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Abstract

A low-protein diet leads to functional and structural pancreatic islet alterations, including islet hypotrophy. Insulin-signaling pathways are involved in several adaptive responses by pancreatic islets. We determined the levels of some insulin-signaling proteins related to pancreatic islet function and growth in malnourished rats. Adult male Wistar rats (N = 20 per group) were fed a 17% protein (normal-protein diet; NP) or 6% protein (low-protein diet; LP), for 8 weeks. At the end of this period, blood glucose and serum insulin and albumin levels were measured. The morphometric parameters of the endocrine pancreas and the content of some proteins in islet lysates were determined. The β-cell mass was significantly reduced ($\cong$65%) in normoglycemic but hypoinsulinemic LP rats compared to NP rats. Associated with these alterations, a significant 30% reduction in insulin receptor substrate-1 and a 70% increase in insulin receptor substrate-2 protein content were observed in LP islets compared to NP islets. The phosphorylated serine-threonine protein kinase (pAkt)/Akt protein ratio was similar in LP and NP islets. The phosphorylated forkhead-O1 (pFoxO1)/FoxO1 protein ratio was decreased by 43% in LP islets compared to NP islets ($P < 0.05$). Finally, the ratio of phosphorylated-extracellular signal-related kinase 1/2 (pErk1/2) to total Erk1/2 protein levels was decreased by 71% in LP islets compared to NP islets ($P < 0.05$). Therefore, the reduced β-cell mass observed in LP rats is associated with the reduction of phosphorylation in mitogenic-related signals, FoxO1 and Erk proteins. The cause/effect basis of this association remains to be determined.

Key words: β-cell mass; Insulin signaling; Low-protein diet; Pancreatic islets; Protein phosphorylation

Introduction

Malnourishment induces functional and structural alterations in the endocrine pancreas in fetal, neonatal (1-4) and adult rats (5-9). We have shown that malnutrition caused by a low-protein diet increases insulin sensitivity in peripheral tissues of adult rats (6,7). Malnourished rats exhibit reduced levels of circulating insulin that may be a consequence of decreased islet responsiveness to glucose (5-7). Reduction of islet size is also observed in these rats (6,9). Other studies have reported alterations in pancreatic islet structure in models of undernutrition. Among these alterations, reduction in β-cell mass and proliferation has been reported, as well as diminished islet vascularization (1-4). Changes in peripheral insulin sensitivity are compensated by reciprocal alterations in islet mass and function (10-13). The interaction between the systemic requirement for insulin and islet function guarantees that blood glucose is kept within physiological ranges in rats when malnutrition is induced by a low-protein diet (6,7). The adaptive modifications in the endocrine pancreas are commonly associated with alterations in the status of the insulin-signaling pathway. Insulin exerts its biological effects by binding to its insulin receptor, which is a hetero-

tetrametric protein with tyrosine kinase activity (14,15). The insulin receptor substrates-1 (Irs-1) and -2 (Irs-2) are high-molecular weight proteins that are phosphorylated at several tyrosine residues by activated insulin receptor protein. These substrates connect to the proteins containing homology domains to Scr2 (SH2), such as the regulatory subunit p85 of phosphatidylinositol 3-kinase (Pi3-k) (16) and adapting proteins such as Grb-2/Sos and Shp-2 (17). The activation of Pi3-k and Grb-2/Sos triggers the signaling cascade of mitogen-activated protein kinase (MAPK) and the sequential activation of the proto-oncogenes Ras and Raf, leading to extracellular-signal regulated kinase-1 (Erk1) and -2 (Erk2) activation. The activation of the insulin-signaling pathway is involved in the regulation of cell differentiation, growth and survival (18).

Reports correlating malnutrition and the insulin pathway during islet compensatory mechanisms are scarce. Here we report the determination of some crucial components of the insulin cascade in a model of hypotrophied pancreatic islets induced by a low-protein diet.

Material and Methods

Material

Collagenase, bovine serum albumin (fraction V), imidazole, ethylene diamine tetraacetic acid, ethylene glycol tetraacetic acid, pepstatin, aprotinin, phenylmethylsulfonyl fluoride, leupeptin, trypsin inhibitor, and Triton X-100 were from Sigma (USA). Western blotting was performed using the Bio-Rad system (USA). All reagents for Western blotting were from Sigma or Bio-Rad. Antibodies against the β subunit of the insulin receptor (IR-β; SC-711), Irs-1 (SC-560) and Irs-2 (SC-9299), serine-threonine protein kinase (Akt; SC-8312), phosphorylated Akt (pAkt; SC-7985, Ser473), Erk1/2 (SC-93), phosphorylated Erk1/2 (pErk1/2; SC-7383), and α-tubulin (SC-8035) were obtained from Santa Cruz Biotechnology (USA). Antibodies against forkhead-O1

(FoxO1; #2488) and phosphorylated forkhead-O1 (pFoxO1; #2486) were from Cell Signaling Technology (USA). The antibody against pancreas duodenum homeobox-1 (Pdx-1) (AB3243) was obtained from Chemicon International, Inc. (USA).

Animals and diet

Male Wistar rats (21 days of age) from the Universidade Estadual de Campinas Animal Breeding Center were maintained at 24°C on a 12-h light/dark cycle. A total of 40 rats were divided randomly into 2 groups (20 per group) respectively fed an isocaloric diet containing 6% protein (low-protein diet, LP group) or 17% protein (control diet, NP group) for 8 weeks. The compositions and differences between diets (6) are described in Table 1. Both diets are isocaloric containing 3.76 kcal/g. During the experimental period, the rats had free access to food and water. The experiments reported here were approved by the Comissão de Ética na Experimentação Animal (CEEA), Instituto de Biologia, UNICAMP.

Metabolic, hormonal and biochemical determinations

On the day after the 8-week treatment, fasted rats (12-14 h) were sacrificed by CO_2 exposure, followed by decapitation. Before sacrifice, blood glucose concentration was measured from the tip tail using a glucosimeter (One Touch, Johnson & Johnson, USA). Serum samples obtained after centrifugation of trunk blood were used for later measurement of insulin levels by radioimmunoassay. The ^{125}I-labeled insulin (human recombinant) for radioimmunoassay was purchased from Amersham Biosciences (UK). Serum albumin levels was also quantified according to manufacturer instructions (*In Vitro* Diagnostica, Brazil) by spectrophotometry (Spectronic® 20 Genesys™, Spectronic Unicam, USA).

Isolation of pancreatic islets

A separate group of fed rats was used for the isolation of pancreatic islets by the collagenase method. Briefly, the pancreas was inflated with Hanks' solution containing 0.8 mg/mL collagenase, and then removed from the rat and kept at 37°C for 23 min. After tissue digestion, the islets were collected manually under a microscope using a siliconized Pasteur pipette and randomly divided into two pools, one for the measurement of islet area "en bloc" and the other for Western blotting experiments.

Islet area, β-cell mass and Pdx-1 cellular distribution

Islet area was measured as previously described (6,19). To study β-cell mass and cellular distribution of Pdx-1, the endocrine pancreas was excised from 4 animals in each group and processed as previously described (19-21). The relative and absolute β-cell mass as well as the islet number per pancreatic area were determined by point-counting morphometry on each pancreas section immunostained for

Table 1. Composition of the control (17% protein) and low-protein (6% protein) isocaloric diets.

Ingredient	Control diet (g/kg)	Low-protein diet (g/kg)
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-93G)	35.0	35.0
Vitamin mix (AIN-93G)	10.0	10.0
L-cystine	3.0	1.0
Choline hydrochloride	2.5	2.5

insulin as previously reported (20,21). A minimum of 500 fields per pancreas were counted. Cell distribution of Pdx-1 was analyzed in sections immunostained for Pdx-1 (21).

Tissue extraction and immunoblotting

Islet protein (100 µg) was used for each experiment as previously reported (20,21). Immunoblotting experiments were performed at least five times. Briefly, the proteins were transferred to a nitrocellulose membrane and checked for transfer efficiency staining with Ponceau S at the end of transfer. The membranes were then blocked with TBST (10 mM Trizma base, 150 mM NaCl, 0.05% Tween 20) plus 5% dried skim milk for 2 h, washed in TBST (3 x 5 min) and incubated overnight with the appropriate primary antibody at room temperature in TBST plus 3% dry skim milk. After washing in TBST (3 x 5 min), membranes were incubated with the appropriate secondary antibody conjugated with HRP for 2 h in TBST plus 1% dried skim milk at room temperature. Antibody binding was detected using the enhanced SuperSignal® West Pico Chemiluminescent Substrate (Pierce, USA), as described by the manufacturer. Blots were scanned (Epson expression 1600) and densitometry of the protein bands was determined by pixel intensity using the Scion Image software (Scion Corporation, USA).

Statistical analysis

All numerical results are reported as means ± SEM of the indicated number (N) of experiments. The Student t-test for unpaired groups was used for comparison between NP and LP data, with the level of significance set at P < 0.05.

Results

Body weight, blood glucose and serum albumin and insulin levels

As stated in Ref. 6, LP rats had a significantly reduced body weight (210 ± 1.8 vs 406 ± 12 g for LP and NP, respectively) and a significant decrease in fasting serum albumin and insulin levels compared to the NP rats (N = 10, P < 0.05). The values for NP and LP were 3.6 ± 0.02 and 3.18 ± 0.06 g/dL for albumin and 0.49 ± 0.01 and 0.26 ± 0.02 ng/mL for serum insulin levels, respectively. The fasting glucose levels were similar in both NP and LP rats (67.1 ± 4.3 and 62.2 ± 3.5 mg/dL, respectively).

Reduced pancreatic islet area and β-cell mass in pancreas from LP rats

The morphometric parameters revealed differences between LP and NP pancreatic islets. The islet area was significantly reduced in LP compared to NP rats (7390 ± 230 vs 15,370 ± 450 µm^2, respectively; N = 400 islets, pooled from 3 rats; P < 0.001). Sections stained for insulin exhibited the pattern of islet size and number in NP and LP rat pancreas (Figure 1A,B). Islets from LP rats seem to be smaller in size compared to those observed in NP rats (compare islets indicated by arrows in Figure 1A and
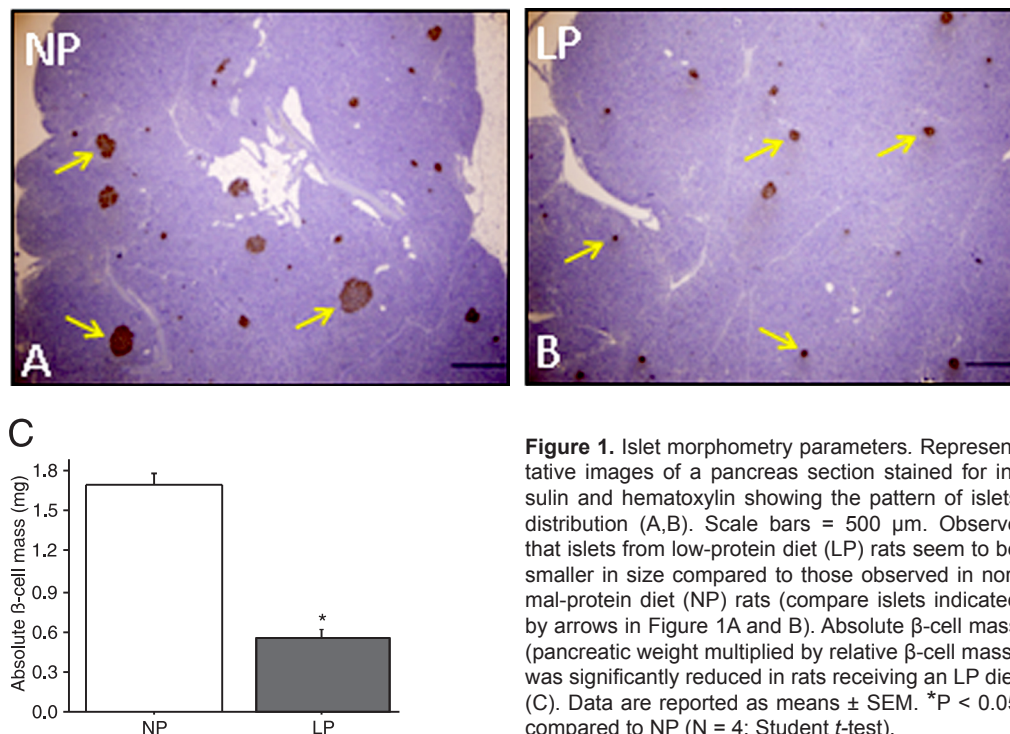

Figure 1. Islet morphometry parameters. Representative images of a pancreas section stained for insulin and hematoxylin showing the pattern of islets distribution (A,B). Scale bars = 500 µm. Observe that islets from low-protein diet (LP) rats seem to be smaller in size compared to those observed in normal-protein diet (NP) rats (compare islets indicated by arrows in Figure 1A and B). Absolute β-cell mass (pancreatic weight multiplied by relative β-cell mass) was significantly reduced in rats receiving an LP diet (C). Data are reported as means ± SEM. *P < 0.05 compared to NP (N = 4; Student t-test).

B). The total pancreas weight was significantly reduced in LP compared to NP rats (0.46 ± 0.04 *vs* 1.35 ± 0.03 g, respectively; N = 4, P < 0.05). Morphometric analysis revealed that the relative β-cell mass was similar in the two groups (1.26 ± 0.07 *vs* 1.18 ± 0.03%, respectively). However, a marked reduction in absolute β-cell mass was observed in LP compared to NP rats after correction for pancreas weight (0.55 ± 0.05 *vs* 1.69 ± 0.08 mg, respectively; P < 0.05, N = 4; Figure 1C). Since LP rats had a reduced body weight, we next normalized the β-cell mass to body weight and observed a reduction in β-cell mass/body

weight of LP compared to NP rats (0.26 ± 0.02 *vs* 0.42 ± 0.02 mg/100 g, respectively; P < 0.05). Finally, the number of islets per pancreatic area was similar in NP and LP rats (1.17 ± 0.06 *vs* 1.53 ± 0.21 islet number/per pancreas area, respectively.

IR-β, Irs-1, and Irs-2 protein content in LP islets

Western blotting experiments were performed to determine whether altered islet size was associated with any modification in insulin receptor and insulin receptor substrate proteins. Figure 2A shows a significant 2.7-fold
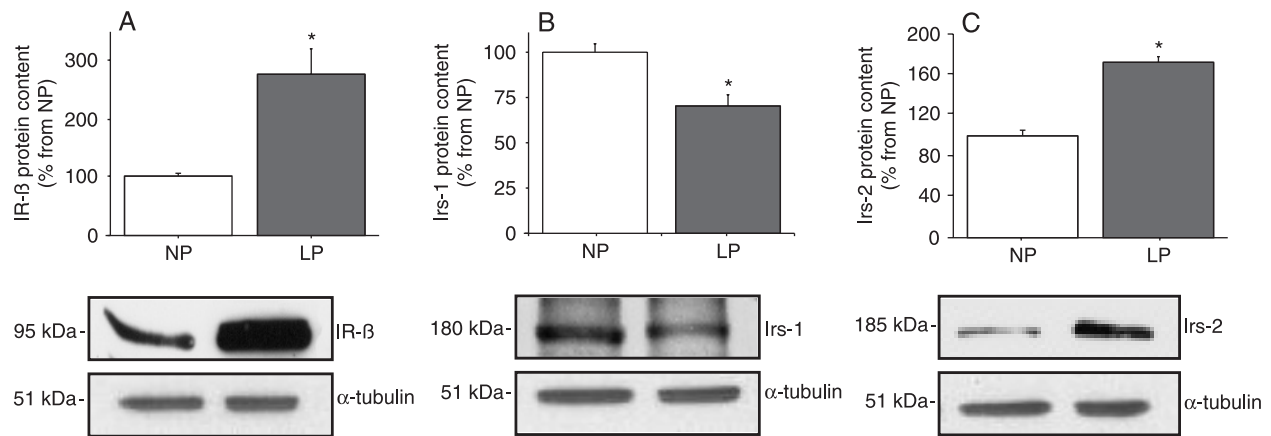

Figure 2. Protein content of *A*, insulin receptor β (IR-β); *B*, insulin receptor substrate-1 (Irs-1), and *C*, insulin receptor substrate-2 (Irs-2) in pancreatic islets from rats receiving a normal-protein diet (NP) and a low-protein diet (LP). A representative control blot for α-tubulin is shown below each panel. Data are reported as means ± SEM for 5 independent experiments. *P < 0.05 compared to NP (Student *t*-test).

Figure 3. Protein ratio of *A*, phosphorylated serine-threonine protein kinase (pAkt)/Akt, of *B*, phosphorylated forkhead-O1 (pFoxO1)/ FoxO1, and of *C*, phosphorylated extracellular signal-regulated kinase 1/2 (pErk1/2)/Erk1/2 in pancreatic islets from rats receiving a normal-protein diet (NP) and a low-protein diet (LP). A representative control blot for α-tubulin is shown below each panel. Data are reported as means ± SEM for 5 independent experiments. *P < 0.05 compared to NP (Student *t*-test).
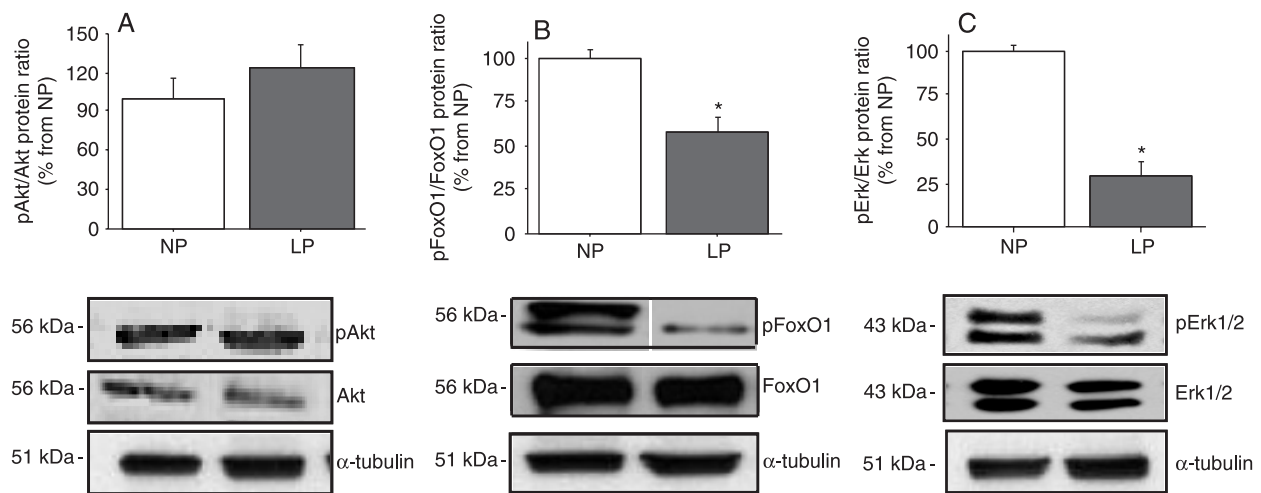
increase in the β subunit content of insulin receptor protein in islets from LP compared to NP rats (P < 0.05). However, the level of Irs-1 protein was significantly lower (30%) in islets from LP rats than in islets from NP rats (P < 0.05; Figure 2B). In contrast, Irs-2 protein content was significantly increased by 1.7-fold in islets from LP rats compared to NP rats (P < 0.05; Figure 2C).

Phosphorylation and total levels of Akt, FoxO1 and Erk1/2 proteins in LP islets

Since the protein phosphorylation status suggests the degree of activity of a protein, we investigated the levels of protein phosphorylation and total content of some important proteins involved in islet growth. Total Akt protein content and pAkt were not different in LP islets compared to NP. The pAkt/Akt protein ratio in islet lysates was similar for the two groups (Figure 3A). The analysis of total FoxO1 protein content revealed similar quantities of protein in NP and LP islets (Figure 3B). However, the pFoxO1 protein levels decreased significantly in LP islets compared to NP (P < 0.05). Thus, an important reduction of 43% in the pFoxO1/FoxO1 ratio was observed in islets from LP rats compared to NP rats (P < 0.05; Figure 3B). The protein levels of total Erk1/2 were similar for NP and LP islets (Figure 3C). This pattern was not observed when the phosphorylated levels of Erk1/2 protein were determined. Rats submitted to a low-protein diet exhibited a significant reduction in pErk1/2 protein levels when compared to NP islets (P < 0.05; Figure 3C). Accordingly, a decrease of 71% in the pErk1/2/Erk1/2 ratio was observed in LP compared to NP rats (P < 0.05; Figure 3C).

Levels of Pdx-1 protein in islet lysates and cellular distribution in pancreatic islets from LP rats

To confirm whether any alteration in Pdx-1 protein content in islets from LP rats may be associated with islet hypotrophy, we determined the levels of this protein. Figure 4A shows similar levels of Pdx-1 protein in both NP and LP islet lysates. Qualitative analysis of Pdx-1 in pancreas sections confirmed the wide distribution of this protein in pancreatic β-cell nuclei and cytoplasm in both groups (Figure
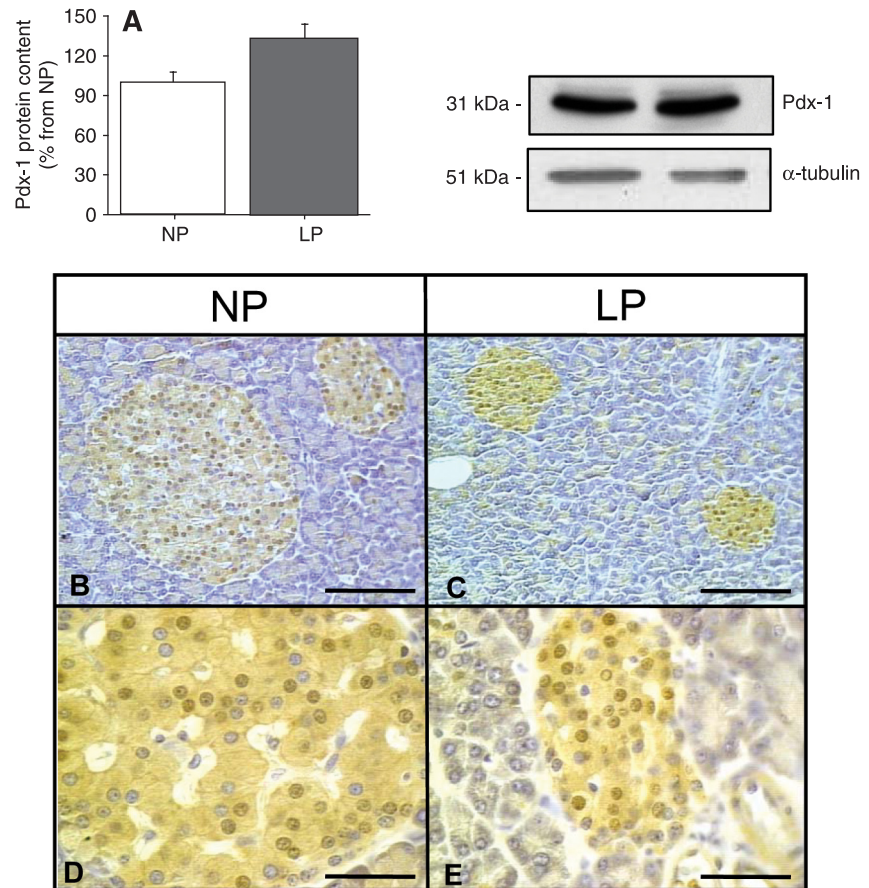

Figure 4. Protein content of pancreatic-duodenal homeobox 1 (Pdx-1) in pancreatic islets from rats receiving a normal-protein diet (NP) and a low-protein diet (LP) (A). A representative control blot for α-tubulin is shown on the right side of *panel A*. The bars are means ± SEM for 5 independent experiments. *P < 0.05 compared to NP (Student *t*-test). *Panels B* and *C* show representative images of pancreatic sections stained for Pdx-1 and hematoxylin, showing the wide distribution of Pdx-1 in pancreatic islets from both groups. Note the similar Pdx-1 protein distribution in NP and LP islets (D, E). Scale bars = 100 μm for B, C and 45 μm for D, E.

4B,C). No apparent differences were noted regarding the cytoplasmic versus nuclear localization of Pdx-1 in LP and NP rats (Figure 4D,E).

Discussion

In this study, we showed for the first time an association between reduced β-cell mass and decreased phosphorylation levels of FoxO1 and Erk1/2 proteins in pancreatic islets of malnourished rats receiving a low-protein diet.

Our data also confirm previous studies (8,9) showing that rats submitted to a low-protein diet exhibit reduced β-cell mass (Figure 1C). An increase in the population of small islets and a decrease in the population of large islets were observed by Tse et al. (9), as also observed in the present study. Smaller islets can be easily observed in

pancreas sections from LP rats (Figure 1B), which were in agreement with the reduced mean islet size. Since the body weight of LP rats was also reduced, we normalized the absolute β-cell mass according to whole body weight. The normalized β-cell mass also indicated a reduced β-cell mass in LP compared to NP rats. Based on these results, we suggest that the hypoinsulinemia observed in LP rats is not attributable solely to decreased islet function (5-7), but rather that the reduced β-cell mass can negatively affect this process.

The insulin and insulin-like growth factor signaling pathways play an important role in the control of β-cell growth and of insulin biosynthesis and secretion (22-24). Analysis of the initial components of the insulin signaling pathway revealed an increase in the expression of IR-β in LP islets compared to NP islets (Figure 2A). Irs-1 protein levels were consistently diminished, whereas Irs-2 was augmented in LP islets compared to NP islets (Figure 2B,C). Modulation of islet functions, such as insulin secretion, involves the participation of Irs-1 protein. Overexpression of Irs-1 in rat insulinoma cells results in increased glucose-stimulated insulin secretion (25). However, Irs-1$^{-/-}$ mice present decreased glucose-stimulated insulin secretion and reduced islet insulin content (26), but also demonstrate a compensatory increase in Irs-2 protein in pancreatic islets, leading to islet hyperplasia (23,27). In contrast to Irs-1, Irs-2 seems to participate mainly in the control of β-cell growth. Irs-2 knock-out mice show islet hypoplasia (28), while overexpression of this protein in pancreatic β-cells results in β-cell proliferation (29). The diminished levels of Irs-1 protein in LP islets support the reduced levels in insulin release, as judged by the circulating insulin levels in these rats. The increase in Irs-2 protein content observed in LP islets may be an adaptive mechanism compensating for the Irs-1 reduction. Although the increase in Irs-2 levels, accompanied by a decrease in islet size, is intriguing, this could be explained by a modification in downstream signaling intermediates.

The Pi3-k cascade involves the activation of several proteins including the serine-threonine protein kinase Akt. Akt has been shown to be important for glucose homeostasis, growth and cell survival (30,31). However, the similar levels of phosphorylated Akt and total Akt (Figure 3A) suggest that this kinase does not participate in the reduced islet size observed here in LP rats.

The forkhead family of the transcription factor FoxO1 plays an important role in the regulation of cell proliferation, apoptosis and senescence (32). Growth factors and insulin pathway components negatively regulate the activity of FoxO1 (32). The activated protein kinases, Pi3-k and Akt, may phosphorylate FoxO1 protein. When phosphorylated, FoxO1 becomes inactive and moves from the nucleus to the cytoplasm (32). Inside the nucleus, FoxO1 appears to be a repressor of Pdx-1 expression (33) and prevents β-cell hyperplasia (34). The decreased phosphorylation of FoxO1 protein in islets from LP rats (Figure 3B) can exert a negative effect on β-cell proliferation, in agreement with the islet hypotrophy observed in these malnourished rats. As mentioned above, the active form (non-phosphorylated) of FoxO1 protein may repress Pdx-1 expression (32,33); however, we did not detect any significant modification in Pdx-1 distribution in pancreatic islet cells nor any differences in Pdx-1 protein expression in LP compared to NP rats (Figure 4A,B). Other signals such as the FoxA2 transcription factor (35), and the transforming growth factor-β and IkB/NFk-B (32) could be involved in the modulation of Pdx-1 or FoxO1 activity, respectively. Thus, the compensatory decrease in the islet area of LP rats seems not to be preferentially mediated by reductions in Akt and Pdx-1 expression. However, we cannot rule out FoxO1 as an important end signaling molecule that could force the hypoplasia of β-cells in LP islets.

We also showed that LP rats exhibited a reduction in phosphorylated Erk1/2 levels in islet lysates (Figure 2C). The MAPK pathway is stimulated by mitogenic factors and its importance in cell proliferation and differentiation has been well documented (36,37). After activation of the insulin receptor, there is an interaction and subsequent phosphorylation of the tyrosine residues of the Shc protein. Once phosphorylated, Shc recruits Grb-2 and activates the Sos-Ras-c-Raf-Mek1/2-Erk pathway (38-40). The reduced phosphorylated state of the Erk1/2 protein supports the decrease in islet area observed in LP rats. Since insulin may act as a positive signal for the activation of the MAPK pathway, the lower levels of circulating insulin observed in LP rats may exert less influence on MAPK activation. Furthermore, neonatal rats from dams receiving a low-protein diet during pregnancy show reduced insulin-like growth factor-2 mRNA and protein levels in pancreatic islet cells (3), supporting the reduced Erk activity observed in the pancreatic islets of LP rats. This, in turn, reinforces the hypotrophy of the pancreatic islets of these rats.

Based on these results, we conclude that reduced levels of FoxO1 and Erk1/2 protein phosphorylation in pancreatic islets can negatively regulate β-cell growth, leading to islet hypotrophy and reduced β-cell mass in malnourished rats.

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