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Differences in the Developmental Expression of Rabbit Cytochromes P-450 2E1 and 2E2

HWEI-MING PENG, TODD D. PORTER, XINXIN DING, and MINOR J. COON

Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, Michigan 48109-0606

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SUMMARY

The alcohol-inducible *CYP2E* subfamily in rabbits contains two genes; *CYP2E1* encodes the cytochrome earlier termed P-450 3a, and *CYP2E2* encodes a cytochrome that is 97% identical in amino acid sequence to cytochrome P-450 (P-450) 2E1. In the present studies, the ontogenic expression of these two cytochromes was examined. In liver, P-450 2E2 mRNA is detectable immediately after birth and reaches slightly greater than the adult level at 2 weeks of age; in contrast, P-450 2E1 mRNA is not detectable until day 14 and increases rapidly to approximately twice the adult level at 5 weeks of age. P-450 2E protein is present in liver immediately after birth, coincident with the appearance of P-450 2E2 mRNA, peaks at 2 weeks, and then, despite the continued elevation in P-450 2E mRNA, decreases to the adult level at 5 weeks. In kidney, P-450 2E2 mRNA is not detectable at any age; P-450 2E1 mRNA, however, is present at 1 week, and the level increases to about half the adult level at 5 weeks of age. P-450 2E protein in this tissue is elevated at 2 weeks, relative to mRNA levels, and reaches approximately half the adult level at 5 weeks. The lack of close correlation between mRNA and protein levels in the liver and kidney of newborn rabbits indicates that the posttranscriptional control of P-450 2E enzyme levels that predominates in adult animals is also operative during the neonatal period. Monooxygenase activities with ethanol and *p*-nitrophenol as substrates reflect the developmental increase in P-450 2E protein, as well as the appearance and levels of spectrally detectable P-450, cytochrome b_5, and NADPH-P-450 reductase in hepatic microsomes. The expression of P-450 2E2, but not P-450 2E1, in early neonates suggests that these two closely related cytochromes may have functional differences that are important during the first few weeks of life.

The alcohol-inducible *CYP2E* subfamily in rabbits contains two genes that are 97% identical in amino acid sequence (1). P-450 2E1 (P-450 3a) is active in the metabolism of a wide variety of commonly encountered xenobiotics, including alcohols, ketones, halogenated alkanes, and nitrosamines (reviewed in Ref. 2); the substrate profile of the recently discovered P-450 2E2 isoform is not yet known. Previous studies in this laboratory revealed that in adult rabbits the two genes are not coordinately expressed or regulated (3). P-450 2E1 mRNA is present in greatest abundance in the liver and is present to a lesser extent in kidney, nasal mucosa, and lung. In contrast, P-450 2E2 expression is limited to the liver and lung, and P-450 2E2 mRNA is present at approximately one half the level of P-450 2E1 mRNA in these tissues. Neither gene is transcribed in testis, ovary, small intestine, or adrenal tissue.

Recently, Bonfils *et al.* (4) identified a P-450 in neonatal rabbit liver that was related to, but distinct from, P-450 2E1. This cytochrome was a principal P-450 in the liver of preweanling rabbits. NH_2-terminal sequence analysis and immunological cross-reactivity with P-450 2E1 antibody indicated strongly that this neonatal cytochrome probably corresponded to P-450 2E2. The finding that this cytochrome, but not P-450 2E1, is abundant in 1–3-week old rabbits suggested that P-450 2E1 and P-450 2E2 might be differentially expressed during ontogenesis. The livers of fetal laboratory animals, including rabbits, are largely devoid of P-450-linked monooxygenase systems (4, 5); the expression of P-450 monooxygenases in these animals begins shortly after birth. In the rat, P-450 2E1 expression is detected within 24 hr of parturition, and the content of P-450 2E1 mRNA and protein increases steadily, reaching adult levels in approximately 2–4 weeks (6, 7). This transcriptional activation correlates with the demethylation of specific methylcytosines in the 5′ region of the gene (7); however, it is not known whether this phenomenon is unique to the rat or may also be common to other species.

In the present study, the ontogenic changes in P-450 2E1 and P-450 2E2 expression in various rabbit tissues were examined. Developmental changes in other components of the hepatic monooxygenase system of rabbits were also investigated. Our findings reveal that P-450 2E2 expression begins at birth, whereas P-450 2E1 expression does not begin until 2 weeks of age. The delayed expression of P-450 2E1, coupled with the diminished expression of P-450 2E2 in adults, suggests

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ABBREVIATION: P-450, cytochrome P-450, NADPH-P-450 reductase, NADPH-cytochrome P-450 oxidoreductase.

that these two closely related cytochromes may have different roles in neonatal and adult animals.

Experimental Procedures

Animals. Late-term pregnant New Zealand White rabbits were obtained from a local supplier and allowed free access to rabbit chow and water. Newborn rabbits were kept with their mothers. Fetuses were removed via caesarean section at approximately day 25 of gestation (the gestational period of the rabbit is 31 days); the sex of fetuses and neonates was not determined. Liver, lung, kidney, and the duodenal portion of the small intestine were promptly frozen in liquid N_2 and stored at −70° until further processing.

Preparation and analysis of RNA. Total RNA was prepared by the method of Chirgwin *et al.* (8), as previously described (3). RNA electrophoresis and hybridization were performed as described (3), with the use of either a ^{32}P-end labeled oligonucleotide complementary to the P-450 2E1 and P-450 2E2 mRNAs (1) or a nick-translated cDNA for rat NADPH-P-450 reductase (9). The integrity of the RNA samples was confirmed by staining of the blots with methylene blue, to disclose the ribosomal RNA subunits, before hybridization. Appropriately exposed autoradiograms were scanned on a laser densitometer (Helena Laboratory, Beaumont, TX) for quantitation.

Preparation and analysis of microsomes. Pooled tissues were disrupted with a Brinkman Polytron homogenizer, and pyrophosphate-washed microsomes were prepared as previously described (3). Protein concentration was determined by the method of Lowry *et al.* (10); total P-450 content in microsomal suspensions was determined according to the procedure of Omura and Sato (11). NADPH-P-450 reductase was assayed by measurement of the rate of reduction of cytochrome *c*, and the amount of reductase was calculated using a turnover number of 4030 min^{-1} (12). The concentration of cytochrome b_5 was determined spectrally, with an extinction coefficient of 185 cm^{-1} mM^{-1} for the absorbance change between 424 and 409 nm (13). Immunoblot analysis of microsomal preparations (10 μg/lane) was carried out as described (14), with a monoclonal antibody to P-450 2E1.

Enzyme assays. The hydroxylation of *p*-nitrophenol to 4-nitrocatechol was determined spectrally, as described (15). The oxidation of ethanol to acetaldehyde was determined by gas chromatography of the headspace gas of the reaction mixture (16).

Results

Developmental activation of the *CYP2E* genes in liver and kidney. An oligonucleotide that hybridizes to both *CYP2E* genes was used to determine the level of P-450 2E1 and P-450 2E2 mRNA in various tissues from fetal and neonatal rabbits. It was shown previously that this oligonucleotide hybridizes to two RNA species from adult liver, which are 2.0 and 1.7 kilobases in size and correspond to P-450 2E1 and P-450 2E2 mRNA, respectively (1). As shown in Fig. 1, P-450 2E2 mRNA was detected at day 1 after birth; the level of P-450 2E2 mRNA increased rapidly in the first 2 weeks and then remained at approximately 1.5 times the adult level between weeks 2 and 5.[1] In contrast, P-450 2E1 mRNA was not detectable in the liver of newborn rabbits until 2 weeks after birth and it then increased to approximately twice the adult level between weeks 3 and 5. This rapid increase in P-450 2E1 mRNA corresponds approximately to the time of weaning, usually at 4 to 6 weeks in rabbits.

P-450 2E1 and P-450 2E2 are immunochemically and electrophoretically indistinguishable and, thus, the combined level of both P-450 2E proteins was determined by immunoblot analysis of microsomes with a monoclonal antibody to P-450 2E1. As shown in Fig. 1, P-450 2E protein was first detected in liver on day 1 after birth. The level of P-450 2E protein increased rapidly between day 1 and week 2 but then decreased to the adult level between weeks 2 and 5, despite a continued increase in combined P-450 2E mRNA during this period. The decrease in P-450 2E protein in pre-weanlings, despite the continued increase in P-450 2E mRNA, indicates that the posttranscriptional control of P-450 2E enzyme levels that is predominant in adult animals (3, 6) becomes active before weaning. Earlier studies from this laboratory demonstrated that only P-450 2E1 is expressed in adult rabbit kidney (1, 3). As shown in Fig. 2, P-450 2E1 mRNA was detected in kidney as early as 1 week after birth, and P-450 2E protein (presumably P-450 2E1) became evident in the second week. The content of RNA and protein in kidney increased between weeks 2 and 5, but the RNA level reached only about 50% of that found in adult animals. Neither P-450 2E1 nor P-450 2E2 mRNA was detected in lung or intestine from fetal or neonatal rabbits (data not shown).

Developmental changes in the level of total microsomal P-450, cytochrome b_5, and NADPH-P-450 reductase in liver. As shown in Fig. 3, spectrally detectable P-450 and cytochrome b_5 were first evident in day 1 neonates. The content of microsomal cytochrome b_5 reached the adult level at about 3 weeks, whereas total P-450 content reached the adult level by week 5. The ontogenic expression of NADPH-P-450 reductase was similar to that of total P-450; as shown in Fig. 4, reductase mRNA and protein were detected in day 1 neonates but not in fetal liver, and the level of both mRNA and protein reached the adult level at about 5 weeks. Overall, the ontogenic increase in the components of the hepatic microsomal monooxygenase system, including P-450, cytochrome b_5, and NADPH-P-450 reductase, occurred largely in parallel and was essentially complete by the fifth week of life.

Developmental changes in hepatic P-450 2E monooxygenase activities. P-450 2E1 is the most active of the known P-450 isozymes in ethanol oxidation (16); however, the activity of P-450 2E2 toward ethanol oxidation has not been examined. In hepatic microsomes from untreated adult rabbits, P-450 2E protein was found to be responsible for about 35% of total microsomal ethanol oxidation, based on immunochemical evidence obtained with an inhibitory antibody that could not distinguish between P-450 2E1- and P-450 2E2-catalyzed oxidation (16). In neonatal rabbits, as shown in Fig. 5, hepatic microsomal ethanol oxidation activity increased rapidly between day 1 and day 21. A corresponding pattern of developmental change was observed for P-450 2E-dependent ethanol oxidation, as determined with the use of an inhibitory antibody to P-450 2E1, indicating that 30–40% of microsomal ethanol oxidation in neonates is catalyzed by P-450 2E enzymes (data not shown). Moreover, hepatic P-450 2E-dependent ethanol oxidation correlated well with the level of P-450 2E proteins, indicating that P-450 2E2 is as active as P-450 2E1 toward ethanol. Similar results were found for the microsomal hydroxylation of *p*-nitrophenol, a low-K_m substrate for P-450 2E1 (15) (Fig. 5). These results indicate that the hepatic P-450 2E monooxygenase system is active from birth, albeit at a low initial level, but with increasing activity up to weaning.

[1]The "spike" in P-450 2E2 mRNA on day 3 was reproducible between samples, but its significance is unclear, because P-450 2E protein levels increased without fluctuation in the first 2 weeks.

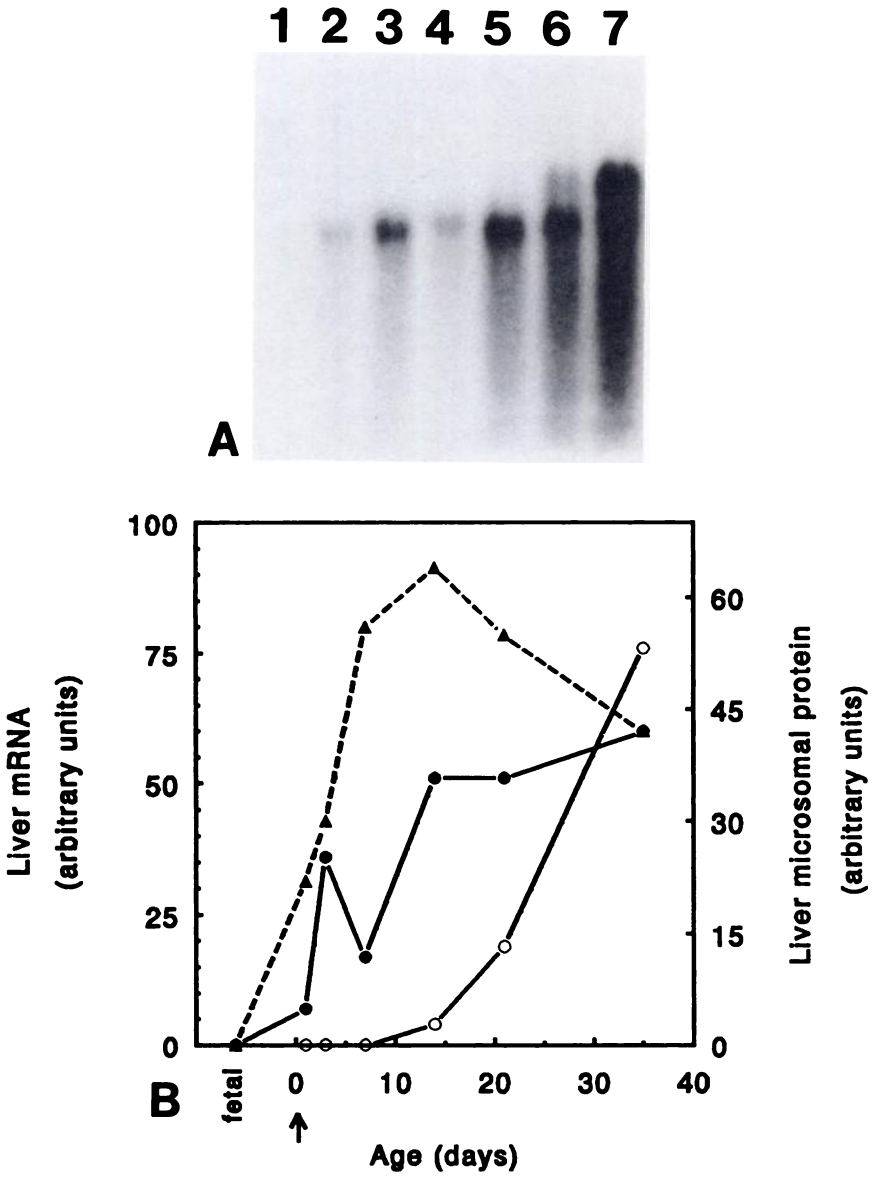

Fig. 1. Developmental activation of the *CYP2E* genes in liver. A, Hybridization of hepatic RNA from neonatal rabbits. Total RNA (10 μg) from the pooled livers of eight fetal or four or five neonatal animals/age group was fractionated by agarose gel electrophoresis and hybridized to a radiolabeled oligonucleotide that recognizes mRNA transcribed from *CYP2E1* and *CYP2E2*. *Upper band* (*lanes 6* and *7*), P-450 2E1 mRNA; *lower band*, P-450 2E2 mRNA. *Lane 1*, fetus; *lane 2*, day 1; *lane 3*, day 3; *lane 4*, week 1; *lane 5*, week 2; *lane 6*, week 3; *lane 7*, week 5. B, Quantitation of liver P-450 2E1 and P-450 2E2 mRNA and P-450 2E protein. The blot in A was scanned by densitometry, and RNA levels were quantitated in arbitrary units. Combined P-450 2E1 and P-450 2E2 protein levels in hepatic microsomes were determined by immunoblot analysis with a monoclonal antibody, followed by scanning densitometry. Microsomal samples were prepared from the same pooled tissues from which RNA was prepared. Day 0 (*arrow*) corresponds to the day of birth; the fetal tissue was obtained 6 days before expected parturition. ○, P-450 2E1 mRNA; ●, P-450 2E2 mRNA; ▲, P-450 2E protein.

Discussion

In the rabbit, several forms of P-450 become elevated during postnatal development, including a form that is very similar to P-450 2E1 (4). The results of the present study establish that this P-450 2E-related, neonatally expressed cytochrome corresponds to P-450 2E2; indeed, in liver the expression of P-450 2E2 can be detected as early as 1 day after birth, whereas P-450 2E1 expression is not evident until 2 weeks of age. Thus, in pre-weanling rabbits P-450 2E2 predominates (Ref. 4 and present work), whereas in adult animals P-450 2E1 mRNA levels exceed P-450 2E2 levels by about 2-fold (3). This difference results from the large increase in P-450 2E1 mRNA that occurs around weaning, while P-450 2E2 mRNA levels remain stable (Fig. 1). The rapid activation of *CYP2E2* transcription at birth, but not that of *CYP2E1*, suggests that *CYP2E2* contains a "parturition response element" not present in *CYP2E1*. Because the structural differences between these two genes consist primarily of a 338-base pair deletion in the 3′ nontranslated segment of the P-450 2E2 mRNA and a divergent segment in the 5′ flanking region between positions −150 and −310 (1), it is possible that these segments are involved in the differential ontogenic expression of these genes.

Dietary changes that accompany weaning may be important determinants of enzyme expression in the liver and other tissues; the shift from high fat breast milk to a vegetable matter diet at weaning is likely to engender changes in a variety of metabolic enzymes, including the P-450s. It is well established that P-450 2E1 levels are increased during physiological and dietary regimens that produce elevated blood ketone levels, such as diabetes (17–19), fasting (20, 21), and, notably, high fat diets (22). Moreover, lipids and ketones are also substrates for this cytochrome; P-450 2E1 is an effective acetone and acetol hydroxylase (23, 24) and is active toward a variety of lipid hydroperoxides (25). Whether P-450 2E2 has a role distinct from that of P-450 2E1 in the disposition of lipids from breast milk remains to be determined but is an interesting question. The possibility that this neonatal P-450 is involved in the metabolism of maternal steroid hormones present in breast milk also warrants consideration.

Present results also illustrate the coordinate postnatal in-

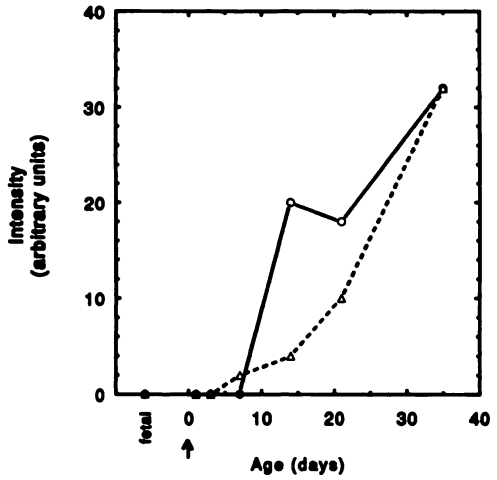

Fig. 2. Developmental activation of the *CYP2E* genes in kidney. Total RNA (20 μg) from pooled tissues was analyzed as described in the legend to Fig. 1; P-450 2E protein levels were determined by immunoblot analysis, as described in the legend to Fig. 1. ○, P-450 2E protein; △, P-450 2E1 mRNA.

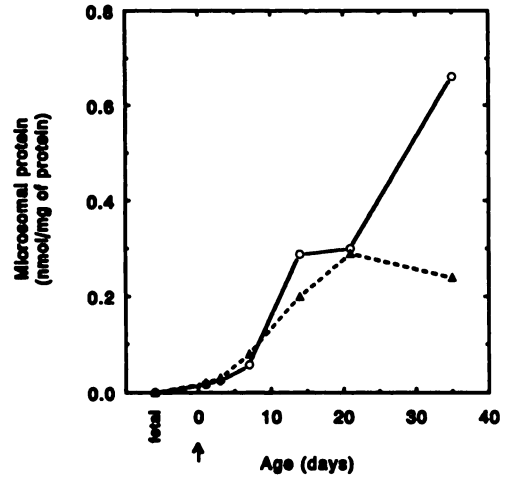

Fig. 3. Developmental increase in spectrally detectable hepatic microsomal P-450 and cytochrome b_5. Microsomes were prepared from pooled tissues from eight fetal or four or five neonatal rabbits/age group. The specific content of total P-450 (○) and cytochrome b_5 (▲) was measured spectrally, as described in Experimental Procedures.

creases in the components of the P-450 monooxygenase system. Overall, the ontogenic increase in these enzymes, including P-450, cytochrome b_5, and NADPH-P-450 reductase, occurs largely in parallel and is essentially complete by weaning. Our results also demonstrate that the hepatic monooxygenase system is active from birth, albeit at a low initial level, but with increasing activity up to weaning. The physiological mechanisms responsible for the synthesis of the P-450 monooxygenase system at parturition are not known but likely include both inhibition and activation at the transcriptional, and perhaps translational, level. In the rat, the expression of P-450 2E1 during the first week of development has been shown to correlate with the demethylation of specific methylcytosines in the 5′ region of the gene (7). A preliminary report on rat *CYP1A1* has indicated that factors present in fetal, but not in adult, liver interact with "negative response elements" in the 5′ region of this gene and may suppress the expression of this P-450 in the fetus (26). Studies are underway in this laboratory to

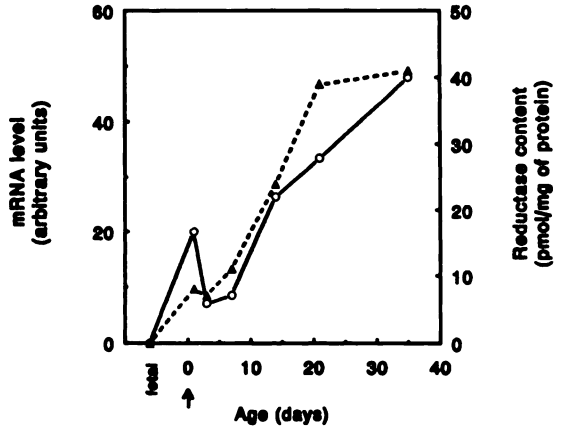

Fig. 4. Developmental expression of NADPH-P-450 reductase in liver. Total RNA (20 μg) from pooled livers was hybridized to a radiolabeled full-length cDNA for rat NADPH-P-450 reductase. The reductase mRNA levels (○) were determined by densitometry. The microsomal content of reductase protein (▲) was determined from the rate of reduction of cytochrome *c*, as described in Experimental Procedures.

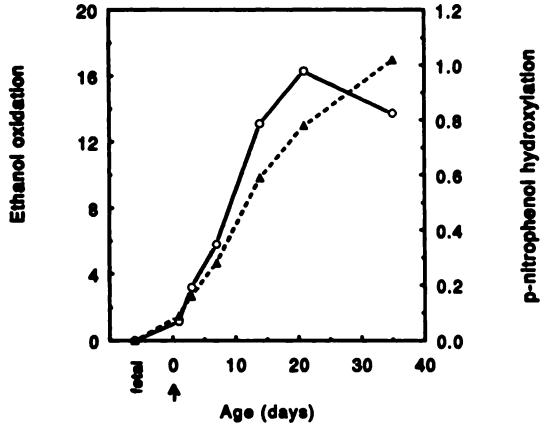

Fig. 5. P-450 2E monooxygenase activities in hepatic microsomes from neonatal rabbits. Reaction mixtures contained liver microsomes from pooled tissues of fetal or neonatal rabbits (2 mg of protein for fetal, day 1, and day 3 samples; 1 mg for all later time-points). Reaction products were measured as described in Experimental Procedures, and activities are expressed as nmol of acetaldehyde formed from ethanol (○) or 4-nitrocatechol formed from *p*-nitrophenol (▲) per min/mg of microsomal protein; the average of duplicate or triplicate determinations is shown.

determine the mechanism of differential transcription of the *CYP2E* genes in the rabbit during development.

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Send reprint requests to: Dr. Minor J. Coon, Department of Biological Chemistry, Medical School, The University of Michigan, Ann Arbor, MI 48109–0606.