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Insulin secretion and GLUT-2 expression in undernourished neonate rats

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Abstract

In previous studies, we verified increased insulin sensitivity in adult male offspring of lactating rats readjusting to lack of insulin secretion reduction brought about by protein restriction during lactation. The present study aims to evaluate the effects of maternal protein undernutrition during lactation on glucose-induced insulin secretion and GLUT-2 expression in β-cells of neonate male and female rats. Lactating Wistar rats were given a protein-free diet during the first 10 days and a normal diet (22% of protein) until weaning. The neonates were separated at birth by sex and diet and studied at 4, 8 and 21 days of lactation. Glucose-induced insulin secretion by pancreatic islets was analyzed by radioimmunoassay and GLUT-2 expression in β-cells by Western blot. Glucose-induced insulin secretion of the undernourished groups was higher than in the control groups except among females. When comparing the male and female groups and the control and undernourished groups, female neonates showed significantly greater insulin secretion than the male group. Also it was noted that undernutrition induced greater GLUT-2 expression. For instance, comparing the undernourished male and female neonates there was an increase in female GLUT-2 expression on day 4. On the other hand, in undernourished male neonates a GLUT-2 expression increased later in lactation. In conclusion, during a short term, maternal undernutrition induces an increase of the glucose-induced insulin secretion only in male neonates and is associated with an increase in GLUT-2 expression in the β-cell.

Keywords: Neonate; Undernutrition; Insulin secretion; GLUT-2 expression

1. Introduction

Members of the GLUT (glucose transporter) protein family facilitate the transport of glucose across mammal cell membranes. These 50-60-kDa glycoproteins supply cellular glucose for ATP (adenosine triphosphate) production and play a critical role in glucose homeostasis, with distinct functional features and tissue distribution [1–3]. In pancreatic β-cells, the glucose uptake is controlled by glucose transporter isoform (GLUT-2), which is essential in the mechanism of glucose-induced insulin secretion [4]. In recent findings, the importance of this mechanism shows that in type 2 diabetes a GLUT-2 down-regulation occurs simultaneously with the loss of glucose-induced insulin secretion [5].

Alterations of the insulin secretion mechanism by reducing the insulin sensitivity in adults and bringing on type 2 diabetes are associated with undernutrition during early life (fetal/neonatal period) [6–8]. The specific mechanisms of the association between early life undernutrition and diabetes mellitus are still being studied. Most studies suggest that the restricted maternal nutrition acting during the period of rapid offspring development causes adaptations that are set by the time of adulthood [9–12].

We recently established an association between maternal protein deficiency during lactation and changes in the glucose homeostasis of the offspring when adulthood was achieved. Basically, in adulthood these animals reduce insulin secretion and, as an adaptation mechanism, there is increased insulin sensitivity [13–15]. Furthermore, alterations in feeding behavior in adulthood were shown to be associated with insulin and leptin concentrations in early life [16]. On reaching adulthood there was a reduction of the systolic and diastolic blood pressure and an increased insu-

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Table 1
Composition of the control and protein-free diets

	Control*	Protein-free†
Ingredients (g/kg)		
Soybean + wheat	220.0	0
Corn starch	676.0	896.0
Soybean oil	50.0	50.0
Vitamin mix‡	4.0	4.0
Mineral mix‡	40.0	40.0
Macronutrient composition (%)		
Protein	22.0	0
Carbohydrate	67.0	88.9
Fat	11.0	11.1
Total energy (kJ/kg)	17038.7	17038.7

* Standard diet for rats (Nuvilab-Nuvital Nutrients LTDA, Paraná, Brazil).
† The protein-free diet was prepared in our laboratory.
‡ Vitamin and mineral mixtures were formulated to meet the American Institute of Nutrition AIN-93G recommendation for rodent diet [18].

lin resistance in the adult female, but not the male, when comparing the blood pressure and glucose homeostasis of the offspring undernourished during lactation [17]. Hence, our previous data strongly suggest that the process of adaptation and setting of new metabolic pathways obtained in early life and maintained throughout life is gender determined.

The effect of the maternal protein deficiency during early lactation on glucose-induced insulin secretion and GLUT-2 expression by pancreatic β-cells of male and female neonates was the aim of this study, for the purpose of evaluating whether the effects of undernutrition were influenced by gender. The results strongly suggest that the effects of undernutrition on these two variables are gender determined. In brief, when the gender was an assumed variable, there was a distinct age relationship in the association of insulin secretion and glucose transporter GLUT-2 of the neonates and the undernutrition of lactating dams.

2. Methods and materials

2.1. Animals and experimental design

Wistar virgin rats (90 days old) were mated and the pregnant dams were randomly housed in individual cages at 23°C on a 12-hour light/dark cycle. The dams were fed *ad libitum* with a 22% protein diet during gestation. After delivery, during the first 10 days of lactation, the control group of dams received a 22% protein diet and the undernourished group received a protein-free diet. Thereafter, all dams were fed a diet with 22% protein until the end of the lactation. Table 1 shows the dietary composition set by recommended standards [18]. Each lactating dam was kept with six pups and they were divided into four groups: control male (CM), undernourished male (UM), control female (CF), and undernourished female (UF). The groups of neonate males and females were analyzed for insulin secretion and GLUT-2 expression by pancreatic islets isolated at 4, 8, and 21 days of lactation. The experimental protocol was conducted in accordance with the local Ethics Committee and with principles set down in the *Guide for the Care and Use of Laboratory Animals* [19].

2.2. Insulin secretion

In vitro insulin secretion was studied using isolated islets by collagenase technique (Boehringer Mannheim, Penzberg, Germany) [20]. A total of 80 islets were placed on Millipore filters in a perfusion system with Krebs solution and mixture of CO_2 (5%) and O_2 (95%), with a multichannel peristaltic pump (Buchler Polystaltic Pump) at a flow rate of 1.0 mL/min. The basal insulin levels were determined during 30 minutes by the infusion of 2.8 mmol/L glucose. After attaining the steady state, 16.7 mmol/L glucose was infused to determine glucose-induced insulin secretion for 50 minutes. The samples were collected in an ice bath and were kept at −20°C for insulin assay. Immunoreactive insulin was measured by radioimunoassay using monoiodated ^{125}I-labeled porcine insulin (Activa, Brazil) as a tracer, guinea pig anti-insulin antibody kindly provided by Dr. Willian Mallaisse (Brussels, Belgium) and purified rat insulin (Novo Nordisk, USA) as standard. Charcoal was used to separate free and bound hormone [21]. The count with ^{125}I was made by a gamma counter (Cobra Autogamma, Packard Instrument Co., Downers Grove, IL). The results were expressed as pmol/L released per 80 islets per 50 minutes.

2.3. Western blot analysis for GLUT-2

GLUT-2 expression was determined by immunoblotting of the total extract of islets [22]. Ten islets of each group were lysed in a buffer containing 50 mmol/L HEPES, 1 mmol/L $MgCl_2$, 10 mmol/L EDTA, and 1% Triton X-100, followed by use of the protease inhibitors aprotinin and leupeptin. Proteins were quantitated by BCA assay using bovine serum albumin (Sigma, St. Louis, MO) as a standard and then analyzed on 12% SDS-polyacrylamide gel. The proteins were then transferred to nitrocellulose filters (Hybond-P, Amersham Pharmacia Biotechnology, USA), and the membranes were incubated with rabbit anti-GLUT-2 1:1000 (Santa Cruz Biotechnology) followed by peroxidase-conjugated donkey antirabbit antibody 1:1000 (Santa Cruz Biotechnology). Immunoreactive bands were visualized by 3.3-diaminobenzidine (Sigma).

2.4. Statistical analysis

All data were presented as mean ± SE. Statistical significance of the results was determined by the one-way analysis of variance (ANOVA) followed by the Newman-

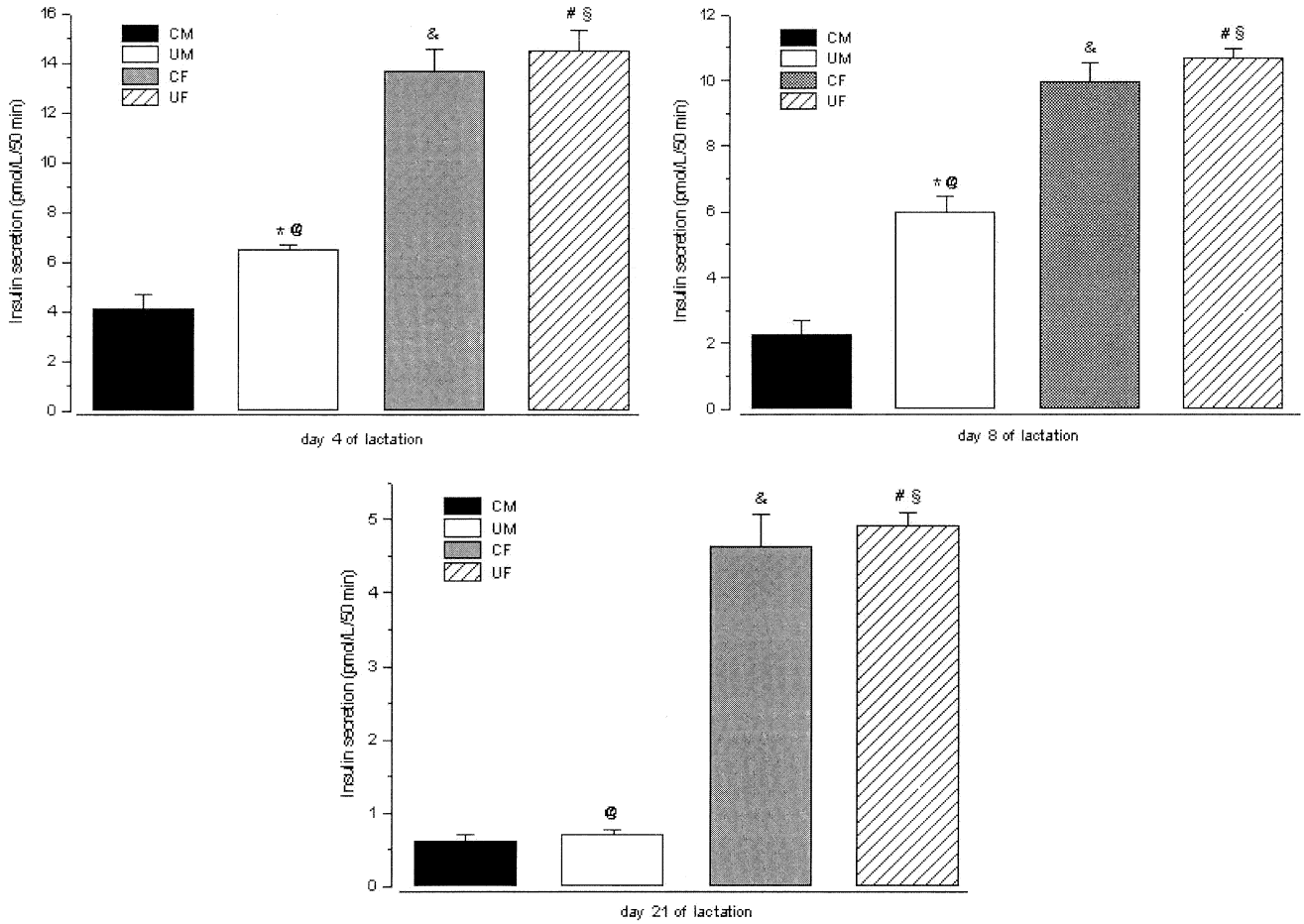

Fig. 1. Insulin secretion by pancreatic islets from neonates males and females on days 4, 8, and 21 of lactation after 16.7 mmol/L glucose stimulus during 50 minutes. Day 4 of lactation: #$P < 0.001$ *vs* CM, &$P < 0.001$ *vs* CM, *$P < 0.05$ *vs* CM, §$P < 0.001$ *vs* UM and @$P < 0.001$ *vs* CF. Day 8 of lactation: #$P < 0.001$ *vs* CM, &$P < 0.001$ *vs* CM, *$P < 0.001$ *vs* CM, §$P < 0.001$ *vs* UM and @$P < 0.001$ *vs* CF. Day 21 of lactation: #$P < 0.001$ *vs* CM, &$P < 0.001$ *vs* CM, §$P < 0.001$ *vs* UM and @$P < 0.001$ *vs* CF. Results are expressed as pmol/L/50 min of insulin release. Mean ± SE of six experiments/group.

Keuls test, with the threshold for significance set at $P < 0.05$.

3. Results

3.1. Insulin secretion

The pattern of insulin secretion by pancreatic islet β-cells was significantly different by gender and treatment group on all days analyzed ($P < 0.0001$). Figure 1A shows that on day 4 of lactation the β-cells of the UM group secreted 57.2% more insulin after 16.7 mmol/L glucose stimulus than did the CM. Females in both groups had a >2-fold greater secretion than males (Fig. 1A). The pattern of secretion observed on day 4 was maintained unchanged until day 8 (Fig. 1B). At day 21 of lactation the UM and CM groups showed no difference in insulin secretion, but females continued having greater secretion than males (Fig. 1C).

Females from both groups showed no differences in insulin secretion for all phases of the study.

3.2. GLUT-2 expression

The expression of glucose transporter GLUT-2 showed significant differences ($P < 0.001$) among the groups evaluated by Western blot analyses of islets isolated from neonate male and female (control and undernourished) on days 4, 8, and 21 of lactation. GLUT-2 was detected in all islet extracts from neonates and islets from control groups, and there were no significant alterations in the GLUT-2 expression during all periods of lactation. On the other hand, increased expression was found in the islets from the undernourished groups (44.7%on average), mainly on day 8 of lactation. However, on day 4 of lactation, when comparing UM and UF groups there was a 50% increase in the GLUT-2 expression in the UF group (Fig. 2A). In contrast, there was an increase of 11% and 25% in GLUT-2 expres-

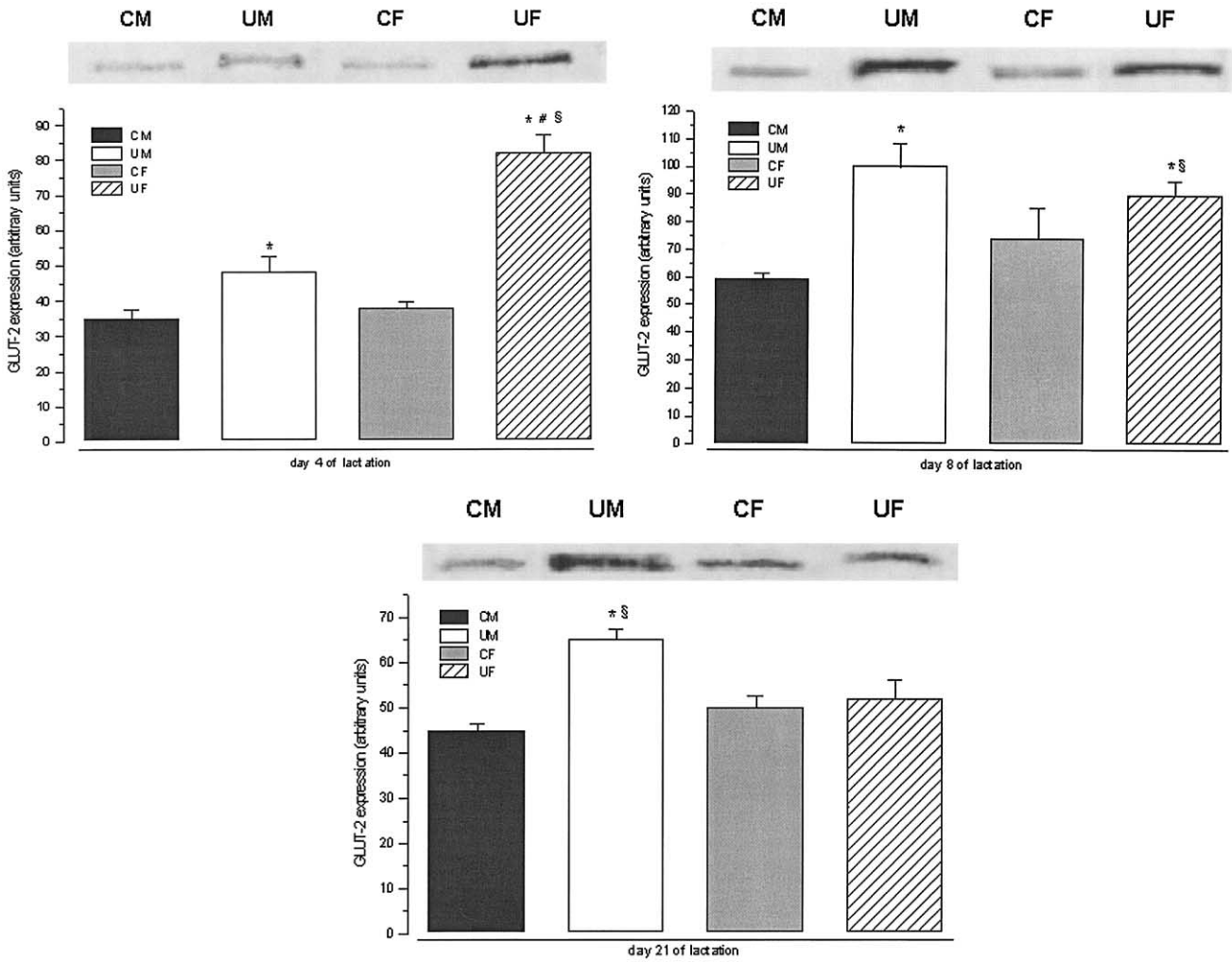

Fig. 2. GLUT-2 expression in pancreatic islets from neonate male and female on days 4, 8 and 21 of lactation. Pancreatic islets were isolated from control male (CM), undernourished male (UM), control female (CF), and undernourished female (UF) groups. Total extracts corresponding to 10 islets were loaded on each lane of gel electrophoresis. GLUT-2 protein was identified as a broad band at 45 kDa by anti–GLUT-2 antibody. This experiment was repeated with another set of three animals, with identical results. *$P < 0.05$ *vs* CM, #$P < 0.05$ *vs* UM, and §$P < 0.05$ *vs* CF.

sion in the UM groups at days 8 and 21, respectively (Figs. 2B, 2C).

4. Discussion

Undernutrition early in life induces pancreatic dysfunction when the animal becomes an adult. This process is probably due to a significant reduction in β-cell proliferation and islet size, causing permanent impairment of the secretory mechanisms. Undergoing structural changes in early life are associated with adaptations to the responsiveness of islets to secretagogues eventually impairing insulin secretion in adulthood [23–25]. Most studies in literature have shown the association between undernutrition and long-term effect on metabolism when undernutrition is induced during fetal or both fetal and neonatal life. However, relatively few studies have observed the effects of undernutrition during early life period (lactation) *per se* on the glucose homeostasis of the neonate and even less is known about the role of the gender in this process.

The present study hypothesized a possible gender role in inducing particular changes obtained by the neonate due to undernutrition. The insulin secretion and the β-cell glucose transporter GLUT-2 were evaluated from neonate pancreas of both sexes of rats undernourished since the beginning of lactation. The idea was to study the effect induced by undernutrition during this period, a period that is critical for the animal's development [9]. For instance, it is well known that during lactation significant structural modifications occur in the pancreas. Basically, the rapidity of islet neogenesis that occurs until weaning is later replaced by a low rate of mitosis found in adult pancreatic β-cells [26]. This fundamental change in cell phenotype, explained by the observed transient wave of apoptosis of islets in rats 2 weeks after birth, generates a cell population controlled by a particular metabolic homeostasis, which in adulthood brings

about modified insulin secretion, sensitivity, or resistance [27]. Also, it is certain that the process of neogenesis and resultant transition to a low level of mitogenesis is modulated by the nutritional value of the milk. In addition, gender was introduced to the study based on recently published data by our group showing that the long-term effect of undernutrition on metabolic homeostasis is determined by gender and changes in insulin secretion mechanisms early in life [17].

This study shows for the first time that undernutrition in early life has a gender-associated effect on insulin secretion and GLUT-2 expression of the neonate. In previous studies using a similar model we demonstrated a reduction of the glucose-induced insulin secretion in offspring male adults from undernourished dams [14,15]. Although the undernourished male group displayed an increase in insulin secretion, no differences were observed in the insulin secretion pattern in the female groups, regardless of whether these rats were undernourished.

There was an increased expression of β-cells of undernourished animals of both sexes when comparing the glucose transporter GLUT-2 in control groups. Our data suggest that the increase in GLUT-2 expression in this period is the main cause of the increased insulin secretion observed in the undernourished males. On the other hand, in the undernourished female group, the results suggest the existence of another mechanism of cellular sinalization that may be enhanced by undernutrition. The uncoupling between the insulin secretion and GLUT-2 may reflect a process of secretion independent of the specific glucose transporter GLUT-2 and also an adaptive process due to undernutrition. Data from the literature show that in animals with GLUT-2–null islets display a lower but sustained glucose uptake and insulin secretion [28], reinforcing the hypothesis that the process found in the undernourished female group is due to a particular glucose uptake.

Despite the lack of significant differences in GLUT-2 expression between male and female rats in the control groups, the females secreted more insulin than did males on all days of lactation studied. Taken together, these results reinforce the hypothetical existence in the females of another mechanism of cellular glucose uptake. For instance, by comparing undernourished male and female, the females on day 4 of lactation increased GLUT-2 expression; however, no significant differences were observed in GLUT-2 expression for days 8 and 21, despite insulin secretion being more expressive in the females.

The quality of maternal milk in the neonatal period is an important modulator for organ development, particularly in the pancreas. Therefore, this suggests that hormones originating in the maternal milk [29–31] may significantly influence the secretory performance in the neonate. In addition, multiple hormonal factors have been implicated in the modulation of neonate development; for example, insulin and the thyroid hormones act directly in the protein synthesis and growth [32,33]. Other hormones such as insulin-like growth factor (IGF) play an important role in the regulation of postnatal development, especially in β-cell growth, maturation and function [34], whereas hormones such as prolactin as well as estrogens act in a gender-dependent manner. The induction of an increased insulin secretion by prolactin is observed only in the female [35]. Also, estrogens in other tissues have been described as increasing the expression of GLUT-1 and GLUT-3, enhancing the insulin gene expression and thereby enabling insulin secretion through the blockage of K-ATP channels and modulating Akt (phospholipase B), a principal enzyme in the insulin-signaling process [36-38]. There is strong evidence to suggest that estrogen could be involved in the gender determination of insulin secretion and GLUT-2 expression, as noted in our results. Further investigation of the involvement of estrogen in these nutritional and gender associations is warranted.

The “thrifty phenotype” hypothesis also proposes a selective distribution of nutrients among the organs by undernutrition that determines the growth and development of some of these organs in detriment to others [39,40]. Thus, organs such as the brain and lungs by enteral nutrition during the postnatal period would receive higher priority during fetal period than would other organs such as the pancreas. We suggest that the increase in insulin secretion in undernourished rats would be an adaptation for the purpose of surviving precarious nutritional conditions, as observed in males. On the other hand, despite the increase in insulin secretion, the similar secretory profile among the female groups suggests that, at least over a short term, a gender determination takes place in adapting the pancreas to respond to nutrient restriction. Thus, the enhanced insulin secretion found in females may occur as an event preceding long-term pancreatic impairment.

In conclusion, the present study demonstrates that maternal undernutrition during early lactation promotes differentiated responses among neonate males and females. Although these data go a long way toward elucidating the gender determination of the prospective effects of undernutrition early in life, the different coupling between insulin secretion and GLUT-2 expression in neonates helps to explain the gender-associated emergence of diseases, such as diabetes mellitus type 2.

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