Reduced Insulin Secretion in Protein Malnourished Mice Is Associated with Multiple Changes in the β-Cell Stimulus-Secretion Coupling

Sergi Soriano, Alejandro Gonzalez, Laura Marroquí, Eva Tudurí, Elaine Vieira, Andressa G. Amaral, Thiago M. Batista, Alex Rafacho, Antonio C. Boscher, Angel Nadal, Everardo M. Carneiro, and Ivan Quesada


The mechanism by which protein malnutrition impairs glucose-stimulated insulin secretion in the pancreatic β-cell is not completely known but may be related to alterations in the signaling events involved in insulin release. Here, we aimed to study the stimulus-secretion coupling of β-cells from mice fed with low-protein (LP) diet or normal-protein (NP) diet for 8 wk after weaning. Patch-clamp measurements in isolated cells showed that β-cells from LP mice had a resting membrane potential that was more hyperpolarized than controls. Additionally, depolarization and generation of action potentials in response to stimulatory glucose concentrations were also impaired in β-cells of LP mice. All these alterations in the LP group were most likely attributed to higher ATP-dependent K⁺ (KATP) channel activity in resting conditions and lower efficiency of glucose to induce the closure of these channels. Moreover, a Western blot analysis revealed higher protein levels of the sulfonylurea receptor of the KATP channel in islets of LP mice. Because β-cell Ca²⁺ signals depend on electrical activity, intracellular Ca²⁺ oscillations were measured by fluorescence microscopy in intact islets, indicating a lower response to glucose in the LP group. Finally, cell-to-cell synchrony of Ca²⁺ signals was analyzed by confocal microscopy. Islets from LP mice exhibited a decreased level of coupling among β-cells, which was probably due to the low expression levels of connexin 36. Therefore, low-protein diet leads to several alterations in the stimulus-secretion coupling of pancreatic β-cells that might explain the diminished insulin secretion in response to glucose in this malnutrition state. (Endocrinology 151: 3543–3554, 2010)

Diabetes is a multifactorial disease that is closely related to nutrition. The incidence of type 2 diabetes mellitus (T2DM) is growing with millions of people diagnosed with this profound metabolic disorder every year. In developed countries, the most common risk factors for the development of T2DM are family history of T2DM and a lifestyle that includes inactivity and overnutrition that lead to obesity (1). On the other hand, in developing countries, malnutrition is also an important risk factor for the susceptibility of multiple disorders including endocrine pancreas dysfunction and diabetes, which particularly affects children during their first year of life (2–4). Early malnutrition promotes adaptive metabolic changes to permit survival in critical conditions (5). One of the main organs affected by malnutrition is the endocrine pancreas which undergoes several structural and functional adap-

Abbreviations: AUC, Area under the curve; GSIS, glucose-stimulated insulin secretion; LP, low protein; NP, normal protein; T2DM, type 2 diabetes mellitus.
tations to maintain glucose homeostasis (6). Among the structural alterations, a marked reduction of the absolute β-cell mass and diminished number of β-cells per islet are observed in endocrine pancreas of malnourished rodent models (7, 8). Additionally, several functional changes have been reported, which include alterations in different signal transduction pathways and failures in glucose-stimulated insulin secretion (GSIS) (9). Depending on the type and intensity of the nutritional deprivation, and particularly if this nutrient deficiency occurs during intrauterine or postnatal periods, there is a progression of the β-cell dysfunction leading to insulinopenia (10). Epidemiological studies have also pointed to the relationship between low weight at birth, which is frequently associated to malnutrition states (11), with higher prevalence of obesity and diabetes in the adult life. Thus, it is necessary to have a better knowledge of the functional adaptations and changes that the endocrine pancreas undergoes during nutritional deprivation states.

We have previously shown that rats submitted to low-protein (LP) diet for 8 wk after weaning exhibit reduced body weight gain, hypoalbuminemia, elevated hepatic glycogen content, and reduced plasma insulin levels (9, 12–14). Islets from these rats are less responsive to glucose and do not exhibit the characteristic insulin secretory biphasic pattern (6, 13–15). Despite these abnormalities in insulin secretion, these animals maintain a normoglycemic state, which has been attributed to enhanced peripheral insulin sensitivity (9, 15). To date, the mechanisms that lead to impaired GSIS during protein malnutrition are not completely known. Given that alterations in the signaling events involved in insulin secretion may be related to the altered GSIS observed in protein-deficiency diets (6), in the present study we explored the stimulus-secretion coupling of β-cells from mice fed with LP diet or normal-protein (NP) diet. Electrophysiology measurements in isolated β-cells revealed several differences between the NP and LP group in terms of the resting membrane potential and the ability of glucose to induce depolarization and action potentials. Analysis of the K\textsubscript{ATP} channel open probability indicated a higher activity in resting conditions and lower competence of glucose to inhibit these channels in the LP group. The ability of glucose to induce intracellular Ca\textsuperscript{2+} signals was also reduced in β-cells from LP mice. Finally, islets from LP mice exhibited a decreased level of cell-to-cell synchrony in the intracellular Ca\textsuperscript{2+} signals, which was likely related to the low expression levels of connexin 36. Thus, protein-deficient diets lead to several alterations in the signaling events involved in GSIS in the β-cell that might explain the reduced insulin secretion in this nutritional state.

### Materials and Methods

#### Animals and diets

All protocols were approved by our Animal Care Committee according to national regulations. Experiments were performed with Swiss albino OF1 mice that were distributed in two groups according to the isocaloric diets: NP diet (17% protein) or LP diet (6% protein). The composition of these diets is described in the Supplemental Table 1 (published on The Endocrine Society’s Journals Online web site at http://endo.endojournals.org). A further description can be found elsewhere (12, 16, 17). Three days before the end of lactation period, a group of mothers was fed with the LP diet before the separation from their pups. After weaning, 21-d-old male pups were fed for 8 wk with either the NP or the LP diet. Thus, all the experiments and analysis in mice were performed at 11 wk old.

#### Plasma levels of insulin, glucagon, protein, and albumin

Blood samples from fasted animals were collected for biochemical analysis, and then total plasma protein (18) and total plasma albumin (19) levels were determined. Plasma insulin and glucagon levels were analyzed by RIA as previously described (20).

#### Glucose and insulin tolerance test

For ip glucose tolerance test, a glucose load of 2 g/kg body weight was administered in overnight fasted mice by ip injection, as previously described (9, 21), and blood samples were collected at 15, 30, 60, 120, and 180 min for measuring plasma glucose. For ip insulin tolerance test, a separated group of fed mice were injected with 0.75 U/Kg body weight of human recombinant insulin (Biohulin R, Biobrás, Brazil). Blood samples were collected before insulin injection and at 15, 30, 60, and 90 min for glucose analysis, as previously shown (21).

#### Islet isolation and cell culture

Adult mice from NP or LP groups were killed at 11 wk old by cervical dislocation, and islets were then isolated by collagenase digestion (22). In some experiments, isolated islets were dispensed into single cells by trypsin enzymatic digestion and then cultured overnight at 37°C in RPMI 1640 (Sigma, Madrid, Spain) supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 11 mM d-glucose (22). Except when indicated, all experiments were done at 37°C.

#### Ca\textsuperscript{2+} signaling measurements by conventional fluorescence microscopy and confocal microscopy

For Ca\textsuperscript{2+} experiments, isolated islets were loaded with either Fura-2 or Fluo-4 (5 μM) for 1 h at room temperature (22). Islets were placed on a perfusion chamber mounted on the microscope stage and perfused at a rate of 1.5 ml/min with a modified Ringer solution containing the following (in mM): 120 NaCl, 5 KCl, 25 NaHCO\textsubscript{3}, 1.1 MgCl\textsubscript{2}, and 2.5 CaCl\textsubscript{2} (pH 7.4), gassed with 95% O\textsubscript{2} and 5% CO\textsubscript{2}. Ca\textsuperscript{2+} signals in whole islets were recorded using an inverted epifluorescence microscope (Zeiss, Axiosvert 200) and a Hamamatsu Digital Camera C4742-95 (Hamamatsu Photonics, Barcelona, Spain). Images were acquired every 3 s using a dual filter wheel (Sutter Instrument Co., Novato, CA) equipped with 340- and 380-nm band-pass filters (Omega Optics, Madrid, Spain). Data were acquired using ORCA software.
Fluorescence records were expressed as the ratio of fluorescence (F) at 488 nm and collect the emission with a band-pass filter at 505–530 nm from an optical probe at 488 nm and collect the emission with a band-pass filter at 505–530 nm from an optical configuration of the system was set to excite the Ca2+ probe at 488 nm and collect the emission with a band-pass filter at 505–530 nm from an optical section of 8 μm (22). As previously reported, individual cells loaded with Fluo-4 were easily identified at the periphery of the islet (22). Fluorescence records were represented as the percent-age of ΔF/ΔF0, where F0 is the fluorescence signal at the beginning of a record and ΔF is F-F0. The oscillatory Ca2+ signal of four to seven individual β-cells were analyzed in different islets. A synchronized cell within an islet was considered when the analyzed cell exhibited coupling of its Ca2+ oscillations with the rest of the cells (22, 25). Nonsynchronized cells were easily identified, because their oscillations were out-of-phase with the rest of Ca2+ signals (25).

Insulin secretion

Groups of five islets were first incubated for 45 min at 37 C in Krebs-bicarbonate buffer containing glucose 5.6 mM equili-brated with a mixture of 95% O2-5% CO2 (pH 7.4). The incubation medium contained in mM: 115 NaCl, 5 KCl, 24 NaHCO3, 1 CaCl2, 1 MgCl2, and albumin (3 g/liter; BSA). The solution was then replaced with fresh buffer containing 3 mM, 1 mM, 2 mM, 2.5 mM, 2 CaCl2, 10 HEPES, and 1.1 MgCl2 (pH 7.4), supplemented with glucose or 500 μM tolbutamide and incubated for 1 h. Insulin was measured by RIA.

Patch-clamp recordings

Isolated cells were cultured overnight as previously described (26). KATP channel activity and membrane potential were recorded using standard patch clamp recording procedures. Currents and membrane potential were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments Co., Sunnyvale, CA). Patch pipettes were pulled from borosilicate capillaries (Sutter Instruments Co.) using a flaming/brown micropipette puller P-97 (Sutter Instruments Co.) with resistance between 3–5 MΩ when filled with the pipettes solutions as specified below. Bath solution contained the following (in mM): 5 KCl, 135 NaCl, 2.5 CaCl2, 10 HEPES, and 1.1 MgCl2 (pH 7.4), supplemented with glucose as indicated. In cell-attached experiments the pipette solution contained the following: 100 mM KCl, 10 EGTA, and 1 MgCl2 (pH 7.2). The pipette potential was held at 0 mV throughout recording. KATP channel activity was quantified by digitizing 60-s sections of the current record, filtered at 1 kHz, sampled at 10 kHz by a Digidata 1322A (Axon Instruments Co.), and calculating the mean NPo during the sweep. Channel activity was defined as the product of N, the number of functional channels, and Popen, the open-state probability. Popen was determined by dividing the total time channels spent in the open state by the total sample time. In the experiments using whole-cell configuration, the pipettes were filled with the following (in mM): 76 K2SO4, 10 NaCl, 10 KCl, 1 MgCl2 and 5 HEPES. Experiments were carried out at room temperature (20–24 C).

Real-time PCR

Total RNA was isolated using RNeasy Plus Mini Kit (Qiagen). Extracted RNA was quantified by OD260/280 measurement. Extracted RNA was used to generate cDNA using Expand Reverse Transcriptase M-MuLV (Roche, Mannheim, Germany). Quantitative PCR assays were performed using LightCycler (Roche). Reactions were carried out in a final volume of 10 μl, containing 200 nM of each primer, 100 nM of endogenous control primer, 1 μl of cDNA, and 1× LightCycler FastStart DNA MasterPLUS SYBR Green I (Roche). Samples were subjected to the following thermal cycle conditions: 10 min at 95 C, 40 cycles (10 sec at 95 C/7 sec at 60 C/12 sec at 72 C), and melting curve from 60 to 95 C with a slope of 0.1 C/sec. Actin was used as the endogenous control for quantification. The resulting values were expressed as relative expression respect to control levels (2–ΔΔct) (27). Primers are described in Supplemental Table 2.

Western blot analysis

Isolated islets were resuspended in 200 μl of Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA). Islets extracts were subjected to SDS-PAGE (10% gels). Prestained SDS-PAGE standards were included for molecular mass estimation. The transfer to PVDF membranes was performed at 125 mA for 90 min in a buffer with 2.5 mM Tris base, 9 mM glycine, 20% methanol. After membranes were blocked with 2% nonfat dry milk, and they were incubated with anti-Kir6.2 antibody (1:200; Alomone Labs), anti-Sur1(C-16) antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), or anti-Actin antibody (1:1000; Sigma-Aldrich), before being incubated with appropriate HRP-conju-gated antibodies. Protein bands were revealed by using the ECL chemiluminescence Reagents kit (Amersham Biosciences, Barcelona, Spain). Intensity of the bands was quantified using Scion image software (Frederick, MD).

Statistical analysis

Some data are shown as mean ± SE. Student’s t test or one-way ANOVA were performed as appropriate with a level of significance P < 0.05.

Results

Glucose homeostasis in mice fed with low-protein diet

Mice that were subjected to LP diet for 8 wk had lower body weight compared with mice under NP diet (37.35 ± 1.13 and 45.58 ± 1.06 g, respectively; n = 12; P < 0.001) (Table 1). Additionally, LP mice had decreased plasma levels of total protein compared with NP mice as well as total plasma albumin (Table 1). All these parameters are in agreement with previous findings in rodents subjected
We have previously demonstrated that rats fed with LP diet are normoglycemic despite a remarkable impairment in GSIS (9). In these conditions, normal glucose levels are attained due to enhanced peripheral insulin sensitivity in these malnourished animals (9). To confirm whether mice fed with LP diet have the same metabolic changes as malnourished rats, we analyzed several in vivo metabolic parameters. Fasting blood glucose concentrations obtained before the glucose tolerance test were similar in the two groups (Fig. 1A). However, fasting plasma insulin levels were significantly lower in the LP compared with the NP group (Fig. 1B). In contrast, plasma glucagon levels were similar in both groups (Fig. 1C), suggesting that islet functional alterations in LP mice were probably occurring only in β-cells. To check peripheral insulin sensitivity and glucose tolerance, we performed an ip insulin and glucose tolerance tests. After the insulin load, the glucose disappearance rate was significantly higher in LP compared with NP mice (Fig. 1D). The augmented insulin sensitivity in LP mice was associated with increased glucose tolerance as judged by ip glucose tolerance test experiments (Fig. 1E). Despite lower plasma insulin levels observed at the beginning of the experiment, LP mice had augmented glucose tolerance compared with NP mice (Fig. 1E). These results indicate that mice fed with a protein-deficient diet have lower plasma insulin levels but higher insulin sensitivity in peripheral tissues, in agreement with previous findings in malnourished rats (6, 9, 15).

When the endocrine pancreas function was analyzed, we observed that insulin secretion from isolated islets in the presence of 3 mM glucose was similar in both dietary groups (Fig. 1F). However, when islets were challenged with stimulatory glucose concentrations (11 and 22 mM), insulin release in LP mice was lower than that in NP mice (Fig. 1F). These results are consistent with previous findings in malnourished rats (9). Given that the mechanisms involved in this altered GSIS in the LP islets have not been studied in detail, the next experiments were designed to analyze the main signaling events involved in β-cell insulin secretion.

### Electrical activity in isolated β-cells from mice under protein-deficient diet

We studied the electrical activity in isolated β-cells using patch-clamp in the whole-cell configuration. In the

### Table 1. Body weight and plasma parameters in the fasted state of NP and LP mice

<table>
<thead>
<tr>
<th>Parameter</th>
<th>NP</th>
<th>LP</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>45.58 ± 1.06</td>
<td>37.35 ± 1.13b</td>
<td>12</td>
</tr>
<tr>
<td>Total plasma protein (g/dl)</td>
<td>4.06 ± 0.16</td>
<td>3.53 ± 0.09a</td>
<td>7</td>
</tr>
<tr>
<td>Total plasma albumin (g/dl)</td>
<td>2.95 ± 0.08</td>
<td>2.56 ± 0.16a</td>
<td>7</td>
</tr>
</tbody>
</table>

Statistical significance vs. control (NP): a P < 0.05; b P < 0.001. NP, Normal protein; LP, low protein.

FIG. 1. Insulin release in response to glucose is reduced in islets from LP mice. A–C, Blood glucose concentrations (n = 8) as well as plasma insulin and glucagon levels (n = 8, n = 9, respectively) were examined in LP and NP mice in the fasted state. D and E, Insulin tolerance test (ITT) and glucose tolerance test (GTT) in LP and NP mice (n = 8, n = 8, respectively). F, Insulin release in response to 3, 11, and 22 mM glucose was measured in isolated islets from both groups (n = 8). Insulin levels at 11 and 22 mM glucose were significantly higher compared with 3 mM glucose in both groups (P < 0.01). G, glucose. Statistically significant comparing LP values with its respective control in NP: *, P < 0.05; **, P < 0.01; ***, P < 0.001.
absence of glucose, the resting membrane potential of NP
β-cells was $-64.67 \pm 1.07$ mV, whereas this parameter
was $-70.61 \pm 1.14$ mV in β-cells from LP mice ($P < 0.01$)
(Table 2). Thus, β-cells from LP mice are more hyperpo-
larized in resting conditions. Figure 2A shows a typical
recording of the NP-β-cell electrical activity in response to
glucose and the KATP channel blocker tolbutamide in NP
mice. In the presence of 8 mM and 11 mM glucose, the
totality of control NP-β-cells from NP mice generated action
potentials that originated from $-46.3 \pm 1.68$ mV and
$-40.49 \pm 1.89$ mV, respectively (Fig. 2, A and B; Table 2).
However, in NP-β-cells from LP mice, 8 mM glucose induced
a small depolarization that was not sufficient to trigger
action potentials in 60% of cases (Fig. 2C; Table 2). Ad-
ditionally, the analysis of the elapsed time from the addi-
tion of 8 mM glucose to the initiation of the depolarization
indicated a 5-fold higher delay ($\approx 338$ sec) in NP-β-cells
from LP compared with NP mice (Table 2). The alterations in
the electrical response of LP-β-cells were also evident in the
presence of 11 mM glucose. The membrane potential at-
tained with 11 mM glucose was also lower in the LP group
than that obtained in control cells (Table 2). When action
potentials took place (Fig. 2, B and D), no differences were
found in the amplitude of the spikes between both groups.
The depolarization by closure of KATP channels with tol-

**FIG. 2.** Glucose-stimulated changes in membrane potential and
electrical activity are impaired in NP-β-cells from LP mice. Membrane
potential ($V_m$) was measured in isolated β-cells from islets of NP and
LP mice using patch-clamp in the whole-cell configuration. A, Repre-
sentative recording in a β-cell of a NP mouse. Glucose at 8 and
11 mM as well as 500 μM tolbutamide induced depolarization and
electrical activity in NP mice. B, The extended trace of a period
indicated in A (*) illustrates the typical action potentials of the β-cell.
C, Representative recording in a β-cell of a LP mouse. Although 8 mM
glucose induced a small depolarization, it was not sufficient to trigger
electrical activity. Glucose at 11 mM and tolbutamide (500 μM)
produced depolarization and action potentials. D, The extended trace
of a period indicated in C (*) illustrates the typical action potentials in
this β-cell. The number of experiments is indicated in Table 2. G, Glu-
cose; Tolb, tolbutamide.

**TABLE 2.** Characteristics of the electrical activity in β-cells of NP and LP mice

<table>
<thead>
<tr>
<th>Condition</th>
<th>β-Cell Type</th>
<th>Membrane Potential ($V_m$, mV)</th>
<th>Increase in Membrane Potential ($\Delta V_m$, mV)</th>
<th>Elapsed Time (min)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM G</td>
<td>NP</td>
<td>$-64.67 \pm 1.07$</td>
<td>$-46.3 \pm 1.68$</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>$-70.61 \pm 1.14$</td>
<td>$-40.49 \pm 1.89$</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>8 mM G</td>
<td>NP</td>
<td>$-46.3 \pm 1.68$</td>
<td>$-40.49 \pm 1.89$</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>$-40.49 \pm 1.89$</td>
<td>$-40.49 \pm 1.89$</td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>11 mM G</td>
<td>NP</td>
<td>$-40.49 \pm 1.89$</td>
<td>$-40.49 \pm 1.89$</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>LP</td>
<td>$-40.49 \pm 1.89$</td>
<td>$-40.49 \pm 1.89$</td>
<td></td>
<td>5</td>
</tr>
</tbody>
</table>

The membrane potential ($V_m$), the increase in membrane potential ($\Delta V_m$), and the elapsed time after the application of stimuli was calculated from the experiments shown in Fig. 2. Statistical significance vs. control (NP): * $P < 0.05$. The $V_m$ obtained with the three stimuli was found significant compared with 0 mM glucose in both groups. For the sake of clarity, statistical symbols were not included. G, Glucose; NP, normal protein; LP, low protein.
butamide led to similar membrane potential levels and triggered normal electrical activity in both groups (Fig. 2, A and C; Table 2), indicating that cells were responsive during these experiments and that failures in the KATP channel were unlikely involved in the lower effect of glucose in the LP group. Because LP β-cells were more hyperpolarized in resting conditions, the increase in membrane potential with tolbutamide (\( \Delta V_m \)) was higher in these cells compared with controls, given that tolbutamide depolarized to the same level in both groups (Table 2). Although a small difference in the time delay of the response to tolbutamide (~5 sec) was found between both groups (Table 2), it may be attributable to small differences in each experiment related to the perfusion rate. Therefore, these results show for the first time that diet protein deficiency can lead to alterations in the typical electrical activity of the pancreatic β-cells in response to glucose.

**K\(_{\text{ATP}}\) channel activity in LP mice**

The K\(_{\text{ATP}}\) channel has a central function in GSIS, transducing the changes in extracellular glucose levels to the depolarization of the β-cell membrane potential, generation of action potentials, and voltage-dependent Ca\(^{2+}\) influx (28). Thus, we measured K\(_{\text{ATP}}\) channel activity in isolated β-cells from LP and NP mice. Figure 3, A and B, shows the basal K\(_{\text{ATP}}\) channel activity in the absence of glucose was higher in LP mice than in NP mice. This result may be related to the more hyperpolarized state found in β-cells from LP mice. The relative quantification indicates higher levels of SUR1 protein in LP mice. G, glucose. *, Statistically significant comparing each value with its control (0 mM glucose). &, Statistically significant comparing LP values with its respective control in NP. * and *, P < 0.05; ** and &&, P < 0.01; ***; P < 0.001.
mice in resting conditions (Table 2), because the $K_{\text{ATP}}$ channel is also responsible for the resting membrane potential in the $\beta$-cell. Interestingly, 8 mM glucose was not capable of decreasing the $K_{\text{ATP}}$ channel activity in LP $\beta$-cells to the same extent as in $\beta$-cells from NP mice (Fig. 3C). Actually, the $\beta$-cell $K_{\text{ATP}}$ channel activity in the presence of 8 mM glucose was 2-fold higher ($P < 0.05$) in LP mice (Fig. 3C), indicating that these channels may explain the reduced effect of glucose on the membrane potential of LP $\beta$-cells (Fig. 2). Consistent with the experiments illustrated in Fig. 2, tolbutamide decreased the activity of $K_{\text{ATP}}$ channels to the same level in both groups (Fig. 3C), indicating that these channels were operative. Thus, these results further suggest that metabolic changes rather than failures in $K_{\text{ATP}}$ channel function are involved in the impaired GSIS found in LP mice.

We also checked the expression of the two $K_{\text{ATP}}$ channel subunits by real-time PCR. No differences were found with the relative mRNA levels in both groups (Supplemental Fig. 1A). A Western blot analysis also revealed similar levels of Kir6.2 (inwardly rectifying potassium channel 6.2) protein content in both groups. However, the protein levels of SUR1 (sulfonylurea receptor 1) were increased 2.77-fold in islets from LP compared with NP mice (Fig. 3D), indicating that SUR1 regulation in LP islets might occur during posttranscriptional states. To further analyze the SUR1 protein increase in LP islets, we performed an immunostaining with isolated islet-cells (Supplemental Fig. 1B). Although we found a trend to increase in the SUR1 labeling intensity of $\beta$-cells from LP mice ($P = 0.054$), this increase was more evident in non-$\beta$-cells, suggesting that the augmented SUR1 levels found in LP islets (Fig. 3D) were mainly due to non-$\beta$-cells. Further experiments should be performed to determine whether the up-regulation of this subunit results in a higher density and/or $K_{\text{ATP}}$ channel function in $\beta$-cells and, particularly, in non-$\beta$-cells.

**Glucose-induced $\text{Ca}^{2+}$ signals are altered in the $\beta$-cells of LP mice**

The stimulus-secretion coupling in pancreatic $\beta$-cells is well established. Glucose-induced depolarization leads to $\text{Ca}^{2+}$ influx through voltage-dependent channels, rise of intracellular $\text{Ca}^{2+}$, and exocytosis of insulin granules (28). To check $\text{Ca}^{2+}$ handling in islets from LP mice, we next measured intracellular $\text{Ca}^{2+}$ signals by fluorescence microscopy. Islets exposed to 11 mM glucose produced a rapid transient rise in $\text{Ca}^{2+}$ followed by oscillations (Fig. 4A). The analysis of the AUC, which gives an estimation of the global $\text{Ca}^{2+}$ entry (23), indicated that intracellular $\text{Ca}^{2+}$ in the presence of 11 mM glucose was decreased in islets from LP mice compared with islets from NP mice ($P < 0.05$) (Fig. 4A). Because glucose-induced $\text{Ca}^{2+}$ signals depend on electrical activity, these findings are in part consistent with the effects on membrane potential (Fig. 2). However, we do not discard other factors such as protein

![FIG. 4](image-url). Glucose-induced $\text{Ca}^{2+}$ signaling is diminished in islets from LP mice. Intracellular $\text{Ca}^{2+}$ signals were measured in intact islets by conventional fluorescence microscopy and Fura-2. Each figure shows a representative trace of the $\text{Ca}^{2+}$ signal in both groups, the NP and LP mice. Either the AUC or the increase of fluorescence ($\Delta F$) indicate the magnitude of the $\text{Ca}^{2+}$ signal during the stimulus (23, 24). A, Exposure of islets to 11 mM glucose produced the characteristic transient rise in $\text{Ca}^{2+}$ followed by oscillations. The analysis of the $\text{Ca}^{2+}$ signals indicated a lower response in LP ($n = 7$) compared with NP mice ($n = 9$). B, Tolbutamide induced similar responses in islets from NP and LP mice ($n = 6, n = 10$, respectively). C, Similar levels of $\text{Ca}^{2+}$ were also obtained with 40 mM $K^+$ ($n = 9$ in NP; $n = 6$ in LP). Statistical significance vs. control (NP): *, $P < 0.05$. 

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kinases that can also modulate glucose-induced Ca\^{2+} signaling. In this regard, it has been reported that protein kinase A levels are reduced in the islets of protein-malnourished rats (12). There were no differences in the Ca\^{2+} signals of both groups when islets were exposed to non-metabolic stimuli such as tolbutamide or 40 mM of K\(^+\) (Fig. 4, B and C). In agreement with these latter experiments, tolbutamide did not produce different profiles of secretion between both groups (Supplemental Fig. 2), in contrast with the effect of glucose (Fig. 1).

Cell-to-cell synchrony among \(\beta\)-cells is reduced in the islets of LP mice

It has been shown that Ca\(^{2+}\) oscillations induced by glucose are synchronous in all \(\beta\)-cells within the mouse islet and this coordination is essential for pulsatile insulin release (31–33). The cell-to-cell communication and the degree of synchrony among mouse \(\beta\)-cells depends on the gap-junctions made of connexin 36 (34). Therefore, we next analyze the level of synchrony among Ca\(^{2+}\) signals in \(\beta\)-cells within intact islets as we have previously performed (22, 25), as well as the mRNA expression of connexin 36 in islets from NP and LP mice. Figure 5A shows individual Ca\(^{2+}\) signals in \(\beta\)-cells from NP islets. Increasing glucose from low (0.5 mM) to stimulatory concentrations (11 mM) produced synchronous Ca\(^{2+}\) oscillations in the totality of \(\beta\)-cells of all the islets analyzed in NP mice (Fig. 5D). Figure 5B shows that \(\beta\)-cells from LP islets also synchronized individual Ca\(^{2+}\) signals in the presence of high glucose in some islets, but we also found several islets with low level of coupling (Fig. 5C). The analysis of the percentage of synchronized cells clearly shows that \(\beta\)-cells from LP islets exhibit a reduction of approximately 30% in the number of coupled cells. Additionally, the relative mRNA levels of connexin 36 were significantly decreased by approximately 35% in LP islets compared with controls (Fig. 5A), suggesting that the lower level of coupling levels of the Ca\(^{2+}\) oscillations in islets from LP mice might be explained by decreased levels of connexin 36.

Glut-2 and glucokinase expression is reduced in islets from malnourished mice

Previous studies in malnourished rodents have shown several alterations related to glucose metabolism including glucose oxidation and utilization as well as changes in the expression of mitochondrial genes (35–39). Consistent

![Image](image_url)
with these previous findings, we observed an approximate 57\% decrease in GLUT-2 expression in LP islets compared with controls (P < 0.05; Fig. 6). Additionally, we found a trend to decrease in the expression of glucokinase (47\%; P = 0.09). In agreement with previous studies of several groups, including ours (36, 38, 39), we also observed that glucose oxidation was reduced (Supplemental Fig. 3). Overall, these findings indicate that glucose metabolism is altered in malnourished animals.

**Discussion**

Protein deficiency has been considered one of the pathogenic factors involved in malnutrition-related diabetes that may occur in developing countries (2). Malnutrition during intrauterine period or during early postnatal life can predispose to development of T2DM and obesity during adulthood (40, 41). We have previously demonstrated that rats subjected to LP diet have several alterations in their metabolic parameters as well as glucose homeostasis (6, 15). In the present work, we show that mice fed with LP diet have similar metabolic abnormalities as those found in malnourished rats including lower body weight, lower total plasma protein, hypoalbuminemia, and lower plasma insulin levels compared with mice fed with NP diet (Table 1; Fig. 1). Because plasma glucagon levels were not altered, it seems that functional changes in LP islets probably occur only in \( \beta \)-cells and in insulin release. The lower plasma insulin levels observed in LP mice were consistent with the reduced GSIS found in isolated islets from these animals. A reduced insulin secretion (6, 7, 39) together with diminished number of \( \beta \)-cells per islet and decreased insulin amount per \( \beta \)-cell (8, 42, 43) have been demonstrated in several models of malnutrition. Despite low plasma insulin levels and a remarkable reduction in GSIS, LP mice had increased glucose tolerance and higher glucose disappearance rate than NP mice as judged by the insulin and glucose tolerance tests. This seems to be a compensatory mechanism in the peripheral tissues to maintain normoglycemia that is also found in malnourished rats (9). We have previously proposed that the increased insulin sensitivity in malnourished rats is due to an increase in insulin-induced tyrosine phosphorylation of the insulin receptor and the insulin receptor substrate-1 in the skeletal muscle (9). However, further studies should be performed to determine what is taking place first, the alteration in the islet secretory response or in the peripheral insulin sensitivity. A time-course study on this subject could be helpful to analyze the temporal and causal relationship between both processes. In any case, we show that LP mice exhibit a coordinated cross talk between islet function and peripheral insulin sensitivity, which allows for the coupling of the insulin demand with the amount of insulin secreted to maintain normal glucose levels. Previous studies supported this idea (9). In this line, we can also suggest that islets from LP mice may have adapted to their smaller body weight, and thus the reduced insulin secretion could be sufficient to maintain glucose homeostasis in response to stimuli. Therefore, all these results indicate that protein deficiency during early age in mice alters glucose homeostasis, diminishing GSIS and increasing peripheral insulin sensitivity. Because malnourished mice have all the metabolic features of most animal models of protein-deficient nutritional status, we used this model to further study the alterations in the stimulus-secretion coupling of pancreatic \( \beta \)-cells.

Several theories have emerged to explain the reduced insulin secretion in response to glucose in animal models of protein-deficient diets. Decreased insulin release has been related to changes in glucose metabolism (35–39, 44), defects in the ability of glucose to increase \( \text{Ca}^{2+} \) uptake in \( \beta \)-cells (6), and alterations in \( K_{\text{ATP}} \) channel pathways (45). However, no study on the electrophysiological properties has been performed in pancreatic \( \beta \)-cells from malnourished animal models. Using the patch-clamp technique, we found that \( \beta \)-cells from LP mice had a more hyperpolarized resting membrane potential than controls in the absence of glucose. This hyperpolarization may be explained by the higher basal \( K_{\text{ATP}} \) channel activity found in LP mice. In addition, LP \( \beta \)-cells exhibited less efficiency to depolarize and generate action potentials as well as a higher time delay for the initiation of depolarization in response to stimulatory glucose levels (Fig. 2; Table 2). The alterations in the generation of action potentials in LP \( \beta \)-cells were consistent with the inability of the sugar to decrease the \( K_{\text{ATP}} \) channel activity to the same extent as that found in NP \( \beta \)-cells (Fig. 3). Glucose effects on membrane potential and electrical activity through the \( K_{\text{ATP}} \) channel occur within a narrow window of channel activity (i.e. 8 mM glucose produces almost 90% inhibition of \( K_{\text{ATP}} \) channels) (29, 30). Thus, the higher (2-fold) \( K_{\text{ATP}} \) channel
activity with glucose in β-cells from LP mice (Fig. 3) may explain the lower glucose sensitivity of the membrane potential in these cells (Fig. 2). The diminished capacity of glucose to affect the stimulus-secretion coupling in LP β-cells might be related to alterations in glucose metabolism. Actually, it has been shown that oxidative metabolism in β-cells from malnourished rats is impaired (36, 38, 39) and might be explained by altered activity of m-GDH and L-leucine transamination to 2-ketoisocaproate (37).

Our results shown in Fig. 6 further indicate that glucose metabolism is altered in malnourished animals. On the other hand, it has been proposed that the defects in insulin secretion in malnourished rats are located in both K\textsubscript{ATP} channel dependent and independent pathways (45). Here, we found that the effect of tolbutamide to induce depolarization, action potentials, and K\textsubscript{ATP} channel inhibition in β-cells (Figs. 2 and 3) as well as insulin secretion in intact islets (Supplemental Fig. 2) was similar in LP and NP mice. Thus, our findings indicate that the alterations in the islets of LP animals might be more related to alterations in glucose metabolism rather than nonfunctional K\textsubscript{ATP} channels.

The K\textsubscript{ATP} channel is essential for proper GSIS by coupling glucose metabolism to electrical activity (28). The channel is composed of the subunit Kir6.2, responsible for ion selectivity, and SUR1, which controls most of the channel sensitivity to nucleotides (46, 47). Interestingly, we found that islets from LP mice exhibit 2.77-fold more SUR1 protein content than NP mice (Fig. 3). Immunocytochemical experiments revealed that, although a trend to increase was also observed in LP β-cells, non-β-cells were mainly contributing to the enhanced SUR1 levels in LP islets. The physiological importance of this difference in islet cells is not known, but it has been suggested in neurons that SUR1 overexpression may enhance coupling with endogenous Kir6.6 subunits, which would elevate the density of K\textsubscript{ATP} channels, leading to hyperpolarization in response to metabolic stress (48). Whether a similar process would occur in islet cells, it would be possible that SUR1 overexpression may contribute to changes in the electrical response as those observed here, although the altered glucose metabolism may have a key role (35, 44).

The alterations in the stimulus-secretion coupling in LP β-cells was also observed in other signaling events involved in GSIS. Actually, β-cells of malnourished mice exhibited lower ability to increase intracellular Ca\textsuperscript{2+} in response to glucose (Fig. 4). However, no differences were observed between both groups in the presence of nonmetabolic stimuli such as tolbutamide and high K\textsuperscript{+} concentrations. These results further point to an abnormal glucose metabolism in LP β-cells (35–39) and are in agreement with our previous work in islets from malnourished rats, which exhibited poor insulin secretory response to nutrients due to altered 45Ca uptake (6).

Proper insulin secretion requires a fine coordination among β-cells within pancreatic islets. One of the most important proteins involved in this coordination is connexin 36, which allows for the formation of gap-junctions that connect adjacent β-cells in the mouse islet (33). This cell-to-cell communication ensures an electrical and chemical coupling, favoring the synchrony of electrical and Ca\textsuperscript{2+} signals between neighboring β-cells (34, 49). In the present work, we found lack of synchrony in 30% of β-cells of LP mice, which might be due the approximate 35% reduction in the relative mRNA levels of connexin 36 (Fig. 5). These results resemble the data published with Cx36\textsuperscript{−/−} mice. Pancreatic islets from these animals displayed irregular Ca\textsuperscript{2+} oscillations and lack of pulsatile insulin release during glucose stimulation (34). Thus, the present work shows for the first time that LP diet leads to alterations in important genes involved in the pancreatic β-cell function as well as changes in several signaling steps involved in GSIS such as electrical activity, Ca\textsuperscript{2+} signals, and islet synchrony. All these alterations in the stimulus-secretion coupling of β-cells from malnourished mice may explain the endocrine pancreas dysfunction and development of diabetes in malnutrition state.

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Address all correspondence and requests for reprints to: I. Quesada, Instituto de Bioingeniería, Universidad Miguel Hernández, Avenida de la Universidad s/n, 03202 Elche, Spain. E-mail: ivanq@umh.es; or E. M. Carneiro, Departamento de Anatomia, Biologia Celular e Fisiologia, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), 13083-970 Campinas, Sá Paulo, Brazil. E-mail: emc@unicamp.br.

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