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Effects of Phenylalanine Loading on Protein Synthesis in the Fetal Heart and Brain of Rat: an Experimental Approach to Maternal Phenylketonuria

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Pregnant rats were loaded with L-phenylalanine, and the distributions of [^{14}C]leucine and [^{14}C]urea into fetal plasma and tissues were examined. Uptake of [^{14}C]leucine into the supernatant and protein fractions of fetal plasma and tissues was low in the rats loaded with phenylalanine. In contrast, [^{14}C]urea was distributed identically in both groups, indicating that maternal hyperphenylalaninemia did not affect blood flow across the placenta. Administration of phenylalanine and *p*-chlorophenylalanine produced amino acid imbalance in fetal tissues. Along with these changes, polysomes of the affected fetal heart and brain disaggregated without changes in the ribonuclease activity. These results indicate that high phenylalanine levels in maternal plasma disturb the active transport of amino acids across the placenta, causing an amino acid imbalance and disaggregation of polysomes in fetal heart and brain. These changes may contribute to the congenital heart disease and mental retardation of maternal phenylketonuria.

After Dent (1957) reported on three nonphenylketonuric but mentally retarded children of a mother with phenylketonuria (PKU), it has become clear that the offspring of a mother with untreated PKU (McKusick 26160) may have mental retardation, microcephaly, congenital heart disease, intrauterine growth retardation, and other malformations (Stevenson and Huntley, 1967; Fisch *et al.*, 1969; Lenke and Levy, 1980). Neonatal mass screening for PKU means that females of normal intelligence with PKU will reach childbearing age in larger numbers in the future, creating new therapeutic problems. According to Lenke and Levy (1980), the incidence of congenital heart disease from maternal PKU was 12.5% when the maternal blood phenylalanine level was 16 mg dl^{-1} or more, and not measurable when it was 10 mg dl^{-1} or less. (The incidence in the general population is 0.8%). Mental retardation due to maternal PKU may be associated with disturbances in

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protein synthesis in the fetal brain during development (Wong *et al.*, 1972; Copenhaver *et al.*, 1973). However, there are few reports on the cause of congenital heart disease in maternal PKU. Here, we investigate amino acid imbalances and protein synthesis in the fetal heart and brain using pregnant rats injected with phenylalanine as a model of maternal PKU.

MATERIALS AND METHODS

Materials

L-[U-^{14}C]Leucine (342 p mCi mmol^{-1}), [^{14}C]urea (60 mCi mmol^{-1}) and L-[U-^{14}C]phenylalanine (10 mCi mmol^{-1}) were obtained from Amersham (Buckinghamshire, UK), and AQUASOL-2 was purchased from New England Nuclear (Boston, USA). Ribonuclease inhibitor was purchased from the Sigma Chemical Co. (St. Louis, USA).

Animals

Sprague–Dawley rats were given water and food *ad libitum* with light–dark intervals of 12 h. Adult female rats were mated with males, and the day when vaginal smears were positive for sperm was considered day 0 of gestation.

Uptake of [^{14}C]leucine and [^{14}C]urea into fetal and maternal tissues

On day 20 of gestation, pregnant rats received a single intraperitoneal injection of 2.5% (w/v) phenylalanine (5 ml per 350 g of body weight) or 0.9% (w/v) NaCl as a control injection, followed by administration of [^{14}C]leucine or [^{14}C]urea (each 0.04 μCi per g of body weight) after 30 min. After 1 h, maternal blood was sampled by heart puncture under light ether anaesthesia. Fetuses were obtained by caesarean section, and blood samples were collected after decapitation. The liver, brain and heart were immediately excised from maternal and fetal rats, rinsed in cold saline and weighed. The liver (1:5, w/v) and other tissues (1:4, w/v) were homogenized in cold distilled water, and the homogenates were centrifuged at 10000 *g* for 30 min at 4°C. The supernatant was mixed with an equal volume of 12% (w/v) trichloroacetic acid (TCA), and centrifuged at 2000 *g* for 15 min. The supernatant was removed, and the protein fractions were washed twice with 5% TCA and dissolved in 1 mol l^{-1} NaOH. The plasma was similarly fractionated into TCA soluble or insoluble fractions. The radioactivity of the supernatant and protein fractions was counted after the fractions had been mixed with AQUASOL-2.

Analysis of polysome profiles

For the experiment on polysome profiles, *p*-chlorophenylalanine, an inhibitor of phenylalanine hydroxylase, was used to maintain high levels of blood phenylalanine. Phenylalanine (2.5% w/v) and *p*-chlorophenylalanine (3.0%, w/v) were dissolved in distilled water separately or together, and were injected intraperitoneally (5 ml per 350 g of body weight). Pregnant rats were divided into three groups. The

rats in group 1 were given both compounds at 21.00 h of day 18 of gestation as the initial load, followed by three injections of phenylalanine at intervals of 12 h; the rats in group 2 were given only *p*-chlorophenylalanine as the initial load, followed by three injections of 0.9% NaCl; the rats in group 3, the control group, were given a total of four injections of 0.9% NaCl.

Polysomes were analyzed by the method of Campagnoni and Mahler (1967) with some modifications, as follows. One hour after the last injection, blood and tissues from maternal and fetal rats were collected as described above and rinsed with cold buffer A (50 mM tris, 50 mM KCl, 10 mM $MgCl_2$, pH 7.6). The tissues were homogenized in 3 volumes of buffer B (50 mM tris, 50 mM KCl, 10 mM $MgCl_2$, 5 units ml^{-1} of ribonuclease inhibitor and 3 mM dithiothreitol, pH 7.6) containing 0.3 M sucrose with a Potter-Elvehjem homogenizer at 0–4°C, and centrifuged at 17 400 *g* for 15 min at 4°C. Postmitochondrial supernatant fluid (PMSF) was used in the ribonuclease assay, and supplemented with 1/10 volume of 11% (w/v) sodium deoxycholate. Purified polysomes were prepared by centrifugation of detergent-treated PMSF through buffer B containing 2 M sucrose at 152 000 *g* for 4 h at 4°C. The resulting pellets were rinsed twice and resuspended in buffer B containing 0.3 M sucrose. Samples with identical absorbance at 260 nm were layered onto 15 ml of a 15–40% (w/v) convex sucrose density gradient in buffer A, and centrifuged at 100 800 *g* for 3 h at 4° C. After centrifugation, the gradients were collected from the top by pumping 50% (w/v) sucrose into the bottom of each gradient tube, with simultaneous recording of absorbance at 260 nm.

Other procedures

Ribonuclease [EC 3.1.27.5] activity in PMSF was assayed by the method of Takahashi (1961). Amino acid analysis was carried out on a Shimadzu HPLC system with fluorimetric detection of *o*-phthalaldehyde. Phenylalanine hydroxylase [EC 1.14.16.1] was assayed by the method of Berry and colleagues (1972) with slight modifications. Protein was measured by the method of Lowry and colleagues (1951) using bovine serum albumin as the standard.

RESULTS

Effect of a single phenylalanine loading

Phenylalanine levels in maternal plasma reached a peak of 1.38 mmol l^{-1} at 30 min, while in fetal plasma, there was a peak of 2.51 mmol l^{-1} at 60 min. Table 1 shows uptake of [^{14}C]leucine into the supernatant and protein fractions of various tissues and plasma. By phenylalanine loading, such uptakes were greatly reduced in the maternal cerebrum and other parts of the brain, and in the fetal brain, heart, liver and plasma. This is in contrast to maternal heart, liver and plasma, in which there were no large changes. The placenta had less [^{14}C]leucine uptake into the supernatant fraction, but incorporation into the protein fraction was little changed. Table 2 shows [^{14}C]leucine distribution in maternal and fetal plasma and in fetal tissues. The results are expressed in terms of the radioactivity of fetal plasma

Table 1 Effects of L-phenylalanine loading on [^{14}C]leucine uptake in supernatant and protein fractions of tissues and plasma

	Control (dpm g wet weight^{-1})	*Phe loading* (dpm g wet weight^{-1})	*Percentage of control*	*p*
Supernatant fractions				
Mother				
Plasma	8430±582 (7)	8814±1435 (7)	104.6	NS
Brain				
Cerebrum	9497±1404 (7)	5623±579 (7)	59.2	<0.001
Other parts	8865±853 (7)	4775±666 (7)	53.9	<0.001
Heart	8471±1112 (6)	7978±2111 (7)	94.2	NS
Liver	17065±1781 (7)	14977±2046 (7)	87.8	NS
Placenta	16542±4257 (6)	11166±2108 (7)	67.5	<0.05
Fetus				
Plasma	12678±1094 (6)	9409±607 (6)	74.2	<0.001
Brain	12724±1937 (7)	5547±877 (7)	58.4	<0.001
Heart	17327±1325 (7)	11073±1002 (7)	63.9	<0.001
Liver	16884±2487 (7)	11690±1262 (7)	69.2	<0.001
Protein fractions				
Mother				
Plasma	2708±505 (7)	2705±364 (7)	99.9	NS
Brain				
Cerebrum	334±29 (7)	212±21 (7)	63.2	<0.001
Other parts	371±64 (7)	252±27 (7)	67.8	<0.005
Heart	395±43 (6)	412±72 (7)	104.3	NS
Liver	1052±241 (7)	930±120 (7)	88.4	NS
Placenta	730±74 (6)	672±106 (7)	92.1	NS
Fetus				
Plasma	1579±264 (6)	1205±249 (6)	76.3	<0.05
Brain	664±192 (7)	291±50 (7)	43.8	<0.001
Heart	1116±156 (7)	642±66 (7)	57.5	<0.001
Liver	1224±225 (7)	842±174 (7)	77.7	<0.01

After phenylalanine loading, pregnant rats were injected with [^{14}C]leucine; samples were obtained 1 h later, and fractionated into TCA-soluble supernatant and TCA-insoluble, protein fractions. One fetal sample was from one litter. Each value is the mean ±SD of 6 to 7 measurements. Numbers of rats used are in parentheses. NS: not significant Phe: phenylalanine

relative to that of the maternal plasma, and of fetal tissue relative to fetal plasma. They showed that phenylalanine loading influenced the transport of [^{14}C]leucine from maternal to fetal blood and from fetal blood to the brain, but did not influence transport from fetal blood to heart and liver. These findings suggest that hyperphenylalaninemia suppresses the transport of [^{14}C]leucine through the placenta and especially to the fetal brain.

Results with [^{14}C]urea are shown in Table 3. The uptake of [^{14}C]urea into the tissues and the plasma was not very different. The fetal plasma/maternal plasma

Table 2 Effects of L-phenylalanine loading: [^{14}C]leucine distribution in plasma and various tissues

Ratios	*Control*	*Phe loading*	*p*
Fetal plasma/maternal plasma	1.50±0.19	1.07±0.19	<0.01
Fetal brain/fetal plasma	0.91±0.17	0.60±0.12	<0.01
Fetal heart/fetal plasma	1.27±0.21	1.19±0.11	NS
Fetal liver/fetal plasma	1.21±0.18	1.22±0.15	NS

Supernatant fractions were isolated from plasma and tissues as described in Table 1, and their [^{14}C]leucine radioactivity was compared. Each value is the mean ±SD for 6 samples

Table 3 Effects of L-phenylalanine loading on [^{14}C]urea distribution in supernatant fractions of tissues and plasma

	Control (dpm g wet weight^{-1})	*Phe loading* (dpm g wet weight^{-1})	*Percentage of control*	*p*
Mother				
Plasma	90338±13290	101754±10209	112.6	NS
Brain				
Cerebrum	21572±4273	23641±1867	109.6	NS
Other parts	18442±2365	18388±1875	99.7	NS
Heart	56030±8027	64807±9482	115.7	NS
Liver	62157±9641	69769±6774	112.2	NS
Placenta	54529±10918	58977±6000	108.2	NS
Fetus				
Plasma	75495±6900	78722±5714	104.3	NS
Brain	27822±4081	26211±3245	94.2	NS
Heart	43402±5919	41867±9161	96.5	NS
Liver	37429±2068	41146±5642	109.9	NS

Pregnant rats were given phenylalanine and [^{14}C]urea, and TCA-soluble supernatant fractions were prepared as described in Table 1. One fetal sample was from one litter. Each value is the mean ±SD of 5 measurements

ratio of [^{14}C]urea was 0.784 in the group given phenylalanine and 0.845 in the controls. Incorporation of [^{14}C]urea into protein fractions was not detected.

Effects of phenylalanine and *p*-chlorophenylalanine loading

p-Chlorophenylalanine reduced maternal phenylalanine hydroxylase activity by 75% of that of the controls (group 1, 1.58±0.37; group 2, 1.88±0.63; and the control group, 6.25±0.50 nmol min^{-1} mg^{-1} of protein, n = 9, mean ±SD). Fetal rats treated with phenylalanine and *p*-chlorophenylalanine (group 1) had mean (±SD) body weights of 2.99±0.15 g, but the fetuses given *p*-chlorophenylalanine alone (group 2) were of normal weight (3.71±0.57 g; the controls weighed 3.67±0.41 g). The phenylalanine level of group 2 began to increase in 12 h and reached a peak (0.996 mmol l^{-1}) at 24 h. The phenylalanine level of group 1 was

much higher at 12 h, at 2.0 mmol l^{-1} (controls, 0.08 mmol l^{-1}). Maternal plasma tyrosine levels did not markedly increase in either group due to the suppressive effect of *p*-chlorophenylalanine.

Figure 1 shows changes in the free amino acid levels in maternal and fetal plasma and in fetal tissues of group 1 compared with the controls, examined 37 h after the initial loading. Ornithine could not be measured because its retention time was

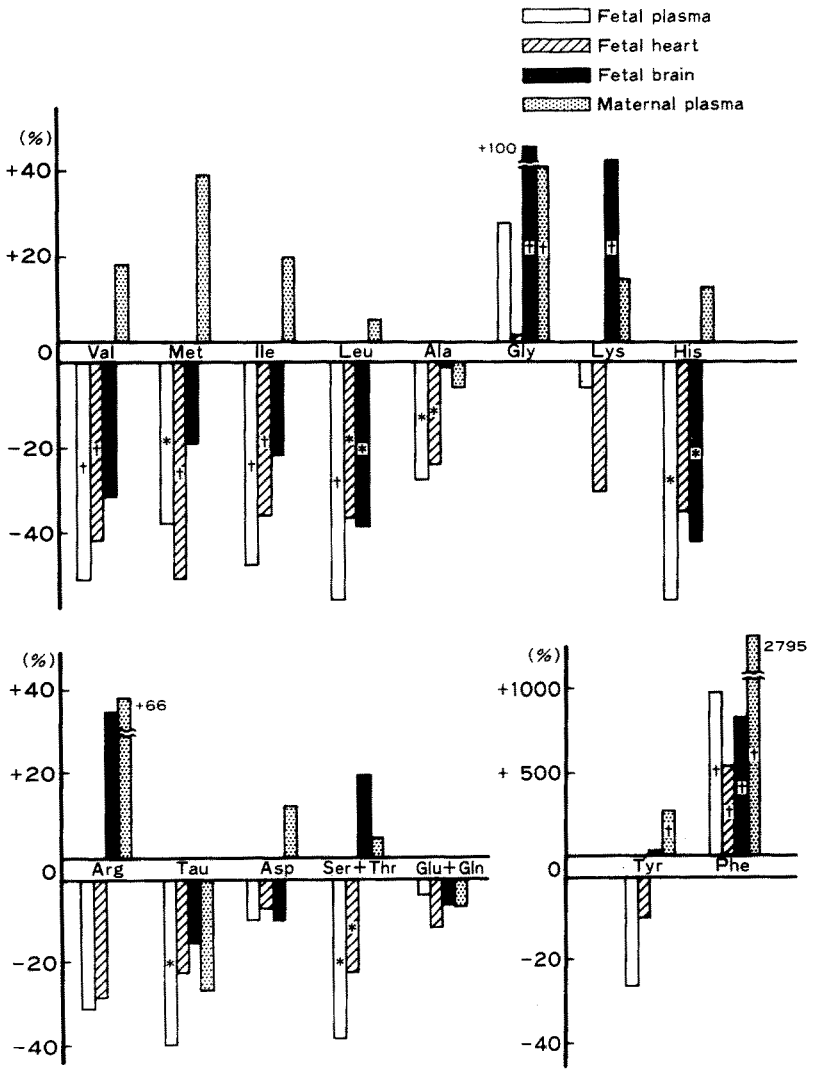

Figure 1 Effects of L-phenylalanine and *p*-chlorophenylalanine loading on free amino acid levels in tissues and plasma. Samples were obtained 60 min after the final injection on day 20 of gestation. Data are means of 6 or 7 measurements and are expressed as differences in percentages between group 1 and the control group (*$p<0.05$; †$p<0.01$). Each fetal sample was from one litter

close to that of *p*-chlorophenylalanine. Fetal plasma in group 1 showed large decreases in the branched-chain amino acids, methionine, alanine, histidine, taurine and serine plus threonine, and decreases in other amino acids except for glycine. Changes in the fetal heart were similar to those in fetal plasma. On the other hand, free amino acid levels in the fetal brain were different from those in the fetal heart and plasma; in the brain lysine and glycine increased, and leucine and histidine decreased.

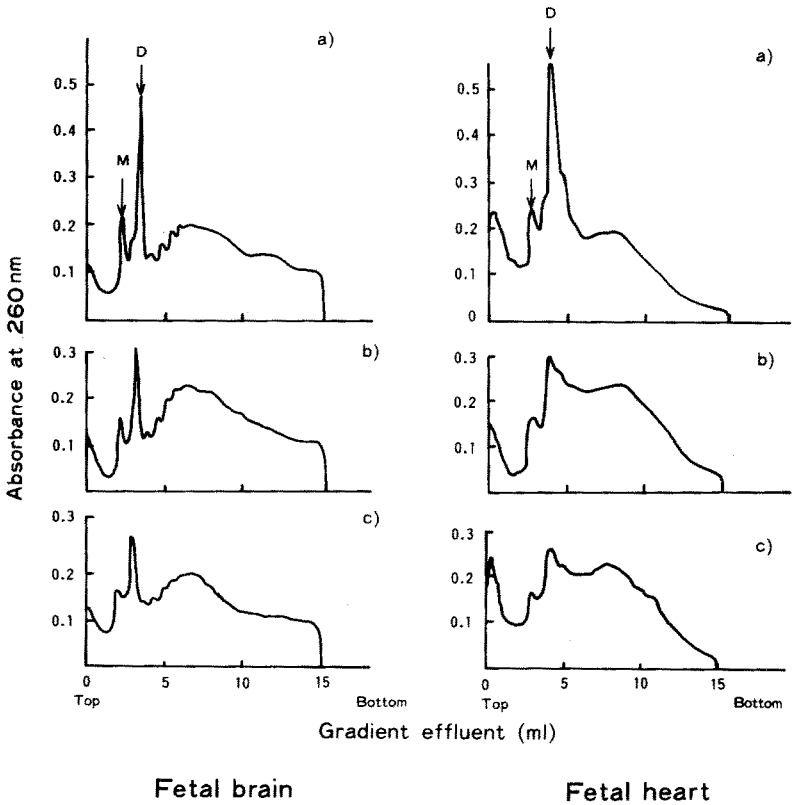

Figure 2 Effects of phenylalanine and *p*-chlorophenylalanine loading on sedimentation of purified polysomes from fetal brain and heart. Pregnant rats were put into 3 groups: (a) group 1 (phenylalanine+*p*-chlorophenylalanine), (b) group 2 (*p*-chlorophenylalanine) and (c) the control group. One hour after the final injection on day 20 of gestation, the brains and hearts were removed from the fetuses. One fetal brain sample was from 2 litters, and one fetal heart sample was from 3 litters. Sucrose density gradient analysis of purified polysomes was carried out as described in the methods section. Each profile was chosen from 3 separate experiments as the most representative
M: monosome, D: disome

Ribosome profiles are shown in Figure 2. Large increases in the disome population and small increases in monosomes were noted in preparations of fetal brains and hearts from group 1. Disomes increased slightly in these organs in group 2. Oligosomes and polysomes from the three groups were not very different. Alkaline

(pH 7.6) ribonuclease activities (mean ±SD) of fetal heart and brain in PMSF preparations were similar in all three groups (fetal heart; group 1, 50.6±3.7, group 2, 45.2±7.2, controls, 48.4±7.7: fetal brain; group 1, 45.2±14.5, group 2 48.5±7.0, controls, 47.7±9.1: acid soluble RNA $\mu g\,mg^{-1}$ of protein, $n = 3$).

DISCUSSION

Animal experiments studying maternal PKU in terms of brain damage have shown the behavioural abnormalities in the offspring (Kerr *et al.*, 1968), amino acid imbalances (Carver *et al.*, 1965), disaggregation of polysomes (Wong *et al.*, 1972, Copenhaver *et al.*, 1973), changes in the amounts of DNA, RNA and protein (Brass *et al.*, 1982; Spero and Yu, 1983), low pyruvate kinase (Wapnir *et al.*, 1977) and decreased transmitter levels (Brass *et al.*, 1982). However, to our knowledge studies concerning congenital heart disease using animal models of maternal PKU have not been reported. We have already studied the effects of phenylalanine loading in pregnant rats on the amino acid balance in the fetal brain (Chow, 1977). Here we examined protein synthesis in both the fetal heart and brain.

Fetal body weights in group 1 were lower than in group 2 or the control group suggesting that the maternal hyperphenylalaninemia induced by loading caused a slower weight gain. This phenomenon may parallel the intrauterine growth retardation in human maternal PKU. Wootton and colleagues (1977) reported that fetal growth and development are regulated by the blood supply through the placenta; we examined the effects of phenylalanine loading on the maternal blood flow through the placenta and on fetal blood circulation using urea (Table 3), an excellent indicator of the total body water compartment (Waddell, 1968). The results indicate that the diminished weight gain of the fetuses in group 1 was not caused by decreased maternal blood flow across the placenta or by impaired fetal blood circulation.

Hyperphenylalaninemia in pregnant rats reduced both [^{14}C]leucine uptake into the plasma and the ratio of fetal/maternal plasma [^{14}C]leucine (Tables 1 and 2). The levels of branched-chain amino acids and methionine in fetal plasma also decreased (Figure 1). These findings show that hyperphenylalaninemia in pregnant rats suppressed active transport across the placenta to the fetuses, especially that of branched-chain amino acids and methionine. Amino acid imbalance in the fetal plasma therefore resulted, which affected the amino acid balance of the fetal brain and heart. In particular, the fetal brain was more affected than other fetal tissues because the transport of [^{14}C]leucine from the fetal blood to the brain was suppressed by phenylalanine loading. This result might mean that fetuses on day 20 of gestation may have something like the blood–brain barrier. Ultimately, [^{14}C]leucine incorporation into protein fractions of the fetal brain and heart decreased with phenylalanine loading.

Copenhaver and colleagues (1973) showed disaggregation of polysome structures in fetal brain following treatment of pregnant animals with *p*-chlorophenylalanine and phenylalanine. Munro (1970) reported that free amino acid pools have a regulatory role in protein synthesis, reflected in polysomal aggregation. Here we

found marked disaggregation of polysomes prepared from the fetal heart and brain in group 1. In contrast, loading of *p*-chlorophenylalanine alone caused little disaggregation. We interpret these phenomena as meaning that maternal hyperphenylalaninemia causes disaggregation of polysomes without the specific effects of *p*-chlorophenylalanine. Alkaline ribonuclease activities of groups 1 and 2 and of the controls were similar. It is therefore unlikely that phenylalanine loading causes the rise in ribonuclease activity that in turn leads to the disaggregation of polysomes. Taub and Johnson (1975) reported that the effect of phenylalanine on the integrity of brain polysomes is independent of ribonuclease action. This suggests that hyperphenylalaninemia in pregnant rats inhibits protein synthesis, presumably because it disturbs amino acid pools in the fetal heart and brain.

Our results suggest that: (1) hyperphenylalaninemia in pregnant rats influences neither maternal nor fetal blood circulation; (2) high phenylalanine levels in maternal blood inhibit active transport of amino acids across the placenta and cause an amino acid imbalance in the fetal plasma and tissues; and (3) this imbalance seems to result in polysome disaggregation without changes in ribonuclease activity in the fetal heart and brain. We suspect that inhibition of protein synthesis may contribute to congenital heart disease and other symptoms in maternal PKU.

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