Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 59 (2010) 635–644
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Augmentation of insulin secretion by leucine supplementation in malnourished rats: possible involvement of the phosphatidylinositol 3-phosphate kinase/mammalian target protein of rapamycin pathway

Eliane Filiputti[a], Alex Rafacho[a,b,1], Eliana P. Araújo[a,b], Leonardo R. Silveira[c], Amon Trevisan[a,b], Thiago M. Batista[a,b], Rui Curi[b,d], Lício A. Velloso[a,b], Ivan Quesada[e], Antonio C. Boschero[a,b], Everardo M. Carneiro[a,b,*]

[a]*Department of Anatomy, Cell Biology and Physiology and Biophysics, Institute of Biology, State University of Campinas (UNICAMP), CEP 13083-970 Campinas, SP, Brazil*
[b]*National Institute of Obesity and Diabetes, CEP 13083-970 Campinas, SP, Brazil*
[c]*School of Physical Education and Sports, University of Sao Paulo (USP), CEP 14040-900 Ribeirão Preto, SP, Brazil*
[d]*Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo (USP), CEP 05508-900 São Paulo, SP, Brazil*
[e]*CIBERDEM and Institute of Bioengineering, University of Miguel Hernández, CEP 03202 Elche, Spain*

Received 23 April 2009; accepted 4 September 2009

Abstract

A regimen of low-protein diet induces a reduction of pancreatic islet function that is associated with development of metabolic disorders including diabetes and obesity afterward. In the present study, the influence of leucine supplementation on metabolic parameters, insulin secretion to glucose and to amino acids, as well as the levels of proteins that participate in the phosphatidylinositol 3-phosphate kinase (PI3K) pathway was investigated in malnourished rats. Four groups were fed with different diets for 12 weeks: a normal protein diet (17%) without (NP) or with leucine supplementation (NPL) or a low (6%)-protein diet without (LP) or with leucine supplementation (LPL). Leucine was given in the drinking water during the last 4 weeks. As indicated by the intraperitoneal glucose tolerance test, LPL rats exhibited increased glucose tolerance as compared with NPL group. Both NPL and LPL rats had higher circulating insulin levels than controls. The LPL rats also showed increased insulin secretion by pancreatic islets in response to glucose or arginine compared with those observed in islets from LP animals. Glucose oxidation was significantly reduced in NPL, LP, and LPL isolated islets as compared with NP; but no alteration was observed for leucine and glutamate oxidation among the 4 groups. Western blotting analysis demonstrated increased PI3K and mammalian target protein of rapamycin protein contents in LPL compared with LP islets. A significant increase in insulin-induced insulin receptor substrate 1–associated PI3K activation was also observed in LPL compared with LP islets. These findings indicate that leucine supplementation can augment islet function in malnourished rats and that activation of the PI3K/mammalian target protein of rapamycin pathway may play a role in this process.

1. Introduction

Impairment of pancreatic β-cell function and/or alteration in β-cell mass is a key factor in the development of type 2 diabetes mellitus [1,2]. However, before the onset of diabetes, the endocrine pancreas undergoes several structural and functional adaptations to maintain glucose homeostasis. These adaptations are observed during pregnancy, aging, obesity, and malnutrition [3-6].

Malnutrition along intrauterine or postnatal periods induces significant structural and functional changes in

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

The experiments with animals are in adherence with the institutional State University of Campinas Committee for Ethics in Animal Experimentation.

* Corresponding author. Tel.: +55 19 3521 6203; fax: +55 19 3521 6185.
E-mail address: emc@unicamp.br (E.M. Carneiro).
[1] Present address: Department of Physical Education, School of Sciences, São Paulo State University (UNESP), CEP 13033-360 Bauru, SP, Brazil.

0026-0495/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2009.09.007

pancreas, liver, muscle, and adipose tissues, which can lead to insulinopenia [7]. We have demonstrated that partial deprivation of protein in diet in pre- or postnatal periods in rats results in a good model to mimic human undernourishment [8-13]. Rats submitted to low-protein diet for 8 weeks exhibit reduced body weight, hypoalbuminemia, normoglycemia, and reduced plasma insulin levels [10,11,14,15]. Islets from these rats are less responsive to glucose and do not exhibit insulin secretory biphasic patterns [6,8,14,15]. These animals, however, show improved peripheral insulin sensitivity, possibly because of cross talk as a result of reduced plasma insulin levels [14,16].

Epidemiologic studies have suggested an association between previous exposure to undernourishment and subsequent development of type 2 diabetes mellitus in adult life, especially when overnutrition is imposed [17-19]. Thus, it is important to seek strategies to improve the islet function and metabolic management in undernourished subjects. Leucine supplementation could be an interesting approach because this amino acid has been shown to ameliorate islet function [20-23]. Leucine acts positively on insulin release by increasing adenosine triphosphate (ATP) in the tricarboxylic acid cycle (TCA) [22]. The first mechanism involves leucine transamination and α-ketoisocaproate formation, which is further oxidized in the mitochondria. Leucine also promotes insulin release via allosteric activation of glutamate dehydrogenase (GDH) that converts glutamate to α-ketoglutarate, which is also oxidized in TCA [24]. Leucine also up-regulates glucokinase, which increases NAD(P)H levels [23] and ATP synthase [23,25], enhancing oxidation of NADH and production of ATP [22,23,25,26]. Moreover, leucine metabolism in β-cells activates secondary signals that stimulate the mammalian target protein of rapamycin (mTOR), a serine/threonine protein kinase, which subsequently phosphorylates and activates the ribosomal protein S6 kinase-1 (S6K-1), resulting in improved protein translation in β-cells [22,26].

We have recently demonstrated a reduction of the nutritionally responsive S6K-1 content in islets from malnourished rats, which exhibit decreased insulin secretion both in vivo and ex vivo [15]. Herein, we sought to study the effects of leucine supplementation on pancreatic β-cell function. For this purpose, insulin secretion was determined in isolated pancreatic islets, as well as glucose, leucine, glutamine oxidation, and components of the phosphatidylinositol 3-phosphate kinase (PI3K) pathway, such as mTOR and S6K-1 protein expression.

2. Material and methods

2.1. Animals and treatment

The experiments described herein were approved by the institutional Committee for Ethics in Animal Experimentation, State University of Campinas. Groups of 5 male Wistar rats (21 days old) from the breeding colony at the State University of Campinas were housed at 24°C on a 12-hour light/dark cycle. The rats were separated into 4 groups and treated for 12 weeks with the following: normal-protein diet (NP)—maintained on an isocaloric diet containing protein (17%); normal-protein diet plus leucine (NPL)—maintained on an isocaloric diet containing protein (17%) plus leucine supplementation during the last 4 weeks; low-protein diet (LP)—maintained on an isocaloric diet containing protein (6%); and low-protein diet plus leucine (LPL)—maintained on an isocaloric diet containing protein (6%) plus leucine supplementation during the last 4 weeks. The compositions of the 2 isocaloric diets are shown in Table 1 Leucine (1.5%) was supplied in the drinking water.

2.2. Body weight, blood glucose, and serum insulin and total protein levels

Absolute body weight was determined before the initiation of diet, on the day before the beginning of the leucine supplementation, and on the last day before killing the rats. On the day rats were killed, rats were first used to measure blood glucose levels, which were obtained from the tail tip, using a glucosimeter (Accu-Check Advantage II; Roche, São Paulo, SP, Brazil). The rats were then killed by exposure to CO_2; the trunk blood was collected; and the serum, obtained after centrifugation of the blood, was used to determine insulin levels by radioimmunoassay [27]. Total serum protein was determined according to the manufacturer's instructions (Bio-Rad Laboratories, Munchen, Germany) using spectrophotometry.

2.3. Intraperitoneal glucose tolerance test

The intraperitoneal glucose tolerance test was performed according to Rafacho et al [28]. Briefly, after food deprivation (12 hours), independent groups of rats were anesthetized (60 mg/kg body weight; with sodium thiopental); and blood samples were obtained from the tail tip for measurement of glucose levels, as described above. Subsequently, a glucose solution (50%) was administered into the peritoneal cavity at a dose of 2 g/kg body weight.

Table 1
Composition of NP and LP diets [43]

Ingredient	NP (17% protein)	LP (6% protein)
	g/kg	
Casein (84% protein)	202.0	71.5[a]
Cornstarch	397.0	480.0[a]
Dextrin	130.5	159.0[a]
Sucrose	100.0	121.0[a]
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-Cysteine	3.0	1.0[a]
Choline chlorhydrate	2.5	2.5

[a] Difference between the 2 isocaloric diets.

Additional blood samples were obtained for determination of glucose concentrations at 15, 30, 60, 90, and 120 minutes after glucose load.

2.4. Intraperitoneal insulin tolerance test

The intraperitoneal glucose tolerance test was carried out according to Rafacho et al [28]. Briefly, after anesthesia of animals as described above, blood glucose (time 0) was measured as described above in independent groups of fasted rats; and the rats received 2 U/kg body weight human recombinant insulin (Biohulin N) from Biobrás (Montes Claros, MG, Brazil) in the peritoneal cavity. Blood glucose concentrations were then determined at 5, 10, 15, 20, 25, and 30 minutes after insulin administration. The constant ratio for glucose disappearance (*k*ITT) was calculated using the formula $0.693/t_{1/2}$; $t_{1/2}$ was calculated using the analysis of the square fall of glucose concentration during the linear phase decay.

2.5. Insulin secretion

Islets were isolated by collagenase digestion of the pancreas, as previously described [29]. For static incubation, groups of 5 islets from each group were first incubated in a 24-well plate for 45 minutes at 37°C in 1 mL Krebs-bicarbonate buffer with the following composition (in millimoles per liter): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, 15 HEPES, and 5.6 glucose, supplemented with 3 g/L of bovine serum albumin and balanced with a mixture of 95% O_2:5% CO_2 to give pH 7.4. This medium was then replaced with the media indicated in the figures for 1 hour. Samples from independent experiments were collected on different days and stored at −80°C. The insulin concentration was determined at the end of the incubation period by the radioimmunoassay method. The total protein content in 100 islets from NP, NPL, LP, and LPL rats did not change statistically (238 ± 21, 225 ± 21, 217 ± 35, and 222 ± 35 μg, respectively). Results were expressed as percentages of NP values.

2.6. Glucose, leucine, and glutamine oxidation

Glucose, leucine, and glutamine oxidation rates were measured in isolated islets, as previously described [30]. Briefly, groups of 30 islets were placed in wells containing Krebs-bicarbonate–buffered media (50 μL) supplemented with trace amounts of D-[U-14C] glucose, D-[U-14C] leucine, or L-[U-14C] glutamine (10 μCi/mL) plus nonradioactive glucose, leucine, or glutamine to a final concentration of 16.7, 20, or 10 mmol/L, respectively. The wells were suspended in 20-mL scintillation vials, which were gassed with 95% O_2 and 5% CO_2 and capped airtight with rubber membranes. The vials were shaken continuously for 2 hours at 37°C in a water bath. After incubation, 0.1 mL HCl (0.2 mmol/L) and 0.2 mL hyamine hydroxide were injected through the rubber cap into the glass cup containing the incubation medium and into the counting vials, respectively. After 1 hour at room temperature, 6 mL of scintillation fluid was added to the hyamine; and the radioactivity was counted. Glucose, leucine, and glutamine oxidation rates were expressed as picomoles per liter islet per hour.

2.7. Western blotting assay

After isolation by collagenase digestion of pancreata and subsequent separation, groups of 300 freshly isolated islets were pelleted by centrifugation and then resuspended in 50 to 100 μL of homogenization buffer containing protease inhibitors, as previously described [31]. The islets were sonicated (15 seconds), and total protein content was determined by the Bradford method. The sample volume was adjusted to provide the same amount of protein added to each lane. Samples containing 70 μg of protein from each experimental group were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, and stained with Ponceau S to confirm the efficiency of the electrotransference. The insulin receptor (IR), insulin receptor substrate 1 (IRS-1), PI3K, mTOR, and S6K-1 proteins were detected in the membrane after 2 hours of incubation at room temperature with a rabbit polyclonal antibody against IR (diluted 1:1000), IRS-1 (diluted 1:1000), PI3K (diluted 1:500), mTOR (diluted 1:1000), and p70S6K-1 (diluted 1:2500). All antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and were diluted in TBS-Tween 20 containing 30 g/L dry skimmed milk. Detection was performed using enhanced chemiluminescence (SuperSignal West Pico; Pierce, Rockford, IL) after incubation with a horseradish peroxidase–conjugated secondary antibody. Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD) of the developed autoradiogram.

2.8. RNA isolation and semiquantitative reverse transcriptase–polymerase chain reaction analysis

Isolation of total RNA and semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) were performed according to a previously described method [28]. Briefly, total RNA from islets was extracted using Trizol (Invitrogen, Carlsbad, CA) reagent as described by the manufacturer. For the PCR, RNA was reverse-transcribed using random primers. The resulting complementary DNA (cDNA) was amplified by RT-PCR using primer sequences and PCR parameters as indicated in Table 2. The PCR was performed in a 25-μL reaction volume containing cDNA equivalent to 3 μg of RNA, 10 mmol/L cold dNTPs per liter (dATP, dCTP, dGTP, dTTP), 50 mmol/L $MgCl_2$, 10× PCR buffer, 10 μmol/L of appropriate oligonucleotides primers, and 2 units of Taq polymerase. The PCR products were separated on 1.5% agarose gel in 1× Tris-Borate-EDTA (TBE) buffer and stained with ethidium bromide. All PCRs included a negative control. The absence of genomic contamination in the RNA samples was confirmed by the RT-negative RNA samples. Subsequent digitalization and measurement of relative band intensities were performed

Table 2
PCR primers used for mRNA amplification

Gene name	Gene bank accession no.	Forward 5′ oligonucleotide	Reverse 5′ oligonucleotide	Product size	Annealing temperature (C°)	Cycles
r Irβ	NM_017071	AGTCATTGTCAGAAAGTTTG	TGTCACGGAATCAATGGTCT	420	55	29
r IRS1	NM_012969	ACCCACTCCTATCCCG	CACCGCTCTCAACAGG	539	59	33
r PI3K	NM_022958	TCTCCTCTCATTACACCAACC	TGCTCTAGGTTTTCCCCATCA	319	59	33
r mTOR	NM_019906	CAAAGAGAAGGGTATGAA	CAAAGAGAAGGGTATGAA	189	58	30
r S6K-1	BC089789	TGGATTGGTGGAGTTTGGG	ATTTGACTGGGCTGACAGGC	531	57	29
r RPS29	NM_012876	AGGCAAGATGGGTCACCAGC	AGTCGAATCATCCATTCAGGTCG	201	57	29

using a Gel Doc documentation system (BioRad Laboratory, Hercules, CA). The results were expressed as a ratio between the target gene amplification and internal control product RPS-29.

2.9. PI3K assay

Aliquots of supernatants containing equal amounts of proteins were incubated overnight at 4°C using antibodies against IRS-1 or the p85 subunit of PI3K (Santa Cruz Biotechnology), and the immunocomplexes were precipitated with a 50% solution of protein A–Sepharose 6MB. In vitro PI3K assays were performed as described [32,33] and allowed the determination of the catalytic activity of insulin-induced IRS-1–bound PI3K. The ^{32}P-labeled 3-P-phosphatidylinositol was quantified by the same software described above for Western blotting assay.

2.10. Statistical analysis

Results are expressed as means ± SEM of the indicated number of experiments. Analysis of variance (ANOVA) for unpaired groups, followed by Tukey posttest, was used for multiple comparisons of parametric data. The significance level adopted was $P < .05$.

3. Results

3.1. Leucine supplementation improved the biochemical parameters in malnourished rats

In accordance with a previous observation [14,15], the body weight and total serum protein were significantly reduced ($P < .05$) after feeding on a low-protein diet; and, although not statistically different, a reduction of 27% was also observed in serum insulin levels in LP compared with NP rats (Tables 3 and 4). These findings indicate the establishment of malnourishment in rats. Leucine supplementation partially recovered the changes in total serum protein induced by low-protein diet. The LPL rats also exhibited a significant increase (2.2-fold) of insulin levels compared with the LP rats ($P < .05$, Table 4). The NPL rats also showed increased serum insulin values compared with the NP rats ($P < .05$, Table 4). These results suggest that leucine supplementation increases insulin secretion under both normal and malnourished conditions.

3.2. Low-protein diet but not leucine supplementation increased glucose tolerance

The NPL rats did not exhibit any alterations in glucose tolerance and insulin sensitivity compared with the NP animals (Table 5). The parameters in LPL rats did not differ from those in LP animals. However, LPL rats showed a significant improvement in glucose tolerance (as judged by the 2.1-fold increase in the constant rate for glucose disappearance [*k*GTT] between minutes 15 and 60 after glucose load) compared with NPL rats ($P < .05$) and a tendency toward improved insulin sensitivity (as judged by the 1.27-fold increase in the *k*ITT along 30 minutes after insulin administration) compared with NPL rats (Table 5). The LP rats showed a partial increase in glucose tolerance (1.45-fold in *k*GTT values) and in insulin sensitivity (1.22-fold in *k*ITT values) compared with NP rats (not significant).

Table 3
Body weight of NP, NPL, LP, and LPL rats

Groups	21 d old	77 d old	105 d old
	Body weight (g)		
NP	56.5 ± 2.6	279.2 ± 24	327.5 ± 13
NPL	58.5 ± 3.2	334.0 ± 22	383.2 ± 20
LP	59.2 ± 3.7	154.0 ± 25[a]	224.7 ± 21[a]
LPL	53.8 ± 8.2	152.8 ± 37[a]	201.6 ± 37[a]

Values are means ± SEM. n = 10. $P < .05$ using ANOVA with Tukey posttest.

[a] Significantly different from the respective control (protein restriction effect).

Table 4
Blood glucose, serum insulin, and total protein levels from NP, NPL, LP, and LPL fasted rats

Groups	NP	NPL	LP	LPL
Glucose (mmol/L)	6.9 ± 0.3	6.8 ± 0.5	7.1 ± 0.7	6.1 ± 0.2
Insulin (nmol/L)	0.15 ± 0.02	0.28 ± 0.03[b]	0.11 ± 0.03	0.25 ± 0.03[b]
Total proteins (g/L)	46 ± 1.0	43 ± 3.0	28 ± 6.0[a]	36 ± 6.0

Values are means ± SEM. n = 10. $P < .05$ using ANOVA with TUKEY posttest.

[a] Significantly different from the respective control (protein restriction effect).

[b] Significantly different from respective control (leucine supplementation effect).

Table 5
The constant rate for glucose disappearance between minutes 15 and 60 after intraperitoneal glucose load (*k*GTT) or during minutes 0 to 30 after intraperitoneal insulin administration (*k*ITT) from NP, NPL, LP, and LPL rats

Groups	NP	NPL	LP	LPL
*k*GTT (% min)	0.83 ± 0.1	0.69 ± 0.1	1.2 ± 0.13	1.49 ± 0.18[a]
*k*ITT (% min)	1.99 ± 0.15	1.92 ± 0.19	2.43 ± 0.08	2.44 ± 0.21

Values are means ± SEM. n = 6. $P < .05$ using ANOVA with Tukey posttest.
[a] Significantly different from the respective controls (protein restriction effect).

Thus, results suggest that the increased serum insulin levels observed in leucine-supplemented rats are not the consequence of a high peripheral insulin demand. Instead, rats fed on a low-protein diet showed increased glucose tolerance and insulin sensitivity, which are in agreement with our previous observations [14,16].

3.3. LPL rats exhibited increased glucose stimulated-insulin secretion

To determine whether increased serum insulin levels in LPL rats were due to an alteration in secretory mechanisms, insulin secretion from isolated islets was investigated. As previously observed [14,15], insulin secretion by islets from LP rats was significantly reduced when stimulated by 16.7 mmol/L glucose compared with NP islets ($P < .05$) (Fig. 1A). The LPL, but not NPL, rats exhibited a significant increase in insulin secretion after glucose (16.7 mmol/L) induction compared with their control group ($P < .05$). Glucose stimulated-insulin secretion (GSIS) was 64% higher in LPL compared with LP islets (Fig. 1A). The insulin release were 13.5 ± 1.0, 15.3 ± 0.7, 7.0 ± 0.9 and 11.5 ± 1.6 ng per islet per hour for NP, NPL, LP, and LPL, respectively. Interestingly, islets from NPL rats exhibited a marked increase in insulin response to 20 mmol/L arginine (8-fold) and 20 mmol/L leucine (3.7-fold) compared with NP islets (0.71 ± 0.06 vs 5.75 ± 0.11 ng per islet per hour for arginine and 2.23 ± 0.34 vs 8.33 ± 1.38 ng per islet per hour for leucine stimulus in NP and NPL islets, respectively) ($P < .05$). The LPL rats showed a 3.7- and 3.4-fold increase in insulin secretion in response to 20 mmol/L arginine and 20 mmol/L leucine, respectively, compared with LP islets ($P < .05$ only for 20 mmol/L arginine) (Fig. 1B and C, respectively). The insulin secretion were (0.32 ± 0.08 vs 1.21 ± 0.15 ng per islet per hour for arginine and 0.55 ± 0.08 vs 1.87 ± 0.16 ng per islet per hour for leucine stimulus in LP and LPL islets, respectively).

3.4. Glucose oxidation is decreased in LPL Rats

To determine whether the increased insulin secretion stimulated by glucose observed in leucine-supplemented rats is associated with augmented glucose oxidation; this parameter was investigated in incubated isolated islets. Glucose oxidation at 16.7-mmol/L glucose incubation was significantly reduced in both NPL and LP islets compared

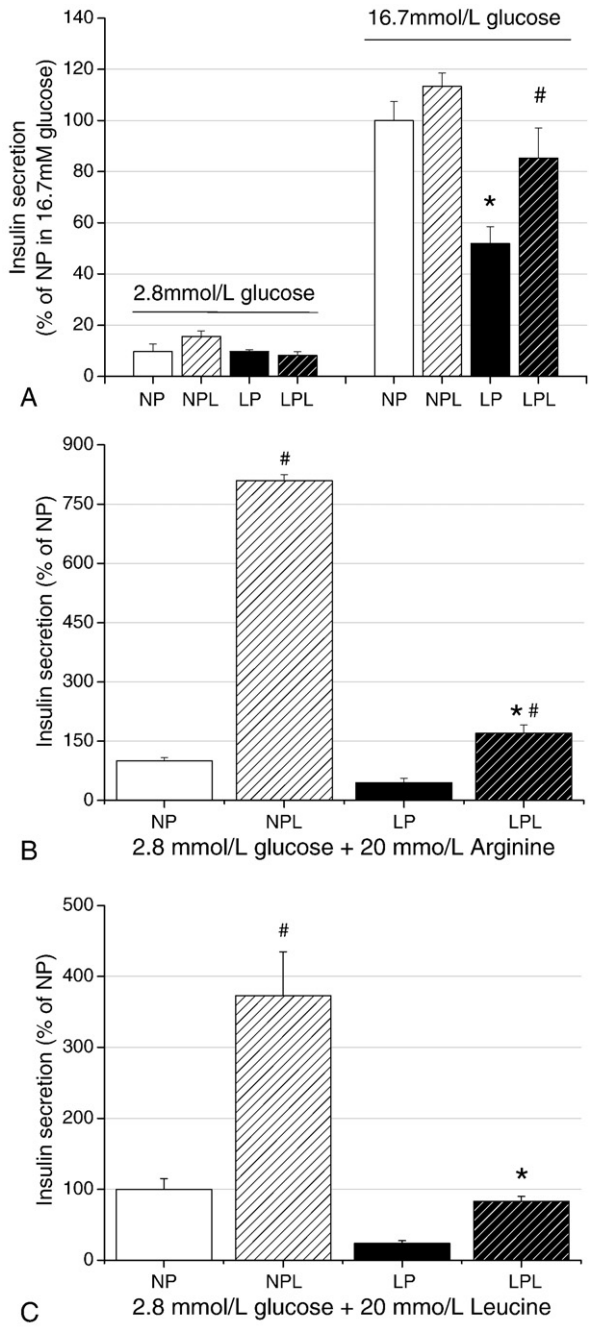

Fig. 1. The LPL rats exhibit increased GSIS. A, At a concentration of 2.8 mmol/L glucose, insulin secretion was similar in all groups. At a stimulatory glucose concentration, the insulin secretion was reduced in LP compared with NP islets and enhanced in LPL compared with LP islets. B, The NPL and LPL rat islets showed a significant increase in insulin secretion in response to 20 mmol/L arginine compared with their respective controls. C, Twenty millimoles per liter of leucine induced a marked increase in insulin secretion in NPL compared with NP islets. Values are means ± SEM. *Significantly different from the respective control (protein restriction effect). #Significantly different from respective control (leucine supplementation effect). n = 10 (2 independent experiments). $P < .05$ using ANOVA with Tukey posttest.

with NP islets ($P < .05$). The LPL rats exhibited an additional reduction of this parameter compared with the LP islets ($P < .05$) (Fig. 2A). Because glucose oxidation was reduced in rats submitted to low-protein diet and/or diet supplemented with leucine, oxidation of this amino acid was measured. Intriguingly, leucine oxidation was not different among the groups (Fig. 2B). Leucine can also act through allosteric activation of GDH, which results in glutamate oxidation. This parameter was not changed under the conditions studied (Fig. 2C).

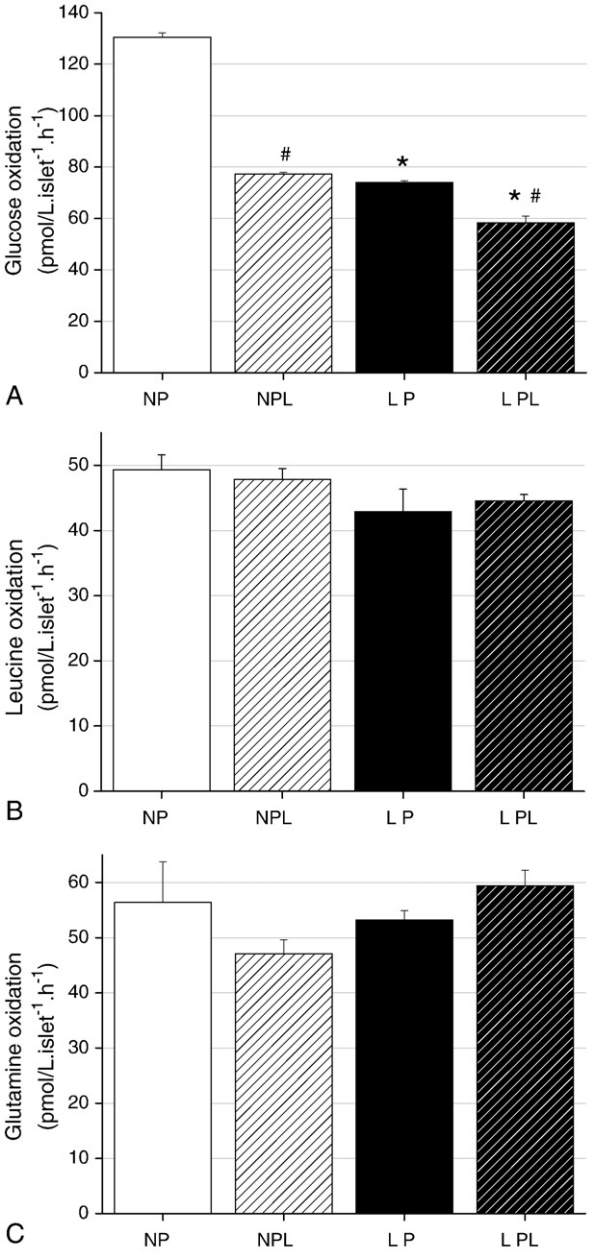

Fig. 2. Decrease of glucose oxidation in isolated islets from NPL, LP, and LPL rats. A, Note the reduction of glucose oxidation in islets from LP rats. Rats supplemented with leucine also exhibited decreased glucose oxidation, and LPL rats exhibited an additional reduction in glucose oxidation compared with LP islets. No difference in leucine oxidation (B) and in glutamate oxidation (C) was observed in islets among the groups. Values are means ± SEM. *Significantly different from the respective control (protein restriction effect). #Significantly different from the respective control (leucine supplementation effect). n = 12 (2 independent experiments). $P < .05$ using ANOVA with Tukey posttest.

3.5. Increased levels of PI3K and mTOR protein in islet lysates from LPL rats

Because glucose, leucine, and glutamate oxidation did not support the elevation in insulin secretion and given that leucine can increase the translational level through the insulin/PI3K/mTOR signaling pathway, we investigated this pathway. The content of the β-subunit of the IR was significantly increased in LP compared with NP islet lysates ($P < .05$) (Fig. 3). Leucine supplementation did not alter the IR expression in LPL and NPL rat islets. The protein content of IRS1 was significantly reduced in LP compared with NP islet lysates ($P < .05$); however, leucine supplementation did not promote any alteration of protein content in either LPL or NPL islets (Fig. 3). A trend toward increased (1.5-fold) PI3K protein levels was observed in islets from LP rats compared with NP islets. Leucine supplementation induced an increase in PI3K content in both NPL (1.5-fold) and LPL (1.44-fold) islets vs NP and LP islets, respectively ($P < .05$ for LPL islets) (Fig. 3). Serine/threonine protein kinase mTOR levels were significantly reduced in islets from LP compared with NP islets ($P < .05$). However, leucine supplementation promoted a 2.5-fold increase of mTOR in LPL compared with LP islets ($P < .05$) and no alteration in NPL compared with NP islets. The ribosomal protein S6K-1 levels were reduced in both LP and LPL islets compared with NP and NPL, respectively ($P < .05$) (Fig. 3). These findings indicate that PI3K and mTOR may be involved in the increased insulin secretion observed in LPL rats.

3.6. Messenger RNA expression in islets from malnourished and leucine-supplemented rats

To verify whether the alterations observed in protein levels were accompanied by changes in protein messenger RNA (mRNA) levels, a semiquantitative PCR analysis was performed. As observed in Fig. 4, there were no alterations in IRβ mRNA levels among the 4 groups. Similarly to observations in protein expression, IRS1 mRNA levels were decreased in LPL compared with NPL ($P < .05$). In contrast to the results for protein levels, PI3K mRNA was markedly reduced in both LP and LPL compared with NP and NPL islets, respectively, and increased in NPL when compared with NP islets ($P < .05$) (Fig. 4). The mTOR mRNA levels followed the same trend of changes observed in protein levels for all groups ($P > .05$). The S6K-1 mRNA levels also showed the same trend of changes observed for S6K-1 protein content, with the exception of NPL rats, which exhibited a significant increase in S6K-1 mRNA levels compared with NP rats ($P < .05$) (Fig. 4). These

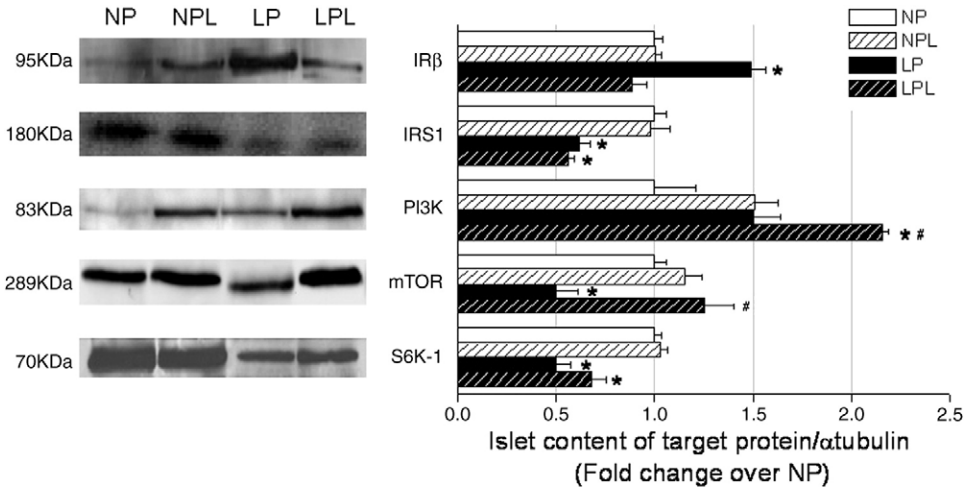

Fig. 3. Increased levels of PI3K and mTOR proteins in islet lysates from LPL rats. A, The LP islets showed increased content of IR and PI3K protein and decreased expression of IRS1, mTOR, and S6K-1 compared with NP islets. However, LPL islets had increased levels of PI3K, mTOR, and, to a lesser extent, S6K-1 compared with LP islets. Values are means ± SEM. *Significantly different from the respective control (protein restriction effect). #Significantly different from respective control (leucine supplementation effect). n = 4 independent experiments. $P < .05$ using ANOVA with Tukey posttest.

findings revealed a positive association between mRNA and protein levels for mTOR and S6K-1, but not for PI3K.

3.7. Increased IRS1-associated PI3K activation in islets from LPL rats

To determine whether the increased PI3K protein content is associated with augmented PI3K activation, islets were incubated for 2 hours in the presence of 100 nmol/L insulin in media containing 5.6 mmol/L glucose; and the IRS1-associated PI3K activity was determined. There was no significant difference in basal IRS1-associated PI3K activity among the groups (Fig. 5). The IRS1-associated PI3K activity was significantly increased in islets incubated in the presence of insulin compared with their basal condition in all groups ($P < .05$). Both LP and LPL islets had a higher IRS1-

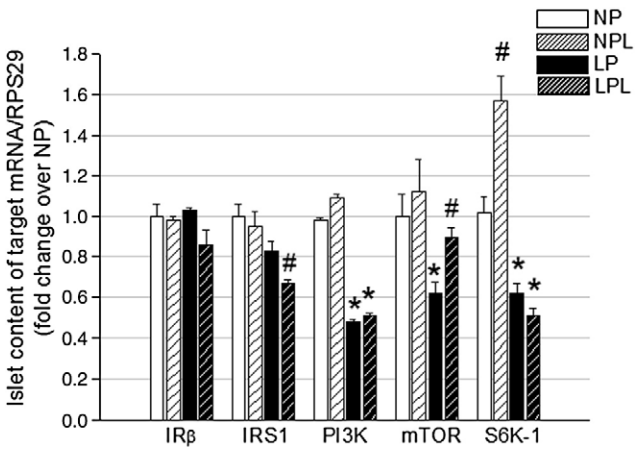

Fig. 4. Messenger RNA levels of insulin signaling components in islet lysates from LPL rats. Semiquantitative RT-PCR from islets for IRβ, IRS1, PI3K, mTOR, and S6K-1. Values are means ± SEM. *Significantly different from the respective control (protein restriction effect). #Significantly different from respective control (leucine supplementation effect). n = 6 (duplicate from 3 different cDNAs). $P < .05$ using ANOVA with Tukey posttest.

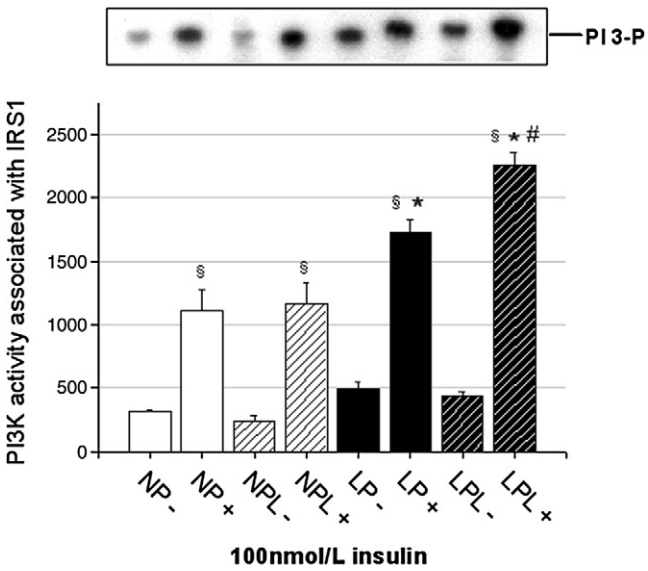

Fig. 5. Increased IRS1-associated PI3K activity in LPL islets. Fluorograph shows the silica TLC plates of insulin-induced, IRS-associated PI3K activity. PI 3-P indicates the migration position of phosphatidylinositol 3-phosphate. Bar graphs depict the relative incorporation of ^{32}P into PI 3-P (means ± SEM). Islets from all groups showed higher levels of IRS1-associated PI3K activity compared with basal status after insulin stimulation. Islets from rats fed on a low-protein diet showed higher levels of IRS1-associated PI3K activity compared with islets from rats fed on a normal-protein diet. Leucine-supplemented rats exhibited an additional increase of islet IRS1-associated PI3K activity compared with LP rats. Values are as follows: §significantly different vs basal value, *significantly different from the respective control (protein restriction effect), and #significantly different from the respective control (leucine supplementation effect). n = 5. $P < .05$ using ANOVA with Tukey posttest.

associated PI3K activity than NP and NPL islet responses. The increase reached 1.5- and 1.9-fold for LP and LPL islets over NP and NPL islets, respectively ($P < .05$, Fig. 5). In addition, IRS1-associated PI3K activity in islets from malnourished rats supplemented with leucine was significantly higher (1.3-fold) than the results observed in LP rats ($P < .05$, Fig. 5).

4. Discussion

The major finding of this study is that leucine supplementation augments the response to glucose in islets from malnourished rats (LPL group) (Fig. 1A), which undergo a reduction in GSIS after low-protein diet treatment (LP group) [11,15]. The increase in insulin secretion observed in LPL islets is also observed in the presence of other nutrients (Fig. 1B and C), indicating increased islet function when compared with low-protein diet. Interestingly, glucose oxidation is reduced not only in LP, but also in LPL and NPL rats (Fig. 2A), indicating that leucine might provide an elevated level of ATP during insulin secretion. Reduced glucose oxidation by islets from malnourished rats has been previously observed [15] and could explain in part the reduced GSIS in these animals. However, intriguingly, LPL and especially NPL islets showed increased GSIS.

Leucine can induce insulin release in the presence of basal or stimulating concentrations of glucose [34,35], a process that has been associated with elevated mitochondrial energy production [22]. Leucine metabolism might supply TCA with different anaplerotic substrates including α-ketoisocaproate, which can be further metabolized to acetyl–coenzyme A and acetoacetate [36]. However, evaluation of leucine oxidation revealed no differences between the 4 groups (Fig. 2B), indicating that use of leucine by this pathway is the same in all of the groups studied.

Leucine may also allosterically activate GDH, the enzyme that converts glutamate to α-ketoglutarate, an important anaplerotic substrate for the second span of TCA. The observation that the β-cell possesses a high level of anaplerotic mitochondrial enzymes, as demonstrated by elevated pyruvate carboxylase as well as GDH activity, suggests that this process is of particular importance for the insulin secretion mechanism [36,37]. As previously reported, an elevated content of TCA intermediates is directly associated with augmented oxidative energy production [38,39]. The reduced glucose oxidation, therefore, may be supported by an elevated expansion of TCA intermediates as a consequence of the leucine-induced effect on GDH [36]. Taken together, these findings suggest that leucine functions as a positive regulator of insulin secretion in malnourished rats.

Importantly, leucine can independently activate the mTOR pathway through multiple mechanisms [40-42]. This biochemical route plays a significant role in protein translation in β-cells, suggesting that elevated anaplerosis may be regulated by mTOR [22]. In agreement, PI3K, mTOR, and, to a lesser extent, S6K-1 protein expression is increased in islets from LPL rats compared with LP animals (Fig. 3). It is known that the nutritional environment plays an important role in the control of gene expression [43-48]. The expressions of several genes and their encoded proteins have been reported to be affected by low-protein diet and/or by leucine supplementation [12,15]. Although PI3K, mTOR, and S6K-1 mRNA levels were reduced in LP islets, leucine supplementation did not exert significant effects, with the exception of mTOR, suggesting that molecular adaptations in LPL rats occurred at the translational level. The main signaling pathway, downstream from the IR, for promoting insulin biosynthesis starts with tyrosine phosphorylation of IRS-1, leading to the sequential activation of PI3K and the downstream effector AKT [46,49]. Akt exerts an important role in protein synthesis through modulation of translational process, including mTOR and its downstream S6K-1 [50]. In previous studies, we have demonstrated reductions in IRS-1 and S6K-1 and increases in IR and PI3K protein expression in islets from LP rats [15]. An increase in the IRS1-associated PI3K activity by insulin and a reduction of mTOR protein expression are also observed in islets from LP rats (Fig. 5). Increased IRS1-associated PI3K activity does not result in an increase of GSIS in LP rats, which may be due, in part, to the reduction of its downstream effectors proteins mTOR and S6K-1. Malnourished rats supplemented with leucine exhibited decreased levels of IR protein; but the content of its downstream effector substrate, IRS-1, was not altered as compared with LP islets. Interestingly, the PI3K/mTOR/S6K-1 pathway seems to be more active in islets from these rats as shown by both protein content and IRS1-associated PI3K activity assay. It is possible that leucine acts on translational processes of islet proteins [51,52]. In addition, it has been shown that leucine can regulate translation initiation through the mTOR signaling cascade, enhancing protein synthesis [22]. The activation of this signaling route has been associated with proliferation and survival [53]. Thus, the improvement in these processes could be related with the enhanced insulin secretion observed in malnourished rats treated with leucine. Actually, inhibition of the mTOR signaling pathway by rapamycin impairs glucose-induced insulin secretion in the pancreatic β-cell [54]. Future research on these aspects is warranted. Moreover, the improvement in protein translation associated with leucine and the mTOR signaling route may also have a positive effect on the synthesis of other functional proteins related with the β-cell function. The increased activity of the PI3K/mTOR/S6K-1 pathway observed in LPL rats could favor the expression and/or activity of target proteins related to the insulin secretion machinery, such as Ca^{2+}-calmodulin kinases, PKC, PKA, and SNARE proteins. It would be interesting to perform studies to analyze whether the expression and/or activity of these proteins is altered after leucine treatment. In addition, malnourished mice supplemented with leucine exhibit an improvement in cytoplasmic

calcium handling after stimulation by glucose and other secretagogues, which are associated with exocytosis and increased GSIS (unpublished observations). The augmented insulin response to arginine in NPL and LPL islets, which depolarizes the β-cell membrane potential, activating Ca^{2+} influx (Fig. 1B), also suggests that leucine may act predominantly at the secretory level instead of in metabolism. Moreover, the elevated PI3K activity (as judged by phosphorylation status) found in LPL rats could also sustain a higher sensitivity to the stimulatory effect of insulin on GSIS by autocrine and paracrine mechanisms [13,55].

In conclusion, leucine augments the islet function in malnourished rats and improves the secretory response to different insulinotropic agents. Moreover, the augmented GSIS response in LPL rats might also be associated with an increased activity of the PI3K/mTOR pathway.

Acknowledgment

This study was supported by grants from FAPESP and CNPq. The authors are indebted to Léscio Teixeira for his technical assistance.

References

[1] Weir GC, Laybutt DR, Kaneto H, et al. B-cell adaptation and decompensation during the progression of diabetes. Diabetes 2001;50: S154-9.
[2] Weir GC, Bonner-Weir S. Five stages of evolving B-cell dysfunction during progression to diabetes. Diabetes 2004;53:S16-21.
[3] Novelli M, De Tata V, Bombara M, et al. Insufficient adaptative capability of pancreatic endocrine function in dexamethasone-treated ageing rats. J Endocrinol 1999;162:425-32.
[4] Nieuwenhuizen AG, Schuiling GA, Moes H, et al. Role of increased insulin demand in the adaptation of the endocrine pancreas to pregnancy. Acta Physiol Scand 1997;159:303-12.
[5] Matschinsky FM, Rujanavech C, Pagliara A, et al. Adaptations of alpha2- and beta-cells of rat and mouse pancreatic islets to starvation, to refeeding after starvation, and to obesity. J Clin Invest 1980;65: 207-18.
[6] Carneiro EM, Mello MAR, Gobatto CA, et al. Low protein diet impairs glucose-induced insulin secretion from and 45Ca uptake by pancreatic rat islets. J Nutr Biochem 1995;6:314-8.
[7] Heywood WE, Mian N, Milla PJ, et al. Programming of defective rat pancreatic β-cell function in offspring from mothers fed a low-protein diet during gestation and the suckling periods. Clin Science 2004;107:37-45.
[8] Latorraca MQ, Reis MA, Carneiro EM, et al. Protein deficiency and nutritional recovery modulate insulin secretion and the early steps of insulin action in rats. J Nutr 1998;128:1643-9.
[9] Latorraca MQ, Carneiro EM, Boschero AC, et al. Protein deficiency during pregnancy and lactation impairs glucose-induced insulin secretion but increases the sensitivity to insulin in weaned rats. Braz J Nutr 1998;80:291-7.
[10] Ferreira F, Filiputti E, Arantes VC, et al. Decreased cholinergic stimulation of insulin secretion by islets from rats fed a low protein diet is associated with reduced protein kinase Cα expression. J Nutr 2003;133:695-9.
[11] Ferreira F, Barbosa HCL, Stoppiglia LF, et al. Decreased insulin secretion in islets from rats fed a low protein diet is associated with a reduced PKAα expression. J Nutr 2004;134:63-7.
[12] Delghingaro-Augusto V, Ferreira F, Bordin S, et al. A low protein diet alters gene expression in rat pancreatic islets. J Nutr 2004;134:321-7.
[13] Araujo EP, Amaral MEC, Filiputti E, et al. Restoration of insulin secretion in pancreatic islets of protein-deficient rats by reduced expression of insulin receptor substrate (IRS)–1 and IRS-2. J Endocrinol 2004;181:25-38.
[14] Giozzet VAG, Rafacho A, Boschero AC, et al. Dexamethasone treatment in vivo counteracts the functional pancreatic islet alterations caused by malnourishment in rats. Metabolism 2008;57:617-24.
[15] Filiputti E, Ferreira F, Souza KLA, et al. Impaired insulin secretion and decreased expression of the nutritionally responsive ribosomal kinase protein S6K-1 in pancreatic islets from malnourished rats. Life Sci 2008;82:542-8.
[16] Reis MA, Carneiro EM, Mello MA, et al. Glucose-induced insulin secretion is impaired and insulin-induced phosphorylation of the insulin receptor and insulin receptor substrate–1 are increased in protein-deficient rats. J Nutr 1997;127:403-10.
[17] Leahy JL. Detrimental effects of chronic hyperglycemia on the pancreatic β-cell. In: Le Roith D, Taylor SI, Olefsky JM, editors. Diabetes mellitus. Philadelphia: Lippincott-Raven; 1996. p. 103-13.
[18] Gepts W, Lecompt PM. The pancreatic islets in diabetes. Am J Med 1981;270:105-9.
[19] Cherif H, Reusens B, Dahri S, et al. A protein-restricted diet during pregnancy alters in vitro insulin secretion from islets of fetal Wistar rats. J Nutr 2001;131:1555-9.
[20] Martin F, Soria B. Amino acid–induced $[Ca^{2+}]_i$ oscillations in single mouse pancreatic islets of Langerhans. J Physiol 1995;486:361-71.
[21] Martin F, Reig JA, Soria B. Secretagogue-induced [Ca2+]i changes in single rat pancreatic islets and correlation with simultaneously measured insulin release. J Mol Endocrinol 1995;15:177-85.
[22] Xu G, Kwon G, Cruz WS, et al. Metabolic regulation by leucine of translation initiation through the mTOR signaling pathway by pancreatic beta-cells. Diabetes 2001;50:353-60.
[23] Yang J, Wong RK, Park M, et al. Leucine regulation of glucokinase and ATP synthase sensitizes glucose-induced insulin secretion in pancreatic beta-cells. Diabetes 2006;55:193-201.
[24] Li C, Najafi H, Daikhin Y, et al. Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets. J Biol Chem 2003;278:2853-8.
[25] Yang J, Wong RK, Wang X, et al. Leucine culture reveals that ATP synthase functions as a fuel sensor in pancreatic beta-cells. J Biol Chem 2004;279:53915-23.
[26] McDaniel ML, Marshall CA, Pappan KL, et al. Metabolic and autocrine regulation of the mammalian target of rapamycin by pancreatic beta-cells. Diabetes 2002;51:2877-85.
[27] Scott AM, Atwater I, Rojas E. A method for the simultaneous measurement of insulin release and B cell membrane potential in single mouse islets of Langerhans. Diabetologia 1981;21:470-5.
[28] Rafacho A, Roma LP, Taboga SR, et al. Dexamethasone-induced insulin resistance is associated with increased connexin 36 mRNA and protein expression in pancreatic rat islets. Can J Physiol Pharmacol 2007;85:536-45.
[29] Bordin S, Boschero AC, Carneiro EM, et al. Ionic mechanisms involved in the regulation of insulin secretion by muscarinic agonists. J Memb Biol 1995;148:177-84.
[30] Malaisse WJ, Sener A, Mahy M. The stimulus-secretion coupling of glucose-induced insulin release. Sorbitol metabolism in isolated islets. Eur J Biochem 1974;47:365-70.
[31] Amaral ME, Ueno M, Carvalheira JB, et al. Prolactin-signal transduction in neonatal rat pancreatic islets and interaction with the insulin-signaling pathway. Horm Metab Res 2003;35:282-9.
[32] Folli F, Saad MJA, Backer JM, et al. Insulin stimulation of phosphatidylinositol 3-kinase activity and association with insulin receptor substrate 1 in liver and muscle of the intact rat. J Biol Chem 1992;267:22171-7.
[33] Velloso LA, Folli F, Sun XJ, et al. Cross-talk between the insulin and angiotensin signaling systems. Proc Natl Acad Sci 1996;93:12490-5.

[34] Rafacho A, Giozzet VAG, Boschero AC, et al. Functional alterations in endocrine pancreas of rats with different degrees of dexamethasone-induced insulin 28 resistance. Pancreas 2008;36:284-93.
[35] Malaisse WJ, Carpinelli AR, Lebrun P, et al. The stimulus-secretion coupling of amino acid-induced insulin release. IV. Ionic response to L-leucine and L-glutamine. Pflugers Arch 1981;391:112-8.
[36] MacDonald MJ, Fahien LA, Brown LJ, et al. Perspective: emerging evidence for signaling roles of mitochondrial anaplerotic products in insulin secretion. Am J Physiol Endocrinol Metab 2005;288:E1-15.
[37] Macdonald MJ, Hasan NM, Longacre MJ. Studies with leucine, beta-hydroxybutyrate and ATP citrate lyase-deficient beta cells support the acetoacetate pathway of insulin secretion. Biochim Biophys Acta 2008;1780:966-72.
[38] Taegtmeyer H, Hems R, Krebs HA. Utilization of energy-providing substrates in the isolated working rat heart. Biochem J 1980;186: 701-11.
[39] Sahlin K, Katz A, Broberg S. Tricarboxylic acid cycle intermediates in human muscle during prolonged exercise. Am J Physiol Cell Physiol 1990;259:C834-41.
[40] Lynch CJ, Fox HL, Vary TC, et al. Regulation of amino acid-sensitive TOR signaling by leucine analogues in adipocytes. J Cell Biochem 2000;77:234-51.
[41] Iiboshi Y, Papst PJ, Kawasome H, et al. Amino acid–dependent control of p70(s6k). Involvement of tRNA aminoacylation in the regulation. J Biol Chem 1999;274:1092-9.
[42] Hara K, Yonezawa K, Weng QP, et al. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem 1998;273:14484-94.
[43] Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123:1939-51.
[44] Bruhat A, Jousse C, Fafournoux P. Amino acid limitation regulates gene expression. Proc Nutr Soc 1999;58:625-32.
[45] Jousse C, Bruhat A, Fafournoux P. Amino acid regulation of gene expression. Curr Opin Clin Nutr Metab Care 1999;2:297-301.
[46] Xu GG, Gao ZY, Borge PD, et al. Insulin receptor substrate 1–induced inhibition of endoplasmic reticulum Ca2+ uptake in beta-cells. Autocrine regulation of intracellular Ca2+ homeostasis and insulin secretion. J Biol Chem 1999;274:18067-74.
[47] Jousse C, Bruhat A, Ferrara M, et al. Evidence for multiple signaling pathways in the regulation of gene expression by amino acids in human cell lines. J Nutr 2000;130:1555-60.
[48] Xu GG, Gao ZY, Borge PD, et al. Insulin regulation of beta-cell function involves a feedback loop on SERCA gene expression, Ca(2+) homeostasis, and insulin expression and secretion. Biochemistry 2000;39:14912-9.
[49] Aspinwall CA, Qian WJ, Roper MG, et al. Roles of insulin receptor substrate–1, phosphatidylinositol 3-kinase, and release of intracellular Ca2+ stores in insulin stimulated insulin secretion in beta-cells. J Biol Chem 2000;275:22331-8.
[50] Hannan KM, Thomas G, Pearson RB. Activation of S6K1 (p70 ribosomal protein S6 kinase 1) requires an initial calcium-dependent priming event involving formation of a high-molecular-mass signalling complex. Biochem J 2003;370:469-77.
[51] Patti ME, Brambilla E, Luzi L, et al. Bidirectional modulation of insulin action by amino acids. J Clin Invest 1998;101:1519-29.
[52] Fafournoux P, Bruhat A, Jousse C. Amino acid regulation of gene expression. Biochem J 2000;351:1-12.
[53] Kwon G, Marshall CA, Pappan KL, et al. Signaling elements involved in the metabolic regulation of mTOR by nutrients, incretins, and growth factors in islets. Diabetes 2004;53:S225-232.
[54] Fraenkel M, Ketzinel-Gilad M, Ariav Y, et al. mTOR inhibition by rapamycin prevents beta-cell adaptation to hyperglycemia and exacerbates the metabolic state in type 2 diabetes. Diabetes 2008;57:945-57.
[55] Aspinwall CA, Lakey JRT, Kennedy RT. Insulin-stimulated insulin secretion in single pancreatic beta cells. J Biol Chem 1999;274:6360-5.