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PHENYLALANINE HYDROXYLASE ACTIVITY IN FOETAL HEPATIC ORGAN CULTURE

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SUMMARY

The biphasic pattern of phenylalanine hydroxylase (L-phenylalanine tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating) EC 1.14.3.1) activity of foetal and neonatal rat liver is not observed in 20- to 22-day-old hepatic explant culture. Two genetic markers of differentiated hepatic tissue were used to monitor hepatic explant: (a) a high baseline tyrosine aminotransferase activity (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5); and (b) hydrocortisone inducibility of this activity. Pre-existing phenylalanine hydroxylase activity of 22-day-old foetal explants decays with a half-life of 6 h. These observations suggest the need for a hepatic or extrahepatic inductive signal for the synthesis of phenylalanine hydroxylase.

INTRODUCTION

Analysis of the genetic control of phenylalanine hydroxylase (L-phenylalanine tetrahydropteridine:O_2 oxidoreductase (4-hydroxylating), EC 1.14.3.1) synthesis and degradation in mammalian tissues has heretofore not been possible. The obstacles have been many, among which are the restriction of this enzymatic activity to hepatic, renal and pancreatic tissues[1] and the lack of cell lines in tissue culture with any phenylalanine hydroxylase activity[2]. The latter deficit has excluded the use of human mutant cells for culture from patients with phenylketonuria, an inborn error of metabolism in which activity of phenylalanine hydroxylase is absent. Liver phenylalanine hydroxylase controls the metabolism of aromatic amino acids of mammalian tissues including brain, both directly and indirectly, as indicated by the multiple biochemical aberrations that result from the block of this enzymatic step in hepatic tissue in phenylketonuria[3].

Insight into mechanisms of control of enzyme synthesis may be gained through study of the process of gene expression[4]. We have previously reported that rat foetal liver has no activity until day 21 when a significant level of phenylalanine hydroxylase activity first becomes detectable[5]. At a gestational age of 22 days and 12 h there is

a second burst of activity to adult male levels, followed by a fall to approximately 25% of the initial value. The low values persist for a little over a week and are followed by a second rise between two and three weeks of age.

The appearance of enzymatic activity on day 21 and the burst of activity on day 22 in the foetus could result from the removal of maternal factors which had heretofore inhibited synthesis, or could be triggered by a foetal inductive signal such as a hormone, or by both factors operating sequentially. In this paper I report observations on the behavior of phenylalanine hydroxylase activity in foetal and neonatal hepatic explant culture. Two important techniques have made these observations possible. Firstly, the synchrony of conception of a group of rats to obtain foetuses and neonates of identical age[5], and secondly, the use of foetal hepatic organ culture to control the environmental conditions of hepatic tissue.

MATERIALS AND METHODS

Materials

Sera were obtained from Grand Island Biological Company. The amino acids, tetrahydropteridine, dibutyryl cyclic AMP, melatonin, hydrocortisone, thyroxin and growth hormone were obtained from Calbiochem. Lysozyme was obtained from Sigma.

Experimental animals and tissue culture

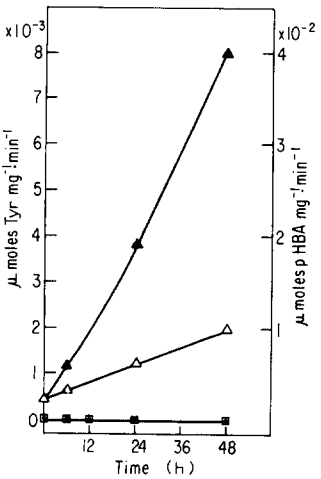
Sprague–Dawley rats were obtained from Holtzman Company. The exact date of conception was determined by a previously published method (Tourian *et al.*[5]). Hepatic explants were cultured by the method of Wicks[6] in minimal essential medium or F-12 medium.

Enzyme assays

Phenylalanine hydroxylase activity on individual foetuses and explant extracts was measured by a modification of a previously published method[5]. All components of the incubation mixture were reduced by a factor of 10 except the tissue extract which was reduced by 5. Phenylalanine activated phenylalanine hydroxylase was measured during the linear burst of activity for 5 min at 25 °C. The range of protein concentration for linear rates of tyrosine formation extends from 0.015 to 6.0 mg of extract protein. The specific activity of this assay is 3-fold greater than another method[7]. Tyrosine was determined by the nitrosonaphthol method[8] using an Aminco-Bowman Spectrophotofluorometer fitted with an adaptor for a No. 4-8114 0.17-ml cuvette. Tyrosine aminotransferase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) activity was measured by the method of Diamondstone[9]. Protein was measured by the method of Lowry *et al.*[10] using lysozyme as standard.

RESULTS

20-day-old foetal liver explants were cultured to determine if phenylalanine hydroxylase activity would spontaneously appear in tissue culture. No detectable activity was seen during 24, 48 and 72 h of explant culture (Fig. 1). The simultaneous determination of tyrosine aminotransferase activity of these same explants showed a high baseline activity and hydrocortisone significantly induced the enzyme. The



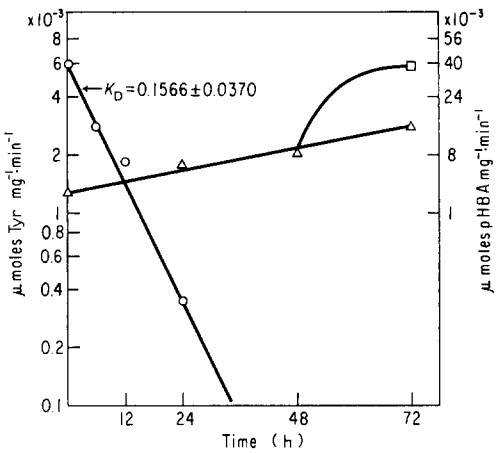


Fig. 1. Tissue culture of 21-day-old rat foetal hepatic explants. Simultaneous measurement of phenylalanine hydroxylase (●—●) and tyrosine α-ketoglutarate transaminase (△—△) on 21-day-old foetal hepatic explants. $2 \cdot 10^{-6}$ M hydrocortisone was added to a second set of explant plates and phenylalanine hydroxylase (□—□) and hydrocortisone-induced tyrosine α-ketoglutarate transaminase (▲—▲) were measured. Each point represents three culture dishes, each containing four bits of 2 mm^3 hepatic tissue determined in duplicate.

Fig. 2. Tissue culture of 22-day-old rat foetal hepatic explants. Simultaneous determination of phenylalanine hydroxylase (○—○) and tyrosine α-ketoglutarate transaminase (△—△) measured by the formation of *p*-hydroxybenzaldehyde (pHBA), in 22-day-old foetal explants. At 48 h of culture $2 \cdot 10^{-6}$ M hydrocortisone was added to the culture dishes. □—□, hydrocortisone-induced tyrosine α-ketoglutarate transaminase. There was no response to hydrocortisone of phenylalanine hydroxylase. Each point represents two duplicates of three culture dishes per point and each plate had four bits of hepatic explant tissue. K_D, the first order rate constant of enzyme decay is computed by the method of least square fit from the differential equation $dC/dt = K_S - K_D C$ (ref. 13).

determination of tyrosine aminotransferase activity in these explants was done for the following reasons: (a) a high level of tyrosine aminotransferase activity and hydrocortisone induction are characteristic of differentiated hepatic tissue and require two separate genetic markers[11]; (b) the half-life of tyrosine aminotransferase is 1.5 h[12]; and (c) cycloheximide blocks the hydrocortisone induction of tyrosine aminotransferase[12].

When 21- or 22-day-old foetal hepatic explant was cultured there was an exponential decay of existing phenylalanine hydroxylase activity (Fig. 2). The half-life of phenylalanine hydroxylase is 6 h in explant culture. K_D, the first order rate constant of enzyme degradation (*i.e.*, the fraction of enzyme molecules destroyed per unit time), is calculated[13] from

$$\frac{dC}{dt} = K_S - K_D C$$

where C is the enzyme activity at any time t and K_S is the rate constante of enzyme synthesis. The assumption made here is that there is no phenylalanine hydroxylase synthesis during the exponential decay of activity in this explant culture. Again simultaneous tyrosine aminotransferase determinations on these explants revealed a temporally rising activity, in addition to hydrocortisone inducibility. Similar results

TABLE I

ADDITIONS TO ORGAN CULTURE

Individual test compounds were added in a final concentration expressed in M or in μg/ml except when indicated otherwise to the organ culture medium, F-12, as indicated. After 24 h in organ culture, the explants were homogenized and phenylalanine hydroxylase activity was determined on the supernatant. Explant viability was monitored by a high baseline tyrosine aminotransferase activity in addition to hydrocortisone induction and cycloheximide suppression of the induction of tyrosine aminotransferase. Each test compound was determined in three culture dishes and each plate had four bits of hepatic explant tissue. Zero hour control of 22-day-hepatic phenylalanine hydroxylase was routinely included to evaluate initial levels of enzyme activity. The number of times each compound was tested in separate experiments is indicated by the bracketed numbers.

Addition	*Quantity*
Hormones	
Growth hormone (1)	10 μg/ml
Insulin (5)	4 μg/ml
Glucagon (2)	10 μg/ml
Thyroxin (1)	$5 \cdot 10^{-7}$
Hydrocortisone (10)	$2 \cdot 10^{-6}$
Insulin + hydrocortisone (2)	5 μg/ml, $2 \cdot 10^{-6}$
Melatonin (2)	$1 \cdot 10^{-5}$ to $1 \cdot 10^{-7}$
Biologicals	
22-day-old pregnant rat serum (1)	15%
Bovine serum (2)	15%
Foetal calf serum (1)	15%
Horse serum (3)	15%
Rat liver extract (1)	15%
Other compounds	
Norepinephrine (1)	$1 \cdot 10^{-5}$
Acetylcholine (1)	$1 \cdot 10^{-5}$
Carbamylcholine (3)	$1 \cdot 10^{-4}$ to $1 \cdot 10^{-8}$
Tetrahydropteridine (1)	$1 \cdot 10^{-3}$
Dibutyryl cyclic AMP (3)	$7 \cdot 10^{-5}$
Phenylalanine (2)	5, 10 × F-12
Tryptophan (2)	2, 5, 10 × F-12
Cu^{2+} (2)	5, 10 × F-12
Fe^{2+} (2)	5, 10 × F-12

were obtained when 50-day-foetal guinea pig liver was studied in organ culture. The gestational period of this mammal is 67 days and its hepatic phenylalanine hydroxylase appears around the beginning of the third trimester of foetal life[7].

The addition of hormones and other compounds such as growth hormone, glucagon, insulin, hydrocortisone, norepinephrine, dibutyryl cyclic AMP, carbamylcholine, melatonin, in addition to sera and other agents listed in Table I to minimal essential medium or F-12 explant medium did not significantly alter the course of the exponential decay of enzyme activity of hepatic explants (Table I).

DISCUSSION

These observations of phenylalanine hydroxylase activity in foetal hepatic culture can be interpreted in the following way: (a) a foetal inductive signal, either of hepatic or extrahepatic origin, is necessary for the synthesis of phenylalanine hydroxylase; (b) the turnover of phenylalanine hydroxylase is very rapid (half-life 6 h), hence to elucidate genetic factors that control synthesis becomes important.

The liver explant technique does have inherent limitations. The explant undergoes both morphological as well as biochemical changes in culture. However, most of the changes are not sufficient to prevent the study of hepatic nucleic acid, protein and enzyme synthesis[6]. Thus, hepatic explant method has been utilized to study the effect of thyroxin on ornithine transcarbamylase synthesis in the amphibian liver[14], the synthesis of glycogen synthetase in chick embryonic liver demonstrating the absolute requirement of insulin for this synthesis[15] and induction of aryl-hydrocarbon hydroxylase[16] and tyrosine aminotransferase[6] in rat foetal liver.

It is likely that the failure of appearance of phenylalanine hydroxylase in 19–20-day-old foetus or its decay in 21–22-day-old hepatic explants is due to the extinction of a differentiated function such as synthesis of phenylalanine hydroxylase. This phenomenon of extinction of a differentiated function could point to an as yet unknown hepatic or extrahepatic physiological signal that specifically evokes the synthesis of phenylalanine hydroxylase during development and is necessary for the maintenance of a high steady state of the enzyme.

If an inductive influence is necessary for a high steady state of phenylalanine hydroxylase activity, then the question arises whether there are variants of phenylketonuria in which an inductive signal for the synthesis of phenylalanine hydroxylase is missing. Such an hypothesis would provide an alternative to the usual considerations of a regulator or structural gene defect in this missing enzyme disease. This suggestion is re-enforced by the knowledge that in the homozygous condition of phenylketonuria the liver has no detectable enzyme activity when measured by the most sensitive radioisotope[1,17–20] or immunochemical[21] methods. Many patients with disorders caused by autosomal recessive mutations have low but detectable levels of functional enzyme activity. The theoretical possibility of such an inductive influence, if confirmed, could have significance for the treatment of phenylketonuria.

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