Functional and Structural Adaptations in the Pancreatic α-Cell and Changes in Glucagon Signaling During Protein Malnutrition

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Chronic malnutrition leads to multiple changes in β-cell function and peripheral insulin actions to adapt glucose homeostasis to these restricted conditions. However, despite glucose homeostasis also depends on glucagon effects, the role of α-cells in malnutrition is largely unknown. Here, we studied α-cell function and hepatic glucagon signaling in mice fed with low-protein (LP) or normal-protein diet for 8 wk after weaning. Using confocal microscopy, we found that inhibition of Ca²⁺ signaling by glucose was impaired in α-cells of LP mice. Consistent with these findings, the ability of glucose to inhibit glucagon release in isolated islets was also diminished in LP mice. This altered secretion was not related with changes in either glucagon gene expression or glucagon content. A morphometric analysis showed that α-cell mass was significantly increased in malnourished animals, aspect that was probably related with their enhanced plasma glucagon levels. When we analyzed the hepatic function, we observed that the phosphorylation of protein kinase A and cAMP response-binding element protein in response to fasting or exogenous glucagon was impaired in LP mice. Additionally, the up-regulated gene expression in response to fasting observed in the hepatic glucagon receptor as well as several key hepatic enzymes, such as peroxisome proliferator-activated receptor γ, glucose-6-phosphatase, and phosphoenolpyruvate carboxykinase, was altered in malnourished animals. Finally, liver glycogen mobilization in response to fasting and the ability of exogenous glucagon to raise plasma glucose levels were lower in LP mice. Therefore, chronic protein malnutrition leads to several alterations in both the α-cell function and hepatic glucagon signaling. (Endocrinology 153: 1663–1672, 2012)
glucose homeostasis and the eventual appearance of diabetes, particularly when these restrictions occur in utero or early in life (1–3).

Although fetal and childhood malnutrition is highly reported in developing countries, this is also an important health problem in developed nations (4). Actually, several studies have found an association between low birth weight and development of T2DM in the adulthood, being fetal malnutrition an important potential cause (4–8). Energy or protein restrictions during fetal and early life stages promote functional and structural changes in the endocrine pancreas to maintain glucose homeostasis in these limited conditions (8, 9). Although these adaptations provide a survival advantage, they can be detrimental with normal or excessive nutrition in the adulthood, leading to enhanced predisposition to develop glucose intolerance and diabetes (8). Thus, a better knowledge of islet adaptations during nutritional deprivation is important for prevention and therapy.

Several studies in animals and humans have demonstrated structural and functional adaptations in the endocrine pancreas as well as in peripheral tissues during nutritional deprivation. In malnourished rodents, these changes include a reduction of β-cell mass (9–11) as well as alterations in the signaling steps involved in glucose-stimulated insulin secretion (GSIS), leading to reduced insulin release (12, 13). Changes in insulin sensitivity have been also observed in peripheral tissues, such as the liver, the muscle, and adipocytes of malnourished rodents (8). All these adaptations and their impact on glucose homeostasis vary depending on the type of diet, the duration of the treatment, and the developmental stage studied (8, 9). In these nutritional-deprived models, aging combined with exposure to normal or excessive nutrition can lead to β-cell failure and insulin resistance, causing glucose intolerance and T2DM (7, 8). In the present study, we focused on a model of protein restriction. Low-protein (LP) diets in rodents usually lead to decreased plasma insulin levels and reduced GSIS in the islets of these mice (12). Despite these alterations, these animals maintain normal glucose levels, which have been attributed to compensation due to enhanced peripheral insulin sensitivity (12, 13).

The regulation of glucose homeostasis depends on the concerted function of both α- and β-cells, which are stimulated by reciprocal glucose levels, as well as the coordinated action of glucagon and insulin on peripheral tissues (14, 15). Actually, the impairment of islet function and the pathophysiology of diabetes are associated to abnormalities in both cell types (16). Despite the importance of glucagon on glucose homeostasis, the vast majority of the above-mentioned studies are focused only on β-cells. Additionally, few studies show altered plasma glucagon levels in human and animals during malnutrition, pointing to altered α-cell function in these conditions (17–19). Thus, in the present study, we have analyzed the structural and functional adaptations of the pancreatic α-cell as well as changes in glucagon action on liver in a mouse model of protein malnutrition. This information will allow a better comprehension of the islet plasticity to manage glucose homeostasis during nutritional deficiencies.

Materials and Methods

Animals

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 following isocaloric diets: normal-protein (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, 20). After weaning, 21-d-old female pups were fed for 8 wk with either the NP or the LP diet.

Plasma levels of insulin, glucagon, and protein

Blood samples from animals were collected for biochemical analysis, and then, total plasma protein levels were determined (12, 21). Plasma insulin and glucagon levels were analyzed by RIA or ELISA as previously described (12, 22).

Islet isolation and cell culture

Adult mice were killed at 11 wk old, and islets were then isolated by collagenase digestion (22). In some experiments, isolated islets were dispersed 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 glucose (22). Except when indicated, all experiments were done at 37 C.

Ca2+ signaling measurements by confocal microscopy

Isolated islets were loaded with Fluo-4 (5 μM) for 1 h at room temperature (22, 23). 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: 120 mM NaCl, 5 mM KCl, 25 mM NaHCO3, 1.1 mM MgCl2, and 2.5 CaCl2 (pH 7.4), gassed with 95% O2 and 5% CO2. Low levels of glucose induce regular Ca2+ oscillations in the α-cell that are inhibited by high glucose concentrations. This typical pattern has been used to identify α-cells within the islet (22, 23). To analyze these Ca2+ oscillations, individual cells within freshly isolated islets were monitored using a Zeiss LSM 510 laser confocal microscope (Zeiss, Oberkochen, Germany). The Ca2+ probe was excited at 488 nm, and emission was collected with a band-pass filter at 505–530 nm from an optical section of 8 μm. Images were collected at 2-sec intervals and treated with a low pass filter. As previously reported, individual cells loaded with Fluo-4 were
easily identified at the periphery of the islet (Supplemental Fig. 1) (23). Fluorescence records were represented as the percentage of ΔF/F_0, where F_0 is the fluorescence signal at the beginning of a record, and ΔF is F – F_0. The frequency of Ca^{2+} oscillations was calculated over a 5-min period of the Ca^{2+} recording, immediately before and 5 min after the application of the stimulus as previously reported (22, 23). A Ca^{2+} oscillation was defined as a rapid increase in the intracellular Ca^{2+} concentration higher than twice the SD of the background signal at the intervals between spikes (22).

**Glucagon secretion and content**

Batches of 15 islets were preincubated for 60 min at 37 C in 0.5 ml of Krebs-Ringer bicarbonate buffer supplemented with 15 mM HEPES, 0.5% BSA, and 5.6 mM glucose (pH 7.4) (22). Then, islets were incubated at 37 C for 60 min with Krebs-Ringer bicarbonate buffer supplemented with different glucose concentrations. Afterward, the medium was aspirated and assayed by RIA (Linco Research, St. Charles, MO). To measure glucagon content, islets were lysed with 50 μl of lysis buffer (70% ethanol, 0.4% HCl at 30%, 24.6% distilled water) and incubated overnight at 4 C. Samples were centrifuged at 2500 rpm for 5 min, and the supernatant was collected for analysis by RIA. Total protein was determined by the Bradford method (22).

**Real-time-PCR**

Quantitative PCR assays were performed using CFX96 Real Time System (Bio-Rad, Hercules, CA). Reactions were carried out in a final volume of 10 μl, containing 200 nM each primer, 100 nM endogenous control primer, 1 μl of cDNA, and 1X iQ SYBR Green Supermix (Bio-Rad). Samples were subjected to the following conditions: 10 min at 95 C, 40 cycles (10 sec at 95 C, 7 sec at 60 C, and 12 sec at 72 C), and a melting curve of 63–95 C with a slope of 0.1 C/sec. The housekeeping gene was glyceraldehyde-3-phosphate dehydrogenase for glucagon expression and ribosomal protein large P0 for the rest of genes, and they were used as the endogenous control for quantification (24). The resulting values were analyzed with CFX Manager version 1.6 (Bio-Rad), and values were expressed as the relative expression respect to control levels (2^{-ΔΔCT}) (24). Primers are described in Supplemental Table 2.

**Western blot analysis**

Livers were lysed in 200 μl of Cell Lysis Buffer (Cell Signaling Technology, Danvers, MA). Extracts were subjected to SDS-PAGE (Mini-ProteinTGX Precast Gel, 4–20% gels; Bio-Rad). Prestained SDS-PAGE standards were included for molecular mass estimation. The transfer to polyvinylidene fluoride 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 5% nonfat dry milk, they were incubated with the following antibodies: anti-cAMP response-binding element protein (CREB) (1:1000), antiphospho-CREB (Ser133) (1:1000), antiprotein kinase A (PKA)-C (1:1000), antiphospho-PKA-C (1:1000), all of them from Cell Signaling Technology, or antiactin (1:1000; Sigma-Aldrich, St. Louis, MO). Then, they were incubated with appropriate horseradish peroxidase-conjugated antibodies (Bio-Rad). Protein bands were revealed by using the enhanced chemiluminescence Western blot substrate (Thermo Fisher Scientific, Madrid, Spain). Bands intensity was quantified using Scion image software (Frederick, MD). In some experiments, glucagon was administrated ip at a dose of 50 μg/kg of body weight in the fed state, and the liver was removed 15 min after the injection (25).

**Quantitative approaches in endocrine pancreas and α-cell mass**

To study the morphometric parameters, pancreases were excised, cleared of fat and lymph nodes, weighed, immersion fixed during 12 h in 4% paraformaldehyde fixative solution, dehydrated, and embedded in paraffin. When the sections achieved the major plane of pancreas area, one section (5 μm) from each block was cut on a rotary microtome and adhered to individual normal or silanized glass. The section was immunoperoxidase stained for glucagon (sc-7779; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (26, 27). α-Cell mass was determined by point counting morphometry on each pancreas section immunostained for glucagon according to previous descriptions with minor modifications (28). Briefly, each section was systematically scored with a grid of 196 points (final magnification, ×200). The numbers of intercepts over α-cells, endocrine non-α-cells, exocrine pancreatic tissue, and nonexocrine pancreatic tissue were counted. The α-cell relative volume was calculated by dividing the intercepts over α-cells by the intercepts over the total pancreatic tissue; the α-cell mass was then estimated by multiplying the α-cell relative volume by the total pancreas weight. A total of 820 and 535 fields was counted for pancreases from control and malnourished mice, respectively. Average α-cell size was measured using the same material used for determination of α-cell mass. Total α-cell area inside the islet was measured with Image-Pro-Plus Media, Cybernetics Program, coupled to an Olympus BX-60 photomicroscope (Olympus, New York, NY), and at least 75 islets per group were sampled. The average cell size was determined by dividing the measured total α-cell area by the number of α-cell nuclei within each total α-cell area sampled, according to previous descriptions (28). Average α-cell number per pancreas area was obtained by counting the total α-cell nuclei within each total α-cell area sampled, and then, the results were expressed as α-cell number per 1000 μm² of α-cell area.

**Glycogen content**

Livers (400 mg) were hydrolyzed with 2 ml of KOH 30% (1 h at 98 C), and then, glycogen was precipitated with 0.2 ml of Na_2SO_4 and 6 ml of ethanol (15 sec at 98 C). The supernatant was discarded, and the pellet was washed three times with 2 ml of water (60 C) and 6 ml of ethanol. Glycogen was resuspended with 25 ml of distilled water (60 C). Glycogen concentration was measured with a colorimetric reaction, adding 15 μl of phenol solution (800 mg/ml), 2 ml of H_2SO_4, distilled water, and boiling 15 min in the bath. Finally, absorbance was read at 490 nm (29).

**Statistical analysis**

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

**Results**

**Body weight and metabolic features of mice fed with LP diet**

The results of Table 1 show that the diet treatment of the present study produced features of a protein-deficient...
nutritional status, similar to those previously published (12, 13). The animals subjected to LP diet exhibited reduced body weight gain as well as reduced plasma insulin and total protein levels. In contrast, these mice had similar plasma glucose levels as those of the control group (Table 1). Although these malnourished animals have several alterations in insulin secretion, this normoglycemic state has been attributed to a compensation by enhanced peripheral insulin sensitivity (12, 13). However, it has not been examined whether α-cells and glucagon action are also involved in these adaptations. These questions will be addressed in the following sections.

Glucose-induced inhibition of Ca\(^{2+}\) signals is attenuated in α-cells of LP mice

To characterize the effect of protein restriction in the α-cell function, we first examined glucose-induced Ca\(^{2+}\) signaling, an essential step of the α-cell stimulus-secretion coupling (15). At low glucose concentrations, pancreatic α-cells exhibit a characteristic pattern of oscillations that is inhibited by high glucose levels (Fig. 1, A and B) (23). Remarkably, although high glucose levels reduced Ca\(^{2+}\) oscillations by approximately 65% in α-cells of controls, this effect was only approximately 35% in LP mice (Fig. 1, C and D). Additionally, the frequency of Ca\(^{2+}\) signals in low glucose concentrations was 25% lower in α-cells of malnourished animals compared with controls (Fig. 1C). In addition to the functional identification of α-cells by their response to 0.5 and 11 mM glucose (23), we also performed some experiments using adrenaline (Supplemental Fig. 2). Similar results were obtained as those of Fig. 1, A–D. Conversely, no differences between both groups were found in the ability of insulin to suppress α-cell Ca\(^{2+}\) signals (Fig. 1, E and F) (30) or adrenaline to induce a Ca\(^{2+}\) transient (Supplemental Fig. 3) (31, 32). Thus, these results indicate that α-cells from malnourished mouse have an altered glucose regulation of Ca\(^{2+}\) signals.

Glucagon expression, content, and secretion in islets of LP mice

Given that α-cell exocytosis is Ca\(^{2+}\) dependent (15, 32), we also examined glucagon release in isolated islets of both groups. Glucose at 5.6 and 11.2 mM was less effective to inhibit glucagon secretion in the islets of LP mice than in controls (Fig. 2A), which was similar to the situation found in Ca\(^{2+}\) signaling shown previously. These alterations were not related with changes in glucagon synthesis, because the expression and protein content of this hormone were similar in both groups (Fig. 2, B and C). Thus, the altered glucose suppression of glucagon secretion seems related with a potential defective glucose-sensing in LP α-cells rather than changes in glucagon synthesis. Measurement of plasma glucagon concentrations revealed higher levels in LP mice in the fasted state and a strong tendency to the increase in fed conditions in LP mice compared with controls (P = 0.0503) (Fig. 2D). As previously shown in malnourished rodents (12, 33), plasma glucose

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**TABLE 1.** Body weight and plasma parameters in the fasted state of NP and LP mice

<table>
<thead>
<tr>
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<th>NP</th>
<th>LP</th>
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<tbody>
<tr>
<td>Body weight gain (g)</td>
<td>32.4 ± 2.4</td>
<td>23.8 ± 2.0*</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>87.2 ± 4.7</td>
<td>99.8 ± 3.8</td>
</tr>
<tr>
<td>Serum insulin (ng/ml)</td>
<td>0.79 ± 0.08</td>
<td>0.36 ± 0.04*</td>
</tr>
<tr>
<td>Total plasma protein (g/dl)</td>
<td>4.81 ± 0.31</td>
<td>3.44 ± 0.07*</td>
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* Statistical significance vs. control (P < 0.05).

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**FIG. 1.** Ca\(^{2+}\) signals in response to glucose and insulin in α-cells of malnourished and control mice. Individual Ca\(^{2+}\) signals were measured in thin optical sections (8 μm) of intact islets using confocal microscopy and the fluorescent probe, Fluo-4. A and B, Representative α-cell Ca\(^{2+}\) signals in response to glucose in NP (A) and LP mice (B). The image illustrates the cell location where the Ca\(^{2+}\) signal was recorded. C, Ca\(^{2+}\) signals frequency in oscillation per minute (n = 36 α-cells for NP; n = 14 α-cells for LP mice). D, Frequency of oscillations in percentage compared with the control condition (0.5 mM glucose). E, Inhibitory effect of insulin on the α-cell Ca\(^{2+}\) signal of a control mouse. F, Frequency of oscillations in the presence of insulin compared with the control condition (0.5 mM glucose) (n = 7 α-cells for NP; n = 15 α-cells for LP mice). Data are means ± se. G, Glucose. Statistically significant: *, P < 0.05. Ns, Nonsignificant.
levels did not differ significantly between both groups (Supplemental Fig. 4).

Pancreatic α-cell mass is increased in islets from malnourished mice

Because nutritional deficiencies can alter cell type distributions within the islet (10, 11), α-cell mass was analyzed in both groups. The dietary treatment did not modify the typical spatial location of α-cells in the periphery of the islet around a β-cell core (Supplemental Fig. 1). Although pancreases were smaller in LP mice, the relative and absolute α-cell mass were found higher than in controls (Fig. 3, A–D). When normalized by body weight, the α-cell mass was also higher in LP mice (Fig. 3E). Although an increase in relative islet mass was observed in LP mice, this increase was not maintained when normalized by the pancreas weight (absolute islet mass) (Supplemental Fig. 5). The proportion of α-cells within the islet was enhanced with the protein restriction (Fig. 3F). The increased α-cell mass seems to be associated to augmented α-cell number rather than α-cell hypertrophy (Fig. 3, G and H). Thus, these results indicate that LP diet induced an increase in α-cell mass.

Glucagon signaling is attenuated in the liver of malnourished mice

Protein malnourished rodents exhibit alterations in both β-cell function and peripheral insulin sensitivity (12, 13). Thus, in addition to study α-cell adaptations to protein restriction, we also analyzed peripheral glucagon action on the liver, the central tissue target of this hormone. Hepatic glucagon action plays a key role in glucose homeostasis, particularly in the adaptive response to fasting (25). In this metabolic situation, hypoglycemia promotes the increase in plasma glucagon levels, inducing gluconeogenesis and glycogenolysis in the liver, which favors hepatic glucose release to maintain normoglycemia (34). The binding of glucagon to its receptor in the liver induces a signaling cascade that involves PKA activation and the subsequent phosphorylation of CREB (15). As shown in Fig. 4, the levels of PKA and CREB phosphorylation with respect to the total protein were similar in both groups during the fed state, when plasma glucagon levels are low (34). As expected, the fasting state, which involves a concomitant rise in plasma glucagon levels (15, 35), was associated with increased PKA and CREB phosphorylation in the control group (Fig. 4). However, the phosphorylation of both proteins in response to fasting was impaired in the LP mice. Similar findings were obtained when a single exogenous glucagon injection was administered in these animals (Fig. 4). Although glucagon administration led to PKA and CREB activation in control mice, these changes were smaller in malnourished mice. Thus, these results point to an attenuated glucagon signaling in the liver of LP animals.
Additionally, we examined other signaling steps in response to fasting that are involved in hepatic glucagon action. Although glucagon receptor (Gcgr) expression was enhanced in response to fasting in the liver of controls, as previously reported (36, 37), this up-regulation was impaired in malnourished mice (Fig. 5A). We also analyzed the effect of fasting on the expression of several glucagon-modulated genes, which are involved in liver glucose metabolism: peroxisome proliferator-activated receptor γ (Ppargc1a) as well as the key gluconeogenic enzymes glucose-6-phosphatase (G6pc) and phosphoenolpyruvate carboxykinase (Pck1) (15, 34). Fasting induced an up-regulation in the expression of the three genes in control animals (Fig. 5, B–D), as previously described (34). However, this up-regulation was not observed in the case of Ppargc1a and G6pc in malnourished mice (Fig. 5, B and C). Although the response of Pck1 to fasting was not altered in LP animals, its expression levels during the fed state were lower than in controls (Fig. 5D). Finally, we also tested whether hepatic glycogen mobilization in response to fasting was also affected in malnourished animals. Hepatic glycogen in the fed state was higher in LP mice than in controls (Fig. 5E), an effect that has been associated with enhanced hepatic insulin sensitivity, which promotes glycogen formation (12, 13, 38). Our above-mentioned results also indicate that glucagon attenuated signaling could also contribute to these enhanced glycogen accumulation in fed state. When challenged with 6 h of fasting, controls exhibited the expected decrease in glycogen (Fig. 5E). However, no mobilization was observed in LP animals (Fig. 5E). When mice were subjected to a more intense fasting (16 h), glycogen decrease was observed in both groups. Thus, all these findings suggest that glucagon signaling as well as fasting adaptations are attenuated.

**FIG. 3.** α-Cell mass is increased in the pancreas of malnourished mice. A, Representative images of pancreas sections stained for glucagon and hematoxylin. Pancreas weight is decreased in LP mice (B), but the relative α-cell mass is augmented (C) compared with controls. D, Increased absolute α-cell mass (pancreas weight multiplied by relative α-cell mass) was observed in LP mice. The absolute α-cell mass normalized by the body weight (b.w.) was significantly increased compared with controls (E). Analysis of α-cell distribution per islet indicated enhanced values for LP mice (F). α-Cell size (G) and number (H) in both groups. Data are means ± SE (n = 5 pancreases for NP; n = 6 pancreases for LP mice). Statistical significance vs. control (NP): *, P < 0.05. Scale bars, 100 μm.
and/or delayed in the liver of mice submitted to protein-deficient diets. These results are consistent with the fact that glucose elevation as a response to a single glucagon injection was smaller in LP animals compared with controls (Supplemental Fig. 6).

Discussion

Caloric and protein nutritional restrictions during intrauterine or early postnatal periods lead to numerous changes in β-cell function and insulin peripheral action (3, 8, 38). Although these changes can provide survival advantages to regulate glucose homeostasis in these conditions, they can be adverse with normal or excessive nutrition in the adulthood, leading to enhanced predisposition to develop glucose intolerance, obesity, and diabetes (8). The dysfunctional behavior of the endocrine pancreas, as well as several forms of diabetes, is frequently associated with nutritional deficiencies, particularly in developing nations, where fetal and infant nutrition are important health problems (1, 2). Although adulthood overnutrition is a key pathogenic factor for T2DM in developed countries, several studies link an impaired β-cell function with in utero nutritional restrictions (5–8). Thus, a better comprehension of islet adaptations during nutritional deprivation is important to handle prevention and therapy strategies.

The vast majority of studies about the endocrine pancreas function during chronic malnutrition have focused on the pancreatic β-cell and insulin actions. We and other groups have previously demonstrated that rodents subjected to LP diet exhibit changes in β-cell mass as well as altered GSIS that leads to reduced insulin secretion (9, 10, 12, 13, 38). Despite lower plasma insulin levels, LP rodents have enhanced peripheral insulin sensitivity, which may allow for the normoglycemic state found in these animals (12, 13, 38, 39). Although glucose homeostasis depends on both α- and β-cell function as well as glucagon and insulin peripheral actions, very few studies have analyzed the role of glucagon secretion in chronic malnutrition. In the present work, we show that dietary protein...
Protein Restriction and α-Cell Function

restriction leads to numerous changes in the pancreatic α-cell: impaired glucose-regulation of Ca\(^{2+}\) signaling and glucagon secretion, altered plasma glucagon levels, increased α-cell mass, attenuated glucagon signaling in the liver, and altered hepatic response to fasting. Therefore, in addition to changes in β-cell function, α-cells exhibit numerous adaptations during chronic malnutrition to fulfill the glucose needs and ensure the normoglycemia found in these malnourished animals. Some similar changes have been described in rats submitted to comparable protocols of LP diets (19, 38, 40). These studies in rats indicated a lower hepatic response to glucagon and/or liver function deregulation in malnourished animals. Additionally, increased plasma glucagon levels have been also observed in chronic malnutrition (18). Although the majority of these previous reports have only focused on glucagon action on hepatic glucose handling, in our current work, we additionally reported 1) adaptations of glucose-regulated Ca\(^{2+}\) signaling and glucagon secretion at the cellular level, 2) alterations in α-cell mass, and 3) changes in glucagon action and fasting responses at the molecular level in receptor and postreceptor steps. Therefore, our present results further support the idea that islet adaptations to chronic malnutrition involve both α- and β-cells (19, 40).

Actually, the final output depends on the insulin to glucagon ratio either in terms of plasma hormonal concentrations or in function (i.e. high glucagon levels, but limited glucagon action leads to a low functional effect) (15). During dietary restrictions in early life periods, these adaptations may be necessary to handle glucose homeostasis and, at the same time, to regulate the anabolic and catabolic functions of both hormones throughout the growth period in these limited conditions.

In the present work, we described in malnourished animals an impaired glucose regulation of both α-cell Ca\(^{2+}\) signaling, an essential step in exocytosis, and glucagon secretion. However, to the best of our knowledge, there are no specific studies describing the α-cell behavior during protein-deficient states, several works in malnourished rats have reported increased plasma glucagon levels and have suggested an altered α-cell function (18, 41). Here, we confirm that pancreatic α-cells present an altered regulation. Additionally, we found an important increase in α-cell mass in malnourished mice. This finding is also new, because to date, nutritional deficiencies have been mainly related with β-cell mass changes (9–11). All these features may contribute to elevated glucagon levels. Regarding the lower ability of glucose to inhibit Ca\(^{2+}\) signals and glucagon secretion in LP islets, it might be related with altered glucose metabolism in the α-cell. In the β-cell, LP diet leads to impaired glucose metabolism, which seems to be involved in the reduced Ca\(^{2+}\) signaling and insulin secretion in the β-cells of these animals (12). Thus, a similar process might be involved in the pancreatic α-cell of LP mice, because glucose metabolism regulates Ca\(^{2+}\) signals and secretion in this cell type (15). Alternatively, given that islets of malnourished mice secrete less insulin in response to glucose (12), it may be possible that α-cells of LP mice are influenced by a lower amount of insulin secreted from surrounding β-cells, and thus, the paracrine inhibition by insulin may be lower (30).

Human and animal chronic malnutrition may lead to different symptoms. However, several metabolic features of protein deficiency in children are similar to those described here in mice, including decreased plasma insulin and albumin levels, reduced body weight, and normoglycemia (17). Interestingly, although plasma glucagon levels in human seem more elevated in caloric restrictions than in protein deficiencies, endogenous glucose production was significantly diminished in the latter, suggesting an impaired hepatic function (17). This is consistent with our present findings. Similarly, several studies reported liver glucagon resistance as well as elevated plasma glucagon levels in protein-restricted rodents (18, 19, 41, 42). Our current work indicates that glucagon resistance and down-regulated hepatic function in malnutrition is likely due to attenuated signaling at the level of the Gcgr, CREB, PKA, and key gluconeogenic enzymes. It has been shown that enhanced glycogen accumulation in malnourished animals is related with increased hepatic insulin sensitivity (19, 20, 38). In addition to the higher glucagon accumulation, we also described an impaired glycogen mobilization during fasting. Our results indicate that, although an increased insulin action may be present, attenuated glucagon signaling in the liver is likely involved in this effect. As we showed here, and consistent with previous findings in protein-restricted rats (19), all these hepatic adaptations seem to be related with a lower efficiency of exogenous glucagon to elevate plasma glucose levels. Although we observe that glucagon signaling is attenuated in the liver, we cannot discard that the lower protein intake in the animals might alter other different routes involved in the hepatic response to fed and fasting states (25, 43). At present, it is difficult to know whether the attenuated glucagon signaling in the liver in LP conditions is related with the increased α-cell response and mass, the interaction between both processes, or the temporal pattern to develop these changes. Additionally, these alterations may be also secondary or related to the changes in β-cell function and peripheral insulin sensitivity observed in these animals to maintain glucose homeostasis (12, 13). Therefore, further research is required to address this complex issue.

Thus, the present work shows for the first time that LP diet leads to numerous alterations in α-cell function as well as...
as changes in glucagon hepatic signaling, which should be considered as an important component of the endocrine pancreas adaptations to chronic malnutrition to regulate glucose homeostasis. All these alterations during early life phases should be also taken into account in the eventual islet dysfunction and development of diabetes during adulthood in malnourished individuals (8). In any case, in the present study, we worked with a model of protein restriction for 8 wk after weaning in mice, and caution should be taken when extrapolating these alterations to other developmental stages with nutritional deprivation either in human or in animal models.

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References

21. Wolfson WQ, Cohn C 1948 Studies in serum proteins; clinical studies employing rapid chemical fractionation procedures, with particular reference to the frequency and significance of hypoalbuminemia. J Lab Clin Med 33:1276–1288
2008 Increased pancreatic islet mass is accompanied by activation of the insulin receptor substrate-2/serine-threonine kinase pathway and augmented cyclin D2 protein levels in insulin-resistant rats. Int J Exp Pathol 89:264–275