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Effects of biotin deficiency on pancreatic islet morphology, insulin sensitivity and glucose homeostasis☆

Elena Larrieta[a], Maria Luisa Lazo de la Vega-Monroy[a], Paz Vital[a], Asdrubal Aguilera[a,b], Michael S. German[c], Mohammed El Hafidi[d], Cristina Fernandez-Mejia[a,*]

[a]Unidad de Genética de la Nutrición, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México/Instituto Nacional de Pediatría, Mexico
[b]Universidad Michoacana de San Nicolás de Hidalgo, Mexico
[c]Center of Regeneration Medicine and Stem Cell Research, University of California, San Francisco, CA, USA
[d]Departamento de Biomedicina Cardiovascular, Instituto Nacional de Cardiología Ignacio Chávez, Mexico

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Abstract

Several studies have revealed that physiological concentrations of biotin are required for the normal expression of critical carbohydrate metabolism genes and for glucose homeostasis. However, the different experimental models used in these studies make it difficult to integrate the effects of biotin deficiency on glucose metabolism. To further investigate the effects of biotin deficiency on glucose metabolism, we presently analyzed the effect of biotin deprivation on glucose homeostasis and on pancreatic islet morphology. Three-week-old male BALB/cAnN Hsd mice were fed a biotin-deficient or a biotin-control diet (0 or 7.2 μmol of free biotin/kg diet, respectively) over a period of 8 weeks. We found that biotin deprivation caused reduced concentrations of blood glucose and serum insulin concentrations, but increased plasma glucagon levels. Biotin-deficient mice also presented impaired glucose and insulin tolerance tests, indicating defects in insulin sensitivity. Altered insulin signaling was linked to a decrease in phosphorylated Akt/PKB but induced no change in insulin receptor abundance. Islet morphology studies revealed disruption of islet architecture due to biotin deficiency, and an increase in the number of α-cells in the islet core. Morphometric analyses found increased islet size, number of islets and glucagon-positive area, but a decreased insulin-positive area, in the biotin-deficient group. Glucagon secretion and gene expression increased in islets isolated from biotin-deficient mice. Our results suggest that biotin deficiency promotes hyperglycemic mechanisms such as increased glucagon concentration and decreased insulin secretion and sensitivity to compensate for reduced blood glucose concentrations. Variations in glucose homeostasis may participate in the changes observed in pancreatic islets.

Keywords: Biotin deficiency; Glucose homeostasis; Islet; Glucagon

1. Introduction

Biotin is a water-soluble vitamin that acts as a prosthetic group in carboxylases, which catalyze steps along various biochemical pathways. Unrelated to this role, several lines of evidence indicate that biotin also regulates gene expression and has a wide repertoire of effects on systemic processes [1–3].

Pioneer studies in biotin-deficient rats led to the discovery of the influence of biotin requirements on gene expression. Since then, biotin deficiency has been a useful tool to unravel the role of biotin deficiency on gene expression and biological functions, such as growth [4–6], immunity [5,7] and reproduction [8,9], as well as its role in carboxylase gene expression [10,11]. In cultured cells, biotin deficiency experiments have shed light on the biotin-signaling pathway [12–18]. The far-reaching effects of biotin deficiency on gene expression have been examined by microarray analysis, which has revealed that thousands of genes are modified in response to this deficiency [19].

Several studies have shown that adequate biotin intake is required to maintain normal glucose metabolism [20–25]. Pioneer studies by Dakshinamurti et al. [20] reported that glucose tolerance test curves of biotin-deficient rats were significantly higher than the curves of nondeficient rats. They further demonstrated that this effect was the result of a reduction in hepatic glucokinase activity [21], a critical enzyme in the regulation of glucose uptake in the liver. Later, with the arrival of molecular biology techniques, Chauhan and Dakshinamurti [22]

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* Corresponding author. Mexico City, CP 04530 Mexico. Tel.: +52 55 5606 35 58; fax: +52 55 56063489.
E-mail address: crisfern@biomedicas.unam.mx (C. Fernandez-Mejia).

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demonstrated that, in starved non-biotin-deficient rats, the effect of biotin on hepatic glucokinase occurs at the level of transcription [22]. This vitamin [17] also regulates the expression of insulin receptor, another essential protein involved in glucose metabolism. In hepatoblastoma cell lines cultured in the absence of biotin, de la Vega and Stockert [17] found that this vitamin is required for insulin receptor translation, suggesting that biotin deficiency may influence insulin sensitivity. Biotin deprivation affects pancreatic islet functions. Studies at our laboratory found that biotin deficiency reduced the expression and activity of pancreatic glucokinase [23], a critical enzyme in glucose-induced insulin secretion [24]. Furthermore, other authors [25] and our group [23] have found that, in islets isolated from biotin-deficient rats, insulin secretion was decreased. Controversial results have been found regarding the relationship between biotin status and hyperglycemia. Studies by Maebashi et al. [26] found that serum biotin concentration was lower in type 2 diabetic patients than in controls. An inverse correlation between serum biotin and fasting blood glucose concentration has also been observed [26,27]. However, studies at our laboratory analyzing lymphocyte propionyl-CoA carboxylase activity, currently considered a more accurate indicator than plasma biotin concentration [28,29], did not find significant differences between mild hyperglycemic type 2 diabetic patients and nondiabetic subjects [30].

To further examine the role of biotin deficiency in glucose metabolism, in this work, we analyzed the effect of biotin deprivation on glucose homeostasis, insulin sensitivity, serum insulin and glucagon concentrations and secretion, as well as pancreatic islet morphology and morphometry.

2. Materials and methods

2.1. Materials

Reagents were obtained from the following sources: biotin-deficient (TD-01363) and biotin control (TD-01362) diets from Harlan Teklad (Madison, WI, USA); Sevorane (sevoflurane) from Abbott Laboratories (Mexico DF, Mexico); Humulin (human regular insulin) from Eli Lilly (Mexico DF, Mexico); ultrasensitive rat insulin enzyme-linked immunosorbent assay (ELISA) and glucagon enzyme immunoassay from ALPCO Diagnostics (Windham, NH, USA); heparin, ultrasensitive streptavidin-peroxidase polymer, 4-chloro-1-naphthol, bovine serum albumin (BSA; fraction V), Ficoll, Triton X-100, Tween-20, paraformaldehyde and poly-L-lysine from Sigma-Aldrich (Saint Louis, MO, USA); D-glucose, Dulbecco's modified Eagle medium (DMEM), dialyzed fetal bovine serum, penicillin, streptomycin and Hank's balanced salt solution from Gibco (Grand Island, NY, USA); collagenase type 4 from Worthington (Freehold, NJ, USA); Paraplast (paraffin) from Sherwood Medical Co. (St. Louis, MO, USA); insulin antiserum from ICN (Irvine, CA, USA); fluorescein isothiocyanate (FITC)-conjugated goat anti-guinea pig IgG and CY5-conjugated goat anti-mouse IgG from Jackson Immunoresearch Laboratories (West Grove, PA, USA); mounting medium from DAKO (Glostrup, Denmark); TRIzol reagent M-MVL Reverse Transcriptase and oligo dT primers from Invitrogen (Carlsbad, CA, USA); TaqMan universal polymerase chain reaction (PCR) master mix, unlabeled PCR primers, and TaqMan MGB probes from Applied Biosystems (Foster City, CA, USA); protease inhibitor cocktail Complete from Roche (Penzberg, Germany); chemiluminescent HRP substrate from Millipore (Bedford, MA, USA); insulin Rβ antibody, α-tubulin antibody and BLOTTO nonfat dry milk from Santa Cruz Biotechnology (Santa Cruz, CA, USA); nitrocellulose membrane 0.45 mm and Bio-Rad protein assay from Bio-Rad (Hercules, CA, USA) and Cell Signaling (Beverly, MA, USA).

2.2. Mice

Mice were handled according to the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* (National Academy of Sciences, Washington, D.C., USA, 1996). The Ethical Committee for Experimentation of the Biomedical Research Institute of the National Autonomous University of Mexico approved all methods used in this study. Three-week-old male BALB/cAnN Hsd mice were obtained from the animal house at the Biomedical Research Institute of the National Autonomous University of Mexico. They were maintained in barrier conditions with 12-h light/dark cycles and allowed to consume water and food *ad libitum* throughout the experiments except during fasting. Mice were divided into two experimental groups and fed one of the two alternative diets: biotin control or biotin deficient (TD-01363), containing 16.4 or 0 μmol biotin/kg diet, respectively, during 8 weeks. Complete information on diet composition has been published elsewhere [7]. These diets contained 30% egg white solids. Egg white contains avidin, a glycoprotein that binds biotin, forming a noncovalent complex that is not absorbed into the blood. The 30% egg white in the diet contributes about 150 mg/kg of avidin. One milligram of avidin is considered to bind 61.4 nmol of biotin. Thus, 150 mg of avidin binds approximately 9.2 μmol of biotin. This makes the TD 01363 diet biotin free. The TD 01362 diet was supplemented with 16.4 μmol biotin/kg; thus, it contained approximately 7.2 μmol of free biotin/kg, enough to provide the daily requirements of the vitamin [31]. A commercial enzyme immunosorbent assay kit [32] confirmed the amount of free biotin in diets. Body weight and food intake were measured every week. After 8 weeks of treatment, mice were food deprived for 16 h and anesthetized with Sevorane; then mice were bled, and the pancreas was removed. Finally, mice were killed by cervical dislocation.

2.3. Blood glucose concentration analysis

Blood glucose concentrations were determined from tail vein samples using a glucometer (Precision QID, MediSense, Inc., Abbott Laboratories Company, Mexico DF, Mexico).

2.4. Serum insulin and glucagon analysis

Blood samples were collected into polypropylene tubes and centrifuged at 10 000*g* and 4°C for 10 min. Sera were separated and stored at −20°C for insulin assays. To obtain plasma, heparin was added to blood samples, and plasma was stored at −20°C for glucagon assay. Insulin and glucagon concentrations were determined with ELISA immunoassay kits. Absorbance was measured using the Labsystems Multiskan MS plate reader (Labsystems, Helsinki, Finland). All measurements were performed in duplicate. The insulin and glucagon detection ranges were 3.47–173.6 and 14.3–2869 pmol/L, respectively.

2.5. Intraperitoneal glucose and insulin tolerance tests

After 8 weeks of diet, mice were deprived of food for 16 h. For the glucose tolerance test, blood glucose concentrations were determined from tail vein samples after 0, 15, 30, 60 and 120 min of an intraperitoneal injection of 2 g/kg glucose, using a glucometer (Precision QID, MediSense, Inc., Abbott Laboratories Company, Mexico City, Mexico). For the insulin tolerance test, fed mice were intraperitoneally injected with 1 IU/kg body weight of regular human insulin. Blood glucose concentrations were measured after 0, 15, 30, 60 and 90 min of an intraperitoneal insulin injection, as reported previously [33].

2.6. Biotinylated pyruvate carboxylase mass abundance

Biotinylated pyruvate carboxylase mass abundance in liver was determined by streptavidin Western blot. Approximately 500 mg of liver was homogenized in 3 ml of ice-cold phosphate-buffered saline (PBS) pH 7.3 with protease inhibitor cocktail Complete using a Polytron (Kinematica AG, Littau, Switzerland) at 4°C for two pulses of 10 s each, with 30-s intervals between pulses. The homogenates were then sonicated (Branson cell disruptor 200, Danbury, CT, USA) with five pulses of 10 s each, with 1-min pauses between pulses, followed by centrifugation at 100 000*g* for 30 min at 4°C. Protein concentration of homogenates was determined by the Bradford assay.

Homogenates (30 μg of total protein) were resolved by 6.5% sodium dodecyl sulfate (SDS) polyacrylamide gel and electrophoresed at constant voltage of 100 V for 150 min. The gel was electroblotted to nitrocellulose membranes at 20 V for 30 min in a semidry transfer camera. Membranes were blocked in 5% dry milk and 0.1% Tween-20 in PBS overnight at 4°C. Afterward, membranes were incubated with streptavidin-HRP in PBS with 0.1% Tween-20 at room temperature for 1 h on an orbital shaker. For pyruvate carboxylase detection, the blot was incubated with 0.05% 4-chloro-1-naphthol and with 0.015% hydrogen peroxide in 15% methanol in PBS. Biotinylated bands were quantified using Image J 1.40 (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

2.7. Insulin receptor protein abundance

Approximately 100 mg of liver was coarsely minced and homogenized with a potter homogenizer in ice-cold lysis buffer containing 1% Triton X-100, 100 mM Tris (pH 7·4), 100 mM Na4P2O7, 100 mM NaF, 10 mM EDTA, 10 mM Na3VO4, 2 mM PMSF and 10 μg/ml aprotinin. Tissue extracts were centrifuged at 12 000*g* for 20 min at 4°C to remove insoluble material, and protein concentration was measured by the Bradford assay [34]. Proteins were resolved by SDS polyacrylamide gel electrophoresis (7.5% acrylamide) in a Bio-Rad miniature slab gel apparatus. Electrotransfer of proteins from the gel to nitrocellulose membranes was performed for 1 h at 100 V (constant) using the Bio-Rad miniature transfer apparatus in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol. To reduce nonspecific binding, the nitrocellulose membranes were incubated overnight with T-TBS buffer (50 mM Tris–HCl, pH 7·6, 150 mM NaCl and 0.1% Tween 20) containing 5% milk. Blots were then incubated overnight with anti-β-subunit insulin receptor. The membranes were washed four times for 5 min each in T-TBS buffer and were then incubated with bovine anti-rabbit IgG HRP in T-TBS containing 3% fat free milk for 2 h at room temperature and then washed again as described above. Bands of IR proteins were detected by autoradiography, and band

Table 1
Effect of 8 weeks of biotin deficiency on body weight, food intake, metabolites and hormones

	Control	Biotin deficient
Fasting serum glucose (mmol/L)	4.5±0.2	3.3±0.2 *
Fed serum glucose (mmol/L)	9.3±0.3	6.3±0.4 *
Fasting serum insulin (pmol/L)	53.2±16.6	11.5±2.9 *
Fed serum insulin (pmol/L)	86.7±26.6	44.0±5.3 *
Fasting plasma glucagon (pmol/L)	68.8±5.5	182.9±39 *
Fed plasma glucagon (pmol/L)	82.3±28.2	87.5±18.1
Body weight (g)	23.4±0.3	19.4±0.3 *
Food intake (g food/mice /day)	3.6±0.3	3.0±0.2
Food intake (g food/g body weight/day)	0.15±.01	0.15±.01

Values are the mean±S.E. Significant differences between biotin-deficient and control mice.
* $P<.05$; $n=4–7$ for insulin, $n=7$ for glucagon and $n=10–16$ for blood glucose.

intensities were quantified by densitometry using Image J 1.40 (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

2.8. Phosphorylated and nonphosphorylated Akt/PKB protein abundance

Liver protein extracts were obtained as described above. Both phosphorylated and nonphosphorylated AKT/PKB protein abundances were determined by Western blot with a commercial assay kit (Phospho-Akt Pathway Antibody Sampler Kit, Cell Signaling Technologies, Beverly, MA, USA) following the manufacturer's instructions.

2.9. Immunohistochemistry and morphometric analysis of pancreas

Pancreases were removed and fixed overnight in 4% paraformaldehyde in PBS, dehydrated and embedded in paraffin. Consecutive 5-μm-thick sections were cut and mounted on glass slides. Sections were deparaffinized, rehydrated, permeabilized and subsequently incubated overnight with guinea pig anti-porcine insulin antibody (1:4000). Then, sections were incubated for 1 h with a secondary goat anti-guinea pig IgG antibody conjugated with FITC for insulin detection (1:100). Afterward, sections were incubated for 3 h with mouse anti-rat glucagon (1:6000) following 1 h with a secondary goat anti-mouse IgG conjugated with CY5 (1:100). After incubation, the sections were washed with PBS and cover slipped with fluorescent mounting medium.

Images of pancreatic islets were obtained using Laser Scanning System LSM 510 (Carl Zeiss, Jena, Germany). Fluorescein isothiocyanate was excited with a 494-nm wavelength, and emitted light was band-passed with a 520-nm filter, while CY5 was excited with a 650-nm wavelength, and emitted light was band-passed with a 670-nm filter.

Morphometric analysis was performed of sections taken from four mice of each group. Sections were analyzed at 100-μm intervals to avoid measuring the same islet twice. Areas of islets, α-cells and β-cells were quantified using Zeiss LSM Image software. Areas were expressed as percentage of the total pancreatic area. Only islets containing 15 or more endocrine cells were measured [35]. Images of entire histological sections were obtained with a digital camera, and total area was measured using a manual optical picture image analyzer.

2.10. Glucagon secretion analysis

Pancreatic islets were isolated by collagenase digestion as previously reported [23]. Groups of 20–30 islets were cultured in biotin-free DMEM medium containing 11 mM glucose, 400 U/ml penicillin, 200 mg/L streptomycin and 10% dialyzed fetal bovine serum. Islets were incubated overnight at 37°C in a humidified atmosphere of 5% CO_2. After incubation, islets were preincubated for 30 min in Hank's solution, 7 mM glucose and 0.5% BSA (w/v). Afterward, Hank's solution was removed, and islets were then incubated in 200 μl of the same solution containing 1 mM glucose for 1 h. Glucagon secretion was determined by ELISA assay.

2.11. Quantitative real-time PCR

Total RNA was isolated from a pooled sample of 200 pancreatic islets using TRIzol according to the manufacturer's instructions. RNA concentration and purity were determined by absorbance at 260/280 nm, and its integrity was confirmed by electrophoresis on 1% denaturing agarose gel. Single-strand cDNA was synthesized from 0.4 μg of total RNA by reverse transcription reaction with 500 U of M-MVL RT and oligo dT primers. cDNA were mixed with TaqMan universal PCR master mix, unlabeled PCR primers and TaqMan MGB probes (FAM dye-labeled). Relative

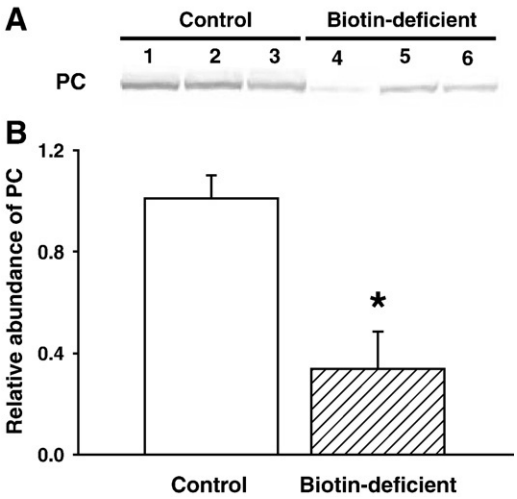

Fig. 1. Effect of biotin deficiency on biotinylated pyruvate carboxylase. (A) Western blot analysis of biotinylated pyruvate carboxylase abundance in the liver of mice fed a control and biotin-deficient diet for 8 weeks. Control mice: lines 1–3; biotin-deficient mice: lines 4–6. (B) Quantification of biotinylated pyruvate carboxylase abundance in the liver. White bars: control group; dashed bars: biotin-deficient group. Data are mean±S.E. of values from three mice of each group. *$P<.05$ vs. control mice.

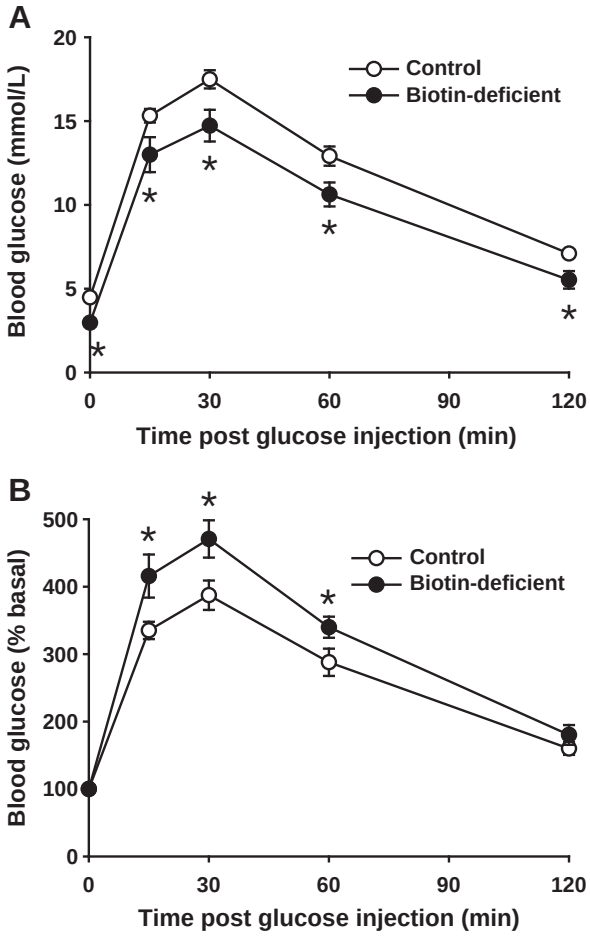

Fig. 2. Effect of biotin deficiency on glucose tolerance. Three-week-old male BALB/cAnN Hsd mice were fed either a biotin-deficient diet or a control-diet during 8 weeks. (A) Intraperitoneal glucose tolerance test. (B) Relative glucose values in intraperitoneal glucose tolerance test. Open circles: control group; closed circles: biotin-deficient group. Data show the mean±S.E. of values from 5–10 mice of each group. *Indicates significant differences ($P<.05$) between groups.

quantifications of mRNA levels for pyruvate carboxylase (Mm00500992_m1) and glucagon (Mm00801712_m1) were determined by real-time quantitative PCR using an ABI Prism 7700 Sequence Detector instrument. The quantitative expression of gene was calculated from the cycle threshold (C_T) value of each sample in the linear part of the curve using the relative quantification method ($2^{-\Delta\Delta C_T}$) [36]. The samples were analyzed in triplicate and corrected for the 18S ribosomal subunit used as internal standard.

2.12. Statistical analysis

The Statview statistical analysis program V.4.5 (Abacus Concepts, Berkeley, CA, USA) was used for statistical analysis. All data presented are the mean±S.E.; *n* denotes the number of evaluated subjects. Statistical significance was assessed by Student's *t* test. *P* values less than .05 were considered statistically significant.

3. Results

3.1. Metabolites, hormones, body weight and food intake

Blood glucose analysis revealed that fasting and fed glucose concentrations in the biotin-deficient group were, respectively, 27% and 32% lower than in control mice (Table 1). Likewise, fasting and fed insulin concentrations in serum were significantly lower (77% and 50%, respectively) than in control mice (Table 1). In contrast with these reductions, fasting glucagon concentration increased 166% in the deficient group (Table 1), while no changes in glucagon concentration between the groups were observed in the fed state. As previously reported [5–7,32], body weight in the deficient group was significantly lower (Table 1) than in the control group, and the amount of food intake/body weight was not significantly different between the groups.

3.2. Pyruvate carboxylase, biotinylated mass and mRNA abundance

Streptavidin Western blot analysis showed 70% reduction of pyruvate carboxylase mass ($P<.02$) in biotin-deficient mice compared to the control group (Fig. 1). In addition, we found that the mRNA expression of pyruvate carboxylase was significantly ($P<.005$) decreased (0.68±0.08-fold change of control mice normalized to 18S rRNA).

3.3. Glucose tolerance test

To further explore the effects of biotin deficiency on glucose homeostasis, glucose tolerance tests were performed. We observed that, compared to controls, biotin-deficient mice presented significantly ($P<.05$) lower glucose concentrations before and after glucose administration (Fig. 2A). In spite of the lower glucose concentration, relative glucose values in the glucose tolerance test revealed that biotin-deficient mice showed decreased ability to reduce blood glucose concentration in response to glucose challenge (Fig. 2B), indicating a failure in glucose disposal.

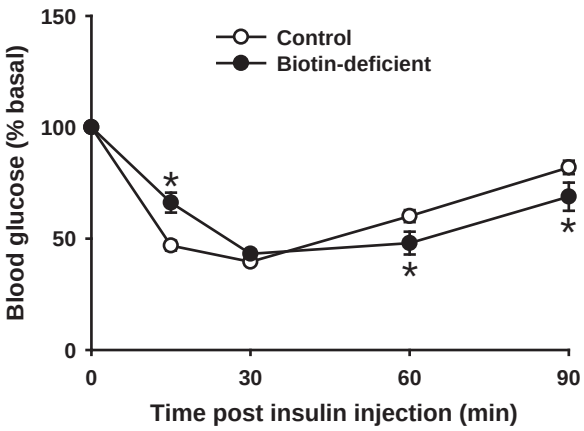

Fig. 3. Effect of biotin deficiency on insulin sensitivity. Relative glucose values in intraperitoneal insulin tolerance test. Open circles: control group; closed circles: biotin-deficient group. Data are the mean±S.E. of values from 5–10 mice of each group. *Indicates significant differences ($P<.05$) between groups.

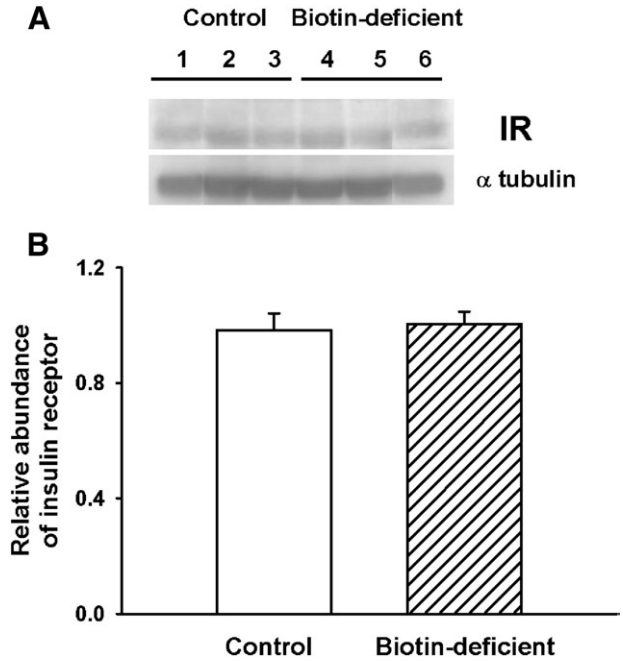

Fig. 4. Effect of biotin deficiency on hepatic insulin receptor protein mass. (A) Representative Western blot analysis of insulin receptor protein mass. (B) Quantification of insulin receptor protein mass abundance. White bars: control group; dashed bars: deficient-diet group. Graph data are means±S.E. of values from 5–10 mice of each group.

3.4. Insulin sensitivity

The insulin tolerance test revealed that, in the control group, blood glucose concentrations decreased about 53% in comparison to basal levels after 15 min of intraperitoneal insulin administration. In contrast, biotin-deficient mice showed a delayed response, reaching a 34% reduction after 15 min of insulin administration ($P<.001$) (Fig. 3). At 30 min, similar reductions were attained in both groups, while at 60 and 90 min, blood glucose concentrations were significantly lower ($P<.001$) in the deficient mice, indicating a reduced recovery of glucose levels after insulin administration.

3.5. Insulin receptor protein mass

Since de la Vega and Stockert [17] found that biotin is required for insulin receptor translation in hepatoblastoma cell lines, we sought to investigate whether the reduction in insulin sensitivity was related to reductions in insulin receptor protein mass. Our studies found no significant differences in insulin receptor protein expression between the control and the biotin-deficient group (Fig. 4).

3.6. Insulin transduction proteins

To determine if reduced insulin sensitivity was the result of changes in insulin signaling, we analyzed the abundance of the insulin transduction protein Akt/PKB (Fig. 5). In the liver, we found reductions of approximately 75% in protein abundance of

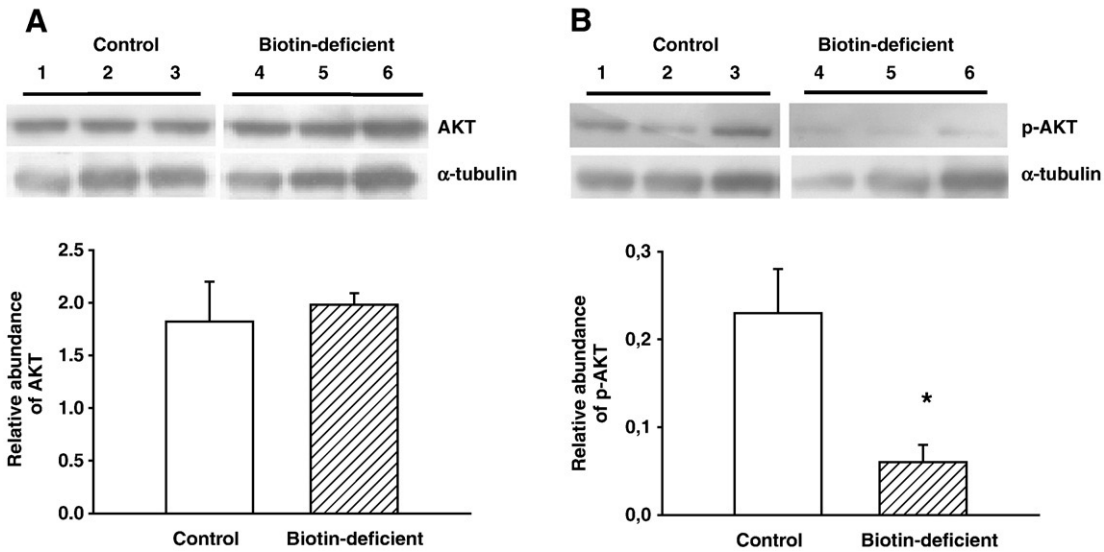

Fig. 5. Effect of biotin deficiency on phosphorylated and nonphosphorylated Akt/PKB. (A) Total Akt. (B) Phosphorylated Akt. White bars: control group; dashed bars: deficient-diet group. Graph data show the mean±S.E. of values from four mice of each group. *Indicates significant differences ($P<.05$) between groups.

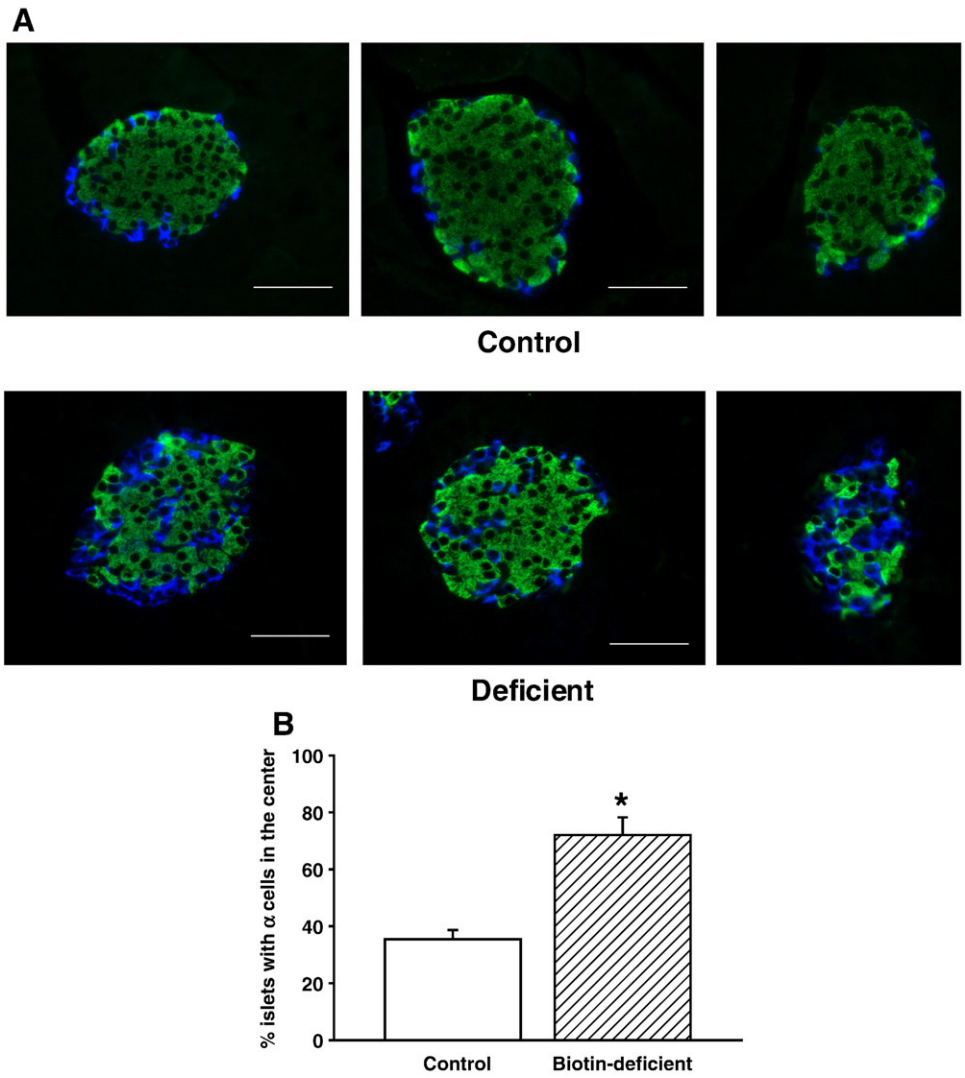

Fig. 6. Effect of biotin deficiency on pancreatic morphology. (A) Islet architecture immunostaining analysis: amplification, 40×. Calibration bar, 50 μm. Insulin (green); glucagon (blue). (B) Percent of islets with α-cells present in the center of the islet. Data show the mean±S.E. of values from 10 mice of each group. *Indicates significant differences ($P<.05$) between groups.

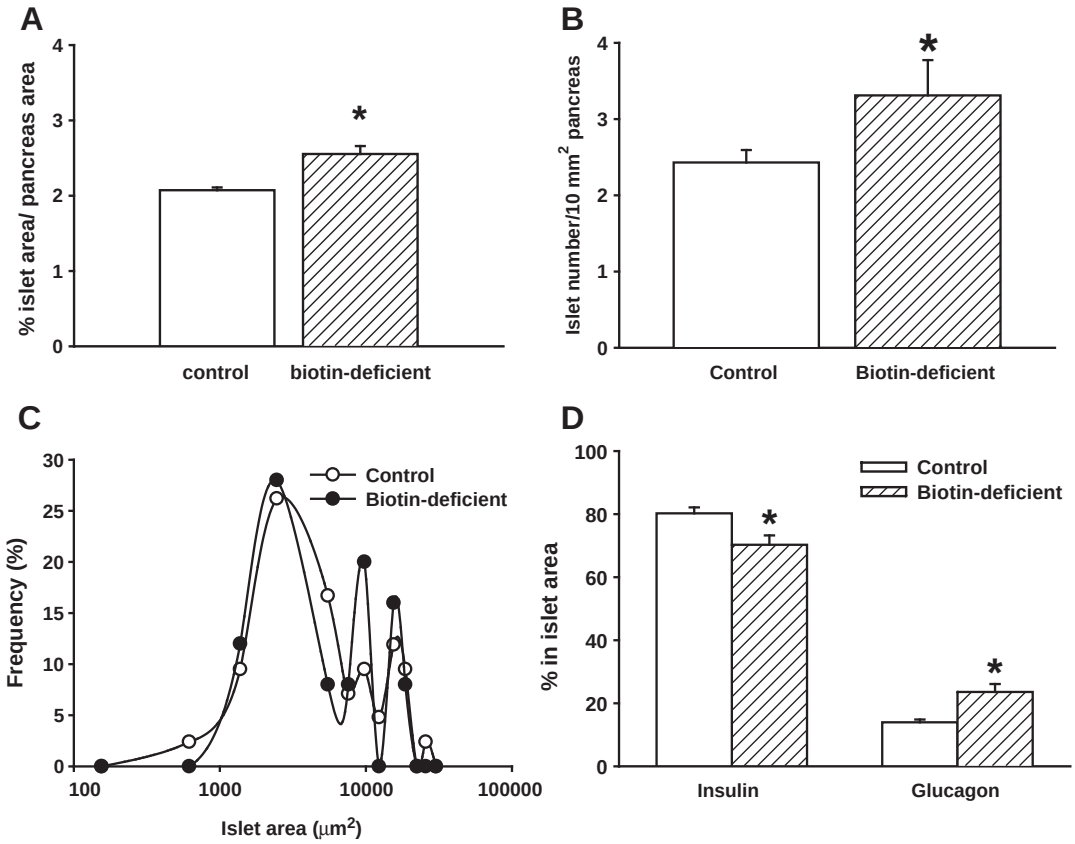

Fig. 7. Pancreatic morphometry analysis. (A) Percentage of islet area per pancreas area. (B) Number of islets per pancreas area. (C) Frequency distribution of islet size. (D) Glucagon-positive area and insulin-positive area per islet area. Data show the mean±S.E. of values from three mice of each group. *P<.05 vs. the corresponding value for control mice.

phosphorylated Akt/PKB, while no significant differences were found in total Akt/PKB protein abundance.

3.7. Morphology and morphometry of pancreas

We explored the effects of biotin deficiency on pancreatic islet morphology. Immunostaining of pancreatic sections with antibodies against insulin and glucagon showed changes in islet architecture of the biotin-deficient mice. In contrast with the normal peripheral distribution of glucagon-containing cells in the islet, in the biotin-deficient group, we found a significant (P<.05) increase in the percentage of α-cells in the islet core (Fig. 6).

Morphometric studies revealed a significant (P<0.05) increase in islet area per pancreas area (Fig. 7A) and in the number of islets per pancreas area (Fig. 7B) in biotin-deficient mice compared to control mice. The predominant size of islets of both groups was in the range between 1000 and 10 000 μm^2. However, two fractions of islets >10 000μm^2 were larger in biotin-deficient mice than in the control group (Fig. 7C). Analysis per islet area revealed a significant (P<0.05) increase in the glucagon-positive area, while the insulin-positive area per islet area was smaller in biotin-deficient mice (Fig. 7D).

3.8. Glucagon mRNA abundance and secretion

Real-time PCR analysis showed that biotin deficiency significantly increased glucagon mRNA abundance (2.3±0.3-fold change of control mice normalized to 18S rRNA). We also found that pancreatic islets isolated from biotin-deficient mice significantly secreted more glucagon than control mice (control: 14.8±2.8; deficient: 27.0±4.2 pg/ml/islet/h) (Fig. 8).

4. Discussion

Several studies have revealed that physiological concentrations of biotin are required for the normal expression of critical carbohydrate metabolism genes and for glucose homeostasis [20–25]. However, the different experimental models and conditions used to reach these conclusions make it difficult to integrate the effects of biotin deficiency on glucose metabolism. In this report, we present an analysis of different aspects of biotin deficiency on glucose metabolism.

Glucose homeostasis is an exquisite mechanism that involves glucose disposal and glucose production. In the fasting state, hepatic gluconeogenesis is critical to maintain normoglycemia. This metabolic pathway requires the catalytic action of the biotin-dependent enzyme pyruvate carboxylase. Consistent with the fall in activity [7] and with the decrease in biotinylated mass and in pyruvate carboxylase mRNA abundance, we found that fasting glucose levels were reduced in biotin-deficient mice compared to control mice.

Glucose tolerance studies have revealed that biotin-deficient mice exhibit lower absolute glucose concentrations than control mice throughout the test. In spite of the decrease observed in sugar concentrations, relative values in glucose tolerance curves showed delayed capacity to decrease blood glucose concentrations in response to sugar challenge in biotin-deficient mice, indicating glucose intolerance. Our findings agree with pioneer observations by Dakshinamurti et al. [20], who found that glucose tolerance was

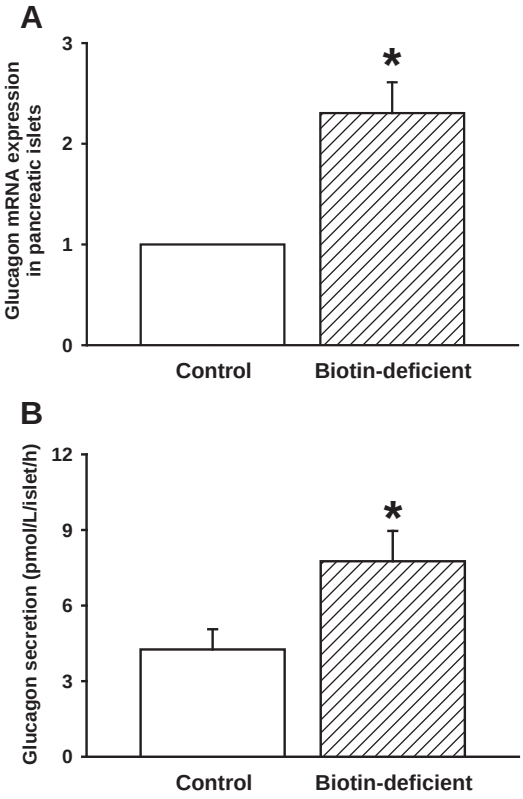

Fig. 8. Glucagon mRNA abundance and secretion. (A) Relative mRNA abundance of glucagon. Glucagon and 18s rRNA were quantified by real-time PCR. Data are expressed as fold change relative to control group. Values show the mean±S.E.M. of four to five pooled samples of 200 islets. (B) Glucagon secretion. Graph data are the mean±S.E. of values from five independent experiments. *Indicates significant differences ($P<.05$) between groups.

impaired in biotin-deficient rats. However, some differences exist between his studies and ours regarding the absolute concentration of glucose. In our studies, we found that, in biotin-deficient mice, glucose concentrations were decreased both at baseline and after glucose challenge. In contrast, in the studies of Dakshinamurti et al., glucose concentrations were higher in biotin-deficient rats than in the control-group throughout the glucose tolerance test. However, since pyruvate carboxylase activity, which decreases during biotin deprivation, is critical to maintain fasting blood glucose levels, it is enigmatic why basal glucose concentration increased in the studies of Dakshinamurti et al.

In glucose tolerance tests, glucose concentrations are the result of both the secretion of endogenous insulin and its action. As found in the present study and in agreement with other reports by others and us [23–25], insulin secretion is impaired in biotin-deficiency conditions. The effects of biotin deprivation on insulin action have not been documented. We found that insulin sensitivity is affected by biotin deficiency, as shown by the slow response of biotin-deficient mice to exogenous insulin observed in the insulin tolerance test. To investigate the mechanisms involved in decreased insulin sensitivity, we analyzed the expression of proteins that participate in the insulin-signaling cascade. Since biotin is required for insulin receptor translation in hepatoblastoma cell lines [17], we first investigated if the effect of biotin deficiency on insulin sensitivity was the result of decreased insulin receptor protein mass. Our data indicate that, in contrast with the reduction observed in hepatoblastoma cell lines, *in vivo* biotin deficiency did not affect insulin receptor protein expression. Instead, we found that phosphorylated Akt/PKB, a key protein involved in insulin sensitivity, dramatically decreased in biotin-deficient mice.

Significant effects of biotin deficiency were found on glucagon production. Our studies revealed that plasma glucagon concentration was approximately two-fold higher in the biotin-deficient group compared to control mice. Increased glucagon concentrations have also been observed in biotin-deficient cockerels. Studies by Klandorf H et al. [37] showed that fasting plasma glucagon significantly increased in the deficient group compared to controls; however, glucose concentrations were similar in both groups, indicating that biotin-deficient cockerels were able to overcome defects in gluconeogenesis induced by biotin deficiency by increasing glucagon levels. In contrast, in our studies, in spite of high plasma glucagon levels, fasting blood glucose concentrations remained low, indicating that, in mice, raised glucagon levels were unable to compensate for reduced gluconeogenesis due to the decrease in pyruvate carboxylase activity. The failure of glucagon to reestablish glucose production was also observed in liver hepatocytes isolated from biotin-deficient rats [38,39]. In these studies, glucagon did not stimulate gluconeogenesis from lactate, while other substrates such as glutamine, dihydroxyacetone or fructose, in which the activity of pyruvate carboxylase is not essentially involved, reactivated glucose production [39].

Regarding islet morphology, the effects of biotin deficiency were striking: in contrast with the normal peripheral distribution of α-cells in the normal islet, in the biotin-deficient group, glucagon-positive cells were spread throughout the islet area. Islet structural differences have been correlated to functional differences [40]. An increased percentage of α-cells in the core of the islet has been observed in many animal models of β-cell deficiency [41–44] and also in conditions of increased demand for insulin, such as in pregnancy and insulin resistance [41]. It is interesting to note that in a recent article [45], decreased pyruvate carboxylase activity, as a result of hyperglycemia, was associated with glucagon-positive cells spread throughout the islet area.

Changes in islet morphometry were also found in the biotin-deficient group. Percentage of islet area with respect to total pancreatic area was increased, most likely attributable to an increment in islet number and size. The glucagon-positive area within the islet was increased as well, while the insulin-positive area was decreased compared to the control group. These results, together with the increase in glucagon mRNA abundance and secretion, may account for the high plasma glucagon concentrations found in biotin-deficient mice. There are several similarities between the effects of biotin deficiency found in the present study and those observed in transgenic models with impaired gluconeogenesis such as PC2 [46] and glucagon receptor KO mice [47]. In these models, defective glucagon-induced gluconeogenesis resulted in reduced blood glucose concentrations in fasted and fed states, as well as increases in plasma glucagon concentrations, and hyperplasia of α-cells. Likewise, we speculate that, as result of biotin deficiency, impaired gluconeogenesis due to reduced pyruvate carboxylase activity in gluconeogenic tissues might be responsible for the changes observed in our studies, such as low blood glucose concentrations in fasted and fed states, increased glucagon concentration as well as α-cell area and conceivably the increase in glucagon mRNA abundance.

In summary, our data suggest that biotin deficiency promotes hyperglycemic mechanisms such as an increase in plasma glucagon concentration, a decrease in serum insulin levels as well as a decrease in insulin sensitivity to overcome the decrease in blood glucose concentrations that resulted from diminished gluconeogenesis due to pyruvate carboxylase activity in gluconeogenic tissues. In addition, as observed in other animal models, changes in glucose homeostasis may participate in morphological changes observed in pancreatic islets. However, we cannot rule out the possibility that the lack of a direct action of biotin on gene expression may also participate in the

effects observed in the present study. Studies specifically designed will be required to discern between the role of biotin as a direct participant in gene expression and the effects caused by the decrease in carboxylase activity.

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