Development of β-cell mass in fetuses of rats deprived of protein and/or energy in last trimester of pregnancy

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Received 22 January 2002; accepted in final form 13 May 2002

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Development of β-cell mass in fetuses of rats deprived of protein and/or energy in last trimester of pregnancy. Am J Physiol Regul Integr Comp Physiol 283: R623–R630, 2002. First published May 16, 2002; 10.1152/ajpregu.00037.2002.—Fetal malnutrition is now proposed as a risk factor of later obesity and type II diabetes. We previously analyzed the long-term impact of reduced protein and/or energy intake strictly limited to the last week of pregnancy in Wistar rats. Three protocols of gestational malnutrition were used: 1) low-protein isocaloric diet (5 instead of 15%) with pair feeding to the mothers receiving the control diet, 2) restricted diet (50% of control diet), and 3) low-protein-restricted diet (50% of low-protein diet). Only isolated protein restriction induced a long-term β-cell mass decrease. In the present study, we used the same protocols of food restriction to analyze their short-term impact (on day 21.5 of pregnancy) on β-cell mass development. A 50% β-cell mass decrease was present in the three restricted groups, but low-protein diet, either associated or not to energy restriction, increased fetal β-cell insulin content. Among all the parameters analyzed to further explain our results, we found that the fetal plasma level of taurine was lowered by low-protein diet and was the main predictor of the fetal plasma insulin level (r = 0.63, P < 0.01). In conclusion, rat fetuses exposed to protein and/or energy restriction during the third part of pregnancy have a similar dramatic decrease in β-cell mass, and their ability to recover β-cell mass development retardation depends on the type of malnutrition used. Moreover, our results support the hypothesis that taurine might play an important role in fetal β-cell mass function.

Epidemiological data in various human populations show that low birth weight and especially thinness at birth are associated with susceptibility to the development of impaired glucose tolerance/type II diabetes in adult life (16, 23, 26, 28, 31, 32). This association has been interpreted as reflecting long-term effects of nutritional factors that reduce fetal growth and impair the development of tissues regulating glucose metabolism (14, 29, 36).

Among these tissues, the endocrine pancreas, and especially β-cells, could suffer from fetal malnutrition. Babies with intrauterine growth retardation have a marked reduction in the size of their endocrine pancreas (41). Animal studies also report that fetal malnutrition is associated with persistently impaired pancreatic β-cell function and development (11, 13). With the use of a low-protein diet during whole rat pregnancy, reduced proliferation rate, size, and insulin content of pancreas islets were observed in fetuses at the end of pregnancy (11, 38).

However, human data did not highlight a clear relationship between body weight or ponderal index (weight/height3) at birth and β-cell function in adult age (8), and human fetal malnutrition was reported to be more strongly related to insulin resistance (5, 9, 10, 23, 26, 31, 40). Some experimental data obtained in adult rats whose mothers were submitted to malnutrition support this hypothesis, revealing an impact on insulin action and fat mass development (17–19). Actually, various patterns of fetal malnutrition were shown to differentially affect adult β-cell mass and then could explain the heterogeneity in the above data. Indeed, a 50% reduction in the mother’s intake during the first 2 wk of gestation did not exert adverse effects on insulin secretion and action in 4-mo-old male offspring (35). However, when such food restriction was applied in the last week of the rat pregnancy, it did significantly affect the pancreatic insulin stores and the β-cell mass in the fetuses or the offspring neonates (1, 13). Moreover, in a previous study, we demonstrated that pancreatic insulin content and β-cell mass in adult age were influenced by the type of malnutrition (energy and/or protein restriction) during the fetal stage of pancreas development, with a particularly deleterious impact of protein deficiency (3).
MATERIALS AND METHODS

Diets

The powdered semisynthetic standard diet contained by weight (g/100 g): 68% starch, 4% cellulose, 5% lipid (corn oil), and 15% protein (casein); and by calories: 72% carbohydrate, 12% lipid, and 15% protein. The powdered semisynthetic low-protein diet contained by weight (g/100 g): 78% starch, 4% cellulose, 5% lipid (corn oil), and 5% protein (casein); and by calories: 83% carbohydrate, 12% lipid, and 5% protein. Energy content per 100-g diet was the same (375 cal) in both diets. Both diets contained 2 g/100 g yeast, a salt mixture (3.5 g/100 g), and a vitamin mixture (2.2 g/100 g) as in Picarel-Blanchot et al. (33).

Animals

Female Wistar rats bred in our colony were housed in a temperature-controlled room with a 12:12-h light-dark cycle (lights on 0700). Weighing 230 to 260 g, they were mated for 1 day before delivery) where fetuses were analyzed. Rats in the first group had their energy restricted to 50% of their pregnancy standard diet intake. Rats in the second group were energy restricted to 50% of their pregnancy intake but were fed a low-protein diet. Rats in the third group were pair fed to control rats with the low-protein diet. Control rats (the fourth group) were given access to standard diet ad libitum throughout pregnancy. Fetuses in the four groups will subsequently be referred to as standard diet restricted (CER), low-protein diet restricted (PER), low-protein pair fed (PR), and control (C) groups, respectively. In each group, four pregnant rats, with litters from 9 to 13 fetuses, were analyzed.
ICN (ref.55–104–1; ICN Pharmaceutical, Orsay, France); it was raised in the guinea pig against porcine insulin. Labeling was performed using a peroxidase-conjugated rabbit anti-guinea pig IgG (ref. PO141; Dako, Trappes, France). The activity was revealed with a peroxidase substrate kit (Vector SG, Biosys-Vector, Compiègne, France). After being stained, sections were mounted in Eukitt. Quantitative evaluation was performed using a computer-assisted image analysis based on an Olympus microscope connected to a Siemens PC computer and using the ImageJ 2000 software (Biocom, les Ulis, France). The area of the insulin-positive cells as well as the area of the total pancreatic cells were evaluated in each stained section. The β-cell relative volume was obtained by calculating the ratio between the area occupied by immunoreactive cells and the area occupied by total pancreatic cells according to stereological methods. The total β-cell mass per pancreas was derived by multiplying the β-cell relative volume by the total pancreatic weight.

Samples and Analytic Techniques

Blood samples in pregnant rats (200 μl for serum and 150 μl for plasma measurements) and in fetuses (whole body blood content for plasma measurements) were immediately put into ice-chilled vials. They were then centrifuged and the plasma or the serum was separated. Plasma glucose concentration was immediately determined in a 10-μl aliquot, and the other plasma or serum aliquots were kept at −20°C until other measurements determination. For glucagon and corticosterone, plasma from three and five fetuses per litter was pooled. The plasma sample for glucagon measurement was mixed with a kallikrein inhibitor (Iniprol, Sano, Sanofi, Libourne, France) before freezing.

Plasma glucose level was determined with a glucose analyzer (Beckman, Palo Alto, CA).

Plasma amino acids and taurine concentrations were measured by ionic exchange chromatography on an automatic instrument using ninhydrine as a reactive agent (Liquimat 4HP, Labotron, France). Serum levels of free fatty acids were obtained by an enzymatic method (NEFA C Wako, Unipath SA, France). Albuminemia was measured with a classical technique using Green of Bromocresol. Immunoreactive insulin in the plasma and fetus pancreases was estimated with purified rat insulin as standard (Novo, Copenhagen, Denmark), and porcine monoiodinated 125I-labeled insulin (32). Charcoal was used to separate free from bound hormone. The method allows the determination of 2 μu/ml (0.08 ng/ml or 14 pmol/l) with a coefficient of variation within and between assays of 10%. Commercial kits for determination of plasma glucagon and corticosterone concentrations were, respectively, from Pharmacia, St-Quentin, France and ICN.

Statistical Analysis

Results are given as means ± SE. ANOVA (Fisher’s test) was used for comparison of unpaired data between groups. A P value <0.05 was considered significant.

Pearson’s correlation coefficient was also used, and stepwise multiple regression models permit to identify the variables independently related to the fetuses’ parameters considered (Statview SE, Abacus Concepts, Berkeley, CA).

RESULTS

Body Weight, Plasma Albumin, and Nutrient Concentrations in Pregnant Rats

On day 14.5 of pregnancy, body weight did not significantly differ among the four groups of mothers (which had similar numbers of fetuses per litter).

In the three experimental groups of rats submitted to a food restriction during the last week of pregnancy, the relative variation for body weight was quite different compared with the C group, with a lower weight gain in the CER and PR groups and a body weight stagnation in the PER group (Table 1). In the four groups, the whole daily food supply was fully ingested.

Biological data related to pregnant rats on day 21.5 of pregnancy are presented in Table 2. Briefly, the plasma albumin level was decreased in the three restricted groups but quite more in the protein-restricted...
PR and PER groups. However, total amino acidemia was not significantly different among the four groups. Free fatty acid concentrations were not significantly different between the restricted groups and the C group. The basal plasma glucose level was similarly decreased in the PR and PER groups without reaching significance compared with the C group. The CER group had values similar to the C group.

**Characteristics of the Fetuses on Day 21.5 of Pregnancy**

**Fetal growth.** The average number of fetuses per mother was similar in all groups studied. Fetuses differed in their body weight value according to the diet changes in the last week of pregnancy (Table 1). The fetuses from the CER, PR, and PER groups had a significantly lower birth weight compared with the C group. No relationship between offspring and mother body weights could be detected. The fetal anonasal length was significantly reduced in the three restricted groups compared with the C group. As the impact of food restriction on fetal size was more marked in the CER group, the fetuses' thickness as described by the Lee index was higher in the CER group than in the other groups.

The three different types of food restriction altered placenta development with a more marked effect in the PR and PER groups than in the CER group. Accordingly, the fetus-to-placenta weight ratio was significantly increased in the PR and PER groups, but not modified in the CER group, compared with the C group.

Concerning the other organ-to-fetal weight ratios in fetuses, no significant difference was seen for kidneys and pancreas among the four groups. The liver-to-fetal weight ratio was significantly reduced in the three restricted groups, but the liver-to-placenta weight ratio did not significantly differ among the four groups.

**Plasma levels of glucose, amino acids, insulin, glucagon, and corticosterone.** The plasma glucose level in pregnant rats was increased when collecting blood samples from fetuses, but this increment was moderate and not significantly different among the four groups (CER: +15%; PR: +17%; PER: +18%; C: +11%). This observation suggests that our sampling methodology is acceptable and allows a reliable comparison of plasma glucose and insulin levels among the different fetuses' groups.

Data are presented in Table 3. Glycemia was lower in the PR group than in the other groups (difference was only significant vs. the C and PER groups, respectively; \( P < 0.05 \) and 0.03). Concerning the insulinemia-to-glycemia ratio in fetuses, it was significantly decreased in the PR group with about half the value found in the CER and C groups (respectively, \( P < 0.03 \) and 0.05). No differences between fetuses from food-restricted and C dams were detected for plasma glucagon and corticosterone.

Blood glucose levels in fetuses, as free fatty acids and amino acid levels in pregnant rats, were not significantly correlated with anthropometric data or insulinemia in the corresponding fetuses.

**Pancreatic Insulin Content and Total β-Cell Mass of the Fetuses on Day 21.5 of Pregnancy**

Fetuses of the CER group exhibited a whole pancreatic insulin content significantly lower than that of the other groups.
C group ($P < 0.01$). When calculating the relative pancreatic insulin content per gram of fetus body weight, a significant difference between the PER group and the C group was also detected ($P < 0.05$; Table 4).

The pancreatic β-cell mass (expressed as absolute value or relatively to fetal body weight) was significantly decreased ($P < 0.001$) to a level similar in the CER, PR, and PER fetuses compared with the C fetuses.

When calculating the mean insulin content-to-β-cell mass ratio, we found that this parameter was significantly higher in the low protein-restricted groups (PR and PER groups) than in the C group. Interestingly, an inverse correlation was present between this ratio and fetal insulinenia or insulinemia-to-glycemia ratio (respectively, $r = -0.73$, $P < 0.001$ and $r = -0.65$, $P < 0.007$) when analyzing the litters of the four groups as a whole.

**Blood Taurine Level in Dams and Fetuses on Day 21.5 of Pregnancy**

Postabsorptive blood levels of taurine were similar in the four groups of pregnant rats (Table 5). In fetuses, a significant decrease was present in the PR and PER groups compared with the C and CER groups, arguing for a specific impact of protein restriction on this parameter.

When pooling the data from the four groups, the fetal blood taurine level did appear as the only predictor of fetal insulinenia or insulinemia-to-glycemia (respectively, $r = 0.63$, $P < 0.009$ and $r = 0.52$, $P < 0.04$).

**DISCUSSION**

Our present data indicate that low energy and/or protein diet in the third part of gestation do not significantly affect basal plasma nutrient levels (glucose, free fatty acids, and pool of amino acids) in pregnant rats when measured in the postabsorptive state. Plasma albumin level was decreased mainly in response to protein restriction. One may retain as important for the interpretation of our data that the CER group exhibits mainly energy deficiency with limited associated protein malnutrition, whereas the PR and PER groups were clearly protein deficient. Our data are therefore consistent with the report by Wade et al. (42) who demonstrated that a 50% reduction in food consumption during 6 wk in nonpregnant rats did not affect serum albumin level, whereas protein restriction (4% protein in the food) added to energy restriction significantly decreased serum concentration of albumin.

As previously reported by our group, the association of protein and energy restriction was more deleterious on body weight growth in pregnant rats (3). Our data suggest that hormonal compensatory changes occur in PER pregnant rats to maintain glycemia and nutrient flux despite the severity of the undernutrition as attested by maternal weight stagnation. This is also illustrated by the lack of additional decrease in the weight of PER fetuses compared with that of PR fetuses, whereas their mothers’ body weight was significantly lower.

In the third part of gestation, placenta development was also found to be mainly dependent on protein supply, thus confirming previous reports in rat and other species (4, 15, 27). A strong positive relationship was detected between plasma albumin level in pregnant rats and placenta weight when pooling the four groups studied as a whole ($r = 0.82$, $P < 0.0001$). Liver development was also mainly significantly altered by

| Table 5. Blood taurine levels on day 21.5 of pregnancy in fetuses and pregnant Wistar rats |
|-----------------|-------|-------|-------|
| **Taurine, μmol/l** | **CER** | **PR** | **PER** |
| Dams | 150 ± 9.4 | 156 ± 18.2 | 201 ± 56.2 |
| Fetuses | 347 ± 18.6 | 282 ± 16.4† | 285 ± 4.1† |

Values are given as means ± SE of each litter mean value (corresponding to the results of 5 fetuses). *$P < 0.05$, †$P < 0.04$ vs. C group; ‡$P < 0.03$ vs. CER group.

**AJP-Regul Integr Comp Physiol • VOL 283 • SEPTEMBER 2002 • www.ajpregu.org**
protein restriction (Table 1). However, fetal growth of the pancreas and kidneys was not specifically altered by protein and/or energy restriction as assessed by the organ-to-body weight ratio measurements. To explain this result, we assume that the liver, which is first and directly connected to the placenta blood flow, could attenuate the deleterious impact of protein restriction. However, the highest value in the fetal body weight-to-placenta weight ratio was found in the PR group, suggesting that compensatory adaptations in nutrient flux through the placenta were probably induced by protein restriction, because no significant changes could be detected in the blood nutrient levels of PR pregnant rats compared with the C rats (see the results in Table 2).

In fetuses, the pool of free amino acids was maintained normal whatever the food-restriction protocol. The same conclusion could be drawn for the pool of essential amino acids (data not shown), whose blood level was similar among the four groups, whereas plasma glucagon or corticosterone levels were not significantly changed by food restriction. Taking into account a possible confounding effect of physiological variations in the 24 to 30 h preceding labor on the above parameters, we may reasonably assume that placenta is able to ensure sufficient nutrient supplies to fetuses even in unfavorable environmental conditions, at least during the third part of gestation.

Despite an unchanged pancreas-to-total body weight ratio in food-restricted fetuses, β-cell mass was highly reduced by both protein and energy restriction at a similar level (≈48% of control data) when adjusting the results on total body weight. However, the relative pancreatic insulin content (insulin content per mg of body weight) was significantly changed (32% decrease) only in CER rats compared with C rats. An increase of the pancreatic insulin content-to-β-cell mass ratio was therefore present in PR and PER fetuses. Moreover, no significant relationship between β-cell mass and pancreatic insulin content could be detected, and fetal insulinemia and insulinemia/glycemia were inversely related to the pancreatic insulin content-to-β-cell mass ratio ($r = -0.73$, $P < 0.001$ and $r = -0.65$, $P < 0.007$, respectively). Taken together, these data suggest that insulin secretion might be impaired in fetuses submitted to protein restriction, and this alteration would be located at the exocytosis step in the insulin secretion cascade and not in the insulin pool of the β-cell. Although this is a speculative assumption, recent experimental data from Cherif et al. (7) support this hypothesis.

A few years ago, the β-amino acid taurine was proposed as an essential amino acid for fetal β-cell function (6). In their study, Cherif et al. (7) found an in vitro insulin secretion defect by islets from low protein-fed rat fetuses, and these defects were corrected when taurine was added to the low-protein diet of dams. Therefore, in the present study, we measured the concentration of taurine in maternal and fetal blood at the end of the malnutrition period. In the pregnant rats, blood taurine level was not significantly changed by the different patterns of food restriction. These results are in opposition with those of Cherif et al. who found a decrease of taurine concentration in the low protein-fed pregnant rats, but at variance with us, they applied their protocol of protein restriction to the whole gestation. However, we also detected a decrease of the taurine level in fetuses whose mothers were submitted to a low-protein diet (PR and PER rats), and this decrease was present independently of energy supply. We also found that the blood taurine level was the main independent predictor of insulinemia or insulinemia/glycemia in fetuses. At the opposite, maternal glycemia did not appear as an independent predictor of fetal insulinemia in our study. These results support the hypothesis that taurine could play a role in fetal β-cell function.

Concerning the impact on β-cell mass, a previous report by Garofano et al. (13), using a 50% energy-restriction protocol in the same period, also showed a significant decrease of β-cell mass and of pancreatic insulin content. Fetal growth retardation was assessed to be ≈18% in their study vs. 15% in the present study. In Garofano’s work, however, analysis was performed at birth and only pups with the lowest weights were selected and kept for further analysis. A 40% decrease in total insulin content of CER pups compared with C was found at birth. This result is very similar to the result of 32% decrease reported in our study for the CER group at day 21.5 of pregnancy. Concerning the absolute β-cell mass, a 35% decrease was reported by Garofano vs. 55% decrease in the present study. This difference could be explained by the difference in the stage of analysis and in the methodology (only 5 to 7 sections were analyzed per pancreas in Garofano’s study).

In previous work, we analyzed the long-term impact in the young adult female offsprings of our different patterns of food restriction in the same experimental conditions. Briefly, weight retardation was rapidly (<15 days) reversed and no significant difference in body weight was therefore detectable among the four groups at the age of 8 wk. The female offspring of mothers who had been malnourished according to different patterns in their third part of pregnancy get limited impairment of their glucose metabolism in adult life without change in insulin action. The whole pancreas development was not dramatically changed despite an increase of the pancreas weight in PER rats (1.25-fold increase) with no impact on β-cell mass or insulin content per milligram of pancreas. On the other hand, the CER rats got an increase, whereas PR rats got a decrease, in the insulin content expressed in micrograms per milligram of pancreas or micrograms per gram of body weight (respectively, +23 and −29%). These data on the pancreatic insulin content are opposite to those found in the present study at the fetal stage, at least for PR and CER rats. In PER rats, the plasticity of the pancreas seems therefore more important as they can totally correct the abnormalities detected during fetal stage. The significant decrease of the β-cell mass (−50%) present in fetuses of the three
restricted groups still persists at the age of 8 wk only in the PR group (28%).

These data demonstrate that strong defects of β-cell mass growth can be partially recovered after birth when the food restriction is not prolonged after a period of fetal malnutrition. However, this relative recovery depends on the pattern of maternal food restriction. In the present study, we did not detect any relevant differences between PR and PER fetuses concerning biological parameters analyzed or fetal growth for whole body or organs. When protein restriction was present, β-cell mass on day 21.5 of pregnancy was not influenced by the level of energy supply. As a matter of fact, the Lee index was increased in CER fetuses compared with the other groups, whereas a stronger relationship was detected in humans between risk of diabetes in adulthood and thinness at birth than with isolated low birth weight (31). We thus may assume that energy restriction during the third part of gestation represents a favorable situation to the recovery of β-cell mass during postnatal growth even when a protein restriction is superimposed. But the determinants of the recovery of β-cell mass and function are presently unknown. A direct modulation of differentiation and/or replication of β-cells could be involved. Persistent alterations of the autonomous nervous system may also be supposed. Some studies indeed demonstrated a long-term impact of protein malnutrition on the reactivity of the sympathetic system (21, 24).

In conclusion, the present study is the first one to investigate the immediate impact of various fetal malnutrition protocols strictly limited to the third part of pregnancy, which corresponds to the crucial period for fetal rat pancreas development. Under these conditions, protein deficiency and/or 50% energy restriction induced a marked impairment of β-cell mass development (−50%) after adjusting the results on fetal growth retardation. Moreover, our results support the hypothesis that the amino acid taurine has an important role for fetal β-cell function, which could explain a differential impact of protein and energy restriction on pancreatic insulin content. Compared with our previous study analyzing the long-term impact of the same patterns of malnutrition, the present results demonstrate that the recovery of the β-cell mass development retardation depends on the type of fetal malnutrition.

This work was partly supported by a grant from the Ministère de l’Education Nationale, de l’Enseignement Supérieur et de la Recherche (#95-G-0103; programme interministèriel “Aliment Demain”).

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