

## Protein deficiency during pregnancy and lactation impairs glucose-induced insulin secretion but increases the sensitivity to insulin in weaned rats

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We studied glucose homeostasis in rat pups from dams fed on a normal-protein (170 g/kg) (NP) diet or a diet containing 60 g protein/kg (LP) during fetal life and the suckling period. At birth, total serum protein, serum albumin and serum insulin levels were similar in both groups. However, body weight and serum glucose levels in LP rats were lower than those in NP rats. At the end of the suckling period (28 d of age), total serum protein, serum albumin and serum insulin were significantly lower and the liver glycogen and serum free fatty acid levels were significantly higher in LP rats compared with NP rats. Although the fasting serum glucose level was similar in both groups, the area under the blood glucose concentration curve after a glucose load was higher for NP rats (859 (SEM 58) mmol/l per 120 min for NP rats v. 607 (SEM 52) mmol/l per 120 min for LP rats;  $P < 0.005$ ). The mean post-glucose increase in insulin was higher for NP rats (30 (SEM 4.7) nmol/l per 120 min for NP rats v. 17 (SEM 3.9) nmol/l per 120 min for LP rats;  $P < 0.05$ ). The glucose disappearance rate for NP rats (0.7 (SEM 0.1) %/min) was lower than that for LP rats (1.6 (SEM 0.2) %/min;  $P < 0.001$ ). Insulin secretion from isolated islets (1 h incubation) in response to 16.7 mmol glucose/l was augmented 14-fold in NP rats but only 2.6-fold in LP rats compared with the respective basal secretion (2.8 mmol/l;  $P < 0.001$ ). These results indicate that *in vivo* as well as *in vitro* insulin secretion in pups from dams maintained on a LP diet is reduced. This defect may be counteracted by an increase in the sensitivity of target tissues to insulin.

### Protein malnutrition: Insulin: Glucose homeostasis

Malnutrition in man and other mammals is associated with impaired insulin secretion and alterations in carbohydrate metabolism (Milner, 1971; Carneiro *et al.* 1995). In adult rats submitted to acute or chronic protein malnutrition during the growing period after weaning, a severe reduction has been observed in insulin secretion in response to glucose and other secretagogues (Okitolonda *et al.* 1987; Carneiro *et al.* 1995; Reis *et al.* 1997). However, glucose intolerance has been found only during the first weeks of protein deprivation (Okitolonda *et al.* 1987; Swenne *et al.* 1987). Protein restriction imposed during fetal life can be detrimental to the development of pancreatic B-cells, thereby leading to permanent insulin deficiency (Snoeck *et al.* 1990; Dahri *et al.* 1991). Furthermore, alterations in the activity of the key hepatic enzymes (glucokinase (EC 2.7.1.2) and phosphoenolpyruvate carboxykinase (EC 4.1.1.49)) that are differentially regulated by insulin have been reported

in the offspring of mothers deprived of protein during pregnancy (Desai *et al.* 1995). Thus, at 21 d of age, protein-deprived pups show decreased glucokinase and increased phosphoenolpyruvate carboxykinase activities, a situation which is indicative of glucose production rather than utilization (Desai *et al.* 1995). Reduced insulin secretion and enzymic changes would be predicted to predispose to diabetes.

The suckling–weaning period is associated with nutritional changes as well as with modifications in the levels of circulating insulin and the action of this hormone (Blásquez *et al.* 1970; Issad *et al.* 1987). Thus, the insulin resistance present in rat sucklings disappears after weaning, and the plasma insulin levels are higher in weaned rats than in sucklings (Issad *et al.* 1987).

In the present study we examined insulin secretion and glucose homeostasis in weaned rats from dams fed on a low-protein (LP) diet during pregnancy and lactation.

**Abbreviations:** LP, low protein; NP, normal protein.

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Offspring from malnourished dams showed a severe reduction in insulin secretion in response to glucose load, *in vivo* as well as *in vitro*. However, no alteration in glycaemia was observed in these animals, indicating a compensatory increase in the sensitivity to insulin in target tissues.

### Materials and methods

Virgin female Wistar rats (90 d old) were obtained from the State University of Campinas animal facilities. Mating was performed by housing females with adult males overnight and pregnancy was confirmed by examining vaginal smears for the presence of sperm. Pregnant females were separated at random and maintained on an isoenergetic diet containing 60 g protein/kg (LP diet) or 170 g protein/kg (normal-protein (NP) diet) from the first day of pregnancy until the end of the lactation period (Table 1). During the experimental period, the dams were fed on their respective diets *ad libitum* and had free access to water.

The animals were kept under standard lighting conditions (12 h light–dark cycle) at a temperature of 24°. The food intake was monitored daily and the dams were weighed weekly. All pups were weighed at birth. Some of the pups (thirty-two from NP mothers, eleven litters; twenty-two from LP mothers, nine litters) were killed for biochemical analyses. Sera from half the NP and LP pups were used for the insulin and glucose determinations, while the remaining sera were used for protein and albumin measurements. The remaining animals (those not used in the previously described analyses; eight litters) were weighed after birth for four consecutive weeks. At 25 d of age, the pups were weaned and maintained on their mothers' diet. Forty-three pups from six NP litters and forty-five pups from six LP litters were killed by decapitation at 28 d for the measurement of liver glycogen (Hassid & Abrahams, 1957) and pancreatic insulin content (Malaisse *et al.* 1967). Blood samples from these animals were also collected and allowed to clot; the sera were subsequently stored at –20° for biochemical analyses. Serum glucose (Trinder, 1969), total serum protein (Wolfson *et al.* 1948), serum albumin (Doumas *et al.* 1971), serum free fatty acids (Regouw *et al.* 1971) and serum insulin (Scott *et al.* 1981) levels were determined. Since the amount of serum obtained from each animal was not sufficient for measurement of all these

biochemical variables, the number of individual experiments varied between groups. Six animals from each of four litters were used for the glucose tolerance test. Eleven NP and thirteen LP rats from the same litters were used for the insulin tolerance test. Finally, isolated islets were obtained from rats of four LP and four NP litters. All experimental procedures were initiated between 08.00 and 09.00 hours.

#### Glucose tolerance test

An oral glucose tolerance test was performed using the 4-week-old male rats. After a 15 h fast, glucose (200 g/l) was administered orogastrically through a catheter at a dose of 2 g/kg body weight. Blood samples were obtained from the cut tip of the tail 0, 30, 60 and 120 min later for the determination of serum glucose and insulin concentrations. The glucose and insulin responses during the oral glucose tolerance tests were calculated by estimating the total area under the glucose and insulin curves respectively, using the trapezoidal method (Matthews *et al.* 1990).

#### Insulin tolerance test

A subcutaneous insulin tolerance test was performed using the 4-week-old male rats, after a 15 h fast. The insulin tolerance test consisted of a bolus injection of insulin (300 mU/kg body weight) beneath the dorsal skin of the fasted animal. Blood samples were obtained from the cut tip of the tail 0, 30, 60, 120, and 180 min later for the measurement of glucose levels. The rate constant for the serum glucose disappearance was calculated using the formula  $0.693/t_{1/2}$  where  $t_{1/2}$  is half-life. The serum glucose half-life was calculated from the slope of a least-square analysis of the serum glucose concentrations from 0–60 min after the subcutaneous injection of insulin, when the serum glucose concentrations declined linearly.

#### Insulin secretion from isolated islets

Islets were isolated by collagenase (*EC* 3.4.24.3) digestion. Briefly, pancreas was inflated with Hanks' balanced salt solution containing 0.7 mg collagenase/ml, excised and then maintained at 37° for 18 min. The digested tissue was then washed four times and the islets separated by hand-picking with the aid of a siliconized stretched Pasteur pipette. Groups of five islets were first incubated for 30 min at 37° in 0.75 ml of a Krebs-bicarbonate buffer with the following composition (mmol/l): NaCl 115, KCl 5, CaCl<sub>2</sub> 2.56, MgCl<sub>2</sub> 1, NaHCO<sub>3</sub> 24, glucose 5.6, supplemented with 3 mg bovine serum albumin/ml, and equilibrated with a mixture of O<sub>2</sub>–CO<sub>2</sub> (95:5, v/v), pH 7.4. This medium was then replaced with fresh buffer and the islets incubated for 1 h in the presence of 2.8 or 16.7 mmol glucose/l. The insulin content of the supernatant fraction at the end of the incubation period was measured by radioimmunoassay (Scott *et al.* 1981).

#### Statistical analysis

The results are expressed as the means with their standard

**Table 1.** Composition of the normal and low-protein diets

Ingredient	Normal protein (170 g protein/kg)	Low protein (60 g protein/kg)
Casein (840 g protein/kg)	202.0	71.5
Maize starch	397.0	480.0
Dextrinized maize starch	130.5	159.0
Sucrose	100.0	121.0
Soyabean oil	70.0	70.0
Fibre	50.0	50.0
Mineral mix (AIN-93)*	35.0	35.0
Vitamin mix (AIN-93)*	10.0	10.0
L-Cystine	3.0	1.0
Choline chlorhydrate	2.5	2.5

\* For detailed composition, see Reeves *et al.* (1993).

**Table 2.** Food and protein intake, weight gain during pregnancy, and litter size for dams fed on a normal (NP) or low-protein (LP) diet† (Values are means with their standard errors for eleven and nine mothers from NP and LP groups respectively)

Dietary group	Food intake (g)		Protein intake (g)		Wt gain (g)		No. of pups (g)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NP	385	9.6	65.8	1.6	117.5	6.2	9.9	1.0
LP	390	21.3	23.4***	1.3	120.4	10.2	11.3	0.7

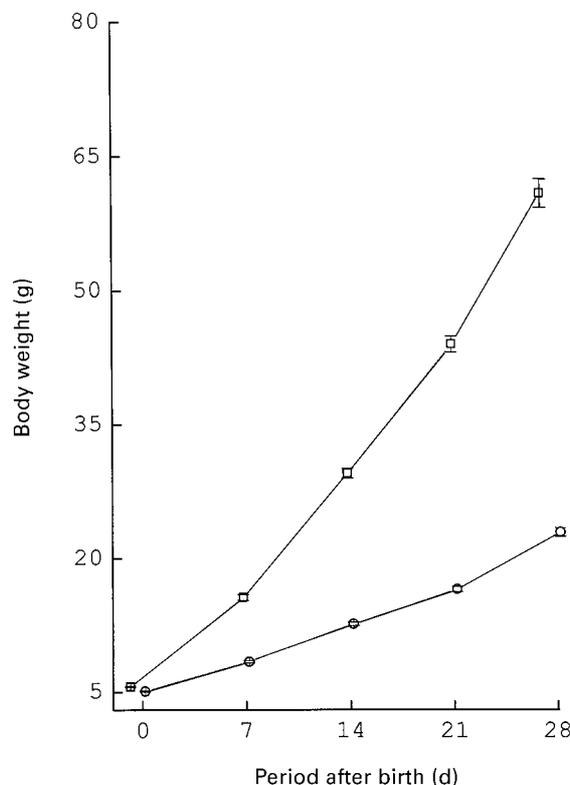
Mean value was significantly different from that for the NP group: \*\*\* $P < 0.001$ . †For details of animals, diets and procedures, see pp. 292–293 and Table 1.

errors for the number of rats indicated. When comparing NP and LP groups, Student's non-paired  $t$  test was used. When comparing the changes in body weight for the pups, Lavene's test for the homogeneity of variance was initially used to check the fit of the data to the assumptions for parametric ANOVA. A Box-Cox transformation was used to correct for variance heterogeneity or non-normality (Sokal & Rohlf, 1981). The data were subsequently analysed by two-way ANOVA followed by the Tukey–Kramer test for multiple comparisons.

### Results

Table 2 shows that of the various variables measured, only the total protein intake was significantly lower in dams maintained on the LP diet compared with the control (NP) group ( $P < 0.001$ ). There was a significant reduction ( $P < 0.01$ ) in body weight and glycaemia of newborn rats from dams fed on the LP diet. However, there was no change in the insulinaemia of newborn rats from protein-restricted mothers (Table 3).

The total food and protein intake during lactation was significantly greater for the NP rats than for the LP rats (intakes for NP rats 787 (SEM 20) g food, 134 (SEM 3.4) g protein,  $n$  6; intakes for LP rats 470 (SEM 32) g food, 28 (SEM 1.9) g protein,  $n$  8;  $P < 0.001$  in both cases). However, when the results were expressed per kg litter body weight, the food intake was significantly greater for the LP group than for the NP group (LP rats 173 (SEM 20) g/kg litter body weight per d,  $n$  8; NP rats 92 (SEM 3) g/kg litter body weight per d,  $n$  6;  $P < 0.001$ ) but, the protein intake was significantly decreased for the LP pups than for the NP pups (LP rats 10 (SEM 1) g/kg body weight per d,  $n$  6; NP rats 16 (SEM 0.1) g/kg body weight per d,  $n$  8;  $P < 0.001$ ).



**Fig. 1.** The body weight of offspring from dams fed on a normal (NP; □) or low-protein (LP; ○) diet during pregnancy and lactation. Values are means with their standard errors represented by vertical bars for sixty-one and sixty-four pups from NP and LP groups respectively. For details of animals and diets, see p. 292 and Table 1.

The body-weight gain of NP pups was significantly higher than that of LP pups during lactation (Fig. 1). Two-way ANOVA revealed a significant main effect of groups (df 1,  $F$  3345.52,  $P = 0.0001$ ) and age (df 4,  $F$  2405.15,  $P = 0.0001$ ), as well as the two-way interaction, group  $\times$  age (df 4,  $F$  273.24,  $P = 0.0001$ ).

By the 28th day of life, the liver glycogen content and serum free fatty acids levels in the fed state were significantly higher for LP pups than for NP pups, while for the total serum protein, serum albumin and serum insulin, the reverse was true (Table 4).

Protein restriction significantly decreased the pancreas

**Table 3.** Body weight, serum glucose, insulin, total protein and albumin levels of newborn rats from eleven and nine mothers fed on a normal (NP) or low-protein (LP) diet respectively during pregnancy† (Values are means with their standard errors for the no. of rats shown in parentheses)

Dietary group	Body wt (g)		Total serum protein (g/l)		Serum albumin (g/l)		Serum glucose (mmol/l)		Serum insulin (nmol/l)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NP	5.6	0.1	19	1	8	1	5.5	0.5	0.26	0.02
	(110)		(16)		(14)		(16)		(11)	
LP	5.0**	0.1	18	1	8	1	4.1**	0.3	0.22	0.04
	(106)		(13)		(14)		(14)		(7)	

Mean values were significantly different from those for the NP group: \*\* $P < 0.01$ . † For details of animals, diets and procedures, see pp. 292–293 and Table 1.

**Table 4.** Liver glycogen, free fatty acid (FFA), total serum protein, serum albumin, serum glucose and serum insulin levels of weaned male rats from dams fed on a normal (NP) or low-protein (LP) diet during pregnancy and lactation†  
(Values are means with their standard errors for the no. of rats shown in parentheses)

Dietary group	Liver glycogen (mg/100 mg)		FFA (mmol/l)		Total serum protein (g/l)		Serum albumin (g/l)		Serum glucose (mmol/l)		Serum insulin (nmol/l)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NP	7.4 (43)	0.4	0.3 (25)	0.02	36 (28)	1	22 (25)	1	7.1 (28)	0.2	0.4 (15)	0.04
LP	10.0*** (45)	0.5	0.5†† (33)	0.04	30†† (28)	1	17†† (27)	0.6	6.4 (28)	0.3	0.1*** (16)	0.02

Mean values were significantly different from those for the NP group: \*\*\* $P < 0.001$ , †† $P < 0.0001$ .  
†For details of animals, diets and procedures, see pp. 292–293 and Table 1.

weight ( $P < 0.01$ ) and the total pancreatic insulin content ( $P < 0.01$ ). However, there was no difference between LP and NP pups when the pancreas weight and pancreatic insulin content were related to body weight or when the pancreatic insulin content was related to pancreas weight (Table 5).

Fasting serum glucose and insulin concentrations obtained before the glucose tolerance test were similar for the two groups of pups. After a glucose load, the areas under the glucose and insulin curves were lower for LP rats than for NP rats ( $P < 0.005$  and  $P < 0.05$  respectively). In addition, after a subcutaneous insulin load, the glucose disappearance rate for the LP group was significantly greater ( $P < 0.001$ ) than that for the NP group, thus indicating an increased sensitivity to insulin (Table 6).

Insulin secretion in the presence of a low concentration of glucose (2.8 mmol/l) was 0.31 (SEM 0.03;  $n$  16) and 0.43 (SEM 0.05;  $n$  13) ng/islet per h for LP and NP rats respectively ( $P < 0.05$ ). When the glucose concentration was increased to 16.7 mmol/l, insulin secretion was 0.81 (SEM 0.10;  $n$  20) and 6.39 (SEM 0.74;  $n$  15) ng/islet per 1 h for LP and NP rats respectively ( $P < 0.001$ ).

## Discussion

Maternal protein restriction during pregnancy and lactation can adversely affect offspring. Hales & Barker (1992) have suggested that poor maternal nutrition, mainly protein deprivation, may favour the appearance of 'syndrome X', characterized by

**Table 5.** Pancreatic insulin content of weaned male rats from dams fed on a normal (NP) or low-protein (LP) diet during pregnancy and lactation (Values are means with their standard errors for the no. of rats shown in parentheses)

Dietary group ....	NP (9)		LP (12)	
	Mean	SEM	Mean	SEM
Wt of pancreas:				
g	0.2	0.02	0.07**	0.01
g/kg body wt	3.7	0.5	3.5	0.3
Insulin ( $\mu$ g):				
per pancreas	11.2	0.9	4.1**	0.4
per g pancreas	55.4	6.8	60.6	5.3
per kg body wt	191.1	15.1	201.3	15.0

Mean values were significantly different from those for the NP group: \*\* $P < 0.01$ .

type 2 diabetes, hypertension and hyperlipidaemia. Consistent with this hypothesis, recent studies in experimental animals have shown that protein restriction during critical periods of development can lead to generalized growth retardation and permanent reduction in the size of organs and tissues, including the pancreas (Desai *et al.* 1996). Changes in the activity of some of the liver enzymes associated with glucose metabolism (Desai *et al.* 1995) and hypertension (Langley-Evans & Jackson, 1996) were also observed.

In relation to maternal weight, we did not observe differences between the two groups until the end of the 4th week of pregnancy. This contrasts with the findings of a previous study, in which maternal weight gain was reduced by 10% in the LP group (Snoeck *et al.* 1990). However, these authors report that the reduction in the weight gain by malnourished mothers occurred only during the last 2 d of pregnancy. At the end of the lactation period, weight gain of LP dams was significantly reduced as compared with that of NP dams (values not shown).

In the present study, we confirmed that a reduction in body weight is associated with low serum glucose levels in newborn pups from protein-malnourished (LP) dams when compared with those from normal (NP) dams. Neonatal hypoglycaemia is a frequent finding in low-weight newborn (Leeun & Vries, 1976), and it has been attributed to the maternal hypoglycaemia (Gruppuso *et al.* 1981). As previously reported (Mello *et al.* 1987), low maternal glycaemia due to malnutrition accounts for the reduced liver glycogen concentration and consequent low serum glucose levels in their offspring. Additionally, long-term maternal hypoglycaemia or reduced fetal glucose supply, due to reduced uterine blood supply during the final third of pregnancy, eventually results in fetal growth retardation (Gruppuso *et al.* 1981). The reduced body weight observed in neonates from dams fed on the LP diet may also be related to a reduced supply of insulin from the mother, since the insulin content of the maternal endocrine pancreas has been shown to be significantly reduced in pregnant rats fed on a low-protein diet (Dahri *et al.* 1995). Insulin exerts a profound effect on the growth of organs and fetal metabolism, as judged by the hyperinsulinaemia and macrosomia of neonates from non-compensated diabetic mothers (Van Assche & Aerts, 1979). Fetal growth induced by insulin is linked to an increase in circulating somatomedins, cross-reaction with growth factor receptors, and a lack of down-regulation of the fetal liver insulin receptors (Roth, 1979; Hill & Milner, 1980; Alvarez & Blázquez, 1984).

**Table 6.** Fasting serum glucose and insulin concentrations, total areas under the glucose ( $\Delta G$ ) and insulin ( $\Delta I$ ) curves obtained from the oral glucose tolerance test, and glucose disappearance rates ( $K_{itt}$ ) calculated using serum samples obtained 0–60 min after a subcutaneous insulin injection in normal (NP) or low-protein (LP) male rats‡

(Values are means with their standard errors for the no. of rats in parentheses)

Dietary group	Glucose (mmol/l)		Insulin (nmol/l)		$\Delta G$ (mmol/l per 120 min)		$\Delta I$ (nmol/l per 120 min)		$K_{itt}$ (%/min)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
NP	4.7	0.8	0.17	0.03	859	58	30	4.7	0.7	0.10
	(6)		(6)		(6)		(6)		(11)	
LP	3.9	0.2	0.10	0.04	607†	52	17*	3.9	1.6***	0.20
	(6)		(6)		(6)		(6)		(13)	

Mean values were significantly different from those for the NP group: \* $P < 0.05$ , \*\*\* $P < 0.001$ , † $P < 0.005$ .

‡ For details of animals, diets and procedures, see pp. 292–293 and Table 1.

Our results demonstrate that the effects of maternal protein restriction were more marked in weaned rats than in newborn rats. Thus, malnourished pups showed a reduction in body weight, while serum protein, albumin and insulin levels were maintained. In weaned protein-deprived rats, these variables were drastically reduced in association with high levels of free fatty acids and liver glycogen concentration. Hypoalbuminaemia, hypoproteinaemia and elevated free fatty acid and liver glycogen levels are features commonly found in malnourished infants and in experimental animals (Milner, 1971; Okitolonda *et al.* 1987; Carneiro *et al.* 1995; Reis *et al.* 1997). During sucking, the growth rate of LP pups was 60 % less than that of NP pups, a finding that agrees with the observation that the post natal period is critical for overall growth (Desai *et al.* 1996). In weaning rats from malnourished dams, the postprandial serum glucose levels were similar to those of NP pups, despite the significantly lower serum insulin levels in the former. These results confirm those of previous studies in fed and fasted malnourished adult rats (Okitolonda *et al.* 1987; Escriva *et al.* 1991). Under fasting conditions, the LP rats in our study had normal serum glucose and insulin levels.

Reduced insulin secretion may result from a decrease in insulin synthesis in malnourished animals. Protein restriction during pregnancy produces a variety of structural changes in the pancreas of the offspring, including a reduction in islet size, a decrease in B-cell proliferation, and a reduction in the size of the islet vascular bed (Snoeck *et al.* 1990; Reusens *et al.* 1997). Our results showed that pups from protein-deprived mothers had pancreas weights and total pancreatic insulin contents which were drastically reduced compared with those of the controls. However, when pancreatic insulin content was expressed relative to body or pancreas weight, no difference was present. Despite this observation, insulin secretion during a glucose load was severely reduced, as previously reported by others (Okitolonda *et al.* 1987; Dahri *et al.* 1991; Reis *et al.* 1997). These observations were confirmed by our experiments with isolated islets. Glucose (16.7 mmol/l) increased insulin secretion, above basal levels (2.8 mmol/l glucose), by 14-fold in NP but only 2.6-fold in LP islets. An adequate explanation for this phenomenon has not yet been proposed. However, it is possible that different steps in the mechanism of insulin

secretion may be altered in islets from malnourished animals. Possible changes include an impaired glucose recognition by B-cells (Dixit & Kaung, 1985), reduced activity of the mitochondrial glycerophosphate dehydrogenase (*EC* 1.1.99.5; Rasschaert *et al.* 1995), and a reduction in  $Ca^{2+}$  uptake by the islets (Carneiro *et al.* 1995). Moreover, long-term exposure to high non-esterified fatty acid levels could also contribute to the inhibition of glucose-induced insulin secretion. The detrimental effects of elevated fatty acids on B-cell function have been demonstrated *in vivo* as well as *in vitro*. The mechanisms involved include the stimulation of fatty acid oxidation (Sako & Grill, 1990), with consequent inhibition of glucose oxidation and ATP generation (Zhou & Grill, 1994).

The paradoxical association of euglycaemia with low serum insulin levels, and the low areas under the glucose and insulin curves during the oral glucose tolerance test in malnourished pups agree with previous reports (Okitolonda *et al.* 1987; Escriva *et al.* 1991). The maintenance of glucose levels in malnourished pups may reflect: (1) an increased sensitivity to insulin and a consequent rise in the glucose uptake by peripheral tissues (Heard *et al.* 1977; Crace *et al.* 1990; Ozanne *et al.* 1996b; Reis *et al.* 1997), (2) an increased ability of insulin to reduce hepatic glucose output (Escriva *et al.* 1991), or (3) the presence of hepatic glucagon resistance with a subsequent decrease in hepatic glucose output (Ozanne *et al.* 1996a). The higher liver glycogen content observed in protein-deprived pups indicates a decreased response to glucagon, perhaps as a result of low hepatic glucose-6-phosphatase (*EC* 3.1.3.9) activity (Heard *et al.* 1977) or fewer hepatic glucagon receptors (Ozanne *et al.* 1996a). The increased serum glucose disappearance rate observed after an insulin load is consistent with an increase in sensitivity to insulin, with increased phosphorylation of the insulin receptor and insulin receptor substrate 1 recently observed in hepatocytes and muscle cells (Reis *et al.* 1997; MQ Latorraca, MAB Reis, EM Carneiro, MAR Mello, LA Velloso, MJA Sand and AC Boschero, unpublished results).

In conclusion, protein restriction in rats during the early period of life can lead to a reduction in insulin secretion with concomitant metabolic alterations. A parallel increase in the sensitivity of target tissues to the hormone may explain the normoglycaemia observed in rats maintained on a low-protein diet.

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