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Decreased insulin secretion in islets from protein malnourished rats is associated with impaired glutamate dehydrogenase function: effect of leucine supplementation

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ABSTRACT

We herein studied the role of glutamate dehydrogenase (GDH), in response to leucine (LEU) supplementation, upon insulin secretion of malnourished rats. Weaned male Wistar rats were fed normal-protein (17%) or low-protein diet (6%, LP) for 8 weeks. Half of the rats of each group were supplemented with LEU (1.5%) in the drinking water for the following 4 weeks. Gene and protein expressions, static insulin secretion, and cytoplasmic Ca^{2+} oscillations were measured. Glutamate dehydrogenase messenger RNA was 58% lower in LP islets, and LEU supplementation augmented it in 28%. The LP islets secreted less insulin when exposed to 20 mmol/L LEU, 20 mmol/L LEU + 2 mmol/L glutamine (with or without 5 mmol/L aminooxyacetic acid, a branched chain aminotransferase inhibitor, or 20 μmol/L epigallocatechin gallate, a GDH inhibitor), 20 mmol/L α-ketoisocaproate, glutamine + 20 mmol/L β-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (a GDH activator), and 22.2 mmol/L glucose. Leucine supplementation augmented insulin secretion to levels found in normal-protein islets in all the above conditions, an effect that was blunted when islets were incubated with epigallocatechin gallate. The glutamine + β-2-aminobicyclo[2.2.1]heptane-2-carboxylic acid–induced increased $[Ca^{2+}]_i$ and oscillations were higher than those for LP islets. Leucine supplementation normalized these parameters in LP islets. Impaired GDH function was associated with lower insulin release in LP islets, and LEU supplementation normalized insulin secretion via restoration of GDH function. In addition, GDH may contribute to insulin secretion through ameliorations of Ca^{2+} handling in LP islets.

1. Introduction

Glutamate dehydrogenase (GDH) is a homohexameric enzyme with 2 isoforms, encoded by 2 distinct genes (*GLUD1* and *GLUD2*), and located in the mitochondrial matrix [1-3]. Glutamate dehydrogenase catalyzes the reversible reaction α-ketoglutarate + NH_3 + nicotinamide adenine dinucleotide phosphate [NAD(P)H] ↔ glutamate +

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$NAD(P)^+$ [1,4-6]. This enzyme is allosterically regulated positively by leucine (LEU) and adenosine diphosphate and negatively by guanosine triphosphate (GTP) [7-10]. Glutamate dehydrogenase is highly expressed in the brain, liver, kidney, and pancreas [2,5,6]. In the brain, GDH participates in the cycling of the neurotransmitter glutamate between neurons and astrocytes [11]. It is also important for ammonia metabolism and detoxification in liver and kidney [12]. In pancreatic β-cells, GDH plays an important role in the regulation of insulin secretion [7]. Inhibition of GDH activity decreases insulin release [6,8,9,13], whereas increasing its activity has been associated with a hyperinsulinism syndrome [14,15].

The participation of GDH in the insulin secretion process has been well documented [7,16]. Mutations in the GDH inhibitory GTP binding domain are associated with the hyperinsulinemia-induced hypoglycemia in children (hyperinsulinism/hyperammonemia syndrome) [14,15,17]. Glutamate dehydrogenase inhibitors such as green tea polyphenol, epigallocatechin gallate (EGCG), and 5′-deoxypyridoxal showed reduced LEU- or β-2-aminobicyclo[2.2.1] heptane-2-carboxylic acid (BCH)–induced insulin secretion [13,18]. Glutamate dehydrogenase (GDH) knockout in mice pancreas was associated with lower insulin release stimulated with glucose and BCH [19]. Finally, GDH superexpression in rat islets and insulinoma-1E (INS-1E) β-cell line was associated with augmented glucose-induced insulin secretion [20].

Although it is widely accepted that GDH plays a role in the amplification of insulin secretion, it is still unclear how this modulation occurs [21]. Glutamate dehydrogenase may regulate glucose-induced secretion, generating glutamate [22,23], suggested to be a metabolic coupling factor, or by the production of anaplerotic compounds that amplify the insulin secretion response [19,24-26]. Branched-chain amino acids stimulate insulin release through the reaction catalyzed by GDH [27,28,29] or by mitochondrial branched-chain aminotransferase (BCATm) [30], generating metabolic signals such as adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH), which in turn control insulin secretion [19,22-26]. Leucine is thought to act in 2 different ways: (*a*) by transferring the amino group to α-ketoglutarate, resulting in the production of α-ketoisocaproate (KIC) and glutamate [31-33], and/or (*b*) by allosteric activation of GDH, augmenting the tricarboxylic acid cycle intermediates [24,26], ATP [7], or glutamate content [22,23].

It is known that protein restriction during early life reduces insulin secretion [34-42]. Proteins involved in insulin secretion such as glucokinase [35], protein kinase Cα [38,41], protein kinase Aα [41], and synaptosomal-associated protein of 25 kd [43], as well as the GDH content, are reduced in islets from protein-malnourished rats [42]. Moreover, LEU supplementation has been associated with the restoration of proteins involved in the secretory machinery, including GDH, improving insulin secretion [40-42]. Here, we observed that, in malnourished rats, the impaired GDH function was associated with lower insulin release. In addition, the restoration of GDH function, induced by LEU supplementation, normalized nutrient-induced secretion. This effect seems to be dependent, at least in part, on ameliorations of Ca^{2+} handling by LP pancreatic islet cells.

2. Materials and methods

2.1. Chemicals

Human recombinant insulin (^{125}I) was purchased from PerkinElmer (Boston, MA). Human recombinant insulin Biohulin N was purchased from Biobrás (Montes Claros, MG, Brazil). Standard commercial kits were used for measurement of plasma total proteins, albumin (both from Laborlab, Guarulhos, SP, Brazil), total cholesterol (CHOL), triglycerides (TG) (both from Roche Diagnostics, Mannheim, Germany), and free fatty acid (FFA; Wako Chemicals, Neuss, Germany). Glutamate dehydrogenase antibody was from Rockland Immunochemical (Gilbertsville, PA). Visualization of specific protein bands was obtained with SuperSignal West Pico from Pierce (Rockford, IL). TRIzol, horseradish peroxidase–conjugated secondary antibody, and Fura-2AM were purchased from Invitrogen (Carlsbad, CA). Fast SYBR Green technology was from Applied Biosystems (Foster City, CA). Primers were purchased from Integrated DNA Technologies (Coralville, IA). All others reagents were purchased from Sigma Chemical (St Louis, MO).

2.2. Animals, diet, and LEU supplementation

All experiments were approved by the University's Committee on Ethics in Animal Experimentation. Weaned Male Wistar rats (21 days old) from the breeding colony at the University of Campinas were housed in standard cages and maintained on a 12-hour light/dark cycle (lights on 6:00 AM to 6:00 PM) and controlled temperature (22°C ± 1°C). The rats were fed on a normal-protein (17%, NP) or low-protein isocaloric diet (6%, LP) for 60 days. Half of the rats from each group were then supplemented with LEU (1.5%) in the drinking water for the next 30 days (NPL and LPL groups). The 2 isocaloric diets' compositions are detailed elsewhere [39].

2.3. Animal features

Body weight and food intake were measured throughout the experimental period (from 21 to 105 days of life). Food intake was expressed by feed efficiency (food intake/body weight) [44]. At the end of the diet treatment and supplementation period, rats were decapitated, their blood was collected, and plasma stored at –20°C. Heart, liver, spleen, kidney, and perigonadal and retroperitoneal fat pad weights were measured. Blood glucose was measured using a glucose analyzer (Accu-Chek Advantage; Roche Diagnostics, Basel, Switzerland) and insulin was measured by radioimmunoassay, as previously reported [45]. Total plasma protein, plasma albumin, CHOL, TG, and FFA were measured using standard commercial kits, according to the manufacturer's instructions. For the intraperitoneal insulin tolerance test, blood glucose (time 0) was measured as described above in independent groups of 6-hour–fasted rats. The rats received 2 U/kg body weight of human recombinant insulin in the peritoneal cavity. Blood glucose concentrations were then measured at 4, 8, 12, and 16 minutes after insulin administration. The constant ratio for glucose disappearance (kITT) was calculated using the

analysis of the square fall of glucose concentration during the linear phase decay, as detailed elsewhere [46].

2.4. Western blotting analysis

Pieces of about 150 mg from the brain, liver, kidney, muscle, and 500 islets were solubilized in homogenization buffer containing protease inhibitors, as previously described [47]. Total protein content was measured by the Bradford method [48]. Samples with 30 μg of protein were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Equal loading was confirmed with Ponceau staining [37,49]. The nitrocellulose membranes were treated overnight with a blocking buffer (5% nonfat dried milk, 10 mmol/L Tris, 150 mmol/L NaCl, and 0.02% Tween 20) and were subsequently incubated with a polyclonal antibody against GDH (1:5000). Detection was performed using enhanced chemiluminescence after a 2-hour incubation with a horseradish peroxidase–conjugated secondary antibody (1:10 000). Band intensities were quantified by optical densitometry (Scion Image, Frederick, MD).

2.5. Islet isolation and insulin secretion

Islets were isolated, as previously described, by collagenase digestion of the pancreas [50]. For static incubation, 5 islets from each group were first incubated for 45 min at 37°C in Krebs-Ringer bicarbonate buffer (KRBB) with the following composition (in millimoles per liter): 115 NaCl, 5 KCl, 2.56 $CaCl_2$, 1 $MgCl_2$, 10 $NaHCO_3$, and 15 HEPES, supplemented with 5.6 glucose and 3 g/L of bovine serum albumin and equilibrated with a mixture of 95% O_2/5% CO_2 to give pH 7.4. This medium was then replaced with fresh buffer, and the islets were incubated for 1 hour with (in millimoles per liter) 2.8 and 22.2 glucose, 2 glutamine (GLN), 20 LEU, 10 β-2-amino-2-norbornanecarboxylic acid (BCH), 20 KIC. Aminooxyacetic acid (AOA) (5 mmol/L) was also used in combination with LEU + GLN. For the experiments with EGCG, islets were preincubated with 20 μmol/L EGCG; the medium was replaced with 20 μmol/L EGCG plus the other compounds cited above. At the end of the incubation period, samples from independent experiments were collected on different days and stored at −80°C. The insulin concentration of the medium was determined by the radioimmunoassay method.

2.6. Cytoplasmic Ca^{2+} oscillations

Fresh pancreatic islets were incubated with Fura-2/AM (5 μmol/L) for 1 hour at 37°C in KRBB buffer containing 5.6 mmol/L glucose, 0.3% bovine serum albumin, and pH 7.4. The islets were washed with the same medium and placed in a chamber at 37°C on the stage of an inverted microscope (Nikon UK, Kingston, UK). Islets were then perifused with KRBB and continuously gassed with 95% O_2/5% CO_2, pH 7.4 containing 2.8 mmol/L glucose, 2 mmol/L GLN, and 10 mmol/L BCH. A ratio image was acquired approximately every 5 seconds with an ORCA-100 CCD camera (Hammamatsu Photonics Iberica, Barcelona, Spain), in conjunction with a Lambda-10-CS dual filter wheel (Sutter Instrument, Novato, CA), equipped with 340 and 380 nm, 10-nm bandpass filters, and a range of neutral density filters (Omega Opticals, Stanmore, UK). Data were obtained using the ImageMaster3 software (Photon Technology International, Birmingham, NJ).

2.7. Real-time polymerase chain reaction

Total cellular RNA was extracted from groups of 500 islets using TRIzol reagent. One microgram of total RNA was reverse transcribed using a reverse transcriptase and random hexamer primers. Real-time polymerase chain reactions (PCRs) were performed in a total volume of 15 μL using the Fast SYBR Green technology. Samples were denatured at 94°C for 10 minutes followed by 40 PCR cycles at 95°C/60°C. The PCR amplifications were performed in duplicate. The purity of the amplified PCR products was verified by melting curves. The expression of the target genes was normalized against the expression levels of the housekeeping gene ribosomal protein S29 (RPS-29). The sequences of the primers used were (5′-3′): GDH forward: GCCACTACAGCGAAGCGG, GDH reverse: CGGGTCTTCAGGTCTTCCAC; RPS-29 forward: TTTTTCCTTGGGCGTCTG, RPS-29 reverse: ACGGAAGCACTGTCGGCACA.

2.8. Statistical analysis

Results are expressed as means ± SEM of the indicated number of experiments. Two-way analysis of variance followed by Newman-Keuls post hoc test was used for comparisons among groups, 1-way analysis of variance followed by Newman-Keuls post hoc test was used for multiple comparisons within a group, and a 2-tailed nonpaired *t* test was used to assess the difference between 2 groups. The significance level adopted was $P < .05$.

3. Results

3.1. Characterization of malnourishment

Fig. 1 (A and B) shows that LP body weight was significantly lower compared with that in NP rats after 29 days ($P < .001$). This difference persisted until the end of the treatment and was not modified by LEU supplementation (NPL and LPL groups) ($P < .001$). The LP group showed increased food efficiency compared with NP rats, and LEU supplementation did not change this parameter (Fig. 1C; $P < .0002$ and $P < .0003$, respectively). Table 1 shows that, except for the heart, the reduction in tissue weight was proportional to that observed for the body weight.

Table 2 shows that fast plasma total protein and albumin were reduced, whereas FFA was increased, in LP compared with NP rats. During the fed state, insulin plasma levels were also reduced in LP rats. Leucine supplementation restored fasted albumin and fed insulin plasma levels in LPL rats. The LP rats showed higher insulin sensitivity, as judged by the kITT; and LEU supplementation reduced this sensitivity in the LPL rats.

3.2. GDH messenger RNA and protein content in tissues from LP supplemented or not with LEU

Fig. 2 shows that the GDH protein content was similar in the brain (A), liver (B), kidney (C), and skeletal muscles (D) in all

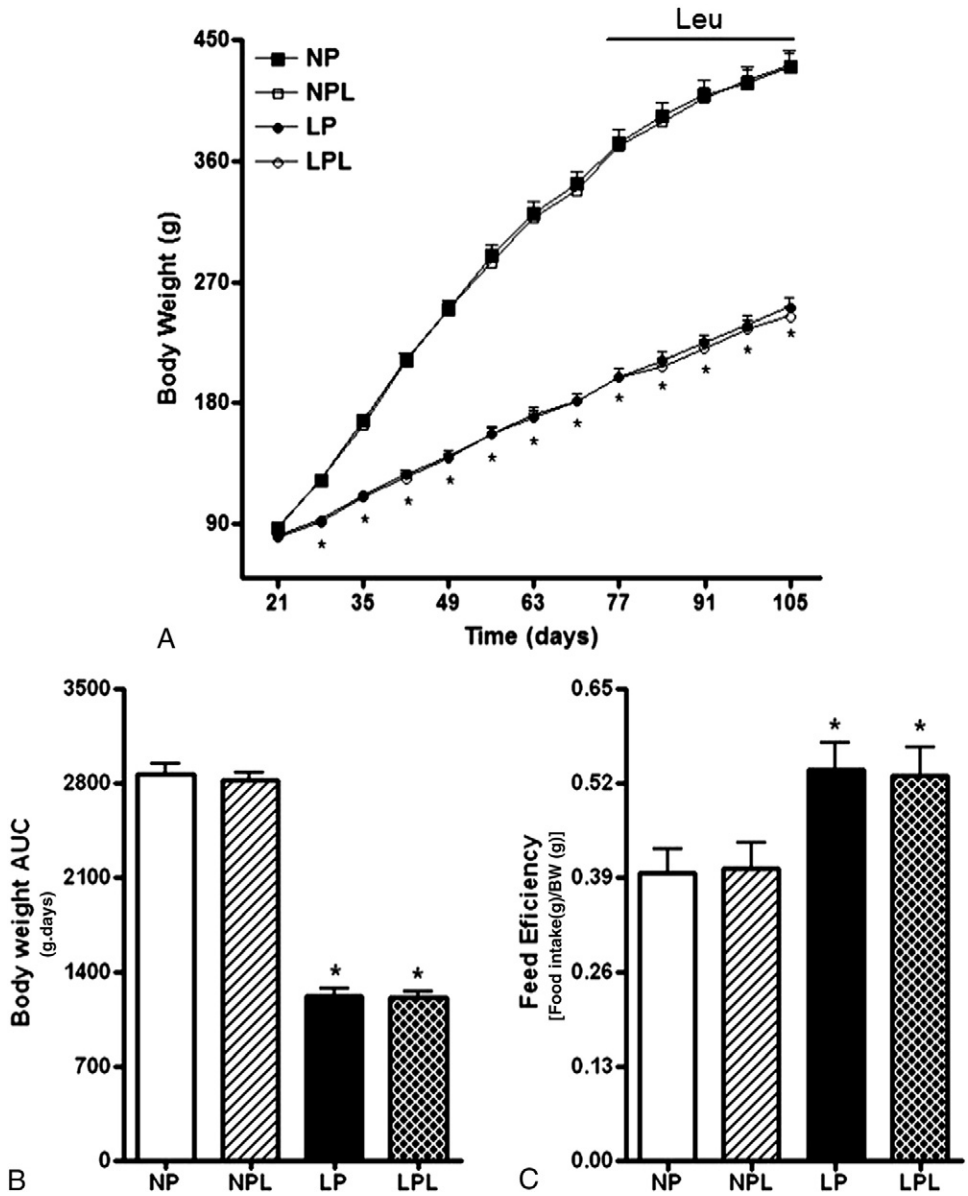


Fig. 1 – Body weight (A and B) and food efficiency (C) of rats fed on an NP or a LP diet without or with LEU supplementation. Values are means ± SEM; n = 15 rats. *Significant difference compared with NP, $P < .05$.

groups. However, and as recently reported, GDH protein and messenger RNA (mRNA) expression was approximately 30% and 60% lower compared with NP islets; and LEU supplementation restored GDH to similar levels observed in NP islets (Fig. 3) [42]. These data indicate that protein restriction affects GDH content only in the endocrine pancreas.

Table 1 – Rat body weight and tissue weight at the end of the treatment

	NP (n = 12)	NPL (n = 11)	LP (n = 11)	LPL (n = 12)
BW (g)	466.4 ± 9.2	467.5 ± 13.0	299.5 ± 11.8*	301.9 ± 13.8*
Heart (mg/g of BW)	2.5 ± 0.06	2.8 ± 0.1	3.3 ± 0.2*	3.2 ± 0.1*
Liver (mg/g of BW)	32.5 ± 1.1	32.0 ± 1.2	30.6 ± 1.3	30.9 ± 1.7
Spleen (mg/g of BW)	2.0 ± 0.06	2.2 ± 0.1	2.4 ± 0.1	2.2 ± 0.1
Kidney (mg/g of BW)	5.6 ± 0.1	5.7 ± 0.2	5.5 ± 0.2	5.6 ± 0.2
RPF (mg/g of BW)	21.4 ± 1.3	19.9 ± 3.5	13.0 ± 1.4*	13.5 ± 1.4*
PGF (mg/g of BW)	20.9 ± 1.1	18.0 ± 2.1	12.5 ± 1.2*	13.7 ± 1.5*

Data are means ± SEM. BW indicates body weight. PGF, perigonadal fat; RPF, retroperitoneal fat.
* Significant difference compared with NP, $P < .05$.

Table 2 – Total plasma protein, albumin, glucose, insulin, TG, CHOL, and FFA concentrations and the kITT from fasted and fed 105-day-old NP, NPL, LP, and LPL rats

	NP (n)	NPL (n)	LP (n)	LPL (n)
Total protein, g/dL	7.6 ± 0.3 (14)	7.6 ± 0.15 (14)	6.3 ± 0.2 (11) *	6.2 ± 0.2 (11) *
Albumin, g/dL	2.6 ± 0.04 (12)	2.7 ± 0.1 (13)	2.4 ± 0.1 (9) *	2.6 ± 0.1 (10) *
Glucose, [a] mg/dL	87.3 ± 2.6 (15)	85.7 ± 3.3 (14)	87.3 ± 3.1 (12)	81.9 ± 3.1 (13)
Glucose, [c] mg/dL	102.7 ± 3.1 (13)	99.1 ± 3.7 (11)	96.3 ± 1.9 (17)	100.3 ± 2.4 (13)
Insulin, [b] ng/mL	1.1 ± 0.2 (14)	1.2 ± 0.3 (12)	0.9 ± 0.1 (14)	0.9 ± 0.1 (11)
Insulin, [c] ng/mL	2.9 ± 0.3(8)	2.9 ± 0.5 (8)	1.1 ± 0.1 (12) *	2.5 ± 0.6 (7)
TG, mg/dL	74.6 ± 13.9 (4)	60.5 ± 5.3 (4)	76.5 ± 7.8 (5)	97.8 ± 7.3 (4)
CHOL, mg/dL	78.90 ± 4.24 (4)	83.0 ± 2.72 (4)	87.40 ± 2.63 (5)	87.0 ± 2.06 (4)
FFA, mmol/L	0.64 ± 0.08 (5)	0.68 ± 0.07 (4)	1.10 ± 0.09 (6) *	0.97 ± 0.09 (5) *
kITT (%/min)	1.95 ± 0.33 (7)	1.39 ± 0.42 (6) *	3.91 ± 0.24 (8) *	2.72 ± 0.21 (7)

[a] Twelve-hour fasted.
[b] Six-hour fasted. Data are means ± SEM.
[c] Fed rats.
* Significant difference compared with NP, $P < .05$.

3.3. *GDH activation and insulin secretion in LP islets*

Fig. 4 shows that LEU-induced insulin secretion was increased in NPL and decreased in LP islets, and that supplementation with LEU restored the insulin secretion in the LPL group. When the islets were challenged with the combination of LEU and GLN, which potentiate secretion [31,51], the insulin secretion pattern was similar to that observed for LEU alone; however, the magnitude of secretion was higher. In the next series of experiments, we stimulated the islets with LEU + GLN in the presence of AOA (aminotransferase inhibitor), observing that the secretion was not altered by the inhibitor. Subsequently, the islets were incubated with LEU + GLN + EGCG (GDH inhibitor), resulting in a significant reduction in insulin secretion in LPL islets. Finally, we stimulated the islets with KIC, observing that the insulin secretion was also reduced in LPL islets. Taken together, these results indicate that LEU supplementation improved LEU-induced insulin secretion by allosteric GDH activation.

To analyze the GDH function, we incubated the islets in the presence of GLN and the nonmetabolizable LEU analogue BCH, with or without EGCG (Fig. 5). Glutamine + BCH stimulated insulin secretion in a similar manner to that of LEU + GLN, with the former combination being 2-fold less effective. Leucine supplementation augmented insulin secretion induced by GLN + BCH (Fig. 5, $P < .009$). However, for all groups, insulin secretion induced by GLN + BCH was 2-fold lower than that for LEU + GLN. In contrast, following the addition of AOA or EGCG to LEU + GLN, the insulin secretion from NP and NPL islets was about 50% lower than that provoked by the combination of EGCG + GLN and BCH (Fig. 5; $P < .05$ and $P < .01$, respectively). The addition of EGCG did not reduce insulin secretion in LP, but significantly reduced it in LPL islets to values comparable to those observed in NP and NPL islets incubated in the presence of EGCG ($P < .01$ and $P > 0.05$, respectively). Because the EGCG inhibitory effect upon insulin secretion is not observed in LP, it seems that the GDH control over insulin secretion is lost in LP islets.

To evaluate whether GDH content and GDH activation were important for glucose-stimulated secretion, we incubated the islets in the presence of glucose + EGCG (Fig. 6). As previously described [40,42], insulin secretion in LP islets was lower; and LEU supplementation augmented it ($P < .0001$ and $P < .005$, respectively). Addition of EGCG did not affect insulin secretion in NP, NPL, and LP islets. However, insulin secretion in LPL was reduced by almost 40% ($P < .0001$), reaching values similar to those observed in LP islets. In conclusion, activation of GDH seems to be important for insulin secretion when stimulated by nutrient secretagogues.

3.4. *LEU supplementation augments intracellular calcium concentration in LP islets*

Fig. 7 shows the Ca^{2+} handling in islets of all groups stimulated with 10 mmol/L BCH. The LP group showed lower intracellular calcium concentration ($[Ca^{2+}]_i$) compared with NP islets (Fig. 7E, $P < .007$), and LEU supplementation restored $[Ca^{2+}]_I$ to levels similar to those observed in NP islets ($P < .04$). Between minutes 0 and 10 of the perifusion period, a reduction in $[Ca^{2+}]_i$ was observed in islets of all groups (Fig. 7A-D); and this reduction could be attributed to Ca^{2+} sequestration by endoplasmic reticulum (ER) due to an increase in cellular ATP content [52]. The total $[Ca^{2+}]_i$ in this period (demonstrated by area under the curve, Fig. 7E) was higher in LP islets. Although not exclusive, this indicates either that the ER from these islets was either not able to take up the cation or that the cells produce less ATP ($P < .001$). These anomalies in Ca^{2+} handling were not observed in LPL, which showed a similar behavior to that for NP islets. Between minutes 10 and 20 of perifusion, the total $[Ca^{2+}]_i$ was lower in LP islets ($P < .02$), but normalized in islets from LEU-supplemented rats.

4. Discussion

In agreement with previous studies [34-40,42,43], in the current study, LP rats showed low body and tissue weight and alterations in several plasma parameters, indicating the efficiency of the treatment. Moreover, we extended our knowledge regarding the impairment of pancreatic function in LP rats and the benefits of LEU supplementation.

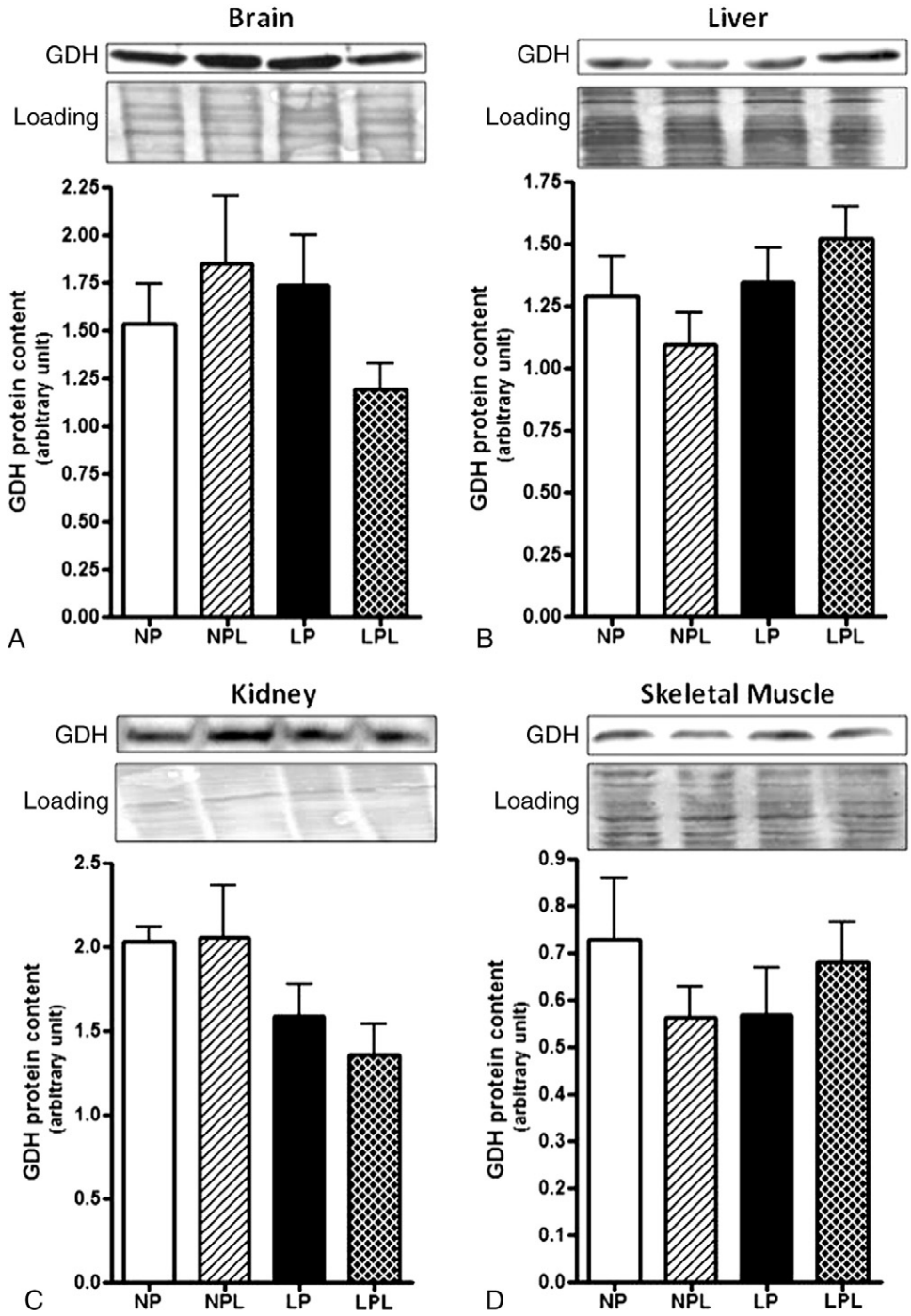


Fig. 2 – Glutamate dehydrogenase protein expressions in the brain (A), liver (B), kidney (C), and muscle (D) from NP, NPL, LP, and LPL rats. The bars represent the means ± SEM of the values, measured by optical densitometry; n = 4. *Significant difference compared with NP, $P < .05$.

Herein, using specific GDH allosteric activators and inhibitors, we provided evidence for at least 2 mechanisms by which LEU supplementation restores insulin secretion in LP islets: (a) by increasing GDH mRNA and protein content and (b) by reestablishing GDH allosteric modulation. However, it should be kept in mind that measurement of GDH activity would strengthen our findings.

Leucine is of major interest because it can act as a pancreatic islet intracellular signaling molecule as well as a metabolic substrate for insulin secretion. These characteristics could provide several benefits for the protein-malnourished state that, ultimately, impairs insulin secretion. In addition, a close association was demonstrated between the specific effects of LEU upon GDH protein content and activity. Leucine supplementation activates the phosphatidylinositol 3-phosphate kinase/mammalian target protein of rapamycin pathway and increases GDH protein content in islets from malnourished rats [40,42]. Leucine-induced intracellular

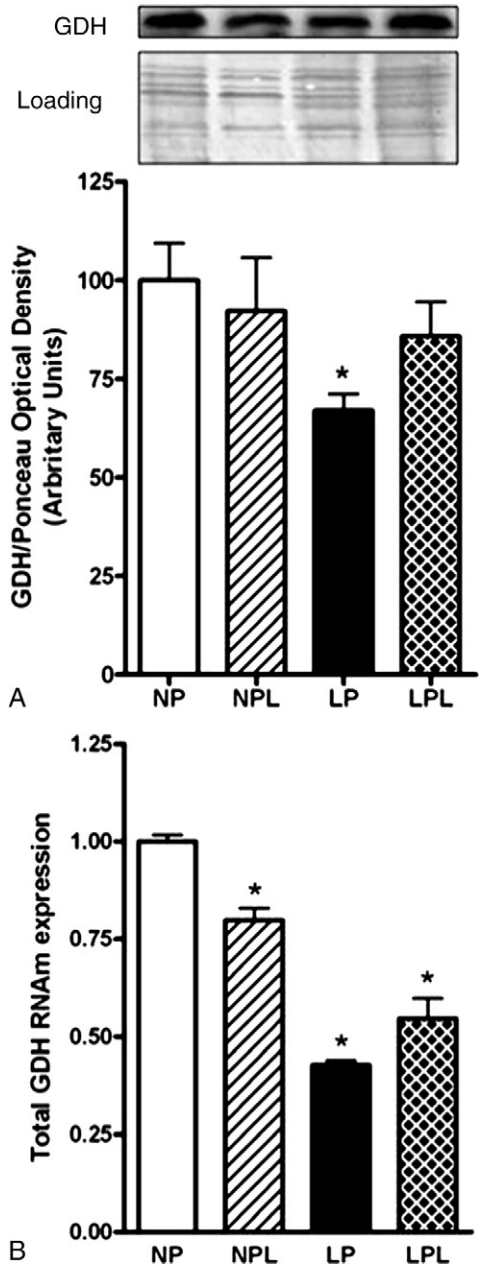


Fig. 3 – Glutamate dehydrogenase protein content (A) and real-time PCR determination of GDH mRNA expression (B) in isolated islets from NP, NPL, LP, and LPL rats. The results from GDH protein content were corrected for Ponceau, whereas GDH mRNA expression was corrected for RPS-29 expression. Both results are shown relative to NP. Values are means ± SEM; n = 5. *Significant difference compared with NP, $P < .05$.

signaling was reported to be linked with GDH function [53]. Thus, improved GDH mRNA and protein content would favor GDH activity. Leucine was chosen for supplementation because of its higher potency in increasing protein synthesis compared with other amino acids such as isoleucine, valine, and GLN in pancreatic islets from rodents [54].

It is well established that LEU is an insulinotropic agent [25,55] and stimulates insulin secretion by its metabolism

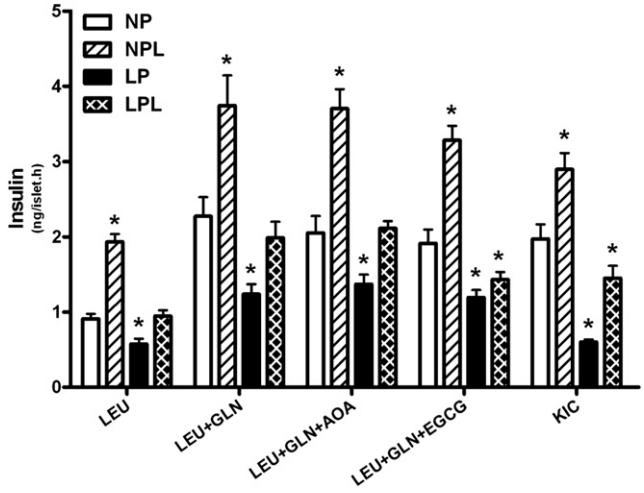


Fig. 4 – Insulin secretion induced by LEU (20 mmol/L), LEU (20 mmol/L) plus GLN (2 mmol/L) with AOA (5 mmol/L) or EGCG (20 μmol/L), and KIC (20 mmol/L) in islets from NP, NPL, LP, and LPL rats. In all conditions, 2.8 mmol/L glucose was present in the incubation medium. Each bar represents means ± SEM from 12 to 24 groups of islets. *Significant difference compared with NP, $P < .05$.

via BCATm or by activating GDH allosterically. There are several metabolic intermediates thought to amplify insulin secretion signaling, the so-called metabolic coupling factors. The main proposed factors are ATP, NADPH, reactive oxygen species, glutamate (GLU), and long-chain acyl-CoAs [56]. It is

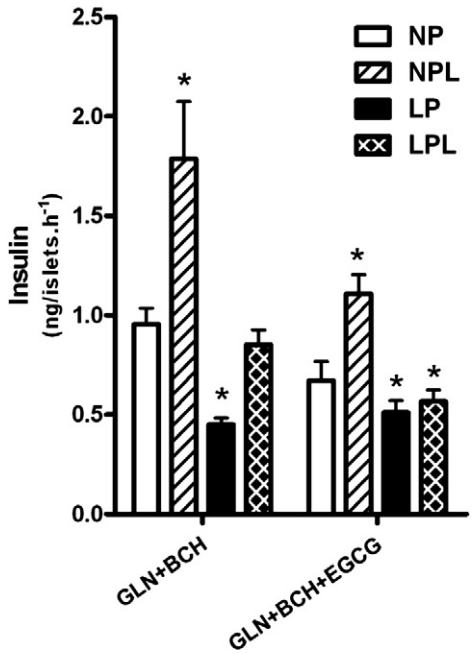


Fig. 5 – Insulin secretion induced by GLN (2 mmol/L) plus BCH (20 mmol/L) with or without EGCG (20 μmol/L) in islets from NP, NPL, LP, and LPL rats. There was no addition of glucose. Each bar represents means ± SEM from 12 to 24 groups of islets. *Significant difference compared with NP, $P < .05$.

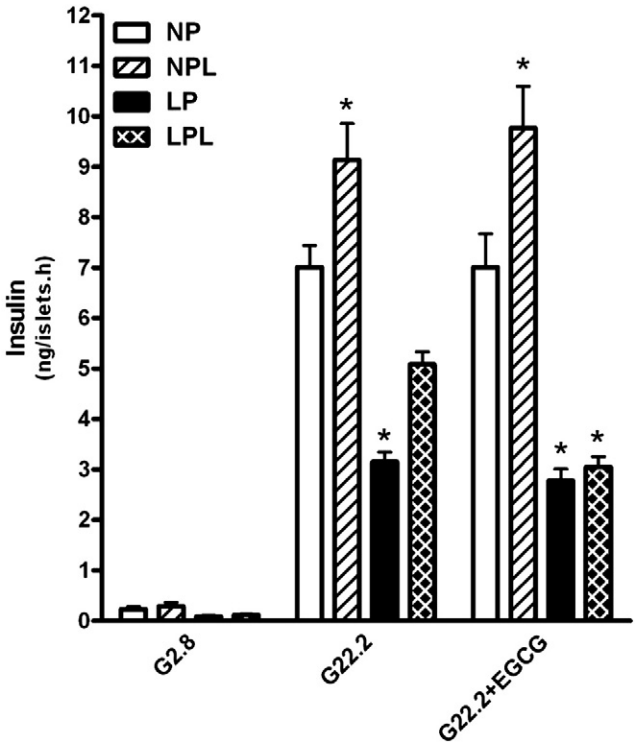


Fig. 6 – Insulin secretion induced by glucose 2.8 mmol/L (G2.8) or 22.2 mmol/L (G22.2) with or without EGCG (20 μmol/L) in islets from NP, NPL, LP, and LPL rats. Each bar represents means ± SEM from 12 groups of islets. *Significant difference compared with NP, $P < .05$.

still under discussion which compound resulting from LEU metabolism is more important for insulin secretion signaling and, consequently, the main LEU-induced insulin secretion pathway.

There is evidence that the GDH reaction modulates insulin release preferentially through the production of GLU, which might be exported from mitochondria as a coupling factor. In permeabilized INS-1 cells, under conditions of fixation $[Ca^{2+}]_i$, addition of glutamate directly stimulates insulin exocytosis independently of mitochondrial function [22]. Moreover, rat islets knocked down for mitochondrial glutamate carrier 1 present reduced insulin secretion when stimulated with glucose [23]. However, this is a controversial issue; and some authors suggest that glutamate is not a second messenger for insulin secretion [57,58]. In fact, increased cellular GLU content, following glucose-stimulated insulin secretion, either in INS-1 cells or isolated rat islets was not found [57]. In addition, rat islets cultured for 4 to 5 days in the presence of 10 mmol/L glucose showed higher GLN oxidation when incubated with BCH plus AOA than just with BCH or AOA, favoring α-ketoglutarate production [33]. Similarly, rat islets incubated with GLN and LEU or BCH augmented GLN oxidation [25]. Furthermore, transgenic mice that express the human GDH H454Y mutation with important modifications in enzyme kinetics show a severe form of the hyperinsulinism/hyperammonemia syndrome. In these mice, stimulation of GDH activity by LEU increases the oxidative deamination of GLU [59].

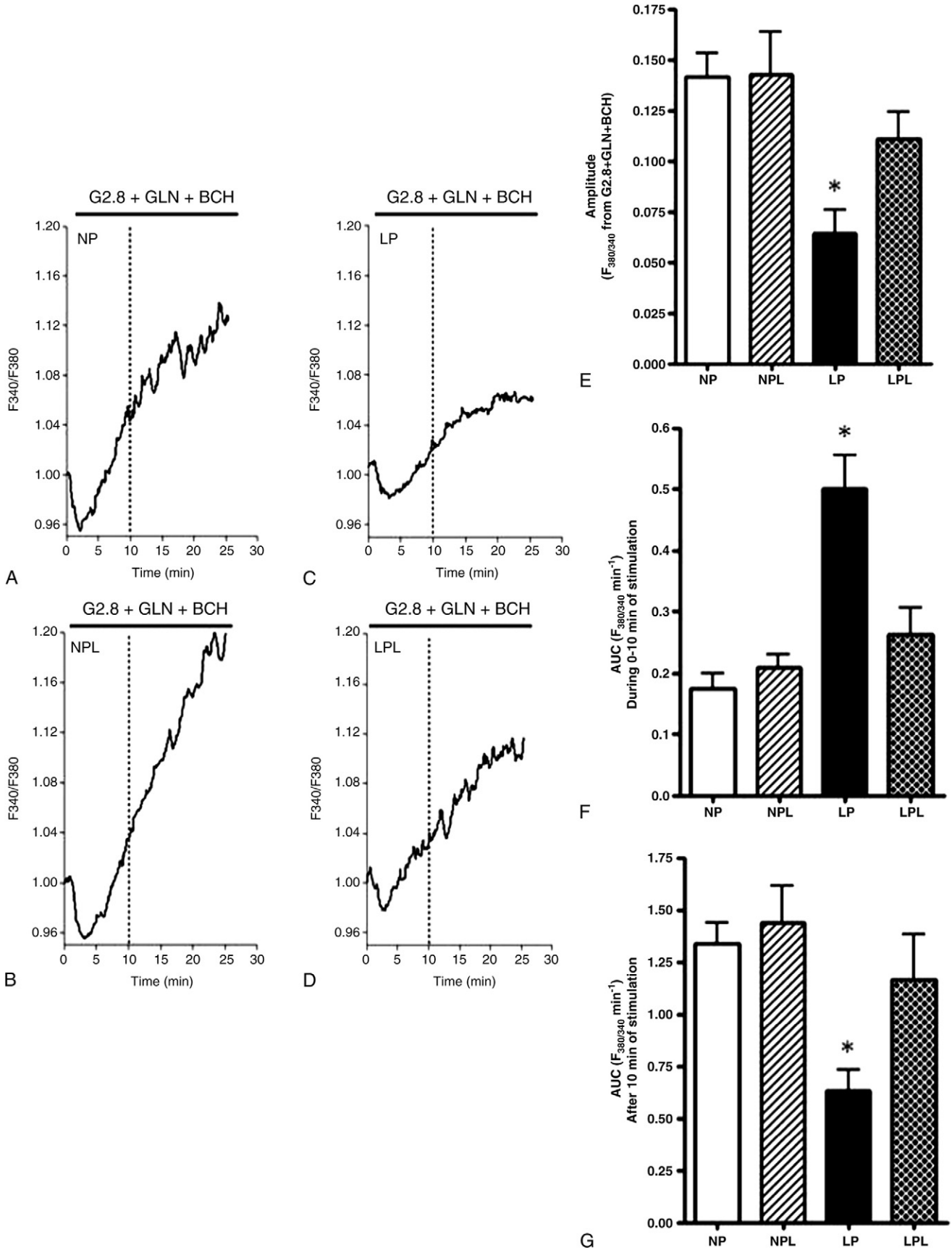
In addition to GDH activation, production of KIC by LEU catabolism is the major result of the reaction catalyzed by BCATm [31]. α-Ketoisocaproate may be converted to acetyl-CoA and acetoacetate by branched-chain ketoacid dehydrogenase complex, signaling insulin secretion [25]. α-Ketoisocaproate and glucose have similar potency upon insulin secretion. Nevertheless, there are studies showing the importance of α-ketoglutarate production by BCATm for insulin secretion; and increased transamination of GLU to α-ketoglutarate leads to hyperinsulinemia [51]. Mice islets knocked out for BCATm secrete less insulin when stimulated with KIC + GLN, but secrete normally when stimulated with dimethyl α-ketoglutarate + GLN or LEU + GLN [31]. Furthermore, α-ketoglutarate production is associated with the increase in NADPH and malonyl-CoA [56].

In the present study, NP islets stimulated with LEU + GLN exhibited similar insulin secretion in the presence of AOA or EGCG. Thus, GDH activation by LEU induces insulin secretion as well as LEU catabolism. This result is in agreement with others that show the importance of GDH activation and α-ketoglutarate production, enhancing the tricarboxylic acid flux and favoring insulin secretion [13,31,59]. In LPL islets stimulated with GLN + BCH, the addition of EGCG reduced insulin secretion, reaching values similar to those observed in LP islets. This reduction is justified because of LEU supplementation restores GDH function.

Insulin secretion in LPL islets was reduced at high concentrations of glucose, associated with EGCG. This is in agreement with a previous report showing impaired insulin secretion in response to high concentrations of glucose in GDH knockout mice islets [19]. For the first time, we reported here that impairment of insulin secretion, induced by glucose in LP rats, is due to the disruption of the GDH control of insulin secretion. We also showed that restoration of insulin secretion, induced by glucose or LEU, in LPL islets arises from the improvement of GDH function by LEU supplementation.

It has been reported that mice islets cultivated with AOA exhibit higher $[Ca^{2+}]_i$ when perifused with the combination of LEU + GLN [32]. Moreover, hyperinsulinemic mice with the GDH mutation in the inhibitory GTP binding domain showed higher $[Ca^{2+}]_i$ compared with control islets when stimulated with GLN [59]. This evidence suggests that GDH may modulate Ca^{2+} handling in pancreatic islets. In addition, LP islets showed reduced $[Ca^{2+}]_i$ when stimulated

Fig. 7 – Representative curves of BCH (10 mmol/L)-induced internal Ca^{2+} concentrations in islets isolated from NP (A), NPL (B), LP (C), and LPL (D) rats. (E) Amplitude and area under the curve of the $[Ca^{2+}]_i$ during the periods from 0 to 10 minutes (F) and 10 to 20 minutes of stimulation period (G) in response to BCH. The experiments were performed in a perifusion system with a KRBB buffer containing 2.8 mmol/L (G2.8) plus 2 mmol/L GLN and 10 mmol/L BCH. Values are the ratio of F340/F380 registered for each group. Data are means ± SEM obtained from 3 independent experiments. *Significant difference compared with NP, $P < .05$.


G2.8 + GLN + BCH
NP
F340/F380
Time (min)
A
G2.8 + GLN + BCH
LP
F340/F380
Time (min)
C
G2.8 + GLN + BCH
NPL
F340/F380
Time (min)
B
G2.8 + GLN + BCH
LPL
F340/F380
Time (min)
D
Amplitude
($F_{380/340}$ from G2.8+GLN+BCH)
NP
NPL
LP
LPL
E
AUC ($F_{380/340}$ min^{-1})
During 0-10 min of stimulation
NP
NPL
LP
LPL
F
AUC ($F_{380/340}$ min^{-1})
After 10 min of stimulation
NP
NPL
LP
LPL
G

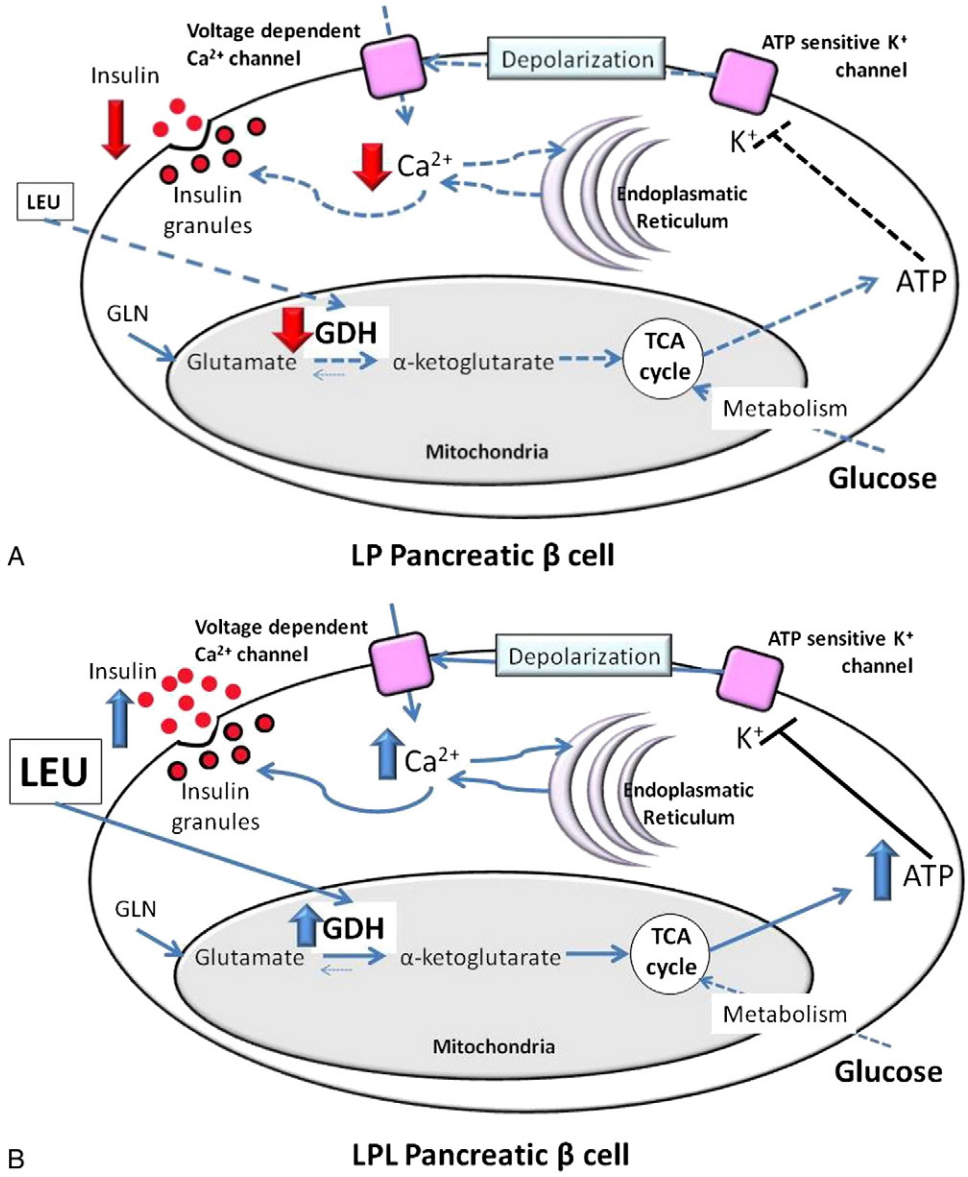


Fig. 8 – Overview of insulin secretion in ß-cells mediated by GDH in LP (A) and LPL (B). The LP diet diminished GDH content and Ca^{2+} handling and impaired insulin secretion (A). Leucine supplementation normalized nutrient-induced secretion by restoration of GDH function (B). Dotted arrows indicate impaired mechanisms by LP diet. Full arrows indicate restored mechanism by LEU supplementation. *LEU* in large letters in the panel indicates the LEU supplementation.

with glucose and other insulinotropic agents [43,60]. Reduction in $[Ca^{2+}]_i$, amplitude of Ca^{2+} oscillation, and lower capacity of ER Ca^{2+} sequestration were also found in LP islets stimulated by GLN + BCH. Leucine supplementation restored these parameters in LPL islets to values similar to those of the NP and NPL groups. Thus, it seems that reestablishment of GDH function is relevant for an adequate Ca^{2+} handling in LPL islets.

In conclusion, the LP diet affects islet GDH content and function, impairing insulin secretion signaling (Fig. 8A). It is conceivable that lower GDH function reduces α-ketoglutarate, ATP production, and Ca^{2+} handling and that LEU supplementation normalizes nutrient-induced insulin secretion by restoration of GDH function (Fig. 8B).

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Conflict of Interests

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 agreement with the institutional University of Campinas Committee for Ethics in Animal Experimentation (no. 2011-11).

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