepatocytes and hematopoietic cells from fetal rat liver.

METHODS

Materials: Earle's Ca^{++}/Mg^{++} free-salt solution and Balanced Salt Solution were from Gibco (Grand Island, NY). Collagenase and Dispase were from Boehringer-Mannheim (Indianapolis, IN), while bovine serum albumin and DNAse I, type II from bovine pancreas were from Sigma (St. Louis, MO). Ficoll-Paque was obtained from Pharmacia (Piscataway, NJ).

Preparation of cell fractions: Pregnant rats (Holtzman Co., Madison, WI) were untreated or received βNF (15 mg/kg, i.p. in corn oil) 24 hr prior to study on days 17-18 gestation. Fetal livers were pooled from 2-3 litters, chopped and suspended in 50 ml of Earle's Ca^{++}/Mg^{++} Free-Salt Solution (CMFS) and washed for 30 min at 37°C. Tissue fragments were then incubated in CMFS containing collagenase (1 mg/ml), bovine serum albumin (10 mg/ml) and DNAse I (0.2 mg/ml) for 45 min at 37°C. Cells were collected by centrifugation at 400g for 5 min at 19°C. The cells were resuspended in CMFS containing Dispase (3 mg/ml) and incubated at 37°C for 1 hr. After filtration through a nylon filter (75 μm), the cells were collected by centrifugation at 400g for 5 min at 19°C. Cells were washed in Earle's Balanced Salt Solution (BSS) 2-3 times and collected by centrifugation. Cells were resuspended in BSS, layered on top of 10 ml Ficoll-Paque and centrifuged at 500g for 20 min at 19°C. Hepatocytes form a layer above the Ficoll while erythroblasts sediment to the bottom of the tube. Each cell fraction was washed in BSS and diluted to 10^8 cells/ml. Cells were identified by histologic examination of smears stained with hematoxylin and eosin (H and E).

Ethoxyresorufin-O-deethylase activity: A 10% homogenate of each cell fraction was prepared in 0.1 M $NaPO_4$ buffer, pH 7.4. Enzyme activity was measured spectrofluorometrically as the formation of resorufin from 7-ethoxyresorufin according to the method of Pohl and Fouts (11), as previously described for fetal tissue (12). Incubation mixtures contained 0.1 M Hepes, pH 7.5, 5 μM ethoxyresorufin, 5 mM NADPH and 0.20% bovine serum albumin in a final volume of 1.0 ml and were run at 37°C for 5-20 min with 0.5-2 mg homogenate protein. Protein concentration was determined by the method of Lowry et al. (13).

Cytochrome and antibody preparations: Cytochromes P-450c and P-450d were purified to apparent homogeneity and characterized as described elsewhere (5,14). Both cytochromes P-450c and P-450d contained only a single band on SDS-polyacrylamide gel electrophoresis (PAGE) even when amounts as high as 10 μg of protein per well were electrophoresed. The specific contents were 18.8 nmoles/mg of protein for cytochrome P-450d and 13.6 nmoles/mg for cytochrome P-450c. Antiserum to P-450d (anti-P-450d) was raised in rabbits. Crossreactivity of anti-P-450d to P-450c was removed by immunoabsorbtion over a column of partially purified P-450c bound covalently to Sepharose gel (14). Immunospecific antiserum to P-450c was similarly immunoabsorbed with solid-phase P-450d.

Electrophoresis and immunostaining: SDS-PAGE was performed as described by Laemmli (15). Proteins were transferred from the gels to nitrocellulose sheets (blotted) which were immunostained by the method of Towbin et al. (16) as outlined by Goldstein and Linko (10) except that 3% Hipure liquid gelatin was used to block nonspecific binding and 4-chloro-1-naphthol was used as the color indicator.

RESULTS

Cell fractions: Photomicrographs in Fig. 1A show the histology of sections of fetal rat liver on day 17 gestation. As described in previous studies of fetal liver (9,17,18), the predominant cell types are extrasinusoidal hematopoietic cells which are small and round, with dense nuclei, high nuclear-cytoplasmic ratio and basophilic cytoplasm. Previous studies have characterized these cells as mainly erythroid, with some megakaryocytes and granulocytes (8,18). In Fig. 1A, nucleated RBC are also present in sinusoids and blood vessels (v). Centrifugation of dispersed fetal liver cells over Ficoll-Paque separated 2 cell fractions, an upper layer at the interface between buffer and Ficoll, and a second layer at the bottom of the

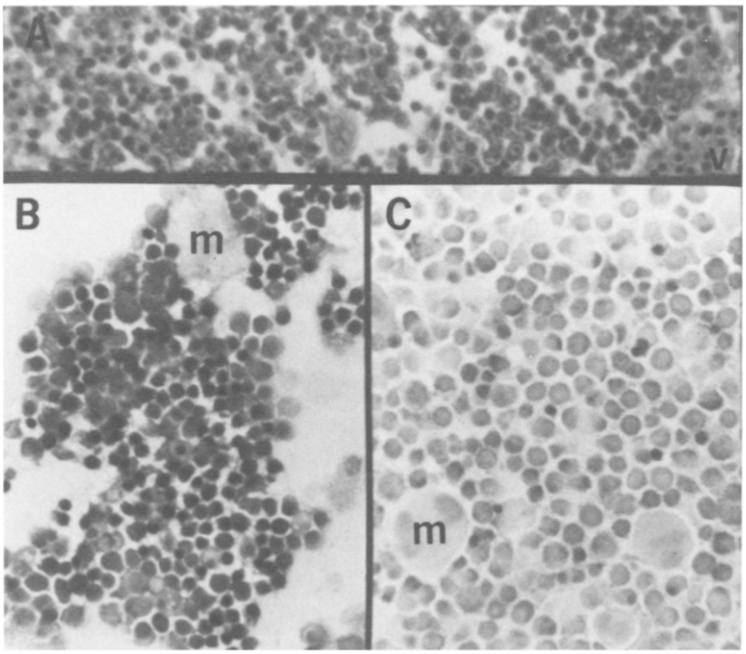

Fig. 1. Photomicrographs of A) fetal liver, day 17 gestation, B) hematopoietic cell fraction, and C) hepatocyte cell fraction. Fetal liver tissue was formalin fixed, paraffin embedded, cut in 7μ sections and stained with H and E. Smears of cell fractions were air dried, fixed and stained with H and E. All magnifications x200.

TABLE 1

Ethoxyresorufin-O-deethylase Activity in Cell Fractions from Fetal Liver

	EROD pmol/mg protein/min	Protein µg protein/10^6 cells
Erythroblasts		
Control (6)	0.07 ± .01	57 ± 16
βNF (4)	19.4 ± 4.2***	60 ± 14
Hepatocytes		
Control (3)	0.12 ± .02	53 ± 5
βNF (4)	27.5 ± 5.1***	95 ± 32

$\bar{X}$ ± SE of (n) experiments, ***$p<0.001$.

tube. The cells in the two layers can be distinguished cytologically in smears stained with H and E. The lower layer (Fig. 1B) contains the hematopoietic fraction with more than 80% erythroid cells, as well as a few larger cells such as immature eosinophils, granulocytes and a rare megakaryocyte (m). These erythroblasts typically exhibit "rouleaux" formation which is characteristic of the erythrocyte aggregating activity of the Ficoll polymer (19). The upper layer of cells (Fig. 1C) is enriched in hepatocytes, large pale cells with pale nuclei, and intermediate size cells which are round with darker nuclei. This fraction also contains large megakaryocytes (m) in addition to a few immature erythroid cells. The relative yield from three litters was 0.23-0.27 $x10^8$ cells in the hepatocyte fraction and 3.34-4.65 $x10^8$ cells in the hematopoietic layer.

Ethoxyresorufin-O-deethylase activity: Data in Table 1 show that βNF administration was associated with a 250-fold increase in enzyme activity in erythroblast and hepatocyte fractions from fetal liver. No significant difference was observed in mg protein/10^6 cells between control and βNF for either cell fraction.

SDS-PAGE and immunostaining: Cytochrome P-450c was not detected on immunoblots of control maternal liver or in erythroblast and hepatocyte cell fractions from control fetuses, as indicated by the absence of staining in the 55,000 molecular weight (M_r) region (Fig. 2A). In contrast, P-450c

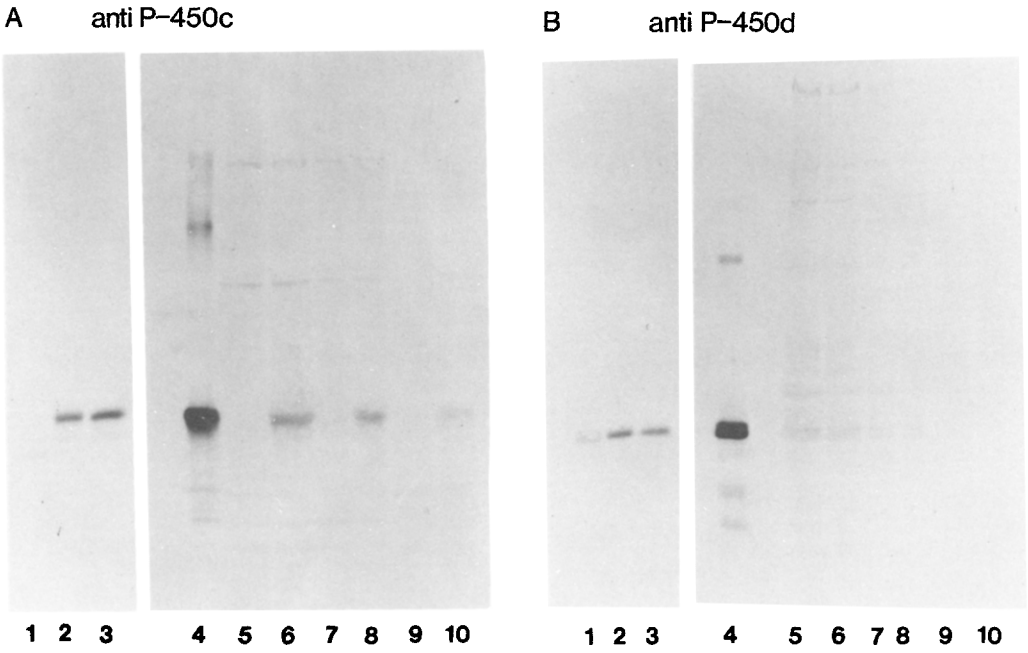

Fig. 2. Identification of cytochromes P-450c (A) and P-450d (B) by SDS polyacrylamide gel electrophoresis and immunostaining.

Lane 1:	Maternal liver microsomes,	Control (5 µg protein)
Lane 2:	" " " ,	βNF (2 µg)
Lane 3:	" " " ,	3MC (2 µg)
Lane 4:	Purified P-450c (A) or P-450d (B), 2 pmoles	
Lane 5:	Erythroblast cell homogenate,	Control (200 µg)
Lane 6:	" " " ,	βNF (200 µg)
Lane 7:	" " " ,	Control (50 µg)
Lane 8:	" " " ,	βNF (50 µg)
Lane 9:	Hepatocyte homogenate,	Control (50 µg)
Lane 10:	" " ,	βNF (50 µg)

was readily detected in βNF-induced maternal liver microsomes (2 µg) and in homogenates of erythroblasts (50 and 200 µg protein) and, to a lesser extent, in hepatocytes (50 µg) from induced fetuses. The lower limit of sensitivity for immunostaining was approximately 0.1 pmole indicating that fetal samples contained at least 2 pmoles/mg protein. Cytochrome P-450d was detected as a 52,000 M_r protein in maternal liver microsomes from control animals and was also highly induced by βNF (Fig. 2B). In contrast to P-450c, no evidence was found for induction of P-450d in erythroblast or hepatocyte fractions. Moreover, immunoreactivity in the 52,000 M_r region in control hepatocytes and erythroblasts was extremely faint compared to maternal liver.

DISCUSSION

The liver has been described as the most important organ in fetal blood cell development due to its large size, immense number of hematopoietic cells and the lengthy period of hepatic hematopoiesis (17,18). While induction by PAH has been studied in total fetal liver (1-4) and cultured fetal hepatocytes (20), the present study is the first to develop procedures for the isolation of induced hematopoietic cells from fetal rat liver. Enzymatic and immunochemical evidence for the induction of P-450c in both hematopoietic and hepatocyte cell populations indicates that the relative poor inducibility of the fetal liver by PAH cannot be explained by the low organ content of hepatocytes. It is noteworthy that P-450c and P-450d are induced coordinately in maternal liver, but not in fetal hepatocytes and erythroblasts. The lack of evidence for induction of P-450d in either cell fraction supports the previous reports of temporal regulation of the two PAH-induced cytochrome P-450 isozymes (1,3,4,7) rather than the predominance of nonhepatic hematopoietic cells. A comparison of immunostaining in the cell fractions from βNF-induced fetuses suggests that P-450c is induced to a greater extent in erythroblasts than in hepatocytes. Similarly, we have reported that microsomes from βNF-induced erythroblast cells are more active than microsomes from source fetal liver in catalyzing the covalent binding of benzo(a)pyrene to DNA, and produce a different profile of DNA adducts (21). Thus the observed induction of metabolic activation by βNF in fetal hematopoietic cells may relate to other reports of PAH-related chemical toxicity to adult bone marrow (22,23), as well as to feto-placental growth retardation and susceptibility to carcinogenesis.

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