Evidence for a Placento-Insular Axis in the Rat Fetus

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Summary. Rat pups delivered by caesarian section one day before term, either remained attached to (experimental group) or were separated from their placenta (control group). Both groups were transferred into an incubator and their metabolic parameters studied. In the control group, plasma amino acid concentration fell from 11.2 to 6.9 mmol/l, plasma insulin fell from 151 to 45 μ U/ml and blood glucose fell from 3.5 to 2.6 mmol/l during the first hour of extrauterine life. In the breathing feto-placental units, plasma amino acid concentration remained at its birth level, plasma insulin remained high (109 μ U/ml) causing rapid hypoglycaemia (1.63 mmol/l). An immediately postnatal glucagon and corticosterone surge was visible only in the experimental group. A partially purified placental extract (molecular weight 6000 to 30000) stimulated insulin secretion in vivo (fasted adult rats) and in vitro (isolated neonatal islets). It is concluded that the placenta maintains fetal hyperinsulinaemia by creating fetal hyperaminoacidaemia and, possibly, by secreting a beta cytotropic factor.

Key words: Rat fetus, feto-placental units, placental extract, glucose, amino acids, non esterified fatty acids, insulin, glucagon, corticosterone, isolated islets, placento-insular axis.

In other species including man, the development of some fetal tissues also seems to be related to the rate of insulin secretion in utero. Macrosomia is a common feature of infants of diabetic mothers. Isolated hyperinsulinaemia during fetal life, as in infants with insulin secreting adenoma, usually yields heavier newborns. Likewise, experimentally produced fetal hyperinsulinaemia in the presence of euglycaemia caused macrosomia in the rhesus monkey [1]. By contrast, fetuses deprived of insulin, such as infants suffering from transient neonatal diabetes [2] or congenital absence of islets of Langerhans [3], are light for gestational age.

If insulin is a fetal growth factor, then more information is required on the regulation of insulin secretion during fetal life. Previous studies on the rat fetus have shown 1) that the high plasma insulin concentration resulted from high rate of production and not slow degradation [4] 2) that the rates of insulin and glucagon secretion by isolated fetal islets could be modulated by fuels and hormones [5] and 3) that the presence of the placenta was essential for maintaining fetal hyperinsulinaemia [4]. The aim of the present study is to examine the mechanism whereby the placenta exerts this action.

Materials and Methods

Animals

Female albino rats weighing 230–260 g were caged overnight with a male so that the duration of pregnancy was known to within 12 h.

Pups delivered by caesarian section one day before term were divided into 3 groups. Group 1 was bled at birth. The cords of the pups of the second group (control) were cut at birth and the pups transferred into an incubator in a warm, humidified, and oxygenated atmosphere. Blood samples were taken after 30 or 60 min of extrauterine life. The pups of the last group (experimental)

During the last 5 days of gestation, the weight of a rat fetus increases six-fold and plasma insulin concentration is higher than in adult rats, peaking at about 500 μ U/ml 2 days before term. Coincidence of rapid body growth and hyperinsulinaemia supports the hypothesis that insulin might be a fetal growth hormone or, at least, a growth factor for sensitive tissues.

Fig. 1. Elution profile of the ultracentrifugation supernatant of placental extract (50 placentas) after G50F Sephadex chromatography. Four ml fractions were collected and their protein content estimated by reading the optical density at $\lambda_{276\text{ nm}}$ (upper panel). Fractions 22–47 were pooled, lyophilised and rechromatographed on G200 Sephadex (lower panel). Buffer was 0.05 mol/l NH_4HCO_3 , pH 8.2

remained attached to their placentas and care was taken not to disturb the circulation of fetal blood through the umbilical cord. The whole feto-placental units (FPU) were removed from the uterus and transferred into the incubator. The timing of blood sampling was the same as for the control group. The pups of the groups 2 and 3 breathed normally and were apparently well oxygenated.

Adult rats were deprived of food for 24 h and anaesthetised with nembutal (60 mg/kg, IP). Placental extract (pool 1–3) was dissolved in 9 g/l NaCl and injected via a jugular vein catheter. Blood samples drawn before and 10, 20 and 30 min after the IV injection and were analysed for blood glucose and plasma IRI concentrations.

Metabolite and Hormone Assays

Fetal blood was collected from the axillary artery. Glucose concentration (20 μl blood) was measured by the hexokinase method. Amino acids were measured on the trichloroacetic acid supernatant of 50 μl plasma using a colorimetric method [6]. Non esterified fatty acids (NEFA) were extracted according to Dole [7] by successively adding to 100 μl plasma 1.5 ml isopropanol-heptane-1 mol/l H_2SO_4 (40–10–1) and, after thorough mixing, 0.9 ml heptane and 0.6 ml distilled water. After centrifugation, 0.7 ml of the upper phase were dried under nitrogen and the NEFA measured by a colorimetric method [8]. Immunoreactive insulin (IRI) was assayed on 20 μl plasma by the double antibody method of Hales and Randle [9] using a rat insulin standard (lot R171, Novo Com-

pany, Copenhagen). Plasma immunoreactive glucagon (IRG) concentration was kindly measured by Dr. R. Assan (Paris) using 30 K antiglucagon serum. Other plasma IRI concentrations were assayed in our laboratory, using 100 μl plasma, the same 30 K antiserum and ^{125}I glucagon prepared by the Novo Co, Copenhagen [10]. Plasma corticosterone concentration (10 μl serum) was measured using a specific radioimmunoassay [11].

Preparation of Islets of Langerhans

One day after birth, rat pups were killed by decapitation and their islets isolated by mild collagenase digestion and microdissection. In each experiment, three lots of 10 islets were incubated 60 min at 37 °C in 2 ml Krebs Ringer bicarbonate buffer supplemented with 10 mg/ml crystallised bovine serum albumin (Sigma Chemical Co, St Louis, Mo) and 2.8 mmol/l D-glucose, with or without 40 mg lyophilised placental extract (pool 2, see below) or 16.7 mmol/l D-glucose. The experimental procedure has been detailed elsewhere [4].

Preparation and Fractionation of a Placental Extract

Approximately 50 rat placentas obtained 2 days before term were homogenised in 50 ml ice-cold 0.05 mol/l NH_4HCO_3 buffer, pH 8.2, using a Polytron homogeniser. The homogenate was centrifuged at 15000 g for 30 min and the supernatant ultracentrifuged at 100000 g for 180 min. The ultracentrifugation supernatant was lyophilised, redissolved in 20 ml ammonium bicarbonate buffer and applied to a $2.7 \times 50\text{ cm}$ G50 fine Sephadex column run with the same buffer. The fractions corresponding to the void volume and the first fractions of the internal volume were pooled and lyophilised. The dry residue was redissolved in 20 ml of ammonium bicarbonate buffer and rechromatographed on a $2.7 \times 50\text{ cm}$ Sephadex G200 gel. The fractions corresponding to the total column volume were pooled and lyophilised (pool 2). The other fractions of the G50 and G200 chromatograms respectively made up pools 1 and 3 (Fig. 1).

Statistical Evaluation of the Results

The Student's *t* test on paired data was applied to evaluate the insulin secretory effect of placental extract pool 2 on isolated neonatal islets. In all other instances, the *t* test was calculated on unpaired data.

Results

Effect of the Placenta on the Metabolic Status of the Pups

As indicated by Table 1, the presence of the placenta caused but a mild metabolic acidosis.

Plasma IRI concentration decreased rapidly in the control group (Table 2). Blood glucose concentration remained at its birth level after 30 min and later decreased. Plasma amino acid concentration immediately fell, reaching half its birth value after 60 min of extrauterine life. In contrast, in the FPU, plasma IRI concentration remained high, blood glucose concentration decreased rapidly reaching

Table 1. Acid-base status of control pups and of feto-placental units. Mean $\pm$ SEM. Number of pups in parentheses

	Control		Feto-placental units	
	30 min (n=5)	60 min (n=5)	30 min (n=4)	60 min (n=5)
pH	7.28 $\pm$ 0.02	7.39 $\pm$ 0.03 ^a	7.24 $\pm$ 0.03	7.32 $\pm$ 0.02
pCO ₂ mmHg	39 $\pm$ 5	42 $\pm$ 4	45 $\pm$ 3	39 $\pm$ 3
base excess mmol/l	-7.7 $\pm$ 2.7	0.4 $\pm$ 1 ^a	-8.2 $\pm$ 1.8	-5.5 $\pm$ 1.8 ^b

Different from the 30 min value: ^a $p < 0.05$

Different from the control pups separated from the placenta after the same length of extrauterine life: ^b $p < 0.05$

Table 2. Plasma metabolite and hormone concentrations during the first hour of extrauterine life in control pups separated from the placenta and in breathing feto-placental units. Mean $\pm$ SEM. Number of pups between parenthesis

	At birth	Control		Feto placental units	
		30 min	60 min	30 min	60 min
Blood glucose mmol/l	3.49 $\pm$ 0.13 (40)	3.48 $\pm$ 0.26 (26)	2.61 $\pm$ 0.24 ^d (17)	2.76 $\pm$ 0.18 ^c (29) ^e	1.63 $\pm$ 0.14 ^d (19)
Plasma amino acids mmol/l	11.2 $\pm$ 0.3 (16)	8.9 $\pm$ 0.3 ^d (16)	6.9 $\pm$ 0.3 ^d (17)	11.8 $\pm$ 0.6 ^b (16)	10.5 $\pm$ 0.2 ^h (16)
Plasma NEFA μ mol/l	205 $\pm$ 17 (11)	243 $\pm$ 30 (8)	233 $\pm$ 18 (8)	228 $\pm$ 21 (8)	267 $\pm$ 33 (9)
Plasma IRI μ U/ml	151 $\pm$ 7 (40)	74 $\pm$ 5 ^d (26)	45 $\pm$ 4 ^d (17)	197 $\pm$ 19 ^h (29)	109 $\pm$ 11 ^d (19)
Plasma IRG ⁱ pg/ml	510 $\pm$ 36 (30)	515 $\pm$ 26 (14)	507 $\pm$ 39 (17)	663 $\pm$ 47 ^a (15) ^e	968 $\pm$ 39 ^d (17) ^h
Plasma corticosterone ng/ml	45 $\pm$ 7 (5)	54 $\pm$ 7 (6)	26 $\pm$ 2 ^a (5)	101 $\pm$ 18 ^a (6) ^e	124 $\pm$ 11 ^d (4) ^h

Different from corresponding value at birth: ^a $p < 0.05$ ^b $p < 0.01$ ^c $p < 0.005$ ^d $p < 0.001$

Different from control pups separated from the placenta after the same length of extrauterine life: ^e $p < 0.05$ ^f $p < 0.01$ ^g $p < 0.005$ ^h $p < 0.001$ ⁱ These IRG determinations were kindly performed by Dr. Assan

1.63 mmol/l after 60 min, and plasma amino acid concentration remained at its birth level.

Plasma IRG and corticosterone concentrations varied little in the control group. On the contrary, a glucagon and corticosterone surge was observed in the FPU.

Effect of Hyperinsulinaemia on the Metabolic Status of Newborn Pups

In order to distinguish better the effects of the placenta and of hyperinsulinaemia in the FPU, one day preterm fetuses received an injection of 0.5 μ g rat insulin at birth via the umbilical vein. The cord was ligated and cut immediately after the injection, and metabolic parameters were measured one h later. Pups of the same litters were used as controls. Sixty minutes after insulin, plasma IRI concentration was still higher in the experimental than in the control group (Table 3). This was associated with a marked hypoglycaemia and a small decrease in plasma amino acid concentration. Plasma IRG concentration was equal in both groups.

Effect of a Placental Extract on Insulin Secretion

As shown by Figure 2 a significant insulin secretory effect was observed only with placental extract pool 2.

Table 3. Effect of IV injection of 0.5 μ g insulin at birth on metabolic parameters measured 60 min later. Mean $\pm$ SEM, number of pups in parentheses

	Control	Insulin injected
Blood glucose, mmol/l	2.92 $\pm$ 0.21 (12)	0.84 $\pm$ 0.08 ^b (13)
Plasma amino acids, mmol/l	5.7 $\pm$ 0.2 (10)	5 $\pm$ 0.1 ^a (12)
Plasma IRI μ U/ml	59 $\pm$ 9 (4)	330 $\pm$ 22 ^b (4)
Plasma IRG pg/ml	237 $\pm$ 20 (10)	232 $\pm$ 22 (10)

Different from control: ^a $p < 0.005$, ^b $p < 0.001$

Table 4. Effect of placental extract (pool 2) and of high glucose on IRI release by isolated islets of 1 day old pups. Mean $\pm$ SEM (n = 15)

	Glucose 2.8 mmol/l	Glucose 2.8 mmol/l + pool 2 ^c	Glucose 16.7 mmol/l
IRI release, μ U/h/islet	39.5 $\pm$ 4.3	62.9 $\pm$ 9.9 ^a	224.9 $\pm$ 11.9 ^b

Different from IRI release in the presence of low glucose:

^a $p < 0.01$, ^b $p < 0.001$ ^c The concentration of placental proteins was 20 mg/ml and corresponded to 2 placenta-equivalents/ml

None of the 3 pools significantly influenced blood glucose concentration.

The insulin secretagogue effect of pool 2 was assessed on isolated neonatal islets, the reactivity of

Fig. 2. Effect of I.V. injection of placental extract (pool 1 ■····■, pool 2 ●—●, pool 3 ▲---▲) on plasma IRI (upper panel) and blood glucose (lower panel) concentrations of nembutal anesthetised fasted adult rats. Each rat received 10 placenta equivalents. Mean $\pm$ SEM, number of experiments between parenthesis. Different from 0 value, * $p < 0.025$ ** $p < 0.005$

which was verified by measuring their insulin response in the presence of a high glucose concentration. As shown in Table 4, the neonatal islets were viable and highly reactive to glucose and pool 2 elicited a significant increase of insulin secretion.

Discussion

The isolated breathing fetoplacental unit is a rather unphysiological model. Blood flow is maintained through the placenta and thus this organ becomes an oxygen consumer. As the pups breathe, an adequate pulmonary blood flow must be maintained by the right heart. Thus, total blood volume, heart work and oxygen consumption are increased. As shown by the almost normal acid-base status of these units, this peculiar condition seems well tolerated during experiments of short duration.

Instead of decreasing rapidly as in pups separated from their placenta, plasma IRI concentration remained as high in the isolated FPU as in those left in utero. This could possibly be explained by patency of porto-caval shunts and decreased extraction of insulin by the liver of the isolated FPU. This possibility appears unlikely because hepatic uptake of [^{125}I] monoiodoinsulin was as rapid and of similar mag-

nitude when the labelled hormone was injected into the vitelline vein of intact eggs in utero as when it was injected into the umbilical vein of breathing pups separated from their placenta (data not shown). We therefore favour the alternative explanation, that the insulin secretion rate is higher in the isolated FPU than in the control pups separated from their placenta.

In pups separated from their placenta, exogenous insulin exerted the expected effects of lowering blood glucose and plasma amino acid concentration. A rapid fall in blood glucose concentration was also observed in the FPU. However, despite hyperinsulinaemia, plasma amino acid concentration remained high. This suggests that, even though separated from the maternal circulation, the placenta released its intracellular amino acids into the fetal blood [12]. Sustained insulin secretion in the face of hyperaminoacidaemia and deepening hypoglycaemia indicates that, of these 2 fuels, amino acids may be more important insulin secretagogue agents at this stage of B cell maturation.

It is possible that the high level of plasma amino acids is not the only factor which maintains high fetal B cell secretion. The placenta could also secrete beta cytotropic factors. A fraction of an aqueous placental extract (pool 2) exerted a moderate but significant insulinogenic activity in vivo and in vitro. This conclusion was reached after verification that pool 2 did not contain insulin, that it did not degrade added insulin and that it did not interfere with the radioimmunoassay of insulin. According to its chromatographic properties, the molecular weight of the active substance was in the range of 6 to 30,000 daltons. Its chemical nature remains to be established. It could be placental lactogen, an insulinogenic fragment of proopiomelanocortin (such as CLIP), or another substance.

Before assigning a physiological role to this insulinogenic substance of placental origin, experimental verification is required that this substance is actually secreted into the fetal blood and that its concentration in the fetal blood is above the threshold of B cell reactivity.

In the control pups the postnatal glucagon surge did not occur immediately after birth as reported by others [13, 14, 15]. Differences in the handling of the newborns could explain these discrepancies. In the present study, the pups were immediately transferred into a warm, humidified and oxygenated atmosphere in order to minimize adrenergic stimulation induced by cold and anoxia. The early glucagon surge observed in the FPU could be due to several factors, one of which might be hypoglycaemia. The stimulation of A cells is not likely due to hypoglycaemia because more profound hypoglycaemia caused by

exogenous insulin did not modify plasma IRG concentration in pups separated from their placenta. This finding corroborates previous data obtained *in vivo* [16, 17] and *in vitro* [5], and strengthens the contention that fetal and neonatal A cells are either slightly or unreactive to glucose. The immediate postnatal glucagon surge of the FPU might rather be explained by persisting hyperaminoacidaemia and/or by the stress imposed by overload of the cardio-pulmonary system [18].

The possible interrelationships between the placenta, the B cells and the insulin-sensitive tissues may therefore be viewed as follows. In utero, the placenta actively transports amino acids [19], and could possibly release a beta cytotropic factor. Both stimulate fetal insulin secretion. High plasma insulin concentration, in turn, favours amino acid and, to a much greater extent, glucose uptake by insulin sensitive tissues [20]. Finally, high glucose utilisation in the fetus steepens the downward glucose gradient between mother and fetus and accelerates its trans-placental passage. In this manner, amino acids and glucose utilisation by the fetus would be coupled by means of the placento-insular axis. The present hypothesis could also explain fetal hypoinsulinaemia in growth retardation due to placental insufficiency.

At birth, when the cord is cut, the placental supply of amino acids and of beta cytotropic factor is interrupted and insulin secretion is turned off.

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