Protein calorie restriction has opposite effects on glucose metabolism and insulin gene expression in fetal and adult rat endocrine pancreas

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Protein calorie restriction has opposite effects on glucose metabolism and insulin gene expression in fetal and adult rat endocrine pancreas. Am J Physiol Endocrinol Metab 286: E542–E550, 2004. First published December 9, 2003; 10.1152/ajpendo.00242.2003.—We previously demonstrated that fetuses from undernourished pregnant rats exhibited increased β-cell mass and hyperinsulinemia, whereas keeping food restriction until adult age caused reduced β-cell mass, hypoinsulinemia, and decreased insulin secretion. Because these alterations can be related to insulin availability, we have now investigated early and long-term effects of protein calorie food restriction on insulin mRNA levels as well as the possible mechanisms that could modulate the endogenous insulin mRNA content. We used fetuses at 21.5 days of gestation proceeding from food-restricted mothers until adult age and with respective controls. Insulin mRNA levels, glucose transporters, and total glycolysis and mitochondrial oxidative fluxes were evaluated. We additionally analyzed undernutrition effects on signals implicated in glucose-mediated insulin gene expression, especially pancreatic duodenal homeobox-1 (PDX-1), stress-activated protein kinase-2 (p38/SAPK2), and phosphatidylinositol 3-kinase. Undernourished fetuses showed increased insulin mRNA, oxidative glucose metabolism, and p38/SAPK2 levels, whereas undernutrition until adult age provoked a decrease in insulin gene expression, oxidative glucose metabolism, and PDX-1 levels. The results indicate that food restriction caused changes in insulin gene expression and content leading to alterations in glucose-stimulated insulin secretion. The molecular events, increased p38/SAPK2 levels in fetuses and decreased PDX-1 levels in adults, seem to be the responsible for the altered insulin mRNA expression. Moreover, because PDX-1 activation appears to be regulated by glucose-derived metabolite(s), the altered glucose oxidation caused by undernutrition could in some manner affect insulin mRNA expression.

glucose metabolism; insulin messenger ribonucleic acid; pancreatic duodenal homeobox-1 levels; insulin synthesis; insulin secretion

It is widely known that dietary influences during early stages of development present a risk factor for the onset of both perinatal and later-life diseases. As indicated by the “thrifty phenotype hypothesis” (21), the endocrine pancreas may be particularly susceptible to the effects of poor maternal nutrition, since fetal and postnatal periods are critical for β-cell development and maturation of pancreatic function. In this way, it has been shown that isocaloric low-protein diets throughout pregnancy impair fetal endocrine pancreas development (35) and the secretory response in pancreatic islets of fetal (12) and adult (33) animals. Similarly, maternal general food restriction in late pregnancy also leads to alterations in β-cell development. Both decreases (5, 16) and increases (2) in pancreatic insulin stores and β-cell mass have been described in fetuses from food-restricted mothers, whereas in the neonatal period a significant and persistent decrease in these parameters has been found at days 1 (16) and 4 (26) of postnatal life. This effect persists until adulthood (26) and can provoke long-lasting consequences in the endocrine pancreas under situations of increased insulin demand, such as aging (17) and pregnancy (6). Consequently, experiments with animal models have provided enough evidence that nutritional intervention leads to different adaptations at cellular (16, 35), biochemical (4, 33, 41), and molecular levels (3). The result indicates a persistent alteration of β-cell mass and several defects in insulin response in food-restricted animals.

Impaired insulin secretion in undernutrition has been related to alterations at different steps in the secretion mechanism itself (4, 33, 41). However, changes in β-cell mass and in insulin release may also result from alterations in insulin biosynthesis, as previously described in models of low-protein diets (3, 35). Long-term regulation of insulin secretion is then controlled by the hormone availability and the β-cell ability to produce it.

Insulin synthesis in β-cells is regulated by nutrients, among which glucose is physiologically the most significant (40). However, mechanisms through which glucose regulates insulin gene expression are not as well understood as those that control insulin secretion. Over short periods of time, glucose regulates insulin biosynthesis mainly by increasing the translation of insulin mRNA; but over longer periods, glucose increases insulin mRNA levels (13). An increase in glucose metabolism generates a sequence of secondary-stimulus coupling factors, which in turn activate downstream signaling elements influencing both insulin gene expression and insulin mRNA translation (31). The homeodomain pancreatic duodenal homeobox-1 (PDX-1) is an important transcription factor in β-cells that seems to play an essential role in linking glucose metabolism with insulin gene transcription (28). PDX-1 DNA-binding activity and insulin promoter activity are modulated via a signaling pathway involving stress-activated protein kinase-2 (p38/SAPK2) (27). Although p38/SAPK2 seems to be activated in response to adverse stimuli and under stress conditions (29), it has also been shown that glucose metabol-
lism is able to modulate the activity of p38/SAPK2 through a cell-signaling pathway that implicates phosphatidylinositol 3-kinase (PI 3-kinase) (42).

Using a rat model based on a protein calorie deficiency [65% reduction in daily food intake (14)], we previously established that β-cell mass and insulin secretion increase in fetuses at 21.5 days of gestation (2) and decrease in 70-day-old adult rats (26). However, the biochemical and molecular adaptations responsible for the alterations we found are currently unknown. Therefore, in this study, we have investigated the early and long-term effects of protein calorie food restriction on insulin mRNA levels as well as the possible mechanisms that could modulate the endogenous insulin mRNA content. For this purpose, glucose transporters (GLUT2 and GLUT1) as well as total glycolysis and mitochondrial oxidative fluxes were evaluated. Moreover, we also analyzed the undernutrition effects on signals implicated in glucose-mediated insulin gene expression, especially PDX-1, p38/SAPK2, and PI 3-kinase.

**MATERIAL AND METHODS**

**Animals and diets.** Wistar rats bred in our laboratory under a controlled temperature and artificial dark-light cycle (from 0700 to 1900) were used throughout the study. Females were caged with males, and mating was confirmed by the presence of spermatozoa in a vaginal smear. Animals were fed a standard laboratory diet (19 g protein, 56 g carbohydrate, 3.5 g lipid, 4.5 g cellulose/100 g plus salt and vitamin mixtures). In both the fetal and adult periods, food restriction was established beginning on day 14 of pregnancy. In the fetal period, the pregnant undernourished group received 35% of the food intake of a pregnant control group until 21.5 days of pregnancy. Then, mothers were put under intraperitoneal pentobarbital sodium anesthesia (4 mg/100 g body wt), and fetal blood was obtained after auxiliary artery incision of fetuses while still connected to the maternal circulation; serum was separated and stored frozen at −20°C until analyzed. Fetuses were individually weighed, and pancreases were dissected, weighed, and extracted for determination of insulin content (see Insulin content and insulin release from isolated pancreatic islets). In the adult period, rats were 65% food restricted during the last week of gestation, and the food restriction was extended during the suckling and adult period until 70 days of life. At this time, the rats were decapitated. Serum was separated and stored at −20°C until analyzed. The pancreas was dissected, weighed, and extracted for determination of its insulin content. Control rats in both periods were given access to food ad libitum. Water was available ad libitum to all groups. Food intake of control and undernourished rats has been given previously (14).

All studies were conducted according to the principles and procedures outlined in the National Institutes of Health Guidelines for Care and Use of Experimental Animals.

**Fetal islet culture and isolation.** Islets from fetal rats (21.5 days of gestation) were obtained according to the method of Hellerström et al. (23). Briefly, 20–24 pancreases were minced in sterile Hanks’ solution. The fragments were transferred to a sterile vial containing Hanks’ solution supplemented with 5–7 mg of collagenase (Boehringer Mannheim, Mannheim, Germany). The vial was shaken for 10 min at 37°C, and the tissue digest was washed three times with Hanks’ solution. The pellet was resuspended in culture medium and transferred to 4–6 plastic dishes containing the same medium. The culture medium consisted of RPMI 1640 (ICN, Nuclear Iberica, Madrid, Spain) with 200 mM L-glutamine, penicillin, and streptomycin and 10% fetal bovine serum (ICN). The islets were maintained at 37°C in an atmosphere of 5% CO2 and the medium was renewed every 48 h. After 5–6 days in culture, the islets were gently detached from the plates, and clean islets were individually transferred under a dissecting microscope and immediately used for experiments. Reversal effects in the islets after 8 days of culture can be disregarded, since all conditions were compared with control plates cultured for the same period.

**Adult islet isolation.** Islets of 70-day-old rats from each group were isolated by the collagenase digestion procedure of Malaisse-Lagae and Malaisse (25). Briefly, islets were isolated from the pancreases of two or three rats and subsequently separated from the remaining exocrine tissue by hand-picking under a dissecting microscope. The islets were immediately used for experiments. Collagenase from Clostridium histolyticum was purchased from Boehringer Mannheim. Hanks’ solution saturated with O2–CO2 (95:5%) was used during the isolation procedure.

**Insulin content and insulin release from isolated pancreatic islets.** For the study of β-cell secretory function, islets in groups of seven were incubated for 90 min at 37°C in plastic microbeakers placed in sealed glass vials and containing 1.0 ml of Krebs-Ringer bicarbonate medium (24) equilibrated against a mixture of 95% O2–5% CO2 and supplemented with 5.0 mg/ml BSA (fraction V; Sigma Chemical, St. Louis, MO) and one of the following: 2.8 or 16.7 mM glucose. At the end of the incubation period, aliquots of the medium were stored at −20°C until assayed for insulin by use of the RIA described below.

Groups of 20 freshly isolated islets were sonicated in acid-ethanol (1.5 ml 12 M HCl/100 ml ethanol) and stored at −20°C for determination of insulin content, which was measured by RIA as described below.

**RNA isolation and Northern blot analysis.** RNA was extracted from pancreas obtained from control and undernourished fetuses and adult rats by the guanidinium isothiocyanate-phenol-chloroform method (11). After quantification, total RNA (30 μg in fetuses and 40 μg in adults) was submitted to Northern blot analysis following the method previously described (38). Insulin cDNA was kindly provided by S. J. Chan [Howard Hughes Medical Institute, Univ. of Chicago (101)] and used as probe. Rat ins1 contains a 399-bp EcoRI-HindIII fragment encoding a preproinsulin I cDNA inserted into a pGEMH2 plasmid. Membranes were autoradiographed, and an 18S probe was used as control for RNA loading. Relative densities of signals were determined by densitometric scanning of the autoradiograms in a laser densitometer (Molecular Dynamics, Sunnyvale, CA).

**Western blot analyses for GLUTs.** Groups of 300–500 islets per condition were frozen in liquid nitrogen and kept at −80°C until analyzed. From GLUT2 and GLUT1 determination the islets were sonicated in 3% SDS, 120 mM Tris-HCl, pH 6.8, 7.5 mM EDTA, 5% 2-mercaptoethanol, 1.5 mM PMSF, and 15% glycerol. Equivalent amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis on 10% polyacrylamide gels at 125 V. Proteins were then electrophoretically transferred to polyvinylidene difluoride (PVDF) filters (PVDF Protein Sequencing Membrane, Bio-Rad Laboratories, Madrid, Spain) at 100 V for 2 h. After transfer, the filters were blocked with 5% (wt/vol) nonfat dry milk in phosphate-buffered saline for 2 h at room temperature. Antibodies against the GLUT2 and GLUT1 were purchased from Biogenesis (Dorset, UK) and were used at dilutions of 1:1,000 and 1:5,000, respectively. The PVDF filters were then washed four times for 10 min at 37°C with phosphate-buffered saline with 0.1% Tween 20 followed by a 1-h incubation with goat anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Sigma BioSciences, St. Louis MO). The PVDF membranes were then washed as indicated above. Detection of antibody-antigen complexes was accomplished by the enhanced chemiluminescence method (BM Chemiluminescence, Boehringer Mannheim). Optical densities of bands corresponding to specific glucose transporters were determined by laser scanning densitometry (Molecular Dynamics). Immunoblots were performed under conditions of linearity according to the amount of protein loaded on the gel. The PVDF filters were finally stained with Coomassie blue to confirm that in the same Western assay equal amounts of protein were analyzed as well as to ensure the heterogeneity of the protein composition pattern in the different samples.
Islet glucose metabolism. Glucose utilization was based on the formation of $^3$H$_2$O from d-[5-$^3$H]glucose, and oxidation of glucose was measured as $^{14}$CO$_2$ production from d-[6-$^{14}$C]glucose. The method used to determine simultaneously the conversion of d-[5-$^3$H]glucose into $^3$H$_2$O and d-[6-$^{14}$C]glucose into $^{14}$CO$_2$ has been reported elsewhere (24). For measurement of glucose utilization and oxidation, groups of 20 islets each were incubated in 40 μl of bicarbonate-buffered medium (23) containing BSA (5 mg/ml), HEPES (10 mM), and the appropriate d-glucose concentration with a tracer amount of both d-[5-$^3$H]glucose and d-[6-$^{14}$C]glucose (40 μCi/ml and 10 μCi/ml, respectively; Amersham Pharmacia). The incubation was at 37°C for 120 min. The islet metabolism was stopped by the addition of 20 μl of a citrate-NaOH buffer (400 mM, pH 4.9) containing antimycin A (0.01 mM), rotenone (0.01 mM), and KCN (5.0 mM). The $^{14}$CO$_2$ was trapped in 0.250-ml hyamine hydroxide (Packard) over a 60-min incubation period at 20°C. After a 20- to 22-h incubation at 20°C, the recovery of $^3$H$_2$O formed by the islets was determined by liquid scintillation counting (1209 Rackbeta; LKB Wallac, Turku, Finland).

The values obtained concerning glucose use were corrected in accordance with the recovery of $^3$H$_2$O from known amounts of $^3$H$_2$O, which in these experiments was 35 ± 1.1%.

Determination of PDX-1, total p85, and p38/SAPK2. The content of PDX-1 and the p85 subunit of PI 3-kinase and p38/SAPK2 were analyzed by Western blot. Protein extracts were obtained from islets sonicated in a homogenization buffer (10 μM leupeptin, 2 mM O-vanadate, 2 mM benzamidine, 10 μg/ml apro tin, and 2 mM phenylmethylsulfonyl fluoride in 12.5 mM EGTA, 1.25 mM EDTA, and 0.25% Triton X-100, pH 7.6). Equal amounts of protein were separated on a 10% SDS-polyacrylamide gel. Proteins were then electrophoretically transferred to PVDF filters and probed with the different antibodies: polyclonal anti-rat PDX-1 (from Chemicon, Temecula, CA), polyclonal anti-rat p85 subunit of PI 3-kinase (from Upstate Biotechnology, Lake Placid, NY), and polyclonal anti-rat p38/SAPK2 (from Santa Cruz Biotechnology, Santa Cruz, CA). The rest of the Western blot procedure was as described for GLUT determinations, with the following antibody dilutions: anti-PDX-1, 1:10,000; anti-p85, 1:2,000; and anti-p38, 1:1,000.

Other analytical procedures. The concentration of protein was determined by the Bradford method (7) using a protein assay (Bio-Rad Laboratories) with gamma-globulin as standard. Immunoreactive insulin in serum samples and islet extracts was determined by RIA using rat insulin as standard (INCSTAR, Stillwater, MN). This method allows the determination of 2.0 ng/ml, with coefficients of variation within and between assays of 10%. Aliquots of 10 μl of Ba(OH)$_2$-ZnSO$_4$ deproteinized blood were obtained from 30-fetal rats, and $^{14}$C was measured as $^{14}$CO$_2$ production from D-[6-$^{14}$C]glucose. The islet metabolism was stopped by the addition of 20 μl of a citrate-NaOH buffer (400 mM, pH 4.9) containing antimycin A (0.01 mM), rotenone (0.01 mM), and KCN (5.0 mM). The $^{14}$CO$_2$ was trapped in 0.250-ml hyamine hydroxide (Packard) over a 60-min incubation period at 20°C. After a 20- to 22-h incubation at 20°C, the recovery of $^3$H$_2$O formed by the islets was determined by liquid scintillation counting (1209 Rackbeta; LKB Wallac, Turku, Finland).

Statistics. Values are given as means ± SE for the number of rats studied. A two-tailed t-test for independent observations was used for comparisons of the two groups. For all other data comparisons, a one-way analysis of variance (ANOVA), followed by the protected least significant difference test, was used.

Table 1. Characteristics of fetuses at 21.5 days of gestation from control or undernourished mothers and control or undernourished 70-day-old rats

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<td></td>
<td>C</td>
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<tr>
<td>Body weight, g</td>
<td>5.04±0.05</td>
<td>4.17±0.09*</td>
<td>274.5±2.0</td>
<td>121.4±7.1*</td>
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<tr>
<td>Pancreas weight, mg</td>
<td>20.01±0.43</td>
<td>17.51±0.53*</td>
<td>860.4±27.1</td>
<td>475.1±29.3*</td>
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<td>Glycemia, mg/100 ml</td>
<td>51.4±3.1</td>
<td>52.1±1.7</td>
<td>80.1±2.1</td>
<td>81.3±1.6</td>
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<td>Insulinemia, μU insulin/ml</td>
<td>171.3±10.2</td>
<td>218.1±9.7*</td>
<td>52.3±5.1</td>
<td>43.9±3.6*</td>
</tr>
<tr>
<td>Islet insulin content, μU insulin/islet</td>
<td>1.54±1.93</td>
<td>3.14±1.11*</td>
<td>2.36±106</td>
<td>1.610±156*</td>
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Values are means ± SE. Number of data, 10–12. *P < 0.05 relative to control rats. C, control; U, undernourished.

RESULTS

Characteristics of undernourished and control rats. Food restriction of pregnant rats during the 3rd wk of gestation provoked a significant decrease in body and pancreas weight in their fetuses compared with those in controls. No change in glycemia was found in undernourished fetuses, but a significant increase in plasma insulin was observed in this group compared with controls. In accord with this increase, the islet insulin content was significantly higher in fetuses from undernourished rats (Table 1).

Adult rats food restricted from day 14 of gestation until 70 days of life showed a significant decrease in body and pancreas weight compared with controls. No significant difference was found in glycemia between the two groups, whereas insulinemia and islet insulin content were significantly lower in undernourished than in control adult rats (Table 1).

Islet insulin secretion. In agreement with the higher insulinemia and islet insulin content found in undernourished fetuses, the output of insulin was significantly higher than in controls, both in basal (2.8 mM) and in stimulating (16.7 mM) conditions. However, insulin release in response to an increase in glucose concentration from 2.8 to 16.7 mM was significantly lower in undernourished adult rats compared with the control group (Table 2).

To compare the relative levels of insulin secretion, the insulin secretion rate was normalized by the islet insulin content. On this basis, fractional insulin secretion was similar in fetal and adult food-restricted and control rats, indicating that undernutrition affects insulin content but not the mechanism of secretory response (Table 2).

Steady-state insulin mRNA levels. In accord with islet insulin content in undernourished fetuses, insulin mRNA levels were elevated fourfold compared with the control group. Similarly, the reduction in insulin content in undernourished adult rats was accompanied by a reduction in insulin mRNA levels compared with controls (Fig. 1).

GLUT2 and GLUT1 protein levels. Islet GLUT2 and GLUT1 protein content was measured by Western blot analysis of homogenates of islets isolated from control and undernourished fetal and adult rats (Fig. 2). The main peptide band observed had an approximate molecular mass of 45 kDa, which is in close agreement with the estimated molecular mass of the transporters. GLUTs consistently did not appear as a single band in freshly isolated adult islets. This represents proteolytic degradation of GLUTs occurring during the isolation procedure and could not be prevented by addition of protease inhibitors to the collagenase solution (37). For this reason, the
sum of all bands was considered for the densitometric analysis. This effect was not observed in the fetus, because islets were obtained directly from culture 6 days after collagenase extraction.

The densitometric analysis of data from five independent determinations (Fig. 2) revealed that the levels of GLUT2 and GLUT1 were similar in control and undernourished animals in both adult and fetal stages.

Glucose metabolism in pancreatic islets. Figure 3 shows the rate of glucose utilization and oxidation in islets of undernourished fetuses. The oxidation in the tricarboxylic acid (TCA) cycle of acetyl residues derived from exogenous D-glucose was measured by the generation of $^{14}$CO$_2$ from D-[6-$^{14}$C]glucose, and glucose utilization was determined on the formation of $^3$H$_2$O from D-[5-$^3$H]glucose. At a low concentration of glucose (2.8 mM) the rate of glucose oxidation was not significantly different in both fetal populations, but at a higher concentration (16.7 mM) it was much lower in the control fetuses than in fetuses from undernourished mothers. At 16.7 mM, the rate of glucose oxidation in fetuses from undernourished mothers increased 2.5-fold, whereas in fetuses from control mothers this increase was only 1.5-fold. In both fetal populations, the rate of glucose utilization increased significantly at 16.7 mM glucose (~2.0-fold in undernourished fetal group and 1.9-fold in control fetuses). No major impairment of glycolytic flux was found in islets from undernourished fetuses compared with control.

To investigate the relationship between glucose utilization and oxidation, the production of $^3$H$_2$O from D-[5-$^3$H]glucose was measured together with the generation of $^{14}$CO$_2$ from D-[6-$^{14}$C]glucose. Fetuses from undernourished mothers showed a preferential stimulation of glucose oxidation relative to total glycolytic rate, as indicated by the increase in the paired ratio found in this group when the glucose concentration rose from 2.8 to 16.7 mM. However, this fact was not observed in the fetal control group, where the paired ratio remained unchanged.

The rate of glucose utilization and oxidation in control and undernourished adult rats is shown in Fig. 4. Glucose oxidation was much lower in islets from undernourished adult rats than in controls at both low and high glucose concentrations. Interestingly, the increase of glucose concentration to 16.7 mM evoked a similar increase of the rate of glucose oxidation in both adult populations (~4.6-fold in control and 4.7-fold in undernourished rats). In undernourished adult rats, the rate of glucose utilization at the two concentrations of the hexose (2.8 and 16.7 mM) was similar to that of controls. Raising the medium glucose concentration from 2.8 to 16.7 mM significantly increased the rate of glucose utilization in both adult populations, control and undernourished, 3.0- and 3.2-fold, respectively. Therefore, no major impairment of glycolytic flux was observed in islets from adult undernourished rats.

At 16.7 mM, the paired ratio between glucose oxidation and glucose utilization was much lower in islets from undernourished adult rats than in controls at both low and high glucose concentrations. This fact indicates that a rise in glucose concentration (from 2.8 to 16.7 mM) stimulates the oxidation of acetyl residues in the TCA cycle relative to total glycolytic rate in islets from control adults but not in islets from undernourished adult rats.

Islet content of PDX-1, total p85, and p38/SAPK2. The levels of PDX-1, total p85, and p38/SAPK2 were evaluated in

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**Table 2. Insulin secretion and insulin secretion rate in islets from control and undernourished fetal and adult rats**

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<td>U</td>
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<tr>
<td>Glucose/2.8 mM</td>
<td>13.6±0.7</td>
<td>20.1±1.6*</td>
</tr>
<tr>
<td>Glucose/16.7 mM</td>
<td>23.1±4.1†</td>
<td>55.2±3.9*†</td>
</tr>
<tr>
<td>Insulin release, % islet insulin content</td>
<td>1.24±0.04</td>
<td>1.18±1.6†</td>
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<tr>
<td>Glucose/2.8 mM</td>
<td>2.85±0.07</td>
<td>3.13±0.12*†</td>
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Values are means ± SE. Number of data, 12–14. *P < 0.05 relative to control rats. †P < 0.05 relative to glucose 2.8 mM within the same group.
islets from control and undernourished fetal and adult rats. The results showed that the islet levels of PDX-1 were similar in control and undernourished fetuses, whereas islet p38 content was increased by food restriction (1.9-fold over control value) in the fetal period (Fig. 5). In contrast, the amount of PDX-1 in the islets from undernourished adult animals was threefold decreased, and no differences were found in the same period in the levels of p38/SAPK2 (Fig. 6). Moreover, food restriction did not alter the islet content of total p85 regulatory subunit protein of PI 3-kinase either in fetal or in adult period.

DISCUSSION

In a previous work (2), we demonstrated that a 65% protein calorie food restriction during the 3rd wk of gestation provoked in pregnant rats impaired insulin secretion in vivo and glucose intolerance at the end of gestation. Moreover, this disturbance of maternal glucose homeostasis in this model led to an increase in β-cell mass and hyperinsulinemia in the fetuses at 21.5 days of gestation. However, from 4 days of life, concordant with the literature (16), we observed decreased β-cell mass and hypoinsulinemia, effects that persisted until adult age (26). Under these conditions, our experimental model shows different responses depending on whether fetal or adult stages are considered. Nevertheless, both periods have in common the existence of alterations at the cellular level and defects in insulin secretion as a consequence of the nutritional intervention. The present study shows how changes at the metabolic and molecular levels seem to be involved in alterations that we found in insulin secretion in the two stages.

The hyperinsulinemia observed in undernourished fetuses appeared to be accompanied by increased islet insulin content and enhanced glucose-dependent insulin secretion in vitro, whereas the adult hypoinsulinemia was accompanied by decreased insulin content and insulin-secretory response in vitro. However, when the insulin secretion rate was normalized with the insulin islet content, the fraction of insulin release in both populations was similar to that in controls. These results indicate that β-cell secretory mechanisms seem not to be altered in undernourished animals, and at the same time, they appear to indicate a regulatory role of insulin content in insulin-secretory response. Similarly, it has been reported that increases in insulin content lead to higher insulin secretion (43), whereas decreased insulin content and proinsulin biosynthesis can be related to decreases in glucose-induced insulin secretion (44).

To determine whether the insulin content alterations that we found could be caused by alterations in insulin mRNA levels, we assessed this parameter at steady state in pancreas of undernourished and control animals, and, in agreement with the above observations, we found it to be higher in undernourished fetuses and lower in undernourished adults compared with their respective controls. The increased mRNA levels in our undernourished fetuses may result from the increased insulin requirements as a consequence of postprandial hyperglycemic episodes generated by the altered glucose homeostasis present in pregnant rats (2). An increase in insulin demand modulated by insulin mRNA levels has been observed in corticosterone-induced insulin resistance and after pancreatec-
tomy (20, 32). However, lower insulin mRNA levels seem to be related to fasting periods and undernutrition (19, 39). It can be deduced from our results that, in both fetal and adult animals, undernutrition affects molecular mechanisms, leading to opposite alterations in insulin mRNA levels and, therefore, in the insulin biosynthesis capacity of the β-cell.

The primary physiological stimulus of insulin transcription and synthesis is glucose (40). Because glucose regulates insulin production by signals coming from the glycolytic pathway (18), we evaluated the adaptive changes of glucose metabolism in islets of fetal and adult undernourished animals. Our aim was to determine the extent to which the glucose metabolism could be related to the alterations that we found in our undernutrition model. The present results indicate that undernutrition did not modify the levels of glucose transporters (GLUT2 or GLUT1) in fetal and adult islets. In addition, the total glycolytic flux measured in both populations revealed no major perturbations in intracellular glucose concentration, which indicates no defects in the glucose-sensing mechanism. However, we found impaired glucose oxidative metabolism in islet cells. In accord with what we have shown related to insulin expression and content, islets from undernourished fetuses exposed to a stimulating concentration of glucose (16.7 mM) significantly increased the oxidation of glucose-derived acetyl residues in the TCA cycle with respect to control values, whereas this pathway appeared severely reduced in islets from undernourished adults. These results suggest that the alterations in insulin gene expression may be related, at least in part, to defects in mitochondrial oxidation of glucose. To our knowledge, this is the first time that a possible relation between glucose oxidation and insulin production has been reported in undernourished rats.

β-Cell mitochondrial metabolism has been proven to be necessary for glucose-induced insulin secretion, but in our fetal and adult undernourished islets, oxidative metabolism in the TCA cycle appeared respectively augmented or diminished, whereas the ratio of insulin secretion remained unaffected. Similar results have been found in other malnourished animal models. Impaired insulin secretion has been related to reduced activity of glycerol phosphate shuttle (34), to defects in ATP-sensitive K⁺ channel pathways (41), and to attenuated islet

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**Fig. 3.** t-[6−14C]glucose oxidation (A), t-[5−3H]glucose utilization (B), and paired ratio between glucose oxidation and utilization (C) in islets from fetuses proceeding from C and U mothers. Islets were incubated for 2 h at 37°C with 2.8 and 16.7 mM glucose. Data are expressed as pmol produced/islet−1 h−1 and represent means ± SE of 16–20 individual observations collected in a series of 4 independent experiments. *P < 0.05 relative to C animals. **P < 0.05 relative to 2.8 mM glucose within the same group.

**Fig. 4.** t-[6−14C]glucose oxidation (A), t-[5−3H]glucose utilization (B), and paired ratio between glucose oxidation and utilization (C) in islets from C and U adult rats. Islets were incubated for 2 h at 37°C with 2.8 and 16.7 mM glucose. Data are expressed as pmol produced/islet−1 h−1 and represent means ± SE of 16–20 individual observations collected in a series of 4 independent experiments. *P < 0.05 relative to C animals. **P < 0.05 relative to 2.8 mM glucose within the same group.
phosphatidylinositol hydrolysis (4), whereas glucose oxidation was not found affected. These results suggest, in accord with those from others (15), a permissive role of mitochondrial glucose oxidation, so additional signals must be involved in glucose induced insulin secretion. In addition, our results concerning alterations in the TCA cycle, leading to insulin changes in synthesis but not in secretion, reveal that, although glucose-induced synthesis and secretion have some common steps in stimulus-coupling signaling, both processes must necessarily diverge at some point. It has been recently shown that the divergence steps seem to be at the level of anaplerosis, downstream of glycolysis and upstream of oxidative phosphorylation (1). The nature of the signals from glucose metabolism linked to insulin biosynthesis and gene expression is not currently well known, but our results seem to place these signals at the TCA cycle level, according to a suggestion of Alarcon et al. (1), who proposed that succinate and/or succinyl-

CoA could be the preferential anaplerotic specific signal for regulating glucose-induced insulin biosynthesis.

The coupling factors linking metabolism to insulin gene expression are not yet completely understood. Insulin gene transcription is regulated by the interaction between several regulatory elements and trans-acting factor (31), of which PDX-1 is the main one in mediating the effect of glucose in insulin gene expression (28). Although PDX-1 protein levels in fetal undernourished islets were found similar to those in controls, the levels of p38/SAPK2, the protein involved in PDX-1 activation (27), were markedly increased. SAPK2 is a member of an expanding family of mitogen-activated protein kinases that is activated in response to adverse stimuli (29). The increased p38/SAPK2 levels suggest that food restriction in our undernourished fetuses could lead to a stress-related response in the islets. Similar results have been found in islets from neonatal rats subjected to a high-carbohydrate diet, where increased mRNA levels and SAPK2 activity were related to a...
stress-like situation provoked by dietary treatment (36). SAPK2 is also implicated in insulin gene transcription (30) and in β-cell proliferation (9). Moreover, it has been shown that overexpression of SAPK2 mimics the effects of glucose and results in a hyperstimulation of the PDX-1 activation pathway (27). Therefore, it is reasonable to suggest that the increased β-cell mass (2) and insulin mRNA levels observed in undernourished fetal pancreases may be mediated by increased p38/SAPK2 protein levels in our model.

Besides stress, glucose metabolism is able to activate p38/SAPK2 via a pathway that involves PI3-kinase (42). Although PI3-kinase levels were not found impaired, it cannot be ruled out that increased glucose oxidation in undernourished fetuses can cause higher activation of PI3-kinase and, consequently, of p38/SAPK2. Even when it is not well known which molecule derived from glucose metabolism might activate PI3-kinase, some studies emphasize again the importance of mitochondrial metabolism by showing the pyruvate, glyceroldehyde, and oxoisocaproate ability to stimulate PDX-1 DNA-binding and insulin promoter activity (42).

On the other hand, until 70 days of life, undernourished animals show a significant decrease in the levels of PDX-1. Recently, Arantes et al. (3) also found decreased levels of PDX-1 protein in islets of rats submitted to a low-protein diet and suggest a link among diminished PDX-1 protein expression and alteration in islet size and functionality. Accordingly, in animal models of type 2 diabetes, reduced PDX-1 activity has been related to impaired glucose-stimulated insulin secretion (8), insulin gene expression, and insulin production (22). It is possible that the loss of insulin mRNA found in adult undernourished animals can be partly attributable to a loss of PDX-1. Similarly to the fetuses, the decrease found in glucose oxidation can also be involved in a lower PDX-1 activation in undernourished adults.

In conclusion, the results of this work indicate that, according to our model, food restriction causes changes in the levels of insulin gene expression and content, leading to alterations in glucose-stimulated insulin secretion. The molecular events, an increase in p38SAPK2 levels in fetuses and a decrease in PDX-1 levels in adults, seem to be involved in the altered insulin mRNA expression. Moreover, because PDX-1 activation appears to be regulated by glucose-derived metabolite(s), the altered glucose oxidation caused by undernutrition could in some manner affect insulin mRNA expression. The study of metabolic and molecular mechanisms involved in alterations caused by undernutrition becomes essential to evaluate the facts that can lead to adult diseases. In this context, our rat model provides an opportunity to assess early and long-term events under physiological conditions.

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