Dynamic Changes in β-Cell Mass and Pancreatic Insulin During the Evolution of Nutrition-Dependent Diabetes in *Psammomys obesus*

Impact of Glycemic Control

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Recent studies ascribe a major role to pancreatic β-cell loss in type 2 diabetes. We investigated the dynamics of β-cell mass during diabetes evolution in *Psammomys obesus*, a model for nutrition-dependent type 2 diabetes, focusing on the very early and the advanced stages of the disease. *P. obesus* fed a high-calorie diet for 26 days developed severe hyperglycemia, β-cell degranulation, and markedly reduced pancreatic insulin content. Reducing calories for 7 days induced normoglycemia in 90% of the animals, restoring β-cell granulation and insulin content. To dissociate effects of diet from blood glucose reduction, diabetic animals received phlorizin for 2 days, which normalized glycemia and increased the pancreatic insulin reserve to 50% of control, despite a calorie-rich diet. During diabetes progression, β-cell mass decreased initially but recovered spontaneously to control levels, despite persistent hyperglycemia. Strikingly, however, β-cell mass did not correlate with degree of hyperglycemia or pancreatic insulin content. We conclude that reduced insulin reserve is the main cause of diabetes progression, whereas irreversible β-cell mass reduction is a late event in *P. obesus*. The rapid recovery of the pancreas by phlorizin-induced normoglycemia implies a causal relationship between hyperglycemia and islet dysfunction. Similar mechanisms could be operative during the evolution of type 2 diabetes in humans. *Diabetes* 54:138–145, 2005

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DAB, 3,3′-diaminobenzidine-tetrahydrochloride; PCNA, proliferating cell nuclear antigen.

Pancreatic insulin reserve is an important parameter of islet function, with tight coupling between insulin secretion and production being necessary for the adequate functioning of the β-cell (rev. in 1). Insulin store size is determined by the balance between the rates of insulin biosynthesis and secretion as well as by the number and volume of the β-cells, i.e., the β-cell mass. In the adult, β-cell mass varies to adapt insulin secretion to long-term changes in insulin demand. This has been demonstrated repeatedly under physiological as well as pathological conditions of insulin resistance (rev. in 2). Although mainly demonstrated in rodents, this also seems to be the case in obese humans (3,4). The deterioration of the metabolic state in type 2 diabetes results mainly from progressive β-cell failure (5,6), hence the importance of determining whether functional β-cell mass is reduced in type 2 diabetes. This question remains controversial. Whereas some authors observed no change in β-cell mass (7–9), recent studies, using a large series of pancreata from patients with type 2 diabetes matched by appropriate control subjects, describe reduced relative β-cell volume or β-cell mass in humans with both impaired fasting glucose and established type 2 diabetes (4,10,11). The finding of reduced β-cell mass already in individuals with impaired fasting glucose has led to emphasizing its pathophysiological importance (9). In their recent evaluation of islets isolated from the pancreas of normal and type 2 diabetic cadaveric donors, Deng et al. (12) observed that in addition to a marked reduction in the recovered islet mass, the islets of type 2 diabetic donors were functionally defective. However, the retrospective nature of these studies did not permit detailed differentiation between various stages of disease progression; furthermore, they did not evaluate the contribution of pancreatic insulin reserve to the β-cell failure.

It is well known that in type 2 diabetes, reduction of insulin demand by diet and physical activity, or by pharmacological manipulations that achieve strict glycemic control, often improves insulin secretion; this demonstrates the adverse effect of the diabetic milieu, in particular hyperglycemia, on the functional β-cell mass (so-called "glucotoxicity") (rev. in 1). Obviously, it is not possible to obtain information on the pancreatic changes

that accompany such effects in humans, neither is it known how β-cell mass and pancreatic insulin reserve evolve during the various stages of human type 2 diabetes, hence the importance of studies in animal models of the disease.

In the present study we have used the *Psammomys obesus* model of type 2 diabetes to evaluate the possible mechanisms of nutrition-induced β-cell dysfunction, focusing on *1*) variations in pancreatic insulin reserve and β-cell mass and *2*) the influence of hyperglycemia on β-cell dysfunction. *P. obesus* seems particularly suitable to address these questions because it is a natural model of nutrition-dependent diabetes, in which hyperglycemia is associated with rapid depletion of pancreatic insulin stores and with temporal changes in β-cell proliferation and death that culminate in disturbed islet morphology (13–17).

RESEARCH DESIGN AND METHODS

P. obesus of both sexes, aged 2.5–3.5 months (Harlan, Jerusalem, Israel), were studied. After weaning at 3 weeks, animals were maintained on a nondiabetogenic low-energy diet containing 2.38 kcal/g (Koffolk, Petach-Tikva, Israel) and exhibited normoglycemia (random nonfasted blood glucose <7.8 mmol/l, Accutrend Sensor; Roche Diagnostics, Mannheim, Germany). Diabetes, defined by blood glucose >8.3 mmol/l, was induced by high-energy diet (2.93 kcal/g; Weizmann Institute of Science, Rehovot, Israel); nearly 90% of the animals developed hyperglycemia within 5 days (16). Only animals that developed hyperglycemia on the high-energy diet were used in our study. They were housed one per cage under standard light conditions (12-h light/dark cycle) and had free access to food and water. Animals were monitored by periodic measurements of body weight and tail blood glucose concentration. At termination of the in vivo studies, animals were anesthetized with ketamine hydrochloride (Ketalar; Parke-Davis, Gwent, U.K.) and exsanguinated by cardiac puncture. The pancreas was rapidly removed, and a sample from the head part was weighed and frozen at −80°C for subsequent determination of insulin content after extraction in acid-ethanol, as previously described (15). The splenic part of the pancreas was weighed and immersion-fixed in 10% formalin or in aqueous Bouin's solution followed by paraffin embedding and sectioning. Blood taken from the heart was used for glucose determination, and the collected sera was stored at −20°C for insulin measurement. All animal studies were approved by the institutional animal care and use committee of the Hebrew University and Hadassah Medical Organization.

Reversibility of diabetes by low-energy diet. Animals in an advanced stage of diabetes (exposure time to high-energy diet 26 ± 1 days; blood glucose >16.7 mmol/l) were switched back to the low-energy diet. Groups of animals were exsanguinated at 0, 2, 4, and 7 days of low-energy diet feeding, and their pancreata were used for determination of insulin content and for immunochemistry. Blood obtained from the heart was used to study blood biochemistry.

Reversibility of diabetes by phlorizin. *P. obesus* were changed from their maintenance low-energy diet to the diabetogenic high-energy diet at the start of the study (day 0) and maintained on this diet throughout. Animals with advanced diabetes after a 20-day high-energy diet were divided into two groups: the test group received three daily injections of phlorizin (0.4 g/kg body wt s.c.; Sigma, St. Louis, MO) for 2 days, whereas the control group received vehicle (propylene glycol, 0.1 ml/100 g body wt); high-energy diet was continued throughout. Animals were exsanguinated 3–4 h after the last injection and studied as above.

Islet morphology. Islet morphology was evaluated in the "reversibility of diabetes by low-energy diet" study by immunostaining of pancreatic sections for insulin. Paraffin sections (7 μm) were deparaffinized, rehydrated, and endogenous peroxidase–blocked by exposure to 3% H_2O_2 for 15 min. Sections were incubated for 1 h at 37°C with guinea pig anti-insulin antibody at a dilution of 1:100 (Sigma); detection was by streptavidin-biotin-peroxidase complex developed with aminoethylcarbazole (Zymed, San Francisco, CA). Sections were counterstained with hematoxylin and mounted in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

Morphometric analysis. Each pancreatic block in the "reversibility of diabetes by phlorizin" study was serially sectioned (7 μm) throughout its length to avoid bias caused by changes in islet distribution or cell composition, and then they were mounted on slides. For each pancreas, seven sections were randomly chosen at a fixed interval through the block (every 35th section). This procedure ensured that the selected sections are representative of the whole pancreas (18). Sections were immunostained for insulin using a peroxidase indirect-labeling technique. Briefly, sections were incubated for 1 h with a guinea pig anti-insulin serum (final dilution 1:1,000; ICN, Aurora, OH). Thereafter, sections were incubated for 45 min with peroxidase-conjugated rabbit anti–guinea pig IgG (final dilution 1:20; Dako, Carpinteria, CA). The activity of the antibody-peroxidase complex was revealed with DAB (3,3′-diaminobenzidine-tetrahydrochloride) using a peroxidase substrate kit DAB (Biosys-Vector, Compiègne, France). A standard concentration of hematoxylin was added as a counterstain. After staining, sections were mounted in Eukitt (Kindler, Freiburg, Germany). Islet cells whose staining ranged from very light to dark brown were considered as insulin-positive cells; therefore, even highly degranulated cells were counted as insulin-positive and included in the calculation of β-cell mass. Quantitative evaluation was performed using an Olympus BH2 microscope connected via a color video camera to a computer, using Imagenia 2000 software (Biocom, Les Ulis, France). The area of insulin-positive cells, as well as that of total pancreatic sections, was evaluated in each section; β-cell area was then determined by calculating the ratio between the areas according to stereological methods. Finally, total β-cell mass per pancreas was derived by multiplying this ratio by the total pancreatic weight.

Individual β-cell area. β-Cell size was measured on insulin-stained sections by evaluating the mean cross-sectional area of individual β-cells. β-Cell nuclei on a random section were counted, and the β-cell area in that section was measured by planimetry, as described above. β-Cell area was divided by the number of nuclei to calculate the area of individual β-cells. It must be recognized, however, that in using this technique, the actual number of β-cells is underestimated because not all cells are sectioned across their nuclei; thus, β-cell size is probably overestimated.

β-Cell replication. Replication was evaluated with an antibody against proliferating cell nuclear antigen (PCNA; 36 kDa), a cyclin essential for DNA replication during the S phase, which marks the proliferating cell compartment in normal tissues. Monoclonal anti-PCNA antibody (clone PC10 from Sigma) was used at 1:1,000 dilution. After antigen retrieval by boiling in 10 mmol/l citrate buffer (pH 6.0) for 10 min in a microwave oven, tissue sections were incubated with antibody for 1 h at room temperature, followed by detection using a streptavidin-biotin-peroxidase complex developed with aminoethylcarbazole (Zymed). Subsequently, sections were incubated overnight at 4°C with a mixture of primary rabbit antibodies to glucagon, somatostatin (Immustain; EURO/DPC, Llanberis, U.K.), and pancreatic polypeptide (BioGenex, San Ramon, CA); detection was performed with streptavidin-biotin-alkaline phosphatase complex (Zymed) developed with 5-bromo-4-chloro-indole phosphate/nitro blue tetrazolium liquid substrate (Sigma). A minimum of 500 nuclei of islet cells surrounded by purple non–β islet cells were counted per section at a final magnification of ×1,000. The ratio of PCNA-positive, presumably β-cell, nuclei to total β-cell nuclei was calculated. Results are expressed as percent positive β-cell nuclei.

β-Cell neogenesis. β-Cell neogenesis was evaluated by determining the area of small β-cell clusters (2–15 β-cells) adjacent to pancreatic ducts (18). Quantification was performed on sections used for β-cell mass measurements (seven different sections per pancreas). Results are expressed as mass of β-cell clusters adjacent to ducts.

Analytical methods. Insulin was measured in serum or pancreatic extracts by radioimmunoassay, using anti-insulin–coated tubes from ICN Pharmaceuticals (Costa Mesa, CA) and ^{125}I-labeled insulin from Linco (St. Charles, MO). Human insulin standard from Novo Nordisk (Bagsvaerd, Denmark) was used for determination of *P. obesus* insulin-like immunoreactivity because it differs from *P. obesus* insulin by only two amino acids (19); cross-reactivity and dilution linearity were comparable to those previously described (20). Hyperglycemic *P. obesus* produce varying amounts of insulin, proinsulin, and proinsulin-conversion intermediates (15), all of which cross-react with our assay antiserum; hence, the term *insulin* is used for the sum of all insulin-like immunoreactive products in this study.

Statistical analysis. Results are expressed as the means ± SE. Statistical significance was determined with the Fisher nonparametric ANOVA test using the StatView statistical software from SAS Institute (Cary, NC). The significance level was set at $P < 0.05$.

RESULTS

Kinetics of diabetes reversal by low-energy diet. To assess whether nutrition-induced β-cell dysfunction was reversible, *P. obesus* with advanced diabetes, which were on high-energy diet for an average of 26 days (blood glucose >16.7 mmol/l during at least two random samples

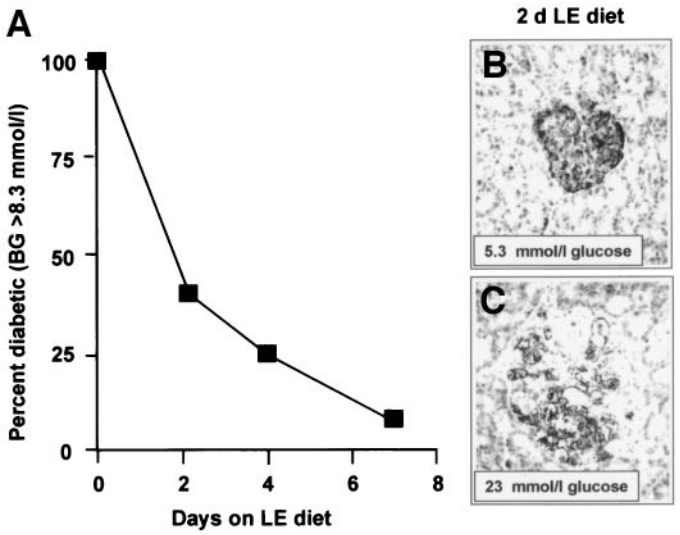


FIG. 1. Time course of diabetes reversal by low-energy (LE) diet. Diabetic animals (blood glucose 23.4 ± 0.8 mmol/l, mean ± SE, $n = 50$) after an average period of 26 ± 1 days on high-energy diet were switched back to the low-energy maintenance diet for up to 7 days. *A*: The percentage of diabetic animals that exhibited nonfasting blood glucose (BG) levels >8.3 mmol/l at different times after the change to low-energy diet. *B* and *C*: Islet morphology of a responsive animal with blood glucose of 5.3 mmol/l (*B*) and of a nonresponsive animal (blood glucose 23 mmol/l) (*C*), both after 2 days (2 d) of low-energy diet. β-Cells were immunostained for insulin and revealed with peroxidase method. Cells were counterstained with hematoxylin. Magnification ×200.

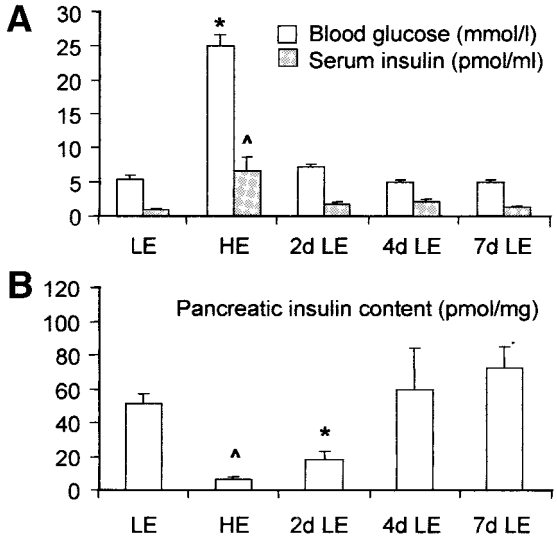


FIG. 2. Characteristics of animals during reversal of diabetes by low-energy (LE) diet. Blood glucose and serum insulin (*A*) and pancreatic insulin (*B*) content are shown for pre-diabetic animals on low-energy diet, diabetic animals after 26-day high-energy (HE) diet, and diabetic animals that were changed back to the nondiabetogenic low-energy diet for 2, 4, and 7 days. Only animals that normalized their blood glucose (<8.3 mmol/l) during reversal of diabetes on the low-energy diet are depicted in the figure ($n = 14$ for low-energy, $n = 16$ for high-energy, $n = 6$ for 2 days low-energy, $n = 9$ for 4 days low-energy, $n = 11$ for 7 days low-energy). Data are means ± SE. *A*: *$P < 0.001$ compared with blood glucose in all other groups; ∧$P < 0.05$ compared with serum insulin in all other groups. *B*: ∧$P < 0.01$ compared with pancreatic insulin content in low-energy, 4 days low-energy, and 7 days low-energy groups; *$P < 0.01$ compared with pancreatic insulin content in low-energy, 4 days low-energy, and 7 days low-energy groups. 2d, 2 days; 4d, 4 days; 7d, 7 days.

1–2 weeks apart), were switched back to low-energy diet. Of the 60 animals that entered the study, 10 died of severe hyperglycemia. The remaining animals were exsanguinated at different times after the change to low-energy diet. Low-energy diet time-dependently reduced the number of diabetic animals (Fig. 1*A*). Moreover, establishment of normoglycemia coincided with regranulation of β-cells and reappearance of normal islet morphology (Fig. 1*B*); this is in stark contrast to islet morphology in animals that did not achieve normoglycemia (Fig. 1*C*).

Figure 2 shows blood glucose and insulin concentrations as well as pancreatic insulin content in all animals that normalized the blood glucose on low-energy diet (6, 9, and 11 animals after 2, 4, and 7 days of low-energy diet, respectively). Attainment of normoglycemia resulted in a concomitant reduction of serum insulin levels (Fig. 2*A*) and a time-dependent replenishment of pancreatic insulin stores, which by day 4 of the low-energy diet reached the levels observed in age-matched control animals maintained throughout on low-energy diet (Fig. 2*B*).

Kinetics of diabetes reversal by phlorizin treatment. The recovery of the islets by reduced caloric intake could result from effects of the low-energy diet per se as well as those of normoglycemia. To differentiate between these effects, *P. obesus* with established diabetes (day 20 of high-energy diet) were treated with phlorizin. Nonfasted hyperglycemic animals (glucose >8.3 mmol/l) were exsanguinated on days 1, 2, and 5 of high-energy diet to characterize their endocrine pancreas during the initial phase of diabetes. Both blood glucose (Fig. 3*A*) and serum insulin levels (Fig. 3*B*) were rapidly increased; this was accompanied by a marked reduction of pancreatic insulin content already at day 1, reaching ≤10% of control level by day 5 of high-energy diet (Fig. 3*C*). In animals with advanced diabetes after 20 days of high-energy diet, phlorizin administration resulted in normalization of blood glucose (Fig. 3*A*). The phlorizin-responsive group did not reduce serum insulin concentrations, despite a fall in blood glucose; however, pancreatic insulin content was restored to ~50% of its pre-diabetic level (Fig. 3).

We found four animals assumed to be in a final, irreversible stage of diabetes. They had marked hyperglycemia (30.6 ± 1.6 mmol/l), significant hypoinsulinemia (0.74 ± 0.49 pmol/l), and near-total depletion of pancreatic insulin content (0.45 ± 0.39 pmol/mg). In two animals given phlorizin, marked hypoinsulinemia and depleted pancreatic insulin content (<0.2 pmol/mg tissue) persisted, despite induction of normoglycemia.

Morphological changes. Prolonged diabetes led to significant changes in islet morphology, including β-cell degranulation and the appearance of vacuolization (Fig. 4*A–C*). The striking feature of islets from phlorizin-treated animals was β-cell regranulation, as evidenced by the presence of a large number of strongly stained islets (Fig. 4*D*). It is noteworthy that this improvement was observed as early as after 2 days of normoglycemia. Conversely, islets in the "end-stage" group exhibited a distorted morphology with marked degranulation and vacuolization (Fig. 4*E*). This is also evidenced by the islet in Fig. 6*D*, with non–β-cells appearing in the center of the islet. The vacuoles seen in islets of the 22-day high-energy pancreata are probably caused by extracted lipids because lipid droplets could be demonstrated in islets from 22-day high-energy animals—but not from 5-day high-energy animals or animals on low-energy diet—by oil red staining of

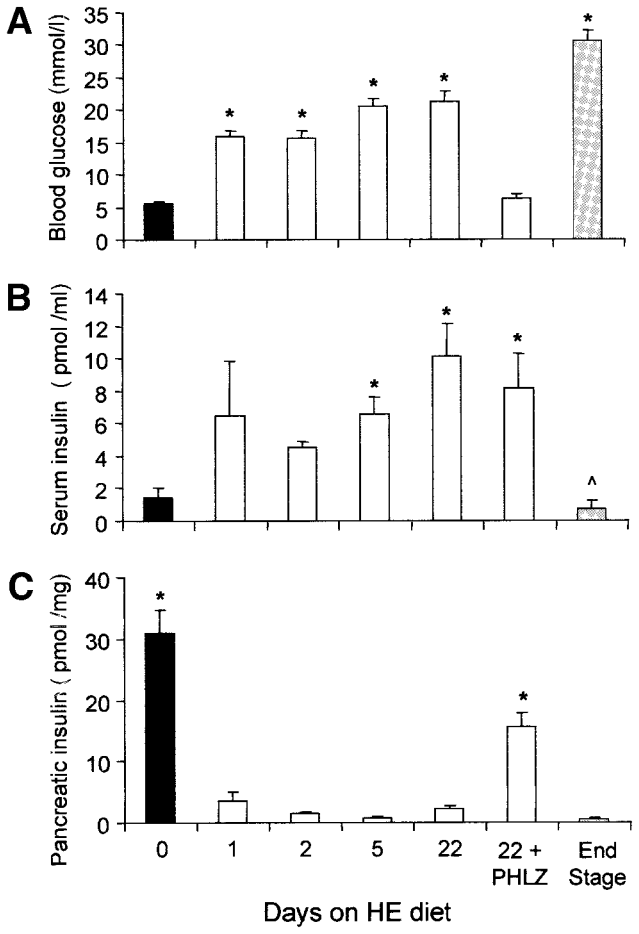


FIG. 3. Characteristics of animals during development of diabetes under high-energy (HE) diet and correction of hyperglycemia by 48 h of phlorizin (PHLZ) administration (subcutaneous injections of 0.4 g per kg, every 8 h). The experimental groups were control *P. obesus* fed low-energy diet (day 0 of high-energy diet) and test animals fed high-energy diet during 1, 2, 5, or 22 days. Phlorizin group animals were fed high-energy diet during 22 days and treated with phlorizin during the last 48 h. All animals in the phlorizin group normalized their blood glucose to <8.3 mmol/l. The end stage group is described in the text. *A*: Blood glucose. **P* < 0.05 compared with blood glucose in day 0 and phlorizin groups. *B*: Serum insulin. **P* < 0.05 compared with serum insulin in day 0; ∧*P* < 0.05 compared with serum insulin in 2, 5, and 22 days of high-energy and in the phlorizin group. *C*: Pancreatic insulin content. **P* < 0.05 for day 0 and phlorizin groups compared with pancreatic insulin content in all other groups. The number of animals per group were *n* = 8 for day 0 of high-energy diet; *n* = 6 for day 1; *n* = 7 for day 2; *n* = 8 for day 5; *n* = 11 for day 22; *n* = 8 for the phlorizin group; and *n* = 4 for the end stage group. Data are means ± SE.

frozen sections, and they could also be demonstrated in epon-embedded semithin sections and in electron microscopy (J. Rahier, Universite Catholique de Louvain, Brussels, Belgium, personal communication).

β-Cell mass and size. Compared with animals on low-energy diet (day 0 on high-energy diet), the β-cell mass decreased as early as 2 days after initiation of the diabetogenic high-energy diet and remained low on day 5 (Fig. 5*A*). However, β-cell mass returned to its pre-diabetic level by day 22, despite persistent hyperglycemia (Fig. 5*A*), although β-cells remained largely degranulated (Fig. 4*C*). When normoglycemia was induced with phlorizin, β-cell mass was equally restored (Fig. 5*A*); however, in contrast to hyperglycemic *P. obesus*, phlorizin-treated animals exhibited β-cell regranulation (Fig. 4, compare *C* and *D*). β-Cell mass dropped dramatically in end-stage animals (Fig. 5*A*). Neither the diet nor phlorizin treatment affected β-cell size (individual β-cell area), which remained similar in all groups (Fig. 5*B*).

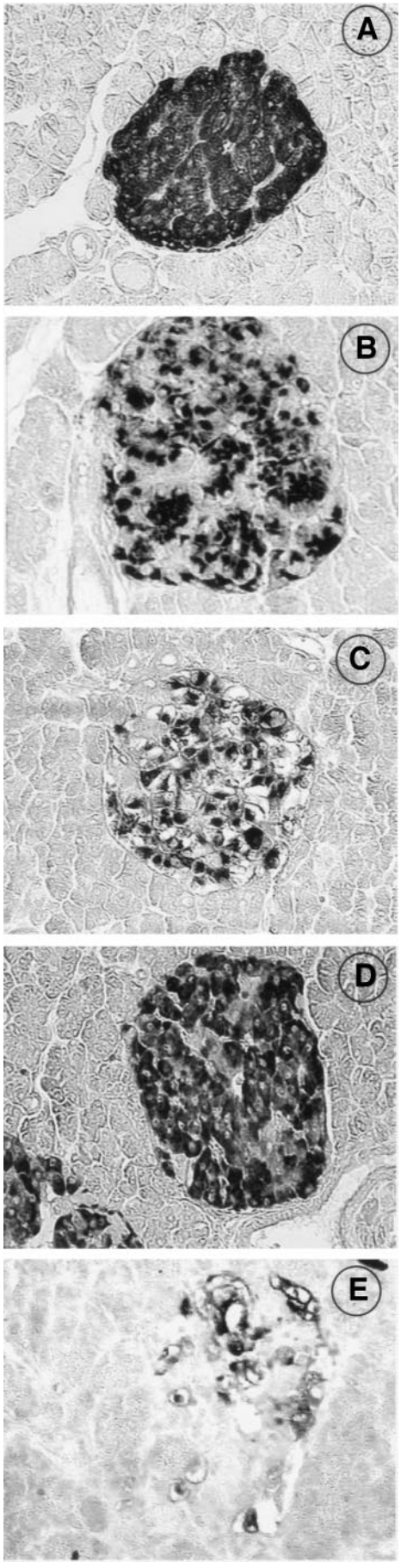
β-Cell replication. The β-cell proliferation rate dramatically increased in 2- and 5-day high-energy diet–fed animals, in a mirror-image of the decrease in β-cell mass (Fig. 6*E*; compare with Fig. 5*A*). After 22 days of high-energy diet, β-cell proliferation remained higher than in low-energy animals but was significantly lower than in the 2- and 5-day high-energy groups; it fell to control values after phlorizin treatment (Fig. 6). In the end-stage group, β-cell proliferation, though lower than after 2 and 5 days of high-energy diet, was not different from the other groups (Fig. 6). Figures 6*A* (low-energy diet), 6*B* (5-day high-energy diet), 6*C* (22-day high-energy diet), and 6*D* (end stage) are illustrative of PCNA staining, with the highest number of PCNA-positive nuclei being observed in the 5-day high-energy diet group.

β-Cell neogenesis. Measured as β-cell clusters (2–15 β-cells) adjacent to ducts, β-cell neogenesis was low in hyperglycemic animals given 1–5 days of high-energy diet (Fig. 7). It increased approximately sixfold after 22 days of high-energy diet and remained high after 2 days of phlorizin treatment (Fig. 7). This impressive increase in a suggested marker of neogenic activity could contribute to the concomitant recovery in β-cell mass. No neogenic activity could be detected in end-stage diabetes (Fig. 7).

DISCUSSION

Impaired insulin secretion is a constant feature of type 2 diabetes, and it is usually regarded as evidence for an intrinsic functional β-cell defect in this disease. Although it was suggested many years ago that reduced β-cell mass could lead to impaired insulin secretion (21), there has been no consensus on this point (3,7–9,22), mainly because most data stem from cross-sectional studies and involve autopsy material with unsatisfactory controls. Recently, a number of well-controlled studies confirmed the loss of β-cells in patients with type 2 diabetes (4,10,11). Strikingly, in obese type 2 diabetes, β-cell volume and presumably β-cell mass was reduced early, already at the stage of impaired fasting glucose (4). Nevertheless, direct evidence for the primary role of decreased β-cell mass in human type 2 diabetes will have to await the advent of noninvasive in vivo methods for β-cell mass measurement. Meanwhile, animal models with type 2 diabetes–like syndromes can be helpful. Thus, the present study, performed in an established animal model of type 2 diabetes, indicates that it is of particular importance not to consider the β-cell mass per se, but rather to take into account the functional β-cell mass, because in addition to the number of insulin-producing cells, β-cell exhaustion and replenishment seem to be crucial factors.

The diabetes model that we have studied here is highly appropriate because of many similarities to the common form of human type 2 diabetes. In particular, both in humans and *P. obesus*, diabetes is associated with obesity, and nutritional factors are involved in both the onset and evolution of the disease (23–25). Moreover, in vitro studies using *P. obesus* and human islets have shown similar sensitivities to glucose-induced β-cell proliferation and



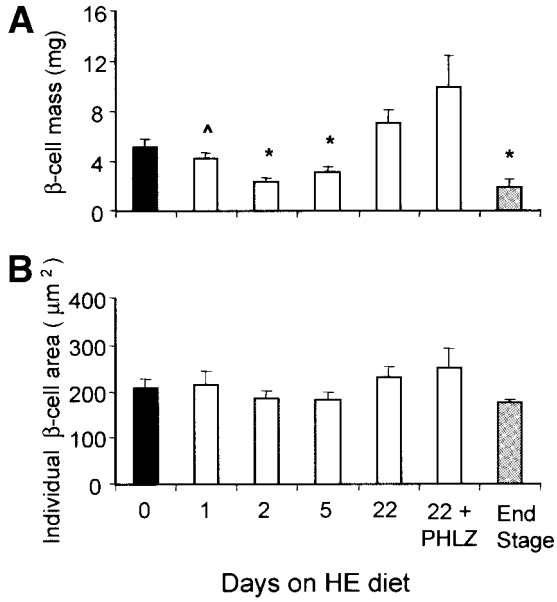


FIG. 5. Total β-cell mass and individual β-cell area in *P. obesus* submitted to different diets and phlorizin as in Fig. 3. *A*: Total β-cell mass (mg/pancreas) in control *P. obesus* fed the low-energy diet (day 0 of high-energy diet) and animals fed the high-energy (HE) diet ± phlorizin (PHLZ). Groups and the number of animals in each group are as shown in Fig. 3. Data are means ± SE. **P* < 0.05 compared with 22-day high-energy diet and PHLZ; ∧*P* < 0.05 compared with phlorizin. *B*: Individual β-cell area (μm²/per β-cell) in the same groups as in *A*.

apoptosis (13,26,27), whereas rat islets differ markedly from human islets in this respect (28,29).

In our previous studies (13,16), moderate caloric increase resulted in rapid development of hyperglycemia in *P. obesus*, with >90% of animals exhibiting high glucose levels by day 5. We now show that hyperglycemia is already apparent by 24 h of high-energy diet feeding (Fig. 3), although only 50% of the animals are hyperglycemic at this time (not shown). The initial hyperinsulinemia that accompanied hyperglycemia was associated with a sharp reduction in pancreatic insulin reserve (Fig. 3*C*), before any change in β-cell mass was observed (Fig. 5*A*). The first important finding of the present study is the very rapid reversal of diabetes by low-calorie diet (Fig. 1), with replenishment of the pancreatic insulin stores in a time-dependent manner (Fig. 2). Several lines of evidence indicate that the loss of insulin stores participates in the deterioration of the insulin response to glucose, and subsequently of glucose homeostasis as a whole. Thus, pancreatic insulin content is dramatically decreased in animals with chronic hyperglycemia (30) and/or insulin oversecretion (31); also, the pancreas of diabetic subjects yields low insulin extracts (32). Prolonged exposure of human pancreatic islets to high glucose in vitro results in

FIG. 4. Islet morphology. *A*: Control *P. obesus* on low-energy diet (day 0 of high-energy diet) with a highly granulated islet. The figure is representative of eight animals. *B* and *C*: A typical feature of degranulated islets in *P. obesus* on high-energy diet for 5 and 22 days, respectively. The figures are representative of pancreatic sections obtained from 8 and 11 animals on high-energy diet for 5 and 22 days, respectively. *D*: Islet regranulation and shape recovery in PHLZ-treated *P. obesus*. The figure is representative of pancreatic sections from eight phlorizin-treated animals. *E*: Islet disorganization and β-cell loss in end-stage *P. obesus*. The figure is representative of four end-stage animals. β-Cells were immunostained for insulin and revealed with peroxidase. Final magnification ×200.

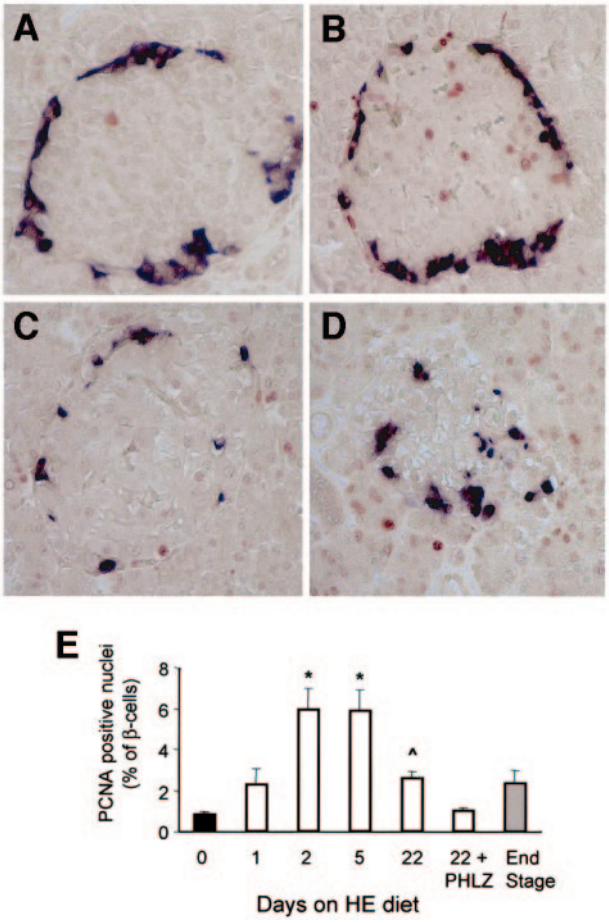


FIG. 6. *A–D*: Islet morphology in animals from the following groups: day 0 of high-energy (HE) diet (*A*), 5-day high-energy diet (*B*), 22-day high-energy diet (*C*), and end stage (*D*). Sections were immunostained with a mixture of antibodies against glucagon, somatostatin, and pancreatic polypeptide (dark purple), and nuclei were immunostained for the PCNA antigen (red). Magnification ×200. The islets exhibit peripheral non-β, hormone-containing cells and PCNA-positive nuclei in the exocrine pancreas and in cells at the center of islets, assumed to be β-cells. Magnification ×200. *E*: β-Cell replication in *P. obesus* submitted to different diets or treatments as in Fig. 3. Data are means ± SE. **P* < 0.05 from all other groups; ∧*P* < 0.05 from 0, 2, and 5 days of high-energy diet and high-energy diet + phlorizin (PHLZ).

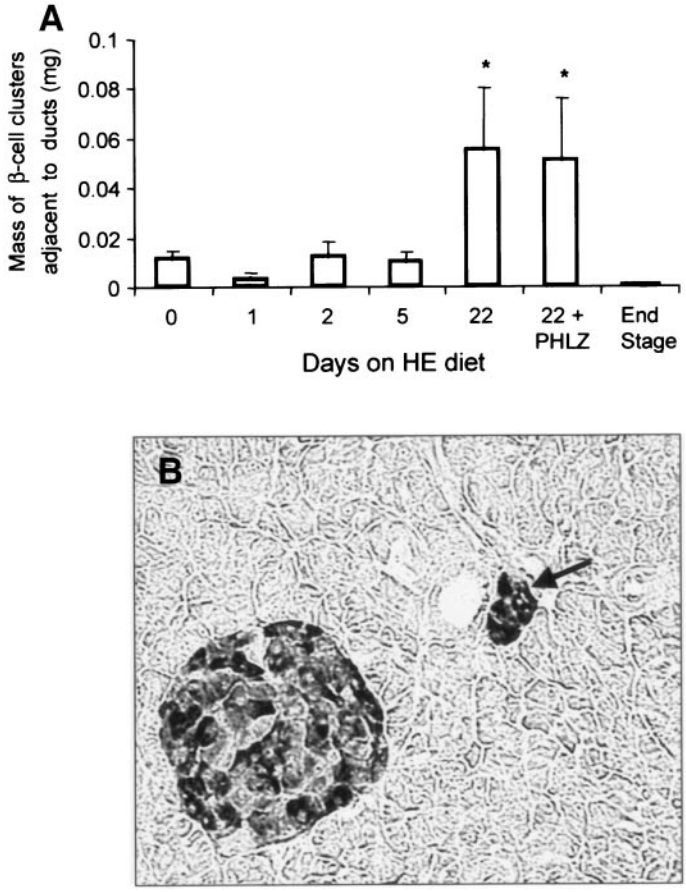


FIG. 7. *A*: Mass of small clusters of 2–15 β-cells adjacent to pancreatic ducts in *P. obesus* submitted to different diets or treatments as in Fig. 3. Results are the means ± SE. **P* < 0.05 from all other groups. *B*: β-Cell cluster adjacent to a duct in the pancreas of 22-day high-energy (HE) diet–fed *P. obesus*, a typical feature of neogenesis in this group and in the high-energy diet + phlorizin (PHLZ) group. β-Cells were immunostained for insulin and revealed with peroxidase.

the exhaustion of insulin stores along with the impairment of β-cell function (33,34). In addition, recent in vitro studies showed that the beneficial effect of diazoxide on insulin secretion was associated with prevention of the insulin store loss occurring during high-glucose exposure (35). Conversely, normalization of glycemia in diabetic nude mice resulted in replenishment of insulin stores of transplanted human islets, with only minimal improvement of their deranged secretory function (36,37). The apparent dissociation between the secretory response of the human islets and their insulin reserve could be a special feature of the experimental system used; although the human islets transplanted into normoglycemic mice had well-preserved insulin release and glucose oxidation in response to glucose, they had low levels of secretory granules and a loss of glucose stimulation of proinsulin biosynthesis. Additional experiments are required to clarify this apparent discrepancy between the present results and the results obtained in transplanted human islets (36,37).

Are loss of pancreatic insulin content and reduction of β-cell mass equivalent with respect to diabetes development? The predominance of variations in insulin content is supported here by the absence of correlation between insulin stores and β-cell mass during the development of the diabetic state and its reversal in *P. obesus*. Thus, depletion of pancreatic insulin occurred already at the initial phase of glucose intolerance (day 1 of high-energy diet) (Fig. 3*C*), before any change in β-cell mass was observed (Fig. 5*A*). Moreover, pancreatic insulin remained very low in high-energy animals independent of the variations in β-cell mass. Strikingly, in 22-day high-energy diabetic animals, the spontaneous return of β-cell mass to control levels was not accompanied by an increase in pancreatic insulin content, with morphologic assessment still showing degranulated β-cells. This suggests that the β-cells that reappeared under conditions of high-energy diet did not achieve functional maturity, probably because of the persistence of hyperglycemia. Indeed, it was shown in previous studies that a 48-h glucose infusion in diabetic rats led to a threefold increase in β-cell mass, but without any improvement in β-cell function (38). The recovery of pancreatic β-cell mass after its initial decrease could result from the early increase in β-cell proliferation (Fig. 6*E*) and the later increase in neogenic activity (Fig. 7). Thus, unlike

recent studies (39) that claim β-cell neogenesis to be minimal in normal adult animals, the diabetic animal model studied here suggests an increase in both β-cell proliferation and neogenesis.

The second important finding in this study is the clear demonstration of a causal relationship between hyperglycemia and islet dysfunction in *P. obesus*. Diabetes could be reversed only if pancreatic insulin stores were replenished to 40–50% of control levels and islet morphology normalized. Thus, the normalization of glycemia with phlorizin was matched in individual animals by normalization of islet architecture and increased insulin content, these parameters seeming to be the sine qua non condition for maintenance of normoglycemia. The observation that partial replenishment of the insulin reserve was sufficient to maintain near-normoglycemia suggests that the depletion of insulin stores becomes functionally important only below a certain threshold.

The effect of glucose on functional β-cell mass homeostasis very likely depends on the duration and level of hyperglycemia. In the present study, the improvement that followed lowering of blood glucose was strikingly rapid: 48 h of phlorizin-induced euglycemia was sufficient to completely reverse the effects of diabetes and restore almost normal islet morphology. This leads us to conclude that the beneficial effect of plasma glucose normalization is mainly attributable to decreased insulin demand and replenishment of insulin stores, rather than drastic changes in β-cell turnover. By the same logic, the induction of hyperglycemia by increased caloric intake seems to us more the result of impaired ability to increase insulin production than loss of β-cells. Indeed, we have previously shown a gradual decrease in insulin mRNA after 1 week of high-energy diet, reaching 15% of control by 3 weeks of hyperglycemia (40). Further studies on insulin gene transcription and translation in *P. obesus* islets exposed to hyperglycemic glucose concentrations supported the hypothesis that the depletion of insulin stores during increased caloric intake results from inadequate regulation of insulin production by glucose (41). To conclude, in the *P. obesus* model of type 2 diabetes, the main expression of β-cell dysfunction is the inability to maintain pancreatic insulin stores in the face of a sustained secretory drive. The pancreas is able to recover from the initial decrease in β-cell mass, albeit only to the level observed in prediabetic normoglycemic animals (i.e., without compensatory expansion of the mass); at later, more advanced stages of diabetes, the initial β-cell failure is accompanied by a second decrease in β-cell mass and complete destruction of islet architecture. This is a state of "no return" from which the animal cannot recover. Thus, in this model, diabetes is reversible by normalization of blood glucose as long as the β-cell mass is preserved. The fast progression of diabetes and its reversal in *P. obesus* illustrates the remarkable plasticity of the endocrine pancreas in terms of both β-cell mass and function. Although *P. obesus* may reflect an extreme situation of energy loss and regeneration of β-cell mass, reflecting a unique adaptive ability to extreme situations of nutrient supply, similar mechanisms could participate in the progression of type 2 diabetes in humans, with hyperglycemia playing a major role in the loss of the functional β-cell mass. New combined therapeutic approaches, addressing simultaneously insulin secretion, insulin production, and β-cell mass, may therefore have higher efficacy in improving metabolic control in human diabetic patients than the currently used pharmacological agents.

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