Microsomal C-25 Hydroxylation of [³H]-Vitamin D₃ by the Fetal and Neonatal Rat Liver

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ABSTRACT. The liver microsomal C-25 hydroxylation of [³H]-vitamin D₃ was evaluated in 19- and 22-day-old rat fetuses, and in 1-, 2-, 3-, 14-, 30-, and 60-day-old pups. The hepatic production of [³H]-25-hydroxyvitamin D₃ by the 19- and 22-day-old fetuses was evaluated at 2.3 ± 0.6 and 5.9 ± 0.6 fmol·min⁻¹·15 mg⁻¹ microsomal protein, respectively. Values stayed unchanged during the 1st day after birth but increased on days 2 and 3 of chronologic age. Thereafter, the activity remained at a plateau of 11.2 ± 0.6 fmol·min⁻¹·15 mg⁻¹ microsomal protein with no further statistically significant increase in the activity of the vitamin D₃-25 hydroxylase through 60 days of chronologic age. The microsomal cytochrome P-450 specific content increased during the perinatal period with values ranging from 0.15 nmol·mg protein⁻¹ in 22-day-old fetuses to 0.44 in 60-day-old rats; the developmental pattern of the cytochrome P-450 was similar to that observed for the vitamin D₃-25 hydroxylase activity. When the amount of [³H]-25-hydroxyvitamin D₃ formed was expressed in relation to the amount of enzyme present in the reaction medium, a constant C-25 hydroxylation capacity in all age groups was observed suggesting that the cytochrome P-450 isoenzyme responsible for the C-25 hydroxylation of vitamin D₃ may be constitutionally determined and that its appearance originates during fetal life. (*Pediatr Res* **19**: 1206–1209, 1985)

Abbreviations

D₃, vitamin D₃
25(OH)D₃, 25-hydroxyvitamin D₃
1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃
D, vitamin D

D is metabolized by sequential hepatic C-25 and renal C-1α hydroxylations to 1,25(OH)₂D, a hormone responsible for the maintenance of normal calcium homeostasis and selected cellular differentiation and maturation events. In pregnancy, both D (1) and 25(OH)D (2) are transferred to the fetus and it is believed that the fetus probably derives 25(OH)D via this transport mechanism (3). In addition, when human premature infants of 31 to 35 wk of conceptual age are challenged with pharmacologic oral doses of D₃, significant amounts of 25(OH)D₃ is detected in their serum (4) suggesting that a D₃ C-25 hydroxylase

activity is present in infants born before term. This observation also raises the question of the time of appearance as well as of the course of maturation of the enzyme during the fetal and neonatal life.

The presence of a D₃ C-25 hydroxylase activity has already been reported in embryonic chick liver (5) but its presence in mammalian fetal and neonatal livers has not yet been investigated. Moreover, in the adult rat and chick, two sites have been reported to possess the capacity to hydroxylate D₃ at C-25. Based on their respective enzyme kinetics, the liver mitochondria are thought to respond to pharmacologic doses of D₃ while the liver endoplasmic reticulum (or microsomal fraction) is believed to be the site predominantly active when small or physiologic amounts of D₃ are present (6).

The purpose of the present series of experiments was to study the development of the hepatic microsomal D₃-25 hydroxylase in rat fetuses and neonates.

MATERIALS AND METHODS

Animals. Male and female Sprague-Dawley rats weighing between 200 to 250 g were ordered (Charles River, St. Constant, Québec, Canada); after an adaptation period of 1 wk, synchronization of the estrous cycle of the female animals was achieved by the subcutaneous injection of two 5 mg·kg⁻¹ dose of Dinoprost (Lutalyse, The Upjohn Company, Kalamazoo, MI) at an interval of 3 days. Female animals were then housed with male rats and vaginal smears were performed every morning to evaluate mating. When positive results were observed, the males were removed immediately and the females were assumed to be at day 1 of gestation. They were then shielded from ultraviolet light and given a D-deficient diet containing 0.4% elemental calcium and 0.2% elemental phosphorus.

Surgical Procedures and Preparation of the Microsomal Fractions.

Fetuses. On days 19 and 22 of gestation, dams were anesthetized with ether and a laparotomy was performed. Fetuses were excised, weighed, and immediately decapitated. The livers were homogenized at 4° C in 4 volumes of 0.25 M sucrose using a Potter-Elvehjem type glass-Teflon homogenizer. Each homogenate was submitted to differential centrifugation as described elsewhere (7) and the postmitochondrial supernatant was centrifuged in a L5-75 ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA); the microsomal fraction was obtained as the 105,000 × g pellet. The microsomal pellet was washed once by resuspension in ice-cold 100 mM buffered sodium pyrophosphate (8) followed by recentrifugation.

Neonates. All animals were born on day 22 of gestation. They were then studied on days 1, 2, 3, 14, 30, and 60 of chronologic age. On the day of the experiment, they were weighed and decapitated and the livers were flushed with ice-cold isotonic saline; the microsomal fractions were prepared as described above.

Determination of the microsomal D₃-25 hydroxylase activity.
All determinations were performed on freshly prepared microsomal fractions. The microsomal pellets were suspended in 40 mM potassium phosphate buffer containing 100 mM sucrose, 1 mM EDTA, and 20 mM dithiothreitol at pH 7.2. The protein content was determined by the method of Lowry *et al.* (9) using bovine serum albumin as standard; cytochrome P-450 content was measured by the method of Omura and Sato (10) using an Aminco DW-2 dual beam spectrophotometer (American Instrument Co., Silver Spring, NY). The increase in [³H]-D₃-25 hydroxylase bioactivity was found to be linear at protein concentrations of 5 to 25 mg per assay tube as well as for a period of up to 100 min. Each incubation tube was prepared with 15 mg microsomal protein and was incubated for a period of 40 min. Saturating amount of D₃ (26 pmol) containing 1×10^5 DPM [1α,2α(n)-³H]-D₃ (Amersham Corporation, Oakville, Ontario, Canada) was dissolved in 95% ethanol: 1,2-propanediol: rat liver cytosol (5:15:80) and preincubated with the microsomal preparation containing 200 μl of D-depleted rat liver cytosol (11) for a period of 1 h at 22° C. The reaction mixture was then brought to 37° C in the presence of 5 μmol mercaptoethylamine hydrochloride (thiol group protector) for a period of 5 min and the incubation was started by the addition of a buffered NADPH generating system (glucose-6-phosphate, 12.4 μmol; glucose-6-phosphate dehydrogenase, 5 U; MgCl₂, 12.5 μmol; NADP, 2.7 μmol). Blank incubations were carried out under identical conditions but without addition of the NADPH generating system. The reaction was stopped by the addition of 15 ml ethyl acetate:toluene (9:1, v:v) and radioinert D₃ (1 μg) and 25(OH)D₃ (2 μg) were added as internal standards and carriers. The final extraction mixture was vortexed with 1 g ammonium carbonate and 1 ml distilled water. Following centrifugation, the organic phase was collected while the aqueous phase was submitted twice to the same extraction procedure with 15 ml ethyl acetate:methanol (9:1, v:v). The combined organic phases were then evaporated under a stream of nitrogen.

Separation of [³H]-D₃ and [³H]-25(OH)D₃. The combined extraction mixtures were applied to Sephadex LH-20 (Pharmacia, Uppsala, Sweden) columns (0.9 × 18 cm) and eluted with chloroform:n-hexane (62:38, v:v) to remove lipid contaminants. Portions containing [³H]-D₃ and [³H]-25(OH)D₃ were collected, dried under nitrogen, dissolved in 250 μl n-hexane:isopropanol (97.5:2.5, v:v) and injected into a Beckman Model 100A high-performance liquid chromatograph (Beckman Instruments, Palo Alto, CA) fitted with a Zorbax-Sil column (25 cm × 4.5 mm ID) (Dupont Instrument, Wilmington, DE). Separation of [³H]-D₃ and [³H]-25(OH)D₃ was achieved at a flow rate of 2.5 ml·min⁻¹ with n-hexane:isopropanol (97.5:2.5, v:v). In this system, [³H]-D₃ eluted at 6.5 min and [³H]-25(OH)D₃ at 19.6 min with no overlap between the two compounds. Fractions of 1 min were collected and counted in 10 ml Biofluor (New England Nuclear, Boston, MA) in a LKB model 1217 RackBeta liquid scintillation spectrometer (LKB-Wallac Ltd, Turku, Finland). The mean recovery after the extraction and separation procedures was 75.1%.

Statistical analysis. All data are expressed as mean ± SEM. Statistically significant differences between group means were analyzed by a one-way analysis of variance (12), and testing for differences between individual group means was done according to the method of Scheffé (13). Regression and break points in the regression lines were calculated according to the methods of Jones and Molitoris (14).

RESULTS

The microsomal [³H]-D₃-25 hydroxylase activity is shown in Figure 1. When expressed in relation to microsomal protein content, the activity was evaluated at 2.3 ± 0.6 fmol·min⁻¹·15 mg protein⁻¹ in preparations obtained from 19-day-old fetuses. It increased to 5.9 ± 0.6 fmol·min⁻¹·15 mg protein⁻¹ in 22-
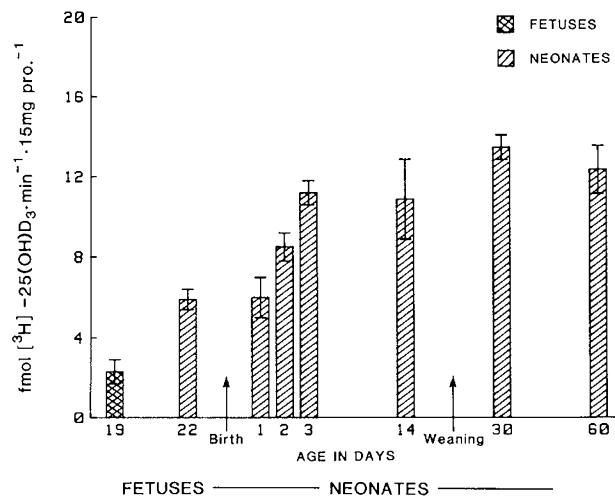

Fig. 1. Hepatic activity of the microsomal D₃-25 hydroxylase. Each incubation tube was prepared with 15 mg microsomal protein and was incubated for a period of 40 min in the presence of 26 pmol [³H]-D₃. Each value represents the mean ± SEM. Statistically significant differences between group means were analyzed by a one-way analysis of variance. F = 7.9308, $p < 0.001$.
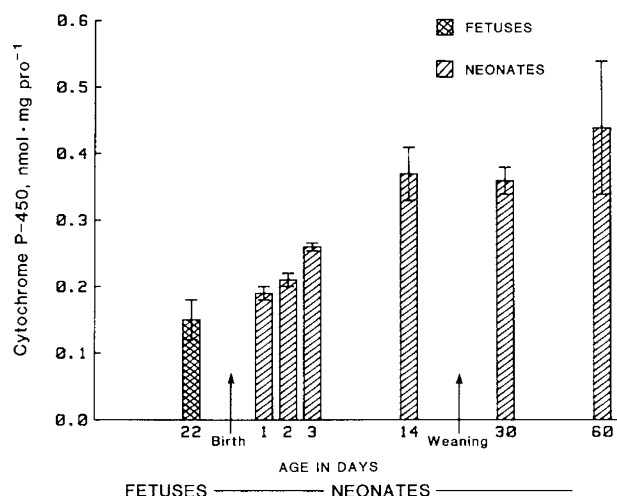

Fig. 2. Liver microsomal cytochrome P-450 specific content. Each value represents the mean ± SEM. Statistically significant differences between group means were analyzed by a one-way analysis of variance. F = 5.6180, $p < 0.001$.

day-old fetuses; this value was similar to the activity observed in postpartum animals of the same conceptual age (1-day-old pups). The enzymatic activity increased by 3 days after birth to a plateau of 11.2 ± 0.6 fmol·min⁻¹·15 mg protein⁻¹.

The specific content of the hepatic microsomal cytochrome P-450 for the different periods of life studied is presented in Figure 2. Microsomes obtained from 19-day-old fetuses contained an unidentified substance with absorption spectrum between 450–500 nm which prevented quantification of the cytochrome P-450. The cytochrome P-450 specific content increased during the neonatal period with maximal value being reached at 2 wk of age. The values observed ranged from 0.15 to 0.44 nmol cytochrome P-450 per mg microsomal protein in 22-day-old fetuses and 2-month-old rats, respectively. When the D₃-25 hydroxylase activity is expressed in relation to the amount of cytochrome P-450 present in the reaction media (specific activity), no significant differences in the specific activity of the D₃-25 hydroxylase were observed among the age groups studied (Fig. 3).

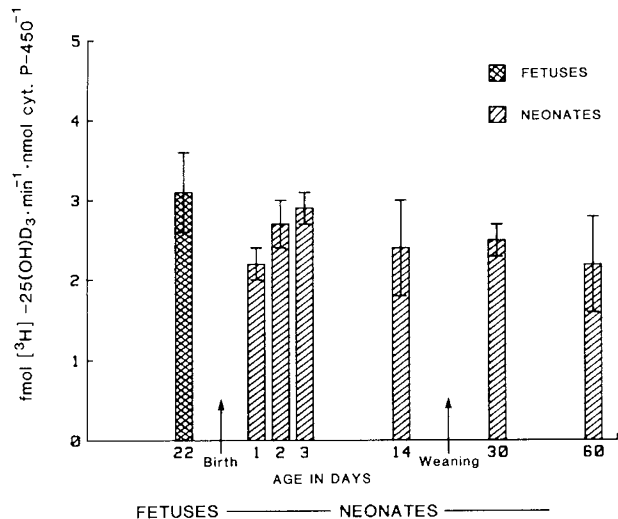Changes in D₃-25 hydroxylase activity as well as in cytochrome

Fig. 3. Specific activity of the liver microsomal D_3-25 hydroxylase. Each incubation tube was prepared with 15 mg microsomal protein and was incubated for a period of 40 min in the presence of 26 pmol [^{3}H]-D_3. The results are expressed as [^{3}H]-25(OH)D_3 formed in relation to the amount of cytochrome P-450 present in each incubation tube. Each value represents the mean ± SEM. Statistically significant differences between group means were analyzed by a one-way analysis of variance. $F = 0.5005$, $p = NS$.

P-450 content through 82 postconceptual days are shown in Figure 4. Statistical analysis of the breakpoints of the regression lines indicated a breakpoint in the ontogeny of the D_3-25 hydroxylase activity at 24.2 days of conceptual age (3.2 days of chronologic age) and a breakpoint in the ontogeny of the cytochrome P-450 at 25.4 days of conceptual age (4.4 days of chronologic age). Moreover, comparison of the slopes of the regression lines revealed a steeper slope in the ontogeny of the D_3-25 hydroxylase activity than in the ontogeny of the cytochrome P-450 content ($p < 0.0005$) during the fetal and early neonatal periods while the ontogeny of the two parameters was found to progress in parallel fashion in the suckling and postweanling periods.

DISCUSSION

The results of the present studies show that the liver capacity for C-25 hydroxylation of physiologic amounts of D_3 is present in the rat during the fetal period. Activity increases linearly to a maximum level by 3 days of chronologic age and remains virtually unchanged for the first 2 months after birth. The progressive increase in enzyme activity during the perinatal period supports the observation, made in human infants, that the C-25 hydroxylation capacity of D_3 might be more related to postconceptual age than to chronologic age (15). This view is further strengthened by the similarity in enzymatic activity observed in 22-day-old fetuses and 1-day-old pups who, in fact, were of the same conceptual age, suggesting that the process of parturition was apparently without stimulating effect on the capacity of the liver to hydroxylate D_3 at C-25. Moreover the steeper slope of regression line of D_3-25 hydroxylase activity relative to that of cytochrome P-450 specific content during the period of onset of both activities (19 to 24 days of conceptual age) suggests that the isoenzyme responsible for the C-25 hydroxylation of D_3 not only appears early in life but seems to evolve more rapidly than other isoenzymes of cytochrome P-450. After reaching peak activity, however, the D_3-25 hydroxylase activity evolves in parallel fashion with the cytochrome P-450 specific content suggesting that the amount of enzyme responsible for the hydroxylation of D_3 at C-25 may be constit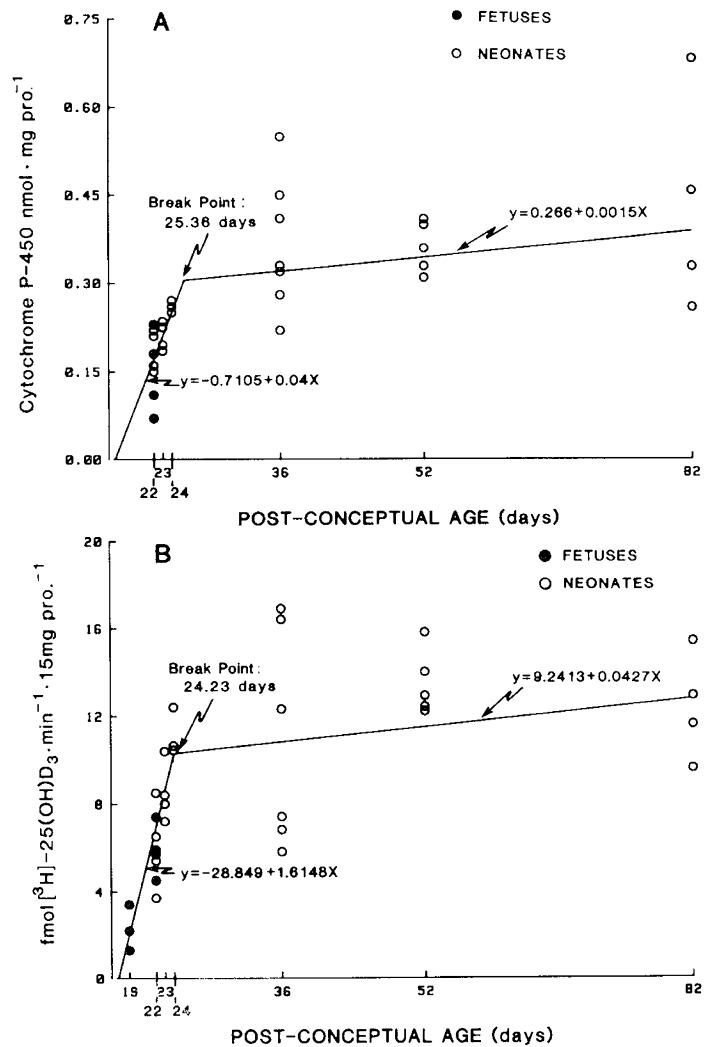utionally determined at a constant fraction of the total cyto-chrome P-450 pool. In reviewing the ontogeny of several hepatic mixed function oxidase activities, Short *et al.* (16) have noted the presence of three distinct patterns of development; one pattern, that of 4-methylcoumarin hydroxylase, was similar to the pattern observed for the D_3-25 hydroxylase in the present studies. It is therefore clear that the ontogeny of the monooxygenase activities is not similar for all substrates and that the D_3-25 hydroxylase seems to belong to a family of isoenzymes whose peak catalytic activity appears very early after birth.

In the present study, when the D_3-25 hydroxylase activity was expressed in relation to the amount of cytochrome P-450 present in the incubation media, the activity was similar in all age groups studied. This suggests the presence of an isoenzyme of cytochrome P-450 which remains unchanged throughout the developmental period and supports the presence of a constitutive form of cytochrome P-450 responsible for the C-25 hydroxylation of D_3 as already reported by Andersson *et al.* (17), and as suspected by Hayashi *et al.* (18).

The total liver capacity to form 25(OH)D_3 in intact animals must also take into consideration the uptake capacity of the liver for D_3. In a previous study (19), it was shown that the maximal uptake capacity of D_3 by the rat liver appeared at the time of weaning. Thus, while the liver oxidative metabolism of D_3 seems to appear relatively early in comparison to its uptake capacity, it might be only at, or near weaning, that sufficient intrahepatic

Fig. 4. Analysis of the ontogeny of the hepatic cytochrome P-450 content (*A*) and of the liver microsomal D_3-25 hydroxylase activity (*B*) from day 19 to day 82 of postconceptual age. The regression lines and the breakpoints in the regression lines were analyzed by the method of Jones and Molitoris (14).

substrate will be available to allow maximal 25(OH)D production. This observation is particularly pertinent in the light of earlier studies in the rat showing that the development of the 1,25(OH)$_2$D intestinal receptor (20), the requirement of 1,25(OH)$_2$D for the intestinal absorption of calcium (21, 22), and the increase in serum 25(OH)D and 1,25(OH)$_2$D (3, 22, 23) all appeared at the time of weaning. These observations suggest that, in the rat, both the vitamin D anabolic pathways and target organ responsiveness seem to be synchronized, and that the full expression of the vitamin D endocrine system seems to coincide with the time of weaning.

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