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Reduced insulin secretion in response to nutrients in islets from malnourished young rats is associated with a diminished calcium uptake

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Changes in ^{45}Ca uptake and insulin secretion in response to glucose, leucine, and arginine were measured in isolated islets derived from 4-week-old rats born of mothers maintained with normal protein (NP, 17%) or low protein (LP, 6%) diet during pregnancy and lactation. Glucose provoked a dose-dependent stimulation of insulin secretion in both groups of islets, with basal (2.8 mmol/L glucose) and maximal release (27.7 mmol/L glucose) significantly reduced in LP compared with NP islets. In the LP group the concentration-response curve to glucose was shifted to the right compared with the NP group, with the half-maximal response occurring at 16.9 and 13.3 mmol/L glucose, respectively. In LP islets, glucose-induced first and second phases of insulin secretions were drastically reduced. In addition, insulin response to individual amino acids, or in association with glucose, was also significantly reduced in the LP group compared with NP islets. Finally, in LP islets the ^{45}Ca uptake after 5 minutes or 90 minutes of incubation (which reflect mainly the entry and retention, respectively, of Ca^{2+}), was lower than in NP islets. These data indicate that in malnourished rats both initial and sustained phases of insulin secretion in response to glucose were reduced. This poor secretory response to nutrients seems to be the consequence of an altered Ca^{2+} handling by malnourished islet cells. (J. Nutr. Biochem. 10:37–43, 1999)

Keywords: protein malnutrition; pancreatic islets; insulin secretion; Ca^{2+} fluxes; rats

Introduction

There are critical periods in embryonic development in which tissues and organs are established. In the endocrine pancreas, the peak pancreatic B-cell mass is determined early in life. Thus, in rats, the number of B cells increases rapidly some days before birth, whereas in humans their development occurs during intrauterine life and infancy.[1]

Alterations in the maternal metabolic milieu during pregnancy influence the development and functional maturation of B cells.[2] In addition, nutritional deprivation before and after birth impairs neonatal B-cell proliferation and reduces B-cell mass, islet size, and islet vascularization.[3,4] Such structural and functional damage during these phases represents a potential hazard for the development of diabetes mellitus in adult life. Because the growth of B cells and insulin secretion during fetal life are predominantly regulated by amino acids, protein restriction in early life may play a major role in the appearance of type 2 diabetes.[5]

Insulin secretion by pancreatic B cells is modulated by nutrients, neurotransmitters, and hormones.[6] Glucose, the major physiologic stimulator of insulin secretion, is transported into the B cell through GLUT2, is phosphorylated by

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Table 1 Composition of the normal and low protein diets (g/Kg)

Ingredient	Normal protein (17% protein)	Low protein (6% protein)
Casein (84% protein)	202.0	71.5
Cornstarch	397.0	480.0
Dextrinized cornstarch	130.5	159.0
Sucrose	100.0	121.0
Soybean oil	70.0	70.0
Fiber	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 chlorydrate	2.5	2.5

*Detailed composition given by Reeves *et al.*[16]

glucokinase, and then undergoes glycolysis and oxidation.[7,8] This leads to an increase in the cytosolic adenosine triphosphate (ATP)/adenosine diphosphate (ADP) ratio, which blocks the ATP-sensitive K^+ channels (K_{ATP}) located at the plasma membrane,[9] and leads to the opening of voltage-dependent Ca^{2+} channels; the latter results in an influx and subsequent elevation of cytosolic calcium ($[Ca^{2+}]_i$).[10] The increase in $[Ca^{2+}]_i$ triggers the exocytosis of insulin-containing granules.[11] In addition, an elevation in $[Ca^{2+}]_i$ activates adenylate cyclase and phospholipase C, with the consequent generation of cyclic adenosine monophosphate (cAMP), diacylglycerol (DAG), and inositol-1,4,5-trisphosphate (IP_3). These second messengers amplify the $[Ca^{2+}]_i$ signal by mobilizing intracellular Ca^{2+} stores and promoting phosphorylation proteins that sensitize the secretory process to Ca^{2+}. Amino acids also stimulate insulin release by increasing the $[Ca^{2+}]_i$.[12,13]

Glucose insensitivity and/or alterations in the dynamics of insulin release induced by glucose have been considered early indicators of defective insulin secretion in diabetes.[14] Disturbances in the regulation of Ca^{2+} fluxes and $[Ca^{2+}]_i$ are also common features in animal models showing alterations in insulin secretion.[15] In the present study, we investigated the effect of protein malnutrition on insulin release and ^{45}Ca uptake by isolated islets during the fetal and suckling periods. We observed that the reduced insulin secretion in islets from weaned, protein deficient rats in response to glucose and amino acids was associated with a diminished ^{45}Ca uptake by these islets.

Methods and materials

Animals

Virgin female Wistar rats (85–90 days old) from the State University of Campinas breeding colony were housed with males until mating occurred. Vaginal smears were examined daily and pregnancy was dated from the first day on which spermatozoa were identified. Pregnant rats were kept in individual cages at 24°C under a 12-hour light:dark cycle. The rats were separated at random and maintained on an isocaloric diet containing 6% protein [low protein (LP) diet] or 17% protein [normal protein (NP) diet] from the first day of pregnancy until the end of the lactation period (*Table 1*).[16] During the experimental period, the dams had ad libitum access to their respective diets and to water. Spontaneous delivery took place on day 22 of pregnancy. At birth, large litters were reduced to eight pups, thus ensuring a standard litter size per mother and uniform lactation demand. Two groups of male Wistar rats aged 28 days old were used in this study: (1) a NP group consisting of the offspring of rats fed a control diet during pregnancy and lactation and (2) a LP group consisting of the offspring of rats fed a LP diet during pregnancy and lactation. All offspring were weaned at the 25th day after birth. The pups were weighed at birth and at the end of experimental period.

Insulin secretion from isolated pancreatic islets

Fed rats were euthanized by decapitation and the pancreas was removed. Islets were isolated by hand-picking after collagenase digestion of the pancreas, following a technique previously described.[17] Groups of five islets were first incubated for 30 minutes at 37°C in Krebs-bicarbonate solution containing 5.6 mmol/L glucose and equilibrated with 95% oxygen (O_2):5% carbon dioxide (CO_2), pH 7.4. The solution was then replaced with fresh Krebs-bicarbonate buffer and the islets were further incubated for 1 hour with the following concentrations of glucose (in mmol/L): 2.8, 5.6, 8.3, 11.1, 16.7, and 27.7. The incubation medium contained (in mmol/L): NaCl, 115; KCl, 5; $NaHCO_3$, 24; $CaCl_2$, 1; $MgCl_2$, 1; and bovine serum albumin (BSA) 0.1% (w/v). The insulin released by each sample was measured as previously described[18] using rat insulin as the standard. The glucose concentration producing a response that was 50% of the maximum (EC_{50}) was calculated as the mean negative logarithm (pD2). In a second series of experiments, the insulin secretion was measured from islets incubated in medium containing the following secretagogues (in mmol/L): glucose, 8.3; leucine, 20; arginine, 20; glucose, 8.3, plus leucine, 20; and glucose, 8.3, plus arginine, 20.

Uptake of ^{45}Ca by isolated islets

Groups of 150 to 200 islets, derived from the same batch of islets, were preincubated for 30 minutes at 37°C in a medium containing 5.6 mmol/L glucose, pH 7.4. The incubation medium contained (in mmol/L): NaCl, 115; KCl, 5; $CaCl_2$, 1; and $MgCl_2$, 1, buffered with 10 mmol/L N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-sodium hydroxide (HEPES) and bubbled with pure O_2. The islets were then incubated for 5 or 90 minutes in 100 μL of the same medium containing $^{45}CaCl_2$ (60 μCi/mL) and increasing concentrations of glucose (2.8–16.7 mmol/L). At the end of incubation, 900 μL of ice-cold medium containing 2 mmol/L $LaCl_3$ (pH 7.4) was added to stop the reaction. After 60 minutes, the medium was removed and an aliquot was saved to calculate the amount of ^{45}Ca in the solution. The islets were subsequently washed three times with fresh, ice-cold La^{3+}-containing medium. The islets were then placed in a petri dish and transferred (groups of eight islets) to counting vials containing 0.5 mL of ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) (0.5 mmol/L). The uptake of ^{45}Ca was expressed as pmol Ca^{2+} per islet per minute.

Islet perfusion

Groups of 20 islets from LP or NP animals were placed on a Millipore SW 1300 filter (8 μm pore) in a perfusion chamber (four chambers for each perfusion), with equal representation between the groups during each perfusion. The perfusion buffer consisted of Krebs-bicarbonate (see above) that was continuously gassed with a mixture of 95% O_2:5% CO_2. The islets were perifused at a flow rate of 1 mL/min with buffer containing 2.8 mmol/L glucose during the first hour in order to equilibrate the system. After this period, samples were collected for 20 minutes. The glucose concentration was then increased to 22.2 mmol/L and the islets perifused for an additional 40 minutes. Perifusate samples were

collected at 2-minute intervals for the determination of insulin levels as previously described.[18]

Statistical analysis

The results are presented as the mean ± SEM for the number of rats or islets (*N*) indicated. When comparing the NP and LP groups, a nonpaired *t*-test was used. When comparing the uptake of ^{45}Ca, Lavene's test for the homogeneity of variance was initially used to check the fit of the data to the assumptions for parametric analysis of variance (ANOVA). These data were log-transformed to correct for variance heterogeneity or nonnormality[19] and then analyzed by two-way ANOVA, followed by the Tukey-Kramer test to calculate individual differences between groups and among glucose concentrations, and to verify the interactions between these factors.

Materials

P collagenase was from Boehringer Mannheim (Indianapolis, IN USA). Dr. Leclercq-Meyer (Faculty of Medicine, Brussels Free University) kindly provided antiserum against insulin. Standard insulin was from Novo-Nordisk (Copenhagen, Denmark). Activated charcoal was from Pfanstiehl Laboratories Inc. (Waukegan, IL USA). Dextran T70 was from Pharmacia (Uppsala, Sweden). Arginine and leucine were from Fluka Biochemik (Switzerland).$^{45}CaCl_2$ and ^{125}I-insulin were from New England Nuclear Co. (Boston, MA USA). Scintillation fluid was from Merck (Darmstadt, FRG). BSA (fraction V), EGTA, and the other chemicals were from Sigma (St. Louis, MO USA).

Results

The low level of protein in the mother's diet did not affect the litter size (11.0 ± 1.1 pups, $N = 4$ dams from the NP group and 11.2 ± 1.3 pups, $N = 4$ dams from the LP group). At birth and at the end of the suckling period, the body weight in the LP group was significantly lower than that in the NP group (5.0 ± 0.01 g and 20.4 ± 0.1 g, $N = 26$; 5.8 ± 0.02 g and 61.3 ± 0.3 g, $N = 26$, respectively; $P < 0.001$).

The insulin secretion by islets from the NP and LP groups over a glucose range of 2.8 to 27.7 mmol/L is shown in *Figure 1*. In both groups, the relationship between glucose concentration and insulin secretion was sigmoidal. However, in the LP group the dose-response curve was shifted to the right compared with the NP group. The basal insulin release (2.8 mmol/L glucose) was significantly lower in islets from the LP group than in those from the NP group (0.3 ± 0.03, $N = 16$ and 0.4 ± 0.1 ng/islet · h, $N = 13$, respectively; $P < 0.05$). Maximal insulin release, obtained at 27.7 mmol/L glucose was 1.7 ± 0.3 ng/islet · h ($N = 12$) and 7.7 ± 0.9 ng/islet · h ($N = 23$) in the LP and NP groups, respectively ($P < 0.0001$). The half-maximal release values were 13.3 mmol/L and 16.9 mmol/L glucose for the NP and LP groups, respectively ($P < 0.0001$).

As shown in *Figure 2*, the insulin secretion in response to glucose, individual amino acids, and a combination of amino acids and glucose was significantly lower in LP islets than in NP islets. In the NP group, insulin secretion in the presence of 8.3 mmol/L glucose was threefold greater than in the LP group (0.9 ±0.2 ng/islet · h, $N = 6$ and 0.3 ± 0.1 ng/islet · h, $N = 6$, respectively; $P < 0.05$). Leucine stimulated insulin secretion by NP islets more potently than

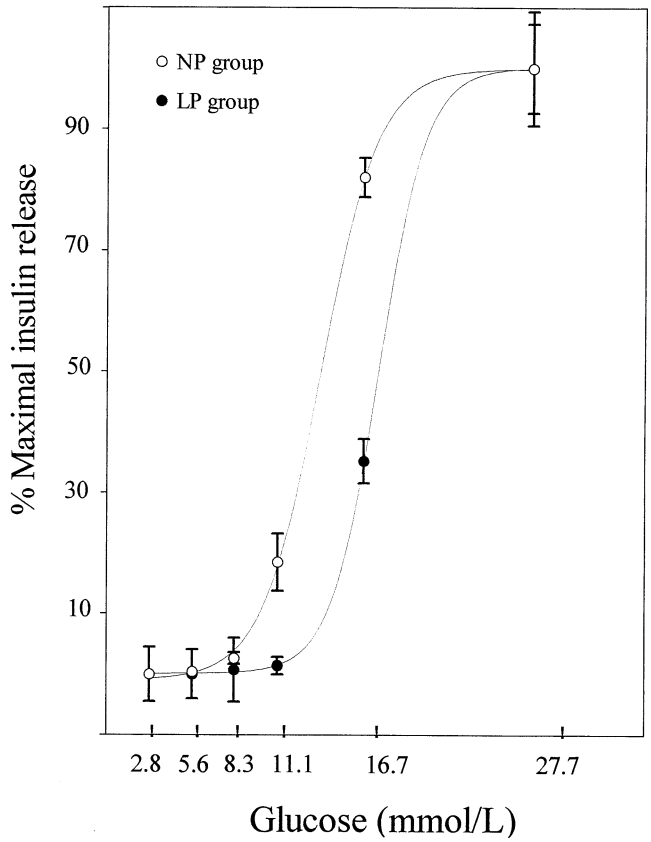

Figure 1 Glucose-stimulated release of insulin by islets from the offspring of mothers fed a normal protein (NP) or low protein (LP) diet during pregnancy and lactation. The islets were incubated for 60 minutes with different concentrations of glucose. Values are the mean ±SEM of four independent experiments expressed as a percent of the maximal insulin secretion in the same experiment. The half-maximal response was obtained with 13.3 mmol/L and 16.9 mmol/L glucose for NP and LP islets, respectively.

did arginine (2.3 ± 0.4 ng/islet · h, $N = 8$ and 1.3 ± 0.1 ng/islet · h, $N = 8$, respectively), whereas in LP islets the insulin secretion in response to these amino acids was similar (0.3 ± 0.1 ng/islet · h, $N = 8$ and 0.2 ± 0.03 ng/islet · h, $N = 8$, respectively). Insulin secretion evoked by a combination of 8.3 mmol/L glucose and 20 mmol/L leucine was significantly higher in the NP group (4.9 and 3.2 times higher, for the NP and LP groups, respectively; $P < 0.0001$). Similar results were obtained for the association of glucose with arginine (3.1 times and 5.4 times in LP and NP islets, respectively, $P < 0.0001$).

The ^{45}Ca uptake by isolated islets after 5-minute and 90-minute incubations is shown in *Figure 3*. After 5-minute incubation (*Figure 3, top*) in a medium containing increasing concentrations of glucose, two-way ANOVA revealed a significant effect of group (df = 1; F = 254.8; $P = 0.00$) and glucose concentration (df = 1; F = 123.7; $P = 0.00$) as well as an interaction between groups by concentration (df = 2; F = 18.5; $P = 0.00$). In basal conditions (2.8 mmol/L glucose), the ^{45}Ca uptake was higher in islets from NP offspring compared with the LP group (0.9 ± 0.03 pmol/islet · 5 min, $N = 24$, and 0.5 ± 0.03 pmol/islet · 5 min, $N = 19$, respectively, $P < 0.0001$). In both groups, the

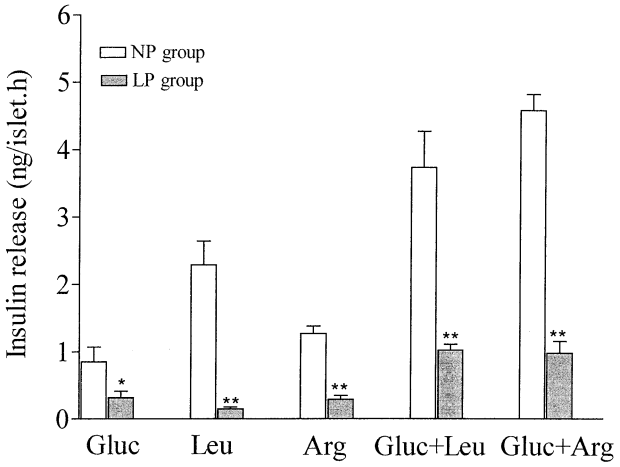

Figure 2 Insulin release by islets in response to various stimuli. The mothers were fed a normal protein (NP) or low protein (LP) diet during pregnancy and lactation. Gluc, 8.3 mmol/L; Leu, 20 mmol/L; Arg, 20 mmol/L; Gluc+Leu and Gluc+Arg, same concentrations as the individual stimuli. Values are the mean ±SEM of six to eight experiments. *$P <$ 0.0001 compared with LP islets.

incorporation of ^{45}Ca in response to 8.3 mmol/L glucose was higher when compared with the basal concentration (1.4 ±0.1 pmol/islet · 5 min, $N = 19$, and 0.9 ± 0.1 pmol/islet · 5 min, $N = 23$, in the NP and LP groups, respectively; $P < 0.0001$). At 16.7 mmol/L glucose, there was an additional increment in the ^{45}Ca uptake (compared with 8.3 mmol/L glucose) in islets from the NP group (2.0 ± 0.1 pmol/islet · 5 min, $N = 19$, $P < 0.001$), but not in those from the LP group (0.9 ±0.1 pmol/islet · 5 min, $N =$ 32). After a 90-minute incubation (*Figure 3, bottom*), two-way ANOVA showed a significant effect of groups (df = 1; F = 6.0; $P = 0.015$), glucose concentration (df = 2; F = 47.2; $P = 0.000$), and an interaction between groups by glucose concentration (df = 2; F = 8.9; $P = 0.0002$). At 2.8 mmol/L glucose, no difference in ^{45}Ca uptake was observed between islets of the two groups (2.3 ± 0.1 pmol/islet · 90 min, $N = 26$, and 2.4 ± 0.2 pmol/islet · 90 min, $N = 24$, respectively). In the presence of 8.3 mmol/L glucose, the ^{45}Ca uptake by islets from the NP and LP groups was not different (3.3 ± 0.3 pmol/islet · 90 min, $N =$ 37, and 2.6 ± 0.3 pmol/islet · 90 min, $N = 16$, respectively). In both groups, the ^{45}Ca uptake increased only between 2.8 mmol/L and 16.7 mmol/L glucose ($P < 0.01$ for the LP group, and $P < 0.0001$ for the NP group). The ^{45}Ca uptake at 16.7 mmol/L glucose was significantly higher in NP islets compared with the LP group ($P < 0.0001$).

The dynamics of insulin secretion by isolated islets is shown in *Figure 4*. At a low concentration of glucose (2.8 mmol/L), the insulin secretion was similar in both groups. The mean secretion of the first 20 minutes of the perfusion period was 6.6 ± 0.5 pg/islet · min, $N = 4$, and 7.9 ± 0.7 pg/islet · min, $N = 4$, for LP and NP islets, respectively). In NP islets, glucose (22.2 mmol/L) elicited a typical biphasic insulin secretion with a rapid first-phase release reaching a peak 19-fold greater than basal (151.5 ± 14.7 pg/islet · min) after 4 minutes of exposure to high glucose. This was followed by a nadir and a slow rising second phase, which

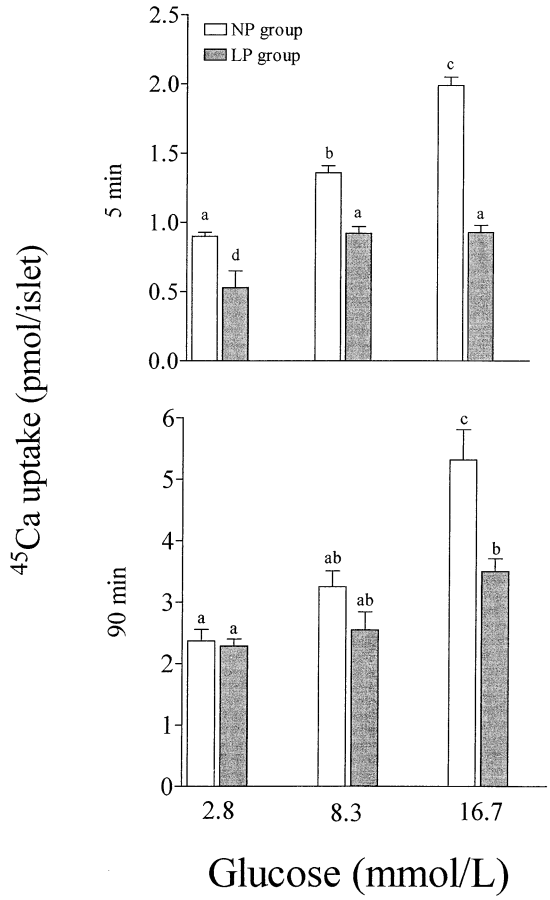

Figure 3 ^{45}Ca uptake by islets from the offspring of mothers fed a normal (NP) or low protein (LP) diet during pregnancy and lactation. The uptake was measured after 5 minutes (*top*) and 90 minutes (*bottom*) incubations in media containing different concentrations of glucose and $^{45}CaCl_2$ (60 μCi/mL). Note difference in the vertical scales. Values are the mean ± SEM of 16 to 26 (5 minutes) and 19 to 32 (90 minutes) experiments. The letters indicate significant differences between groups and conditions.

reached constant values 24 minutes after the introduction of 22.2 mmol/L glucose to the perfusion medium. The pattern of insulin secretion by LP islets was different, with an immediate and small peak of insulin release attaining 11.4 ± 6.5 pg/islet · min (twofold increase over basal levels; $P <$ 0.01) that was maintained thereafter. Maximal insulin secretion was reached 18 minutes after the exposure to 22.2 mmol/L glucose (13.1 ± 3.3 pg/islet · min; $N = 4$). Throughout exposure to high glucose concentration, the insulin secretion by LP islets (10.1 ± 0.7 pg/islet · min; $N =$ 4) was significantly lower than for NP islets (64.5 ± 6.5 pg/islet · min, $N = 4$; $P < 0.0001$).

Discussion

Protein malnutrition during pregnancy is known to affect the structure of the endocrine pancreas[3] and to impair insulin secretion by fetal islets stimulated in vitro with glucose, leucine, and arginine.[20] In the present study, the administration of a LP diet to the mother was extended up to the end of the suckling period and resulted in pups with

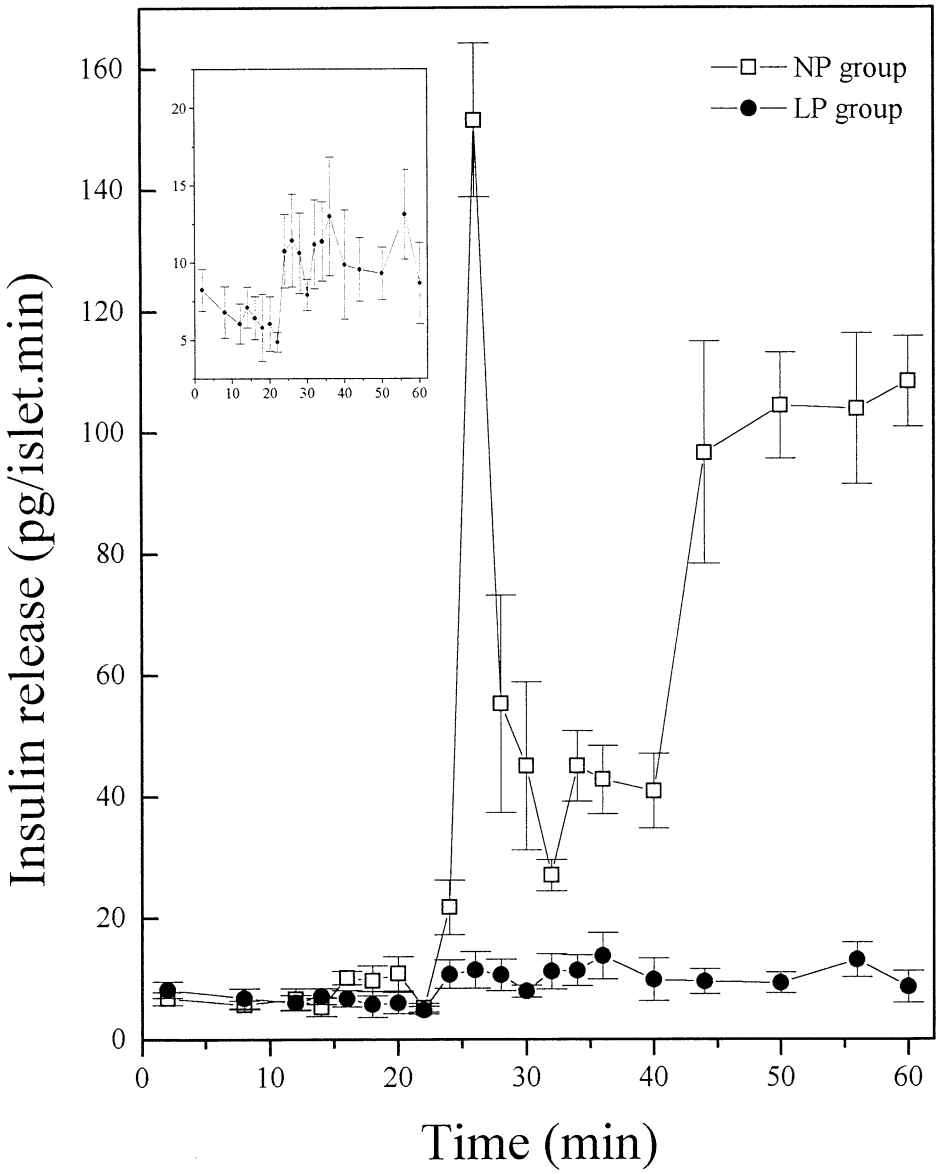

Figure 4 Kinetics of the glucose-induced insulin release by islets from the offspring of mothers fed a normal protein (NP) or low protein (LP) diet during pregnancy and lactation. The glucose concentration from the start until the 20th minute was 2.8 mmol/L. Thereafter the concentration was raised to 22.2 mmol/L until the end of the experiment. The inset shows the insulin secretion from LP islets. Each point represents the mean ± SEM of four experiments.

a reduced body weight at birth and at weaning. An elevated free fatty acid level, decreased serum insulin level in the fed state, and a poor in vivo insulin response to glucose were observed in this experimental model.[21]

In accordance with in vivo data, islets from protein-deprived rats showed impaired insulin secretion in response to glucose, as well as an increased EC_{50} value, as previously described for adult rats submitted to protein malnutrition after weaning.[22] Different, but not exclusive, explanations have been proposed for the reduced glucose-induced insulin release. These include: (1) a smaller size and/or cell volume of B cells,[23] (2) an inappropriate recognition of glucose as a stimulus due to glucoreceptor underexpression and/or decreased metabolism of glucose (substrate site),[24] (3) an alteration in mitochondrial glucose oxidation due to impaired activity of the B-cell mitochondrial glycerophosphate dehydrogenase, possibly associated with other enzymatic anomalies,[25] and (4) a diminished capacity of glucose to increase the Ca^{2+} uptake and/or to reduce Ca^{2+} efflux from B cells.[22]

In our animal model, protein deficiency did not reduce the pancreatic insulin stores[21] and in the present study LP islets exhibited reduced sensitivity to glucose (increased EC_{50}). This finding supports the idea that the decreased response to glucose and other stimuli cannot be explained by differences in islets size and/or cell volume, but is caused by a defect in the secretory mechanism. The impaired response to leucine and arginine, however, indicates that the restriction of secretion must involve some common step in the stimulus-secretion coupling. Leucine is metabolized

independently of glycolysis before entering the oxidative cycle,[26] but is similar to glucose in its effect on K^+ and Ca^{2+} permeability.[12] Arginine exerts an insulinotropic action by its entry via the cationic amino acid transporter 2A,[27] and by its accumulation inside B cells, thereby provoking membrane depolarization and Ca^{2+} entry through voltage-dependent Ca^{2+} channels.[13] An increase in $[Ca^{2+}]_i$ has been shown to play a crucial role in the regulation of insulin secretion by pancreatic B cells in response to various stimuli, including nutrients.[28] For these reasons, we investigated the Ca^{2+} handling in response to glucose after 5 minutes and 90 minutes of incubation to examine the influx and retention, respectively, of ^{45}Ca. The concentration response curves for ^{45}Ca in the presence of increasing concentrations of glucose paralleled those for insulin secretion. The ^{45}Ca uptake curve for malnourished islets was shifted to the right in relation to that for normal islets. Islets from malnourished rats generally exhibited a low ^{45}Ca influx at all glucose concentrations, and the increase in ^{45}Ca uptake over basal at 16.7 mM glucose was lower than in the NP group. These data confirmed previous reports[22,29,30] that pointed to abnormalities in the Ca^{2+} fluxes and in $[Ca^{2+}]_i$ as possible causes for a reduced insulin secretion in response to different nutrients in malnourished animals.

Because the ability of glucose to modulate K^+ permeability in islets from malnourished rats was not affected,[29] alterations of the K_{ATP} channel could be ruled out. The blockade of K_{ATP} channels, which provokes insulin secretion when Ca^{2+} is present, involves the metabolism of glucose and inhibition of fatty acid oxidation.[31] We speculate, therefore, that the unresponsiveness and insensitivity of LP islets to glucose and amino acids reflect activation of the glucose–fatty acid cycle[32] caused by the chronically elevated levels of serum fatty acids. A metabolic shift to fatty acid oxidation may accelerate the production of acetyl-CoA and result in inhibition of the glycolytic flux and in a reduction of the cytosolic levels of long-chain acyl-CoA. These long-chain esters produce or activate second messengers (e.g., DAG) that stimulate enzymes (e.g., protein kinase C isoforms) and exocytosis.[33]

The kinetics of insulin secretion exhibited by our results were entirely consistent with this hypothesis. Normal islets responded to high glucose with a considerable increase in insulin secretion in a clear biphasic pattern. In malnourished islets, glucose evoked an immediate but smaller peak of insulin release that was maintained thereafter. Curiously, the kinetics of insulin release displayed by our malnourished animals were comparable to those exhibited by islets from adult rats subjected to starvation[34] and by suckling neonate islets from rats receiving a high-fat diet.[35] In the latter animals, alterations in the insulin release pattern were attributed to an inhibitory effect of fatty acid oxidation on glucose metabolism. The biphasic pattern of insulin secretion reflected the release of two different pools of granules and was associated with the electrical activity of the membrane, which requires changes in $[Ca^{2+}]_i$ and second messengers. The first phase of secretion is produced predominantly by ion fluxes and corresponds to the release of granules located near the plasma membrane. The second phase is regulated by the cAMP and phosphoinositol pathways and results from the mobilization of granules located in a reserve pool.[36,37] Calcium has a role in regulating both phases through its direct effect on the exocytotic process and by modulating the cAMP and phosphoinositol pathways.[36] Hence, the suppressed Ca^{2+} influx associated with an alteration in phosphoinositol metabolism following the activation of fatty acid oxidation would contribute to abnormal kinetics of insulin release.

In conclusion, our results indicate that in islets derived from malnourished rats, the poor secretory response to glucose and amino acids may occur through altered Ca^{2+} homeostasis. Abnormalities in Ca^{2+} handling may be, at least in part, a consequence of alterations in nutrients metabolism as a result of long-term exposure to fatty acids.

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