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Effects of biotin supplementation in the diet on insulin secretion, islet gene expression, glucose homeostasis and beta-cell proportion☆

M.L. Lazo de la Vega-Monroy[a], E. Larrieta[a,1], M.S. German[b], A. Baez-Saldana[c], C. Fernandez-Mejia[a,*]

[a]Unidad de Genética de la Nutrición, Departamento de Medicina Genómica y Toxicología Ambiental, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México/Instituto Nacional de Pediatría, Mexico City, 04530 México
[b]Diabetes Center/Center of Regeneration Medicine and Stem Cell Research, University of California San Francisco, San Francisco, CA 94143, USA
[c]Departamento de Biología Celular y Fisiología, Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, 04510 México

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Abstract

Besides its role as a carboxylase cofactor, biotin has a wide repertoire of effects on gene expression, development and metabolism. Pharmacological concentrations of biotin enhance insulin secretion and the expression of genes and signaling pathways that favor islet function *in vitro*. However, the *in vivo* effects of biotin supplementation on pancreatic islet function are largely unknown. In the present study, we investigated whether *in vivo* biotin supplementation in the diet has positive effects in rodent pancreatic islets. Male BALB/cAnN Hsd mice were fed a control or a biotin-supplemented diet over 8 weeks postweaning and tested for glucose homeostasis, insulin secretion, islet gene expression and pancreatic morphometry. Insulin secretion increased from the islets of biotin-supplemented mice, together with the messenger RNA (mRNA) expression of several transcription factors regulating insulin expression and secretion, including forkhead box A2, pancreatic and duodenal homeobox 1 and hepatocyte nuclear factor 4α. The mRNA abundance of glucokinase, Cacna1d, acetyl-CoA carboxylase, and insulin also increased. Consistent with these effects, glucose tolerance improved, and glucose-stimulated serum insulin levels increased in biotin-supplemented mice, without changes in fasting glucose levels or insulin tolerance. Biotin supplementation augmented the proportion of beta cells by enlarging islet size and, unexpectedly, also increased the percentage of islets with alpha cells at the islet core. mRNA expression of neural cell adhesion molecule 1, an adhesion protein participating in the maintenance of islet architecture, decreased in biotin-supplemented islets. These findings provide, for the first time, insight into how biotin supplementation exerts its effects on function and proportion of beta cells, suggesting a role for biotin in the prevention and treatment of diabetes.

Keywords: Biotin supplementation; Insulin secretion; Beta cell gene expression; Beta-cell proportion; Islet architecture

1. Introduction

Increasingly, vitamins are being recognized as mediators of gene expression [1,2], illuminating the connection between nutritional signals and biological functions. Vitamin research has identified important transcription factors [3] and has led to the development of new therapeutic agents for different diseases [4–6] including diabetes [7,8]. Although less is known about water-soluble vitamins as genetic modulators, evidence of their effects on gene expression is increasing.

Biotin is a water-soluble vitamin that acts as a covalently bound coenzyme of carboxylases. Unrelated to this role, pharmacological concentrations of biotin modify gene expression (reviewed in Ref. [9]) and have a wide repertoire of effects on systemic processes (reviewed in Ref. [10]). DNA microarray studies and high-throughput immunoblotting studies have aided in the identification of thousands of genes whose expression is modified by biotin at both the transcriptional and the posttranscriptional levels [11,12]. Critical genes for glucose homeostasis, such as hepatic glucokinase [13] and insulin receptor [14], increase their expression in response to biotin supplementation, while the expression of several gluconeogenic

Abbreviations: Foxa2, forkhead box A2; Hnf4α, hepatocyte nuclear factor 4α; Ncam1, neural cell adhesion molecule 1; PDX-1, pancreatic and duodenal homeobox 1; PPARα, peroxisome proliferator activated receptor alpha; PPARγ, peroxisome proliferator activated receptor gamma; SREBF1, sterol regulatory element binding transcription factor 1.

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* Corresponding author. Tel.: +52 5255 5606 35 58; fax: +52 5255 56063489.
E-mail address: crisfern@biomedicas.unam.mx (C. Fernandez-Mejia).
[1] Present address: Unidad de Biología Molecular y Medicina Genómica, INCMSZ, Mexico City, México.

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genes in diabetic rats is decreased by pharmacological concentrations of biotin [15,16].

Studies in vitro by others [17,18] and our group [19,20] have consistently found that acute exposure to pharmacological doses of biotin enhances glucose-stimulated insulin secretion. Treatment of cultured islets with 1 to 10 μmol/l biotin for 30 min to 24 h increase insulin secretion both at basal (5.5 mmol/l) and stimulatory (16 mmol/l) glucose concentrations [17,19,20]. The impact of biotin on insulin secretion is dose dependent [18] and unique among the B vitamins [17].

Pharmacological concentrations of biotin *in vitro* increase the expression of genes that are critical for maintaining the differentiated phenotype of the beta cell, preserving beta-cell mass (proportion of beta cells) and glucose-stimulated insulin secretion [19–22]. Culturing isolated rat islets with biotin increases the expression of pancreatic and duodenal homeobox 1 (*Pdx-1*) [22], a critical transcription factor for the expression of insulin and for several genes involved in insulin synthesis and secretion (reviewed in Ref. [23]). Studies by others using the RIN1046-38 insulinoma cell line [21] and by our group using isolated islets from rats [19,20] have found that pharmacological concentrations of biotin augment the expression of beta-cell glucokinase, the rate-limiting enzyme in glucose-stimulated insulin secretion [24] and a determinant factor in beta-cell regeneration [25]. We have also found that the mechanism by which biotin increases the expression of *Glucokinase* involves enhancing insulin secretion through the cyclic guanylate monophosphate (cGMP)/protein kinase G (PKG) signaling pathway, which increases ATP levels and, thus, beta-cell membrane depolarization. Insulin secretion, in turn, increases beta-cell *Glucokinase* messenger RNA (mRNA) expression via autocrine stimulation of Phosphoinositol-3-kinase (PI3K)/Akt signaling [19].

In line with these effects of biotin on glucose metabolism and insulin secretion, pharmacological doses of biotin lowered hyperglycemia and ameliorated diabetes in some [26–28] but not all [29] studies done in humans. In a group of patients with type 1 diabetes, supplementation with 16 mg/day of biotin with removal of insulin treatment for 1 week considerably decreased hyperglycemia [26]. In Japanese patients with type 2 diabetes [27] and in patients undergoing hemodialysis [28], pharmacological doses of biotin improved glucose tolerance.

Biotin supplementation also ameliorated hyperglycemia in animal models of diabetes. In KK mice, a genetically diabetic model of moderate hyperglycemia and insulin resistance, biotin administration for 10 weeks lowered postprandial glucose levels and improved both glucose tolerance and insulin sensitivity [30]. In spontaneously type-2 diabetic Otsuka Long-Evams Fatty (OLETF) rats, dietary biotin supplementation also improved glucose tolerance [31].

The evidence that biotin has favorable effects on glucose metabolism has led to the development of commercially available diabetes medications [8,32], containing pharmacological amounts of biotin 40- to 166-fold increase compared with the reference dietary intake of 30 μg/day [33]. However, in spite of the importance of the pancreatic islet in maintaining normal glucose homeostasis, no studies have addressed the effect of *in vivo* biotin supplementation on the pancreatic beta cell. Since beta-cell compensation is critical to prevent diabetes development [34], in the present study, we investigated the effects of biotin supplementation on beta-cell function, gene expression and beta-cell proportion.

2. Materials and methods

2.1. Animal model and experimental design

Three-week old male BALB/cAnN Hsd mice from the animal facility at the Biomedical Research Institute of the National Autonomous University of Mexico were maintained in barrier conditions under 12-h light/dark cycles, and allowed free access to water and food throughout the experiments, except during fasting. The mice were handled according to the principles of laboratory animal care (National Institutes of Health publication no. 85-23, revised 1985, http://grants1.nih.gov/grants/olaw/references/phspol.htm). All procedures were approved by the Ethical Committee for Experimentation of the Biomedical Research Institute of the National Autonomous University of Mexico. The mice were fed for 8 weeks with one of the following diets: biotin-control (TD-01362) or biotin-supplemented diet (TD-01363), containing 1.76 and 97.70 mg of free biotin/kg diet, respectively (Harlan Teklad, Madison WI, USA). Complete information on diet composition has been published elsewhere [35]. After 8 weeks of feeding, the mice were food deprived for 16 h, anesthetized with Sevorane (Sevofluorane, Abbott Laboratories, Mexico DF, Mexico), and blood and pancreas were extracted. Finally, the mice were killed by cervical dislocation.

2.2. Blood insulin and biotin measurements

Blood samples were collected and centrifuged at 10,000×*g* and 4°C for 10 min. Sera were stored at −20°C until used. Insulin concentrations were determined with Ultrasensitive rat insulin EIA enzyme-linked immunosorbent assay (ELISA) kit (ALPCO Diagnostics, Windham, NH, USA) according to the 25-μl sample protocol provided by the manufacturer. Serum biotin was quantified using Biotin ELISA kit (ALPCO Diagnostics). Absorbance was measured using the Labsystems Multiskan MS plate reader (Labsystems, Helsinki, Finland). All measurements were performed by duplicate.

2.3. Blood glucose measurements

Blood glucose concentrations under fasting or fed conditions were determined from tail vein samples using a portable glucose meter (Precision QID, MediSense, Inc., Abbott Laboratories).

2.4. Glucose-stimulated insulin secretion

Groups of 20–30 pair-sized islets were isolated as previously reported [36] and were cultured in biotin-free Dulbecco's modified Eagle medium (11 mmol/l glucose, 400 U/ml penicillin and 200 mg/l streptomycin and 10% dialyzed fetal bovine serum; Gibco, Grand Island, NY, USA). After overnight incubation at 37°C in a humidified atmosphere of 5% CO_2, islets were preincubated for 30 min in Hanks' balanced salt solution (HBSS) with 0.5% bovine serum albumin (wt/vol) and 3 mmol/l glucose. Islets were then incubated for 1 h in HBSS containing 5.6 or 15.6 mmol/l glucose. Insulin in the media was measured using ultrasensitive rat insulin EIA ELISA kit (ALPCO Diagnostics).

2.5. Quantitative real-time polymerase chain reaction

Total RNA isolation and relative quantifications of mRNA were determined by real-time quantitative polymerase chain reaction using an ABI Prism 7700 Sequence Detector instrument (Applied Biosystems, Foster City, CA, USA) as previously reported [36]. See electronic support material (ESM) Table 1 for complete information on the primers used. Samples were analysed by triplicate and corrected for the 18S ribosomal subunit RNA used as internal standard.

2.6. Glucose and insulin tolerance tests

All glucose and insulin tolerance tests were performed between (10:00–11:00 AM). For glucose tolerance test, the mice were fasted overnight (16 h) before the test. Glucose (2g/kg body weight) was injected intraperitoneally, and blood samples were taken before and at 15, 30, 60, 90 and 120 min after the injection. Blood glucose was measured with a glucometer (Precision QID, MediSense, Inc., Abbott Laboratories Company). In addition, blood was collected from the tail vein during the glucose tolerance test for serum separation and insulin measurement.

For insulin tolerance tests, the mice had free access to food until the test. Insulin [1 IU/kg body weight of soluble human insulin Humulin (Eli Lilly, Mexico DF, Mexico)] was injected intraperitoneally, and blood samples were taken before and at 15, 30, 60 and 90 min after the injection of insulin. Blood glucose concentrations were measured with a glucometer as described above. Areas under the curves (AUCs) were calculated using Microcal Origin 6.0 software (Microcal Software, Inc., Northampton, MA, USA).

Table 1
Effect of 8 weeks of biotin supplementation on blood glucose and insulin concentrations

Measurement	Control	Biotin supplemented
Fasting blood glucose (mmol/l)	4.9±0.1	4.9±0.2
Fed blood glucose (mmol/l)	7.6±0.4	7.8±0.2
Fasting serum insulin (pmol/l)	50.6±7.3	54.5±2.8
Fed serum insulin (pmol/l)	76.6±6.4	85.4±3.5

Values are means±S.E.M. $n=4$–5 for insulin; $n=10$ for blood glucose.

2.7. Immunohistochemistry and morphometric analysis

Immunohistochemistry and morphometric analysis on pancreas sections were performed as previously reported [36]. Briefly, slides with pancreatic sections were deparaffinized, rehydrated, permeabilized with 0.3% Triton X-100 (Calbiochem, Darmstadt, Germany) and blocked with 3% normal goat serum (Microlab, Aguascalientes, Mexico). Sections were incubated overnight with guinea pig antiporcine insulin antibody (1:1000; Linco, St. Charles, MO, USA) and mouse antiglucagon antibody (1:6000; Sigma, St. Louis, MO, USA). Secondary goat antiguinea pig immunoglobulin G (IgG) fluorescein isothiocyanate-conjugated (1:200) and antimouse IgG Cy3-conjugated (1:800) antibodies (Jackson Immunoresearch Laboratories, West Grove, PA, USA) were used. Slides were imaged using an Olympus 1X70 microscope (Tokyo, Japan) with an attached Hamamatsu camera (Hammamatsu City, Japan). Images were taken with identical configuration settings.

Areas of islets, beta cells, alpha cells and total pancreatic sections were quantified, and relative beta-cell and alpha-cell volume was calculated as the ratio of total cell area/pancreas area. Cell distribution was assessed by quantification of the percentage of islets with one or more alpha cells located at least three cell widths from the mantle. All image analyses were done using Image J 1.40 software (Research Services Branch, National Institute of Mental Health, Bethesda, MD, USA).

2.8. Pyruvate carboxylase activity

Pyruvate carboxylase activity was measured in islets by radioenzymatic assay as previously reported [29], using $NaH^{14}CO_3$ (0.265 GBq/mmol). Protein content was quantified by Bradford assay. Enzymatic activity is reported as nanomoles per liter of fixed $^{14}CO_2$ per minute per milligram of total protein.

2.9. Statistical analysis

All data presented are the mean±S.E.M.; *n* denotes the number of evaluated subjects. Statistical analysis was performed using Statview V.4.5 (Abacus Concepts, Berkeley, CA, USA). The data were analyzed by Student's *t* test or two-way analysis of variance. *P* values of less than .05 were considered statistically significant.

3. Results

3.1. Effects of biotin supplementation on body weight, food intake and biotin levels

Over 8 weeks of biotin supplementation, the average food intake was 0.12±0.015 and 0.12±0.003 g of food (g of body weight)$^{-1}$ day^{-1} in the control and supplemented groups, respectively. Thus, during this period, the daily ingestion of biotin was 0.84±0.1 and 48.16±1.1 μmol of biotin (kg body weight)$^{-1}$ day^{-1} in the control and supplemented groups, respectively. Serum biotin in biotin-supplemented mice was significantly increased compared with control mice (590.6±6.5 nmol/l for biotin supplemented vs. 55.2±17.4 nmol/l for control, $P<.05$). Biotin supplementation did not modify body weight (control: 24.7±0.2 g, biotin supplemented: 25.1±0.5 g).

3.2. Effect of biotin supplementation on insulin secretion from isolated islets

To evaluate whether biotin supplementation *in vivo* can increase insulin secretion as observed in studies *in vitro*, glucose-stimulated insulin release from islets isolated from control and biotin-supplemented mice was measured. We found that insulin secretion was increased in islets isolated from biotin-supplemented mice, being more pronounced at 5.6 mmol/l glucose [control 0.13±0.02 and 0.22±0.04, biotin supplemented 0.22±0.02 and 0.30±0.03 ng of insulin (islet)$^{-1}$ h^{-1} for low and high glucose, respectively] (Fig. 1).

3.3. Expression of genes involved in beta-cell function in response to biotin supplementation

To further analyze the effect of biotin supplementation on beta-cell function, we examined insulin gene expression levels, as well as the expression of several genes involved in insulin transcription, glucose sensing and metabolism and glucose-induced insulin secretion.

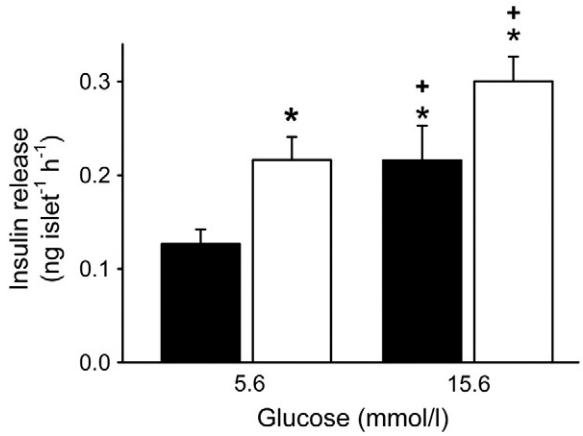

Fig. 1. Biotin supplementation increases insulin secretion. Glucose-induced insulin release from cultured pancreatic islets of control (black) or biotin-supplemented (white) mice. Data represent mean±S.E.M. Results are the mean of four independent experiments. *$P\leq.05$ compared with basal secretion of the control (5.6 mmol/l). +$P\leq.05$ compared with secretion at 5.6 mmol/l for the same group.

Biotin supplementation doubled insulin 2 (*Ins2*) gene expression (1.9±0.3-fold) and increased approximately 70% the expression of *Glucokinase* (1.7±0.1 fold change) (Fig. 2A). Levels of the *Cacna1d* mRNA and of the biotin-dependent enzyme acetyl-CoA carboxylase 1 (*Acc1*) were also increased (2.4±0.7 and 2.3±0.1-fold increase, respectively) (Fig. 2A). Expression of the mRNAs encoding glucose

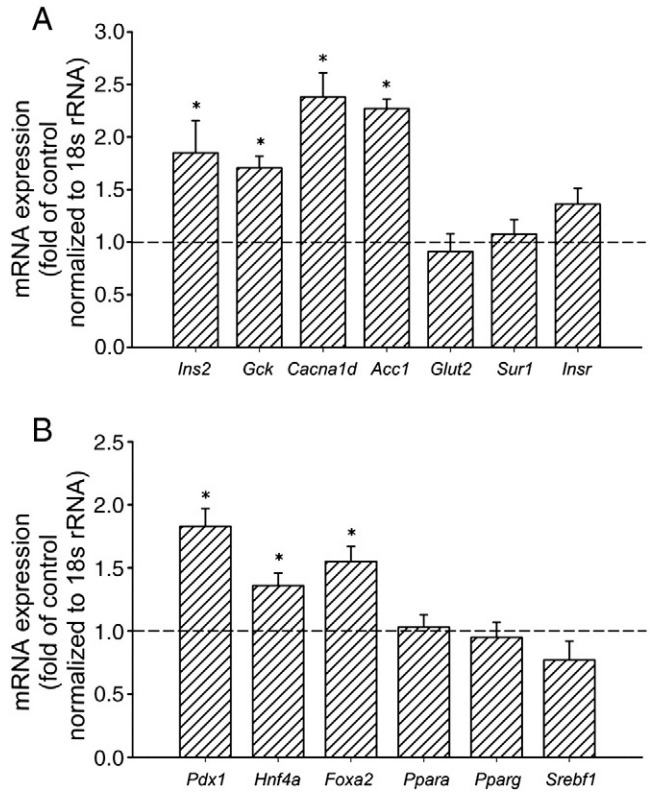

Fig. 2. Biotin supplementation increases expression of genes involved in beta-cell function. The mRNA expression of genes participating in glucose sensing, beta-cell metabolism and glucose-induced insulin secretion (A) and transcription factors involved in beta-cell function (B) was determined from islets of control and biotin-supplemented mice. Expression levels are presented as fold changes for biotin-supplemented islets relative to control values (dashed line). Data are mean±S.E.M. *$P\leq.05$ compared with basal expression. $n=5$–8 pools of 200 islets per group.

transporter Glut2 (*Slc2a2*), sulfonylurea receptor SUR1 (*Abcc8*) and the insulin receptor (*Insr*) did not change significantly (Fig. 2A).

Expression levels of several transcription factors regulating the insulin gene expression, namely, *Pdx1*, forkhead box A2 (*Foxa2*) and hepatocyte nuclear factor 4α (*Hnf4a*) increased significantly in islets from biotin-supplemented mice (1.8±0.1, 1.6±0.1 and 1.4±0.1 fold changes, respectively) (Fig. 2B), whereas sterol regulatory element binding transcription factor 1 (*Srebf1*) (also known as *Srebp1c*), peroxisome proliferator activated receptor alpha (*Ppara*) and peroxisome proliferator activated receptor gamma (*Pparg*) gene expression did not change.

3.4. Effect of biotin supplementation on glucose homeostasis and insulin secretion in vivo

Next, investigations were carried out to ascertain how the changes induced in the pancreas by *in vivo* biotin supplementation influenced glucose homeostasis in the intact animals. No differences in fasting and fed blood glucose and insulin concentrations between the biotin-supplemented and the control groups were found (Table 1). However, in the glucose tolerance test, blood glucose concentrations fell more rapidly at 30 and 60 min postglucose injection in the biotin-supplemented animals (Fig. 3A). The mean total AUC for the glucose tolerance test was significantly lower in the biotin-supplemented group (1397.2±29.1 mmol/l×min for the biotin-supplemented group vs. 1259.8±23.2 mmol/l×min for the control group, $P<.05$).

The improved glucose tolerance in the biotin-supplemented mice was not due to increased insulin sensitivity since these animals had no change in insulin tolerance relative to the control group (Fig. 3B), suggesting that biotin supplementation may improve glucose tolerance by increasing insulin secretion.

To test for augmented insulin secretion, serum insulin levels were measured during the glucose tolerance test. Biotin-supplemented mice had higher serum insulin concentrations at 5, 25, 45 and 90 min after glucose injection than did the control mice (Fig. 3C).

3.5. Effect of biotin supplementation on alpha- and beta-cell proportion and islet composition

The effects of biotin supplementation on islet morphology and cell proportion had not been previously assessed. The insulin immunopositive (beta cells) and glucagon immunopositive (alpha cells) areas in pancreatic sections from control and biotin-supplemented mice were quantified. Biotin supplementation approximately doubled beta-cell proportion, expressed as total beta-cell area per pancreas area (relative volume). Biotin supplementation also increased alpha-cell and total islet area per total pancreatic area (Fig. 4A). No differences were observed in pancreatic weight between biotin-supplemented and control mice (163±6 mg for biotin supplemented vs. 166±7 mg for control).

To ascertain whether the increment in the proportion of beta and alpha cells observed in biotin-supplemented mice was due to changes in islet size, islet number or islet composition, we measured these parameters in pancreatic sections. Biotin supplementation did not significantly modify islet number per total pancreatic area (control: 6.0±0.5 islets/10 mm^2 vs. supplemented: 6.9±0.7 islets/10 mm^2) but increased the average size of individual pancreatic islets by approximately 75% (Fig. 4B and C), particularly augmenting the proportion of islets larger than 10,000 μm^2 (Fig. 4D). Despite their larger size, biotin-supplemented islets showed no significant change in the proportions of alpha and beta cells relative to control islets (control: 77%±1.1% beta cells and 21.7%±1.1% alpha cells vs. biotin supplemented: 74.4%±1.1% beta cells and 24.0%±1.2% alpha cells).

Surprisingly, the morphologic analysis showed that islets of biotin-supplemented mice had a modified islet architecture compared with control islets (Fig. 5A). Instead of the typical islet architecture with the beta cells located at the core of the islet and alpha cells at the periphery in the biotin-supplemented group, we observed a high proportion of islets with alpha cells at the center of the islet (40% above control) (Fig. 5B). To determine the mechanisms involved in this effect, we examined different aspects that have been associated with changes in islet architecture: (1) increased metabolic demand [37], (2) pyruvate carboxylase activity changes [38] and (3) changes in the expression of adhesion molecules [39,40].

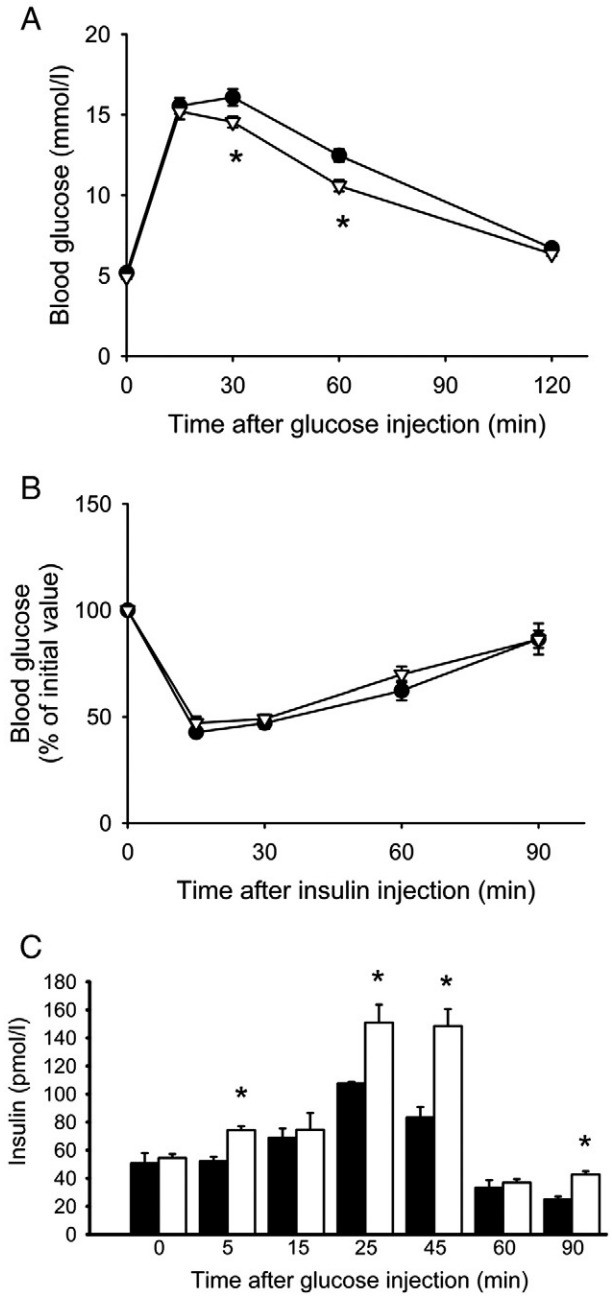

Fig. 3. Biotin supplementation improves glucose tolerance and serum insulin without increasing insulin sensitivity. (A) Blood glucose concentrations during glucose tolerance test (ip injection of 2 g/kg of glucose). (B) Percentages of basal (0 min) blood glucose levels during insulin tolerance test (ip injection of 1 U/kg insulin). (C) Serum insulin concentrations during glucose tolerance test. Black: control mice; white: biotin-supplemented mice. Values are means±S.E.M. $n=18$ mice per group for tolerance tests; $n=3$–4 mice per group for serum insulin. *$P\leq.05$ compared with the control.

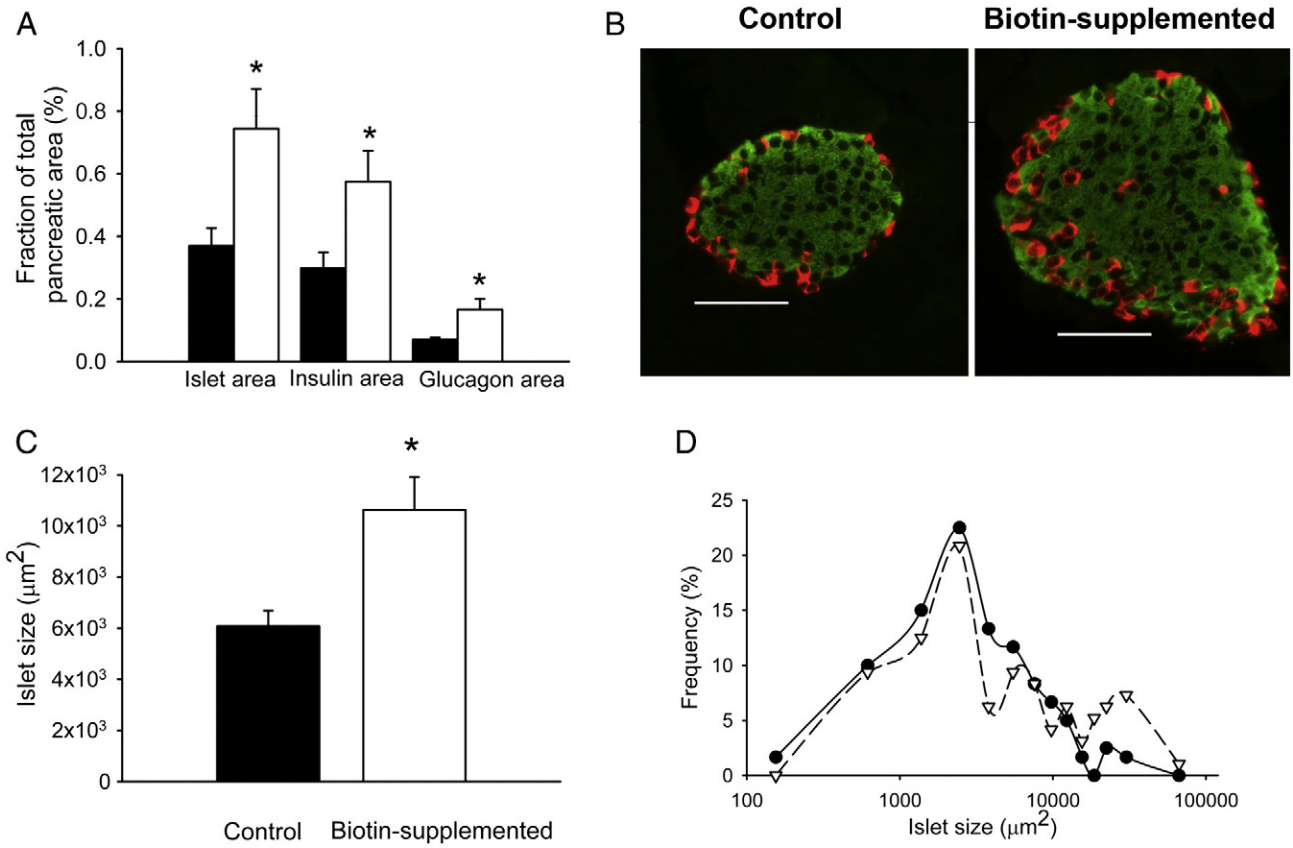

Fig. 4. Biotin supplementation increases the proportion of alpha and beta cells by augmenting islet size. (A) Morphometry from pancreatic sections of control (black) and biotin-supplemented (white) mice was performed after 8 weeks of diet administration. Values are mean percentages±S.E.M. $n=5$–6 mice per group. *$P\leq.05$ compared with the control group. (B) Immunofluorescence images of pancreatic islets from control (left) and biotin-supplemented (right) mice pancreas sections stained for insulin (green) and glucagon (red). Scale bar represents 50 μm. (C) Islet average size (mean area±S.E.M.); *$P\leq.05$ compared with the control. (D) Frequency distribution of islet size expressed as percentage of total number of islets measured. Black circles: control mice; white triangles: biotin-supplemented mice; $n=120$ islets (control) and 96 islets (biotin supplemented).

3.6. Effect of biotin supplementation on body weight, glucose levels or insulin tolerance over 8 weeks on diet

No changes in glucose levels (Fig. 6A and B), body weight (Fig. 6C) or insulin tolerance AUC (Fig. 6D) were found in the biotin-supplemented group compared with the control group at any time point measured.

3.7. Effect of biotin supplementation on pyruvate carboxylase expression and activity

To investigate whether the effects of biotin supplementation on the islet were related to pyruvate carboxylase, a biotin-dependent enzyme, mRNA expression of the pyruvate carboxylase (*Pcx*) gene and pyruvate carboxylase activity was measured, from islets of both the control and the biotin-supplemented mice. We observed no significant differences in the expression (Fig. 6E) or the enzyme activity (Fig. 6F) of pyruvate carboxylase between the groups.

3.8. Effect of biotin supplementation on the expression of adhesion molecules

We investigated the effect of biotin supplementation on the mRNA expression of genes involved in maintaining islet architecture and morphology, such as neural cell adhesion molecule 1 (*Ncam1*) and *Cadherin 1* (*Cdh1*, also known as E-cadherin). The data showed that biotin supplementation significantly decreased *Ncam1* mRNA expression (27±5% below control, $P<.05$). There was no difference in the abundance of *Cdh1* mRNA between the biotin-supplemented and the control groups (Fig. 7).

4. Discussion

Robust beta-cell function and mass are essential for preventing the development of diabetes [34]. Strategies toward enhancing these attributes will aid in the fight against this disease. Several studies *in vitro* have shown that pharmacological concentrations of biotin increase insulin secretion [17–20] and the expression of *Glucokinase* [19,20] and *Pdx1* [22], proteins that favor function and preservation of beta cells [23–25]. In the current study, we examined whether chronic, *in vivo* biotin supplementation could produce changes that promote beta-cell function. We found that biotin supplementation over 8 weeks produces several effects on beta-cell proportion and function and that these effects influence favorably glucose tolerance. These results support observations in both diabetic mouse models [30] and patients [27], which found that 2 months of pharmacological doses of biotin decreased hyperglycemia.

The data obtained demonstrated that *in vivo* biotin supplementation increases insulin release from isolated islets. This finding is in line with *in vitro* studies performed by our group [19,20] and others [17,18] showing that biotin can potentiate glucose-stimulated insulin secretion. It is interesting to note that the increase in insulin secretion in islets isolated from biotin-supplemented mice was more pronounced at 5.6 mmol/l glucose. It has been shown that an increase in glucokinase activity lowers the threshold of

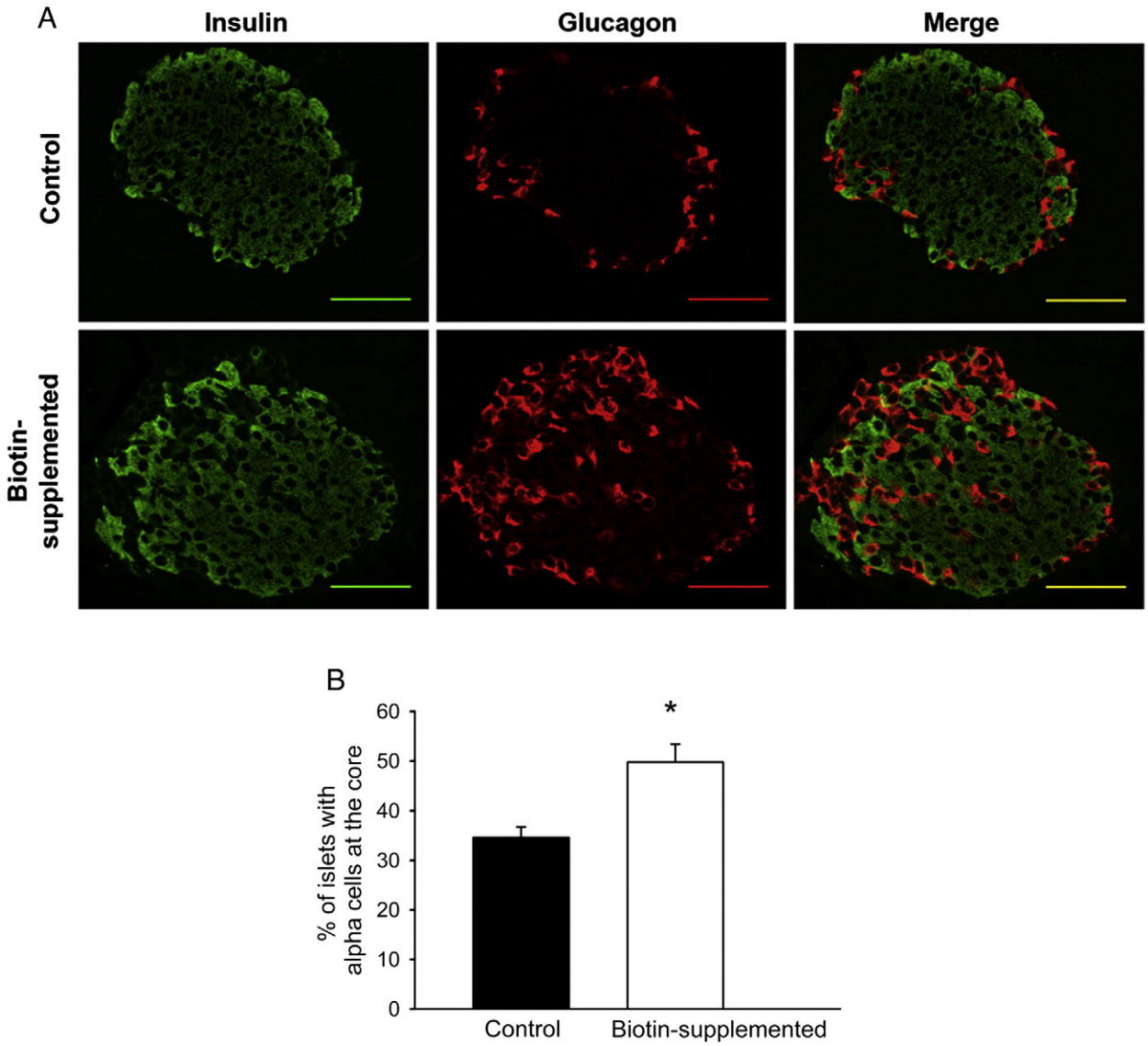

Fig. 5. Biotin supplementation modifies islet architecture. (A) Pancreatic sections of control and biotin-supplemented mice after 8 weeks of diet administration were immunostained with anti-insulin and antiglucagon antibodies. Glucagon-positive cells are found scattered in the islet in biotin-supplemented islets. Scale bar represents 50 μm. (B) Percentage (mean ±S.E.M.) of islets with more than one glucagon-positive cell at the core (fourth layer or further from the mantle) of the islet. *P<.05 compared with the control group. n= 8 mice per group.

glucose-stimulated insulin release, without increasing the maximal secretory activity of the beta cell [41], as observed in our results. Given that biotin supplementation increases glucokinase expression and activity, as reported in the present and other studies [19,20], it is plausible then that increased glucokinase expression could participate in the enhanced effect of biotin supplementation on insulin secretion.

The increasing effect of biotin on insulin secretion was paralleled by increased expression of several genes that regulate insulin secretion. As previously found in studies *in vitro* [19,20], we found that biotin supplementation increased *Glucokinase* mRNA expression, an enzyme considered the glucosensor of the pancreatic beta cell [24]. We also found that biotin supplementation produced a substantial increase in the mRNA abundance of *Cacna1d*, which codes for a voltage-dependent L-type calcium channel subunit importantly controlling Ca^{2+} entry during insulin secretion [42]. In addition, biotin increased the mRNA abundance of *Acc1*, an enzyme involved in the metabolic coupling occurring during the K_{ATP}-independent mechanism of insulin secretion [43]. These results suggest that, in addition to enhancing glucose catabolic flux through glucokinase increase, biotin could also be enhancing insulin release by modulating calcium entry and favoring amplifying signals.

Maintenance of a highly specialised phenotype is essential for beta-cell function [34]. Foxa2, PDX-1, and HNF4α constitute an important part of the beta-cell transcriptional networks essential for the expression of genes involved in insulin synthesis and secretion, as well as in maintenance and proliferation of the beta cell (reviewed in Ref. [44]). Low expression of these factors occurs in beta-cell failure during the progression toward diabetes [34]. In view of the fact that biotin increased the expression of these transcription factors, the data suggest that biotin supplementation may render beta cells more capable to cope with increased insulin demands. It is important to mention that biotin had no effect on transcription factors whose altered expression has been associated with glucotoxicity and beta-cell failure, such as decreases in PPARα [34,45] and increases in PPARγ [34,45] and SREBP1c [46].

Consistently with the increasing effect of biotin on insulin secretion and the expression of genes that participate in insulin secretion, our studies revealed that the AUC of glucose tolerance test was decreased in response to the biotin-supplemented diet.

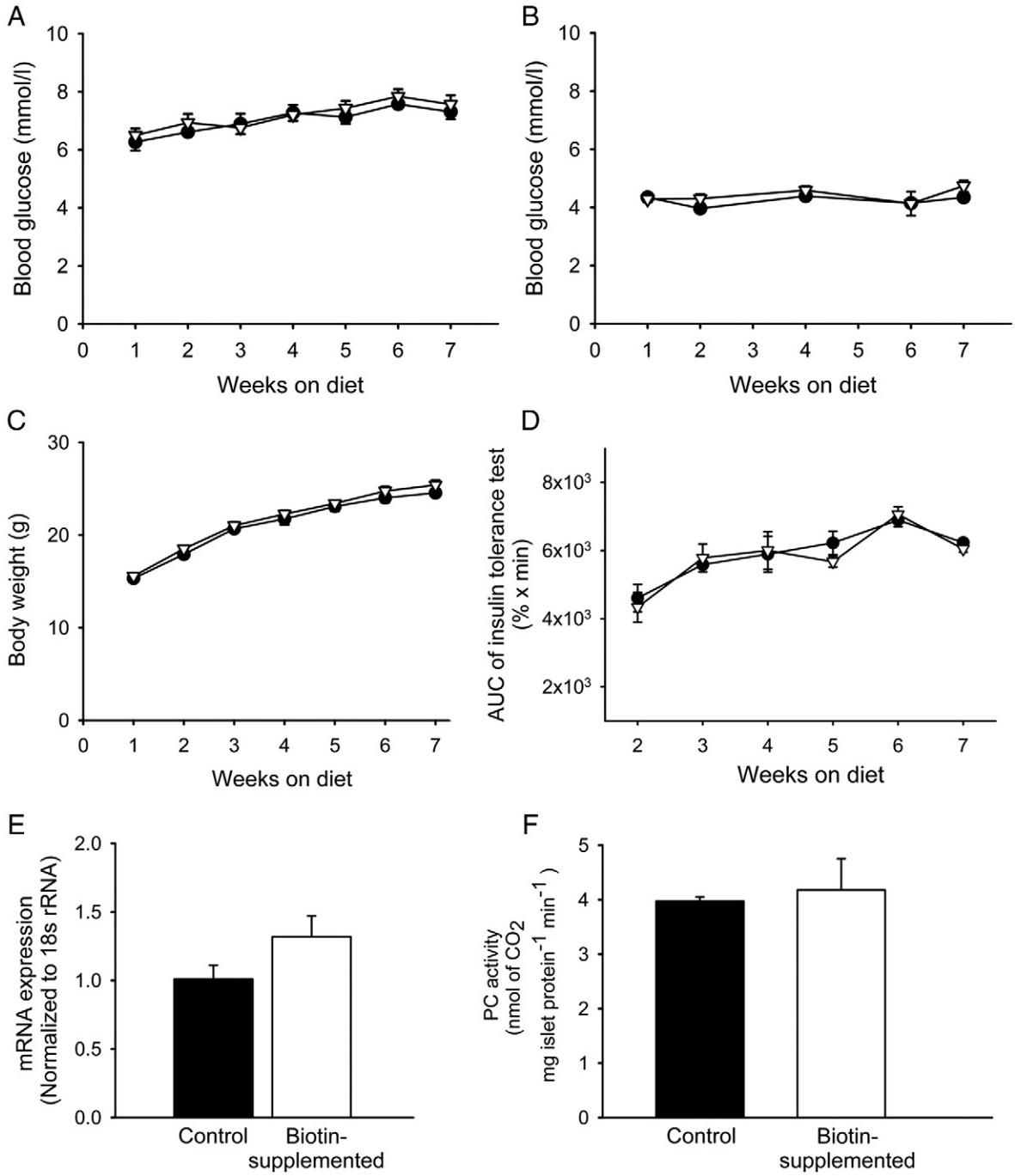

Fig. 6. Biotin supplementation does not modify blood glucose levels, body weight, insulin tolerance, pyruvate carboxylase gene expression or pyruvate carboxylase activity. Fed (A) and fasted (B) blood glucose, body weight (C) and insulin tolerance AUC (D) were monitored throughout the whole period of control (black circles) and biotin-supplemented (white triangles) diet administration. $n=7$–11 mice in each group for body weight and glucose levels; $n=4$–8 mice in each group for insulin tolerance tests. (E) mRNA expression of pyruvate carboxylase was determined for control (black) and biotin-supplemented (white) islets. Data are mean±S.E.M. $n=6$–8 pools of 200 islets per group. (F) Islet pyruvate carboxylase enzymatic activity in control (black) and biotin-supplemented (white) mice. Values are mean±S.E.M. $n=5$ pools of 100 islets per group.

Notwithstanding with these effects, fasting and fed serum insulin levels were unchanged. Indeed, in spite of the present results and a large body of evidence from *in vitro* studies [17–20] that have clearly demonstrated that biotin can potentiate glucose-stimulated insulin secretion, no study had been able to demonstrate increased serum insulin levels in response to biotin supplementation [27,30,31]. By analyzing insulin secretion after a glucose challenge, we unveiled that biotin supplementation induced a considerable increase in serum insulin concentrations. These results demonstrate for the first time that, in fact, biotin supplementation is able to increase blood insulin levels in vivo.

Studies in KK mice, a diabetic model of moderate hyperglycemia and insulin resistance, have found that biotin supplementation with 2 or 4 mg of biotin/kg of body weight increases insulin tolerance [30]. Contrary to these results, we found that biotin supplementation improves glucose tolerance by increasing insulin secretion rather than improving insulin sensitivity. The reasons for this discrepancy may underlie in the use of a model with genetic susceptibility to

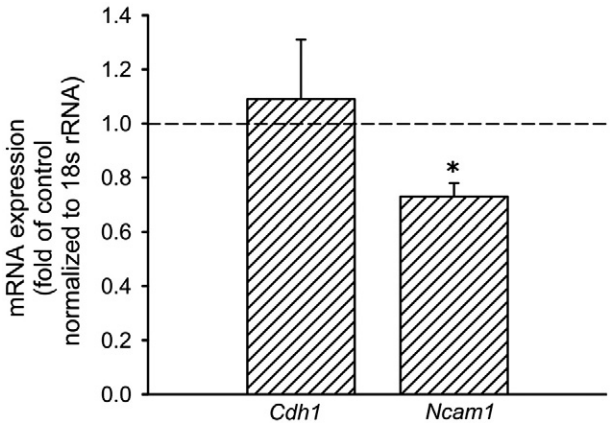

Fig. 7. Biotin supplementation modifies *Ncam1* and does not change *Cdh1* gene expression. mRNA expression of adhesion molecules for control and biotin-supplemented islets. Results are presented as fold change relative to control values (dashed line). Data are mean±S.E.M. $^{*}P \leq .05$ compared with basal expression. $n = 5–6$ pools of 200 islets per group.

develop insulin resistance and diabetes [30] vs. our model with normal insulin sensitivity.

Positive effects of biotin were also observed on the beta-cell relative volume. The morphologic analysis in the present study shows that, besides enhancing beta-cell function, biotin supplementation in the diet notably increases the proportion of beta cells. This increment is likely due to augmented islet size since no changes in islet number or composition were found. Particularly, biotin increased the proportion of large islets (>10,000 μm^2). There is evidence indicating that islets of different sizes are heterogenic in their function and properties [47]. Whether the selective size increase may indicate that some islets are more sensitive to biotin supplementation than others is an interesting question that will require further studies.

Beta-cell proportion is regulated during adulthood by an equilibrium between proliferation and apoptosis [48]. Biotin supplementation has shown to increase proliferation in choriocarcinoma cells [49] and to decrease apoptosis in retinal cells [50]; however, the mechanisms by which biotin exerts these effects are, at present, unknown. The finding that biotin supplementation increases *Pdx1* and *Glucokinase* expression, along with our previous observation indicating that biotin increases autocrine insulin action [19], mechanisms known to participate in the maintenance and proliferation of beta cells [25,51] and antiapoptotic actions [52], suggests that these mechanisms may account for the effects of biotin on beta-cell proportion. Because beta-cell turnover is low in the adult stage [53–55], biotin supplementation might have caused the increase in islet size during an earlier age, where changes in islet maturation [56] and cell proliferation [55] are more likely to occur. Current studies in our laboratory are determining this issue. Also, we are determining the implications of increased alpha-cell proportion.

Surprisingly, the studies revealed that biotin supplementation modified islet architecture. An increase in insulin secretion, together with an increase in beta-cell proportion and islet architecture modification, occurs physiologically in models with increased metabolic demand, such as pregnancy, obesity and insulin resistance states [37]. In addition, a recent study has shown that low islet pyruvate carboxylase activity in diabetic mice models is associated with islet morphologic changes and increased proportion of beta cells [38]. However, none of these effects appear to be involved in the islet architectural modification produced by biotin supplementation since no changes in body weight, blood glucose levels or insulin resistance were found at any point during the 8 weeks of experimentation. Also, no changes in pyruvate carboxylase activity or mRNA expression were found. These results support multiple studies suggesting that the effects of biotin supplementation on systemic processes are independent from its role covalently bound coenzyme of carboxylases (reviewed in Refs. [9] and [57]).

The changes produced by biotin supplementation on islet architecture might be related to decreased expression of *Ncam1*, an adhesion molecule whose ablation causes alterations in islet architecture, rendering high percentages of islets with alpha cells at the core of the islet [39], as observed in our present investigation. The lower *Ncam1* mRNA expression could account for this modification seen in biotin-supplemented islets since a moderate decrease in *Ncam1* expression such as 50% may generate a substantial increase in the percentage of islets with altered architecture [39]; however, in contrast with the detrimental effect of *Ncam1* ablation on glucose tolerance and insulin secretion [39], biotin supplementation does not appear to produce these unfavorable effects.

The evidence provided in the present report on the effects of biotin supplementation on beta-cell function and mass, together with previous reports of its hypotriglyceridemic effects [58,59], suggests that biotin may be used alone or in combination with other novel therapeutic agents proposed for glycemic control that have shown poor durability and undesirable secondary effects in humans, such as glucokinase activators [60]. However, potential concerns may exist regarding detrimental effects of long-term biotin supplementation on pancreatic islets and the increase in beta- and alpha-cell proportion. It has been acknowledged that pharmacological doses of biotin are harmless [61]. Administration of biotin in a range between 20 and 200 mg/day in patients is used in the treatment of inborn errors of biotin metabolism without reported harmful effects [62]. Regarding glucose homeostasis, studies in diabetic patients found that the fasting blood glucose concentrations attained with 2 months on biotin treatment remained within a constant range over an extended period of 48 months of biotin treatment [27]. In addition, clinical aggravation and undesirable side effects were not observed in that study. Toxicity studies have found that biotin is not mutagenic and is genotoxic only at concentrations fourfold to fivefold higher than the serum biotin attained with pharmacological concentrations of biotin [61]. Although scientific literature supports the safety of pharmacological concentrations of biotin, further studies will be required to ascertain the effects of long-term biotin supplementation on beta- and alpha-cell mass vis-à-vis to establish the use of biotin as a therapeutic strategy.

In conclusion, our data demonstrate that biotin supplementation produces *in vivo* changes in the pancreatic islets that increase insulin secretion, islet gene expression, islet cell relative volume and glucose tolerance, attributes that are known to decrease during the progression to diabetes [34,48]. Altogether, our results indicate that vitamin could preserve beta-cell proportions and maintain beta-cell function, a major strategy in the fight against diabetes.

Supplementary materials related to this article can be found online at http://dx.doi.org/10.1016/jnutbio.2012.03.020.

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