Hyperglycemia-Induced β-Cell Apoptosis in Pancreatic Islets of *Psammomys obesus* During Development of Diabetes

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The gerbil *Psammomys obesus* develops nutrition-dependent diabetes associated with moderate obesity. The disease is characterized by initial hyperinsulinemia, progressing to hypoinsulinemia associated with depleted pancreatic insulin stores. The contribution of changes in β-cell turnover to insulin deficiency was investigated in vivo during transition to overt diabetes. Normo glycemic diabetes-prone *P. obesus* animals who were given a high-calorie diet developed hyperglycemia within 4 days, which was found to be associated with a progressive decline in pancreatic insulin content. This was accompanied by a transient increase in β-cell proliferative activity and by a prolonged increase in the rate of β-cell death, culminating in disruption of islet architecture. The hypothesis that "glucotoxicity" was responsible for these in vivo changes was investigated in vitro in primary islet cultures. Exposure of islets from diabetes-prone *P. obesus* to high glucose levels resulted in a dose-dependent increase in β-cell DNA fragmentation. In contrast, high glucose levels did not induce DNA fragmentation in rat islets, whereas islets from a diabetes-resistant *P. obesus* line exhibited a reduced and delayed response. Aminoguanidine did not prevent glucose-induced β-cell DNA fragmentation in vitro, suggesting that formation of nitric oxide and/or advanced glycation end products plays no major role. Elevated glucose concentrations stimulated β-cell proliferation in both rat and *P. obesus* islets. However, unlike the marked long-lasting effect in rat islets, only a transient and reduced proliferative response was observed in *P. obesus* islets; furthermore, β-cell proliferation was inhibited after prolonged exposure to elevated glucose levels. These results suggest that hyperglycemia-induced β-cell death coupled with reduced proliferative capacity may contribute to the insulin deficiency and deterioration of glucose homeostasis in *P. obesus.* Similar adverse effects of hyperglycemia could play a role in the evolution of type 2 diabetes in genetically susceptible individuals. *Diabetes* 48: 738–744, 1999

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BrdU, 5-bromo-2′-deoxyuridine; DP, diabetes-prone; DR, diabetes-resistant; HE, high = energy; IRI, immunoreactive insulin; LE, low-energy; TUNEL, dUTP-X 3′ nick end-labeling.

Type 2 diabetes is characterized by insulin resistance and impaired insulin secretion. Although the relative contribution of insulin resistance versus insulin deficiency remains a matter of controversy, there is considerable evidence that deficient β-cell function plays a dominating role in the establishment of overt diabetes (1,2). No changes in islet cell morphology have been documented in early type 2 diabetes, when β-cells already show the inability to secrete adequate amounts of insulin in the face of insulin resistance; yet, in the advanced stages of the disease, β-cell mass decreases (3–5). This is in contrast to islets of normal individuals that adapt to physiological (e.g., pregnancy, obesity) or pathological (e.g., growth hormone, cortisol excess) states of insulin resistance by increasing both the β-cell function and mass (6).

The etiology of the impaired β-cell function in type 2 diabetes is not clear. In humans, chronic increase in secretory demand, such as that induced by elevated glucose concentrations, may promote β-cell dysfunction; this could be partially corrected by strict glycemic control (7–12). For obvious reasons, no longitudinal studies exist on changes of β-cell mass during the evolution of type 2 diabetes in humans. Moreover, it is not known whether glucose per se, which has been implicated in the generation of reduced secretory function in human islets (13–15), might also have adverse effects on β-cell turnover, thus contributing to the overall reduced β-cell functional mass in advanced type 2 diabetes.

We have tested the hypothesis that hyperglycemia adversely affects β-cell turnover in an animal model of type 2 diabetes, *Psammomys obesus.* This desert gerbil does not develop diabetes in its natural habitat where it feeds on the low-calorie salt bush plant *(Atriplex halimus)*; however, in captivity when fed a high-energy (HE) diet, it shows propensity to develop a type 2 diabetes-like syndrome associated with moderate obesity (16–18). The observation that not all animals develop hyperglycemia on an HE diet (19) enabled the selection by assorted breeding of two lines of *P. obesus*, one diabetes-prone (DP) and another partially diabetes-resistant (DR) (20,21). When *P. obesus* of the DP line are changed from a low = energy (LE) diet to an HE diet, they develop hyperglycemia, initially accompanied by hyperinsulinemia and increased relative levels of insulin precursors, later progressing to hypoinsulinemia with reduced pancreatic insulin stores (19,22,23). The analogy to the evolution of obesity-associated type 2 diabetes in humans, coupled with the observation that insulin resistance is an innate property of both the DP and DR lines of

TABLE 1
Characteristics of *P. obesus* during the evolution of nutrition-induced diabetes

	HE diet (days)						
	0	4	7	11	14	20	30
Age (days)	90 ± 5	87 ± 6	88 ± 5	93 ± 3	91 ± 4	85 ± 2	96 ± 5
Body weight (g)	160 ± 11	163 ± 24	170 ± 10	182 ± 10	181 ± 7	181 ± 12	190 ± 12
Glucose (mmol/l)	5.3 ± 0.5	15.2 ± 4.0*	14.5 ± 4.5*	20.8 ± 1.6*	18.5 ± 1.6*	22.8 ± 4.1*	26.6 ± 2.1*
TG (mmol/l)	0.64 ± 0.08	1.22 ± 0.36	0.85 ± 0.13	1.15 ± 0.12*	1.04 ± 0.16*	19.3 ± 9.0*	24.6 ± 11.4*

Data are means ± SE of 4–7 animals in each group. *P < 0.05 relative to animals on LE diet (day 0).

P. obesus (24), renders this animal an attractive model for longitudinal studies of the pancreatic dysfunction.

RESEARCH DESIGN AND METHODS

Animals. Male Sprague-Dawley (SD) rats (age 1.5–2.0 months) and *P. obesus* of both sexes (age 2.0–3.5 months) from the DP and DR lines of the Hebrew University colonies were obtained from Harlan (Jerusalem, Israel). After weaning, DP *P. obesus* were maintained on an LE diet containing 2.38 kcal/g (Koffolk, Petach Tikva, Israel) until the start of the experiments, whereas DR *P. obesus* were maintained on an HE diet containing 2.93 kcal/g (Weizmann Institute, Rehovot, Israel) to identify animals that develop diabetes and exclude them from the study (~30–40% of the animals in the DR colony). All nonfasted animals with random blood glucose concentrations <7.8 mmol/l (Glucometer Elite, Bayer Diagnostics, Elkart, IN) were considered nondiabetic. SD rats were maintained on a standard laboratory diet. Food and water were given ad libitum. For longitudinal studies of pancreatic histology, DP *P. obesus* were switched to an HE diet and killed on days 4, 7, 11, 14, 20, and 30. Age-matched control animals were kept on an LE diet. *P. obesus* were anesthetized with ketamine (Ketalar, Park-Davis, Gwent, U.K.) and exsanguinated by cardiac puncture. The collected serum was stored at –20°C for glucose, immunoreactive insulin (IRI) (25), and triglyceride (TG) (GPO-Trinder kit, Sigma, St. Louis, MO) determinations. The animal studies were approved by the Institutional Animal Care and Use Committee of the Hebrew University and Hadassah Medical Organization.

Analysis of pancreases. The pancreas was rapidly removed, and a sample (32 ± 4 mg) was frozen at –70°C for subsequent determination of IRI content, as described (22). The remaining piece was immersion-fixed in 10% phosphate-buffered formalin followed by paraffin embedding. Multiple sequential sections (5 µm) were processed for evaluation of β-cell apoptosis or proliferation, as outlined below.

Islet isolation. Islets were prepared by collagenase digestion (Collagenase P, Boehringer Mannheim, Mannheim, Germany), as described (22,26). Islets free of exocrine tissue were obtained by repeated hand-picking under a stereomicroscope.

Islet culture. Details of the procedure have been described (25,26). Islets pooled from two or three animals were suspended (10–15 islets/ml) in RPMI-1640 medium (11.1 mmol/l glucose) containing 100 U/ml penicillin, 100 µg/ml streptomycin, 250 ng/ml amphotericin B, 10% fetal calf serum (FCS) (Biological Industries, Beit Haemek, Israel), and 40 µg/ml gentamicin. Of the islet suspension, 2 ml were plated onto 35-mm plates coated with extracellular matrix derived from bovine corneal endothelial cells (26). Two days after plating, when most islets were attached and had begun to flatten, the culture medium was changed to RPMI-1640 containing 3.3, 5.5, 11.1, or 33.3 mmol/l glucose, 33.3 mmol/l glucose supplemented with aminoguanidine (0.5–1 mmol/l), or 5.5 mmol/l glucose together with 27.8 mmol/l L-glucose (to match osmolarity). The medium was changed twice weekly.

β-Cell replication. For in vivo replication studies, a monoclonal antibody to Ki-67 was used. Ki-67 is a nuclear antigen expressed by proliferating cells used as a marker for late G1, S, G2, and M phases of the cell cycle (27–29). Paraffin sections were deparaffinized and rehydrated, and endogenous peroxidase was blocked by exposure to 0.3% H_2O_2 for 15 min. The slides were then rinsed in distilled water and heated in 10 mmol/l citrate buffer (pH 6.0) at 92°C for 10 min in a microwave oven for antigen retrieval. After cooling at room temperature for at least 20 min, the sections were rinsed in distilled water and washed three times with phosphate-buffered saline (PBS) and incubated with monoclonal mouse anti-Ki-67 antibody (Zymed, San Francisco, CA) for 1 h at room temperature, followed by detection using a streptavidin-biotin-peroxidase complex developed with aminoethylcarbazole (Zymed). Subsequently, sections were incubated for 30 min at 37°C with guinea pig anti-insulin antibody diluted 1:50 (Dako, Glostrup, Denmark), followed by a 10-min incubation with a 1:10 dilution of fluorescein-conjugated rabbit anti-guinea pig antibody (Dako) and staining with hematoxylin. Control Ki-67 staining was performed on sections from human intestine and different tumors.

For in vitro replication studies, 5-bromo-2′-deoxyuridine (BrdU, 10 µmol/l) was added to the culture medium 16 h before the termination of the experiment. Cultured islets were then fixed in 4% paraformaldehyde (30 min, room temperature) followed by permeabilization with 0.5% Triton X-100 (4 min, room temperature). Cultures were double-labeled for BrdU and insulin by 10-min exposure to 0.5% guinea pig serum, followed by incubation (30 min) with mouse anti-BrdU antibody (1:50 dilution, Sigma) and 15-min incubation with Cy3-conjugated sheep anti-mouse antibody (1:100 dilution, Sigma), all at 37°C. Subsequently, the cells were stained for insulin as above. The cells and tissue sections were embedded in Kaiser's glycerol gelatin (Merck, Darmstadt, Germany).

DNA fragmentation. The free 3′ OH strand breaks resulting from DNA degradation were detected with the terminal deoxynucleotidyl transferase–mediated dUTP-X 3′ nick end-labeling (TUNEL) technique (30). Pancreas sections were deparaffinized, rehydrated, and incubated with 20 µg/ml proteinase K (Boehringer Mannheim) for 15 min at 37°C. Islet cultures were fixed and permeabilized as described above. Both tissue sections and cultured islets were then labeled by the TUNEL reaction according to the manufacturer's instructions (In Situ Cell Death Detection kit; Boehringer Mannheim). The preparations were then rinsed with Tris-buffered saline (pH 7.4) and incubated (10 min, room temperature) with 5-bromo-4-chloro-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma), followed by staining for insulin and detection as described above under "β-cell replication," or detection using a streptavidin-biotin-peroxidase complex developed with aminoethylcarbazole (Zymed). The sections were counterstained with hematoxylin.

For positive controls of the TUNEL reaction, DNA fragmentation was induced in cultured rat and *P. obesus* islets by 16-h incubation with 1 mmol/l streptozotocin

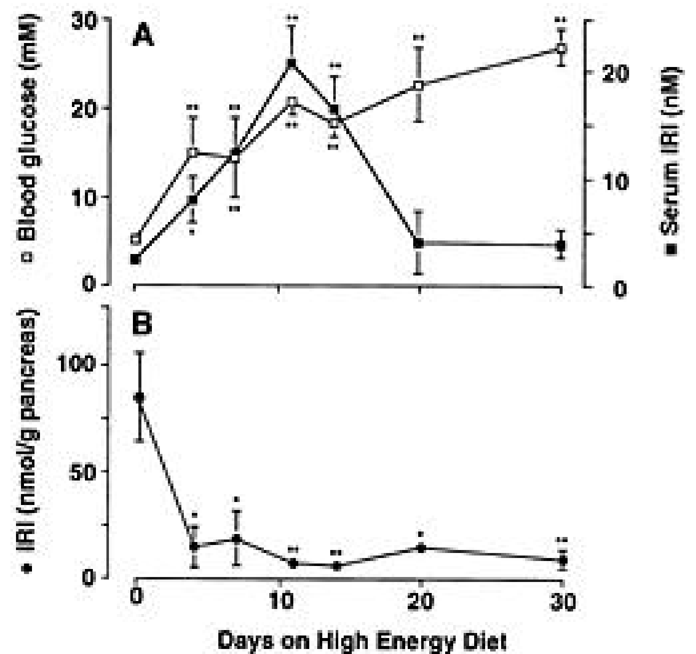

FIG. 1. Longitudinal changes in metabolic parameters in DP *P. obesus* in which diet was switched from LE (day 0) to HE diet. Characteristics of these animals appear in Table 1. *A*: Blood glucose and serum IRI; *B*: Pancreatic IRI content per g tissue. Results are mean ± SE of 4–7 animals. *P < 0.05 and **P < 0.01 relative to animals on LE diet (day 0).

(Sigma) (31), or by incubating the cells for 1 h at 42°C in RPMI supplemented with 10 mmol/l HEPES (pH 7.4). As an additional positive control, tissue sections and cultured islets were exposed to 1 µg/ml DNase I (Sigma) for 10 min at room temperature. As a negative control, the terminal deoxynucleotidyl transferase enzyme was omitted from the reaction.

Because the TUNEL assay detects DNA fragmentation associated with both apoptotic and nonapoptotic cell death (32), additional criteria were used to assess β-cell apoptosis in vivo and in vitro. In tissue sections, β-cells were classified as apoptotic when cells labeled with anti-insulin antibody exhibited sharply demarcated and condensed TUNEL-positive nuclei. In islets cultured on extracellular matrix-coated plates, in parallel to the TUNEL reaction, we used the DNA-binding dye propidium iodide (Sigma) to assess the effects of glucose on necrosis. Unfixed cultured islets were incubated for 10 min on ice with 10 µg/ml propidium iodide (PI) in PBS, washed with PBS, and embedded in glycerol. The samples were immediately evaluated by fluorescent microscopy for positively stained necrotic nuclei.

Data presentation and statistical analysis. Data are expressed as mean ± SE. Statistical differences between groups were determined using the nonparametric Mann-Whitney *U* test. When multiple comparisons were performed, the data were analyzed by the nonparametric Kruskal-Wallis test. A *P* value <0.05 was considered significant. In studies involving tissue sections, all the islets from one animal were considered as a single observation. Cultures and tissue sections were

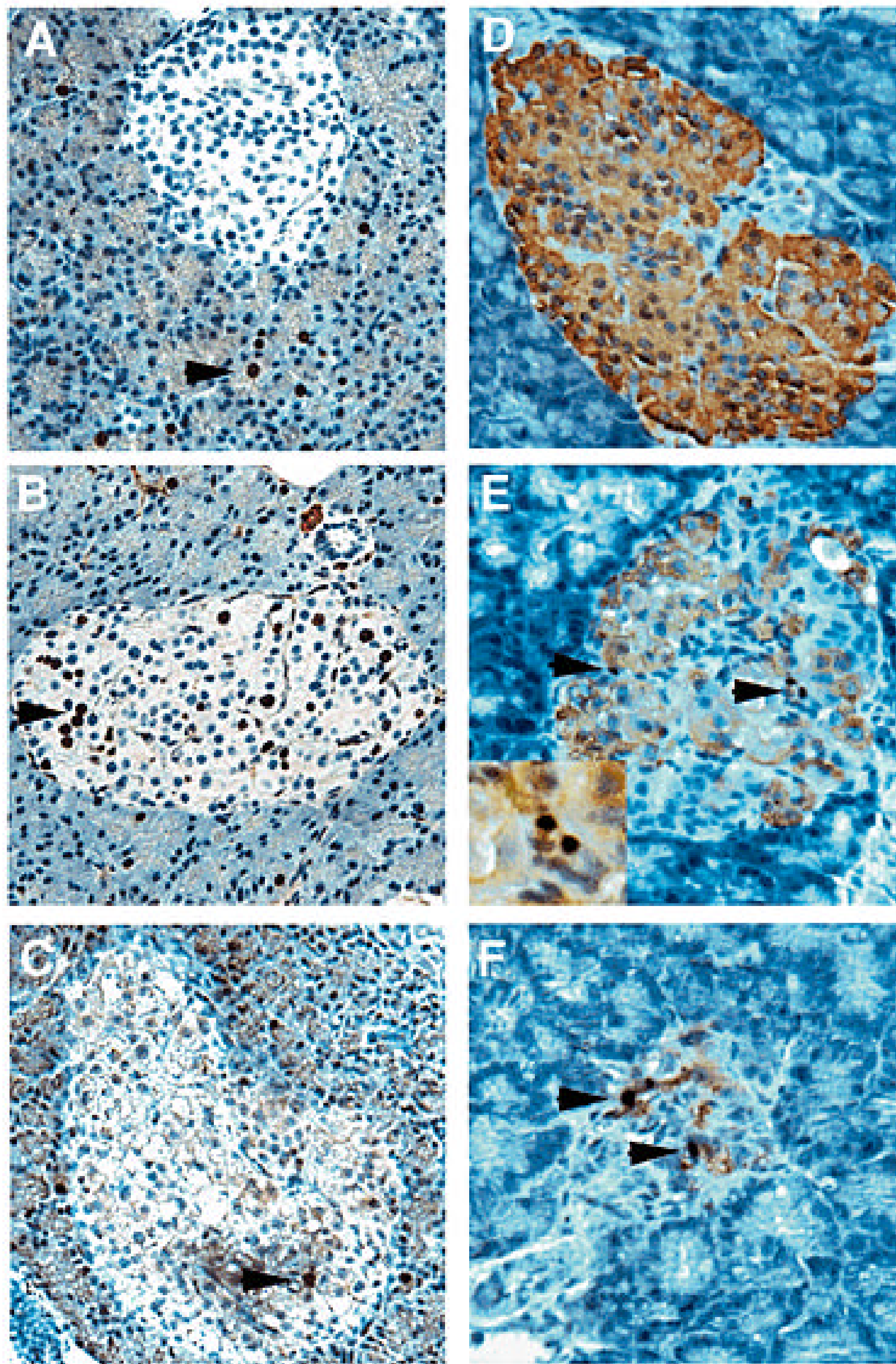

FIG. 2. Photomicrographs of pancreatic sections of prediabetic and diabetic *P. obesus* immunostained for the Ki-67 nuclear antigen, TUNEL, and insulin. Ki-67 staining (peroxidase) of sections from pancreases of prediabetic *P. obesus* on LE diet (*A*) and of diabetic animals on an HE diet for 7 (*B*) and 20 (*C*) days. Double immunostaining for the TUNEL reaction (alkaline phosphatase) and insulin (peroxidase) in sections from pancreases of prediabetic *P. obesus* (*D*) and diabetic animals on HE diet for 20 (*E*) and 30 (*F*) days. The arrows mark the following: an exocrine cell stained positive for Ki-67 (*A*); β-cell doublets stained positive for Ki-67 (*B*); a β-cell stained positive for Ki-67(*C*); TUNEL-positive β-cell doublet and a single TUNEL-positive cell, all with condensed nuclei (*E*); and TUNEL-positive β-cells with enlarged nuclei (*F*). Light microscopy, ×200. The insert in Fig. 2*E* is a magnification of the TUNEL-positive β-cell dublet shown near the center of the islet.

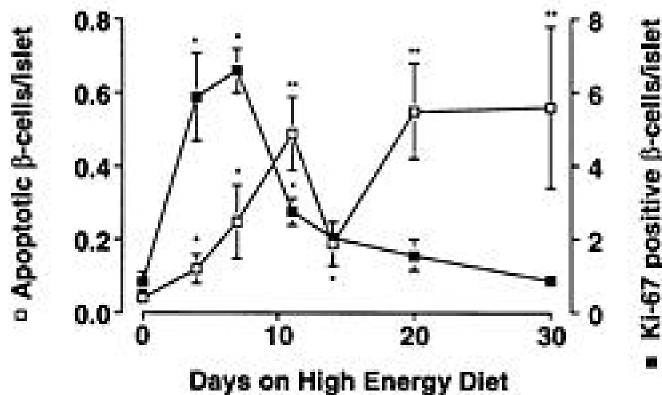

FIG. 3. Time course of HE diet-induced β-cell apoptosis and proliferative activity in the islets of animals described in Table 1. Adjacent sections from each pancreas were analyzed for DNA fragmentation by the TUNEL assay, and for proliferative activity by anti-Ki-67 staining. Results are mean ± SE for 4–7 animals at each time point. One section per animal containing an average of 40 islets was analyzed in each assay. **$P < 0.05$ and **$P < 0.01$ relative to animals on an LE diet (day 0).

analyzed in a randomized manner by a single investigator (M.Y.D.) who was blinded to the treatment conditions. In studies involving cultured islets, care was taken to score islets of similar size. Because of technical limitations, only islets attached in the center of the dish were analyzed.

RESULTS

Induction of diabetes. In the diabetes-prone *P. obesus,* transition from the LE to HE diet resulted in a progressive increase in body weight (Table 1). Blood glucose increased from a mean 5.3 mmol/l in prediabetic animals to 15.2 mmol/l by day 4 of an HE diet, reaching 26.6 mmol/l by day 30 (Table 1 and Fig. 1*A*). Serum immunoreactive insulin (IRI), already elevated on the LE diet (2.5 ± 0.7 nmol/l), increased sharply and remained elevated for the first 2 weeks on the HE diet, then declined to initial levels despite continuing hyperglycemia (Fig. 1*A*). Pancreatic IRI content rapidly declined during the first 4 days of an HE diet, reaching 10–20% of that in prediabetic animals for the remaining period of the study (Fig. 1*B*). Serum triglycerides augmented slightly from 0.64 ± 0.08 mmol/l in prediabetic *P. obesus* to 1.15 ± 0.12 mmol/l ($P < 0.05$) by the 2nd week of an HE diet; however, by the 3rd week, levels increased markedly to 19.3 ± 9.0 mmol/l and persisted at this level to the end of the study.

β-Cell proliferation and apoptosis during development of diabetes. During the prediabetic stage on an LE diet, only few β-cell nuclei stained for the cell proliferation marker Ki-67 (0.9 ± 0.3 β-cells/islet), whereas many Ki-67 positive nuclei were apparent in the exocrine pancreas (Fig. 2*A*). The switch to the HE diet was accompanied by a rapid increase in proliferative activity, reaching a maximum by days 4–7 (an approximate sevenfold increase as compared with animals on an LE diet, Fig. 3). After this point, proliferative activity declined, reaching baseline values by 20 days.

Almost no TUNEL-positive β-cells were observed in islets of prediabetic *P. obesus* on an LE diet (Figs. 2 and 3). The β-cell death rate was significantly increased as early as the 4th day on an HE diet, reaching an initial peak by day 11 and a plateau by day 20 (3-, 12-, and 14-fold increase in rate of apoptosis on days 4, 11, and 20, respectively). After 20–30 days of an HE diet, islets of severely hyperglycemic *P. obesus* exhibited both apoptotic and necrotic β-cell death (Fig. 2*F*). β-Cell death lead to destruction of the islets with the appearance of vacuolization (Fig. 2); yet, no signs of insulitis were observed.

Glucose-induced proliferation and DNA fragmentation in cultured islets. Cultured islets from *P. obesus* and rats exhibited variable degrees of DNA fragmentation when exposed to a low-glucose concentration (3.3 mmol/l): 13.2- and 4.7-fold increase after 3 days and 3.3-, and 2.0-fold after 9–10 days in DP *P. obesus* and rat islets, respectively, relative to islets at 5.5 mmol/l glucose (not shown).

Exposure of islets from DP *P. obesus* to elevated glucose concentrations resulted in a dose-dependent increase in the number of β-cells with TUNEL-positive nuclei (Fig. 4). The increase was 3.2- and 6.1-fold after 3 days (Fig. 5) and 2.2- and 3.8-fold after 9–10 days (Fig. 6) at 11.1 and 33.3 mmol/l glucose, respectively, relative to islets at 5.5 mmol/l glucose. In contrast, glucose did not induce DNA fragmentation in rat islets at either 3 days (not shown) or 9–10 days (Fig. 6). Islets from the partially resistant line of *P. obesus* exhibited a delayed and reduced response to elevated glucose levels: no significant increase after 3 days (not shown) and only 2.1- and 2.3-fold after 9–10 days at 11.1 and 33.3 mmol/l glucose, respectively (Fig. 6). An interesting feature of the DNA fragmentation in β-cells was the appearance of TUNEL-positive

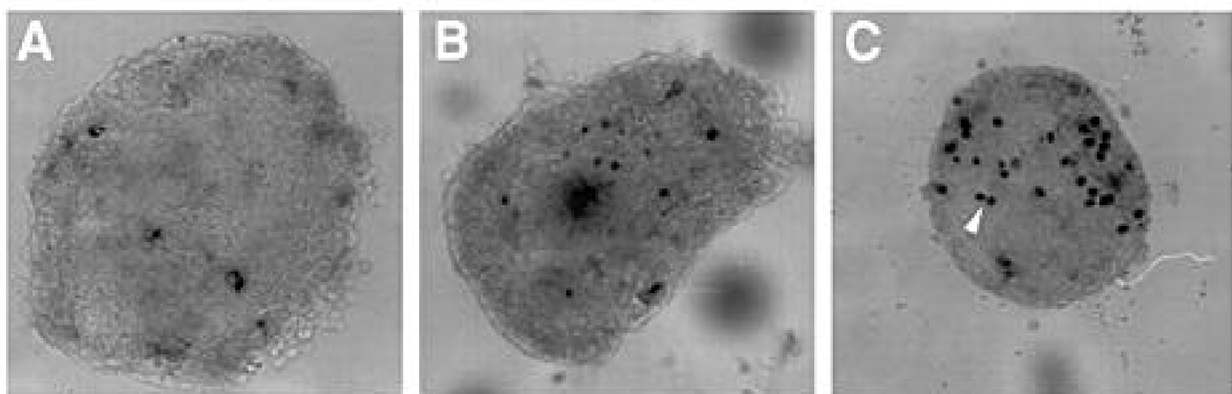

FIG. 4. Photomicrographs of TUNEL-positive nuclei in cultured *P. obesus* islets following exposure to various glucose concentrations. Islets derived from prediabetic *P. obesus* were plated on extracellular matrix-coated dishes and exposed to media containing 5.5 (*A*), 11.1 (*B*), or 33.3 mmol/l (*C*) glucose for 10 days. The arrow marks a nucleus doublet staining positive for the TUNEL-alkaline phosphatase reaction (light microscopy, ×200).

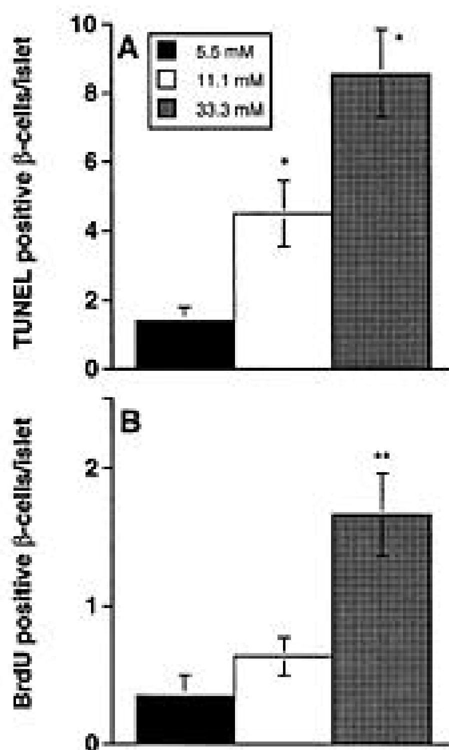

FIG. 5. Glucose-induced β-cell DNA fragmentation (*A*) and proliferative activity (*B*) in cultured islets of DP *P. obesus*. Islets were cultured for 3 days in 5.5, 11.1, and 33.3 mmol/l glucose. Results are depicted as mean ± SE of the number of TUNEL-positive or BrdU-positive β-cells per islet. The mean number of islets scored for DNA fragmentation and BrdU uptake was 31 and 33, respectively. Islets were isolated from eight animals. $*P < 0.005$ and $**P < 0.02$ relative to islets at 5.5 mmol/l glucose.

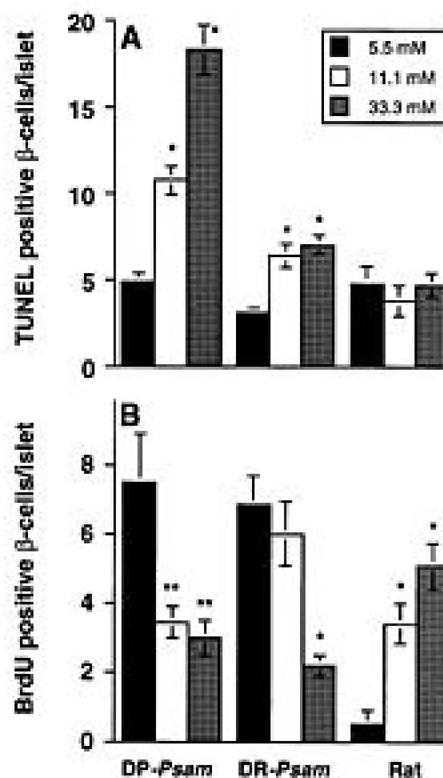

FIG. 6. Glucose-induced β-cell DNA fragmentation and proliferative activity in cultured islets of DP and DR *P. obesus* and in islets of SD rats. Islets were cultured for 9–10 days in 5.5, 11.1, and 33.3 mmol/l glucose. Results are mean ± SE of the number of TUNEL-positive (*A*) and BrdU-positive β-cells (*B*) per islet. The number of islets scored for the TUNEL reaction and BrdU incorporation, respectively, was as follows 64–100 and 30–46 in DP *P. obesus*, 57–65 and 57–59 islets in DR *P. obesus*, and 30–51 and 27–51 in rats. Islets were islolated from 22 DP *P. obesus*, 8 DR *P. obesus* and 12 rats. $*P < 0.001$ and $**P < 0.01$ relative to islets at 5.5 mmol/l glucose derived from the same line of animals.

nucleus doublets suggestive of postmitotic DNA fragmentation (Fig. 4). To exclude a nonspecific effect of the high concentration of D-glucose (33.3 mmol/l), osmolarity was corrected with L-glucose. A 10-day exposure to 27.8 mmol/l L-glucose together with 5.5 mmol/l D-glucose resulted in a β-cell DNA fragmentation rate similar to that induced by 5.5 mmol/l glucose alone (not shown). To assess whether glucose-induced β-cell DNA fragmentation was mediated by nitric oxide production and/or by nonenzymatic glycation, we used aminoguanidine, an inhibitor of nitric oxide synthase (33) and nonenzymatic glycation (34,35). Addition of this agent at concentrations of 0.5 or 1 mmol/l did not prevent the β-cell DNA fragmentation induced by 33.3 mmol/l glucose (not shown).

Exposure of cultured rat or *P. obesus* islets to increasing glucose concentrations (from 5.5 to 33.3 mmol/l glucose) for up to 10 days had no effect on propidium iodide uptake into the cultured cells (not shown).

The 3-day exposure to increasing concentrations of glucose induced an initial dose-dependent proliferation of β-cells in cultured islets of DP *P. obesus* (1.8- and 4.7-fold at 11.1 and 33.3 mmol/l glucose, respectively, relative to islets at 5.5 mmol/l glucose, Fig. 5); this was completely abolished by 9–10 day exposure to elevated glucose levels (Fig. 6), turning into a 50 and 60% inhibition at glucose concentrations of 11.1 and 33.3 mmol/l, respectively. Cultured islets from DR *P. obesus* exhibited a higher threshold for the inhibitory effect of chronically elevated glucose, showing no inhibition at 11.1 mmol/l glucose, but nearly 70% inhibition at 33.3 mmol/l glucose (Fig. 6). In striking contrast, 9- to 10-day exposure of cultured rat islets to elevated glucose concentrations stimulated β-cell proliferation markedly (7.1- and 10.6-fold at 11.1 and 33.3 mmol/l glucose, respectively) (Fig. 6).

DISCUSSION

This study shows that nutrition-induced diabetes in the DP line of *P. obesus* is a very rapid event. The HE-diet–induced hyperglycemia was accompanied by initial elevation of serum IRI, which progressed to severe hypoinsulinemia by the 3rd week on HE diet, paralleled by marked elevation of serum triglycerides (Table 1, Fig. 1). The inability of the β-cells to cope with the increased secretory demand was also evident from the severe depletion of pancreatic IRI content, which accompanied the establishment of hyperglycemia and preceded the decrease in serum IRI (Fig. 1). Similar depletion of islet IRI content was previously described by us in nonfasted diabetic *P. obesus* and in islets from prediabetic *P. obesus* cultured in the presence of high glucose levels (22,23,25); in both cases, it was associated with increased relative concentrations of proinsulin and its conversion intermediates, suggesting that the chronic glucose-driven increase in secretory demand is detrimental to β-cell function in DP *P. obesus*. However, the ability to secrete adequate amounts of insulin also depends on the presence of an appropriate β-cell mass. β-cell mass is determined by the balance between β-cell proliferation/neogenesis and β-cell death (6).

Because reduction in β-cell mass has been documented in obese diabetic patients compared with obese nondiabetic control subjects (4), we studied β-cell proliferation and death during the evolution and progression of diabetes in the moderately obese *P. obesus*. Whereas only a short lasting increase in β-cell proliferative activity accompanied nutrition-induced hyperglycemia, a progressive increase in the rate of β-cell death by probable apoptosis (up to 14-fold above the prediabetic baseline) was evidenced throughout the 30 days of the HE diet, culminating in large areas of vacuolization and severe destruction of the islet architecture. During the final stages of the disease when the animals exhibited hypertriglyceridemia in addition to extreme hyperglycemia, swollen nuclei stained positive for the TUNEL reaction were also evident, suggesting that necrotic β-cell death coexists with β-cell apoptosis during the last stages of the disease. Thus, reduced compensatory proliferative response coupled with increased rate of β-cell death contribute to the reduced insulin content and secretory dysfunction in the diabetic syndrome of *P. obesus*.

Two recent studies suggested a similar failure of β-cell mass to compensate for insulin resistance in another model of type 2 diabetes, the male Zucker diabetic fatty (ZDF) rat, in which extreme obesity is due to mutations in leptin receptors (36, 37). In one of these studies, β-cell apoptosis could not be detected in situ during the evolution of diabetes (38), whereas in the other increased fragmentation of DNA extracted from islets of ZDF rats upon the transition from prediabetes to diabetes suggested the presence of apoptosis (39). Fat deposition in the islets of ZDF rats has been implicated in β-cell dysfunction (40,41), and the increased whole islet DNA fragmentation in this model was suggested to result from lipotoxicity mediated by ceramide and nitric oxide production (39). Unlike the ZDF rat in which diabetes was associated with elevated serum free fatty acids and triglycerides with a marked increase in islet content of triglycerides (40,41), hyperlipidemia was not apparent in the initial stages of hyperglycemia in *P. obesus*: serum triglycerides increased only in the advanced hypoinsulinemic stage of the disease (after 20 days of an HE diet), whereas similar levels of triglycerides (0.08–0.14 µg/islet) were detected in islets from rats, prediabetic *P. obesus*, and diabetic *P. obesus* on an HE diet for 12 days (not shown). Moreover, β-cell dysfunction was evident on day 4 of an HE diet, while the proliferation rate declined after 1 week of an HE diet, and the increased rate of β-cell death was associated with the elevated blood glucose levels throughout the study. These observations make it unlikely that the initial surge of apoptosis and/or the decreased rate of proliferation associated with diabetes in vivo were due to "lipotoxicity"; we therefore tested whether the hyperglycemia per se was responsible for the observed changes in islet cell turnover in *P. obesus.*

When islets from prediabetic DP *P. obesus* were exposed to elevated glucose concentrations in primary cultures, they mimicked the changes observed in vivo during the evolution of diabetes: a glucose-dependent increase in the rate of β-cell proliferation, observed following a 3-day exposure to elevated glucose concentrations, turning into inhibition of DNA synthesis after 9–10 days of glucose exposure. In contrast, glucose-induced DNA fragmentation was evident throughout the entire period. Unlike the stimulatory effect of glucose on DNA fragmentation, elevated glucose did not promote propidium iodide uptake into cultured *P. obesus* islets, excluding a direct effect of elevated glucose concentrations on necrotic cell death in vitro. Taken together, these studies suggest that the increased β-cell DNA fragmentation observed on prolonged exposure of cultured *P. obesus* islets to elevated glucose levels is a result of glucose-induced β-cell apoptosis. Although DNA fragmentation seemed specific to D-glucose and was not mimicked by elevated L-glucose levels, the failure of aminoguanidine, an inhibitor of nitric oxide synthase and of advanced glycosylation end-product formation in islets (33,34) to influence the increase in TUNEL-positive β-cells does not support a major role for the formation of nitric oxide and advanced glycation end products in glucose-dependent β-cell DNA fragmentation, at least in DP *P. obesus*. The molecular mechanisms of this effect remain to be determined.

The in vitro glucose effect on β-cell DNA fragmentation was also tested in islets derived from *P. obesus* of the partially diabetes-resistant line: these exhibited delayed and reduced DNA fragmentation in response to high glucose levels, paralleled by an inhibition of β-cell proliferation upon prolonged exposure to 33.3 mmol/l glucose, but not to 11.1 mmol/l glucose. However, only few animals of the DR line were available for our studies due to low fecundity, lower survival rate, and the fact that only ~60% are resistant to diet-induced hyperglycemia (20,21); we have therefore used SD rats as an additional reference rodent. In contrast to islets of DP *P. obesus*, and in accordance with other studies (42), β-cell DNA fragmentation was insensitive to elevated ambient glucose in cultured rat islets, whereas a pronounced effect on β-cell proliferation (~11-fold at 33.3 mmol/l glucose) was observed. Thus, whether the net β-cell mass increases or decreases in response to high glucose (hyperglycemia) seems to be determined by both genetic and species-related factors.

Although various agents including several reducing sugars have been shown to induce apoptosis in normal islets and in transformed β-cell lines (31,43,44), to our knowledge, this is the first demonstration of glucose-induced β-cell apoptosis. The apparently low rate of apoptosis in vivo compared with the in vitro observations may be due to the rapid kinetics of the final stages of the apoptotic process (detected by the TUNEL reaction) and the fast removal of apoptotic bodies by macrophages (45). Despite these limitations, by using an animal model of type 2 diabetes in which the transition from normoglycemia to hyperglycemia is controlled by the diet and occurs over a matter of days, we were able to capture and analyze β-cell death in situ and obtain data highly suggestive of β-cell death caused by apoptosis.

In previous studies, we demonstrated that prolonged exposure to elevated glucose concentrations caused deterioration of β-cell secretory activity in islets from prediabetic *P. obesus*, whereas in islets from the diabetes-resistant SD rats, glucose induced an adequate output of fully processed insulin (25). This supports the hypothesis that genetic susceptibility to diabetes may confer sensitivity to the adverse effects of hyperglycemia on β-cell secretory function. In light of our present observations, we suggest that genetic susceptibility to type 2 diabetes may be associated with increased sensitivity to yet another process of "glucotoxicity," in which chronic hyperglycemia leads to a progressive loss of β-cell mass by inducing β-cell death, without sufficient compensatory increase in the rate of β-cell production. The identification of this novel pathway of glucotoxicity should promote studies on

the mechanisms involved and hopefully lead to means that prevent the detrimental decrease in β-cell mass in type 2 diabetes.

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REFERENCES

1. Cerasi E: Insulin deficiency and insulin resistance in the pathogenesis of NIDDM: is a divorce possible? *Diabetologia* 38:992–997, 1995
2. Taylor SI, Accili D, Imai Y: Insulin resistance or insulin deficiency: which is the primary cause of NIDDM? *Diabetes* 43:735–740, 1994
3. Saito K, Yaginuma N, Takahashi T: Differential volumetry of A, B and D cells in the pancreatic islets of diabetic and non-diabetic subjects. *Tohoku J Exp Med* 129:273–283, 1979
4. Kloppel G, Lohr M, Hablich K, Oberhilzer M, Heinz PU: Islet pathology and pathogenesis of type I and type II diabetes revisited. *Surv Synth Path Res* 4:110–125, 1985
5. Clark A, Wells CA, Buley ID, Cruickshank JK, Vanhegan RI, Matthews DR, Cooper GJ, Holman RR, Turner RC: Islet amyloid, increased A-cells, reduced B-cells and exocrine fibrosis: quantitative changes in the pancreas in type 2 diabetes. *Diabetes Res* 9:151–159, 1988
6. Vinik A, Pittenger G, Rafaeloff R, Rosenberg L, Duguid W: Determinants of pancreatic islet mass: a balance between neogenesis and senescence/apoptosis. *Diabetes Rev* 4:235–263, 1996
7. Boden G, Ruiz J, Kim CJ, Chen X: Effects of prolonged glucose infusion on insulin secretion, clearance and action in normal subjects. *Am J Physiol* 270:E251–E258, 1996
8. Seaquist RE, Kahn SE, Clark PM, Hales N, Porte DJ, Robertson PR: Hyperproinsulinemia is associated with increased β-cell demand after hemipancreatectomy in humans. *J Clin Invest* 97:455–460, 1996
9. Kosaka K, Kuzuya T, Akanuma Y, Hagura R: Increase in insulin response after treatment of overt maturity-onset diabetes is independent of the mode of treatment. *Diabetologia* 18:23–28, 1980
10. Yoshioka N, Kuzuya T, Matsuda A, Iwamoto Y: Effect of dietary treatment on serum insulin and proinsulin response in newly diagnosed NIDDM. *Diabetes* 38:262–266, 1989
11. Glaser B, Leibovich G, Nesher R, Hartling S, Binder C, Cerasi E: Improved beta-cell function after intensive insulin treatment in severe non-insulin-dependent diabetes. *Acta Endocrinol (Copenh)* 118:365–373, 1988
12. Ilkova H, Glaser B, Tunckale A, Bagriacik N, Cerasi E: Induction of long-term glycemic control in newly diagnosed type 2 diabetic patients by transient intensive insulin treatment. *Diabetes Care* 20:1353–1356, 1997
13. Davalli AM, Ricordi C, Socci C, Braghi S, Bertuzzi F, Fattor B, DiCarlo V, Pontiroli AE, Pozza G: Abnormal sensitivity to glucose of human islets cultured in high glucose medium: partial reversibility after an additional culture in normal glucose medium. *J Clin Endocrinol Metab* 72:202–208, 1991
14. Eizirik DL, Korbutt GS, Hellerstrom C: Prolonged exposure of human pancreatic islets to high glucose concentrations in vitro impairs the β-cell function. *J Clin Invest* 90:1263–1268, 1992
15. Ling Z, Pipeleers DG: Prolonged exposure of human β-cells to elevated glucose levels results in sustained cellular activation leading to a loss of glucose regulation. *J Clin Invest* 98:2805–2812, 1996
16. Adler JH, Kalman R, Lazarovici G, Bar-On H, Ziv E: Achieving predictable model of type 2 diabetes in the sand rat. In *Frontiers in Diabetes Research: Lessons from Animal Diabetes III*. Shafrir E, Ed. London, Smith-Gordon, 1991, p. 212–214
17. Shafrir E, Gutman A: *Psammomys obesus* of the Jerusalem colony: a model for nutritionally induced non-insulin-dependent diabetes. *J Basic Clin Physiol Pharmacol* 4:83–99, 1993
18. von Dorsche HH, Schafer H, Titlbach M: Histophysiology of the obesity-diabetes syndrome in sand rats. In *Advances in Anatomy Embryology and Cell Biology*. Beck F, Hild W, Kriz W, Pauly JE, Sano Y, Schiebler TH, Eds. Berlin, Springer-Verlag, 1994, p. 1–95
19. Kalderon B, Gutman A, Levy E, Shafrir E, Adler JH: Characterization of stages in development of obesity-diabetes syndrome in the sand rat *(Psammomys obesus)*. *Diabetes* 35:717–723, 1986
20. Kalman R, Ziv E, Adler JH, Lazarovici G, Bar-On H: The efficiency of the sand rat metabolism is responsible for the development of obesity and diabetes. *J Basic Clin Physiol Pharmacol* 4:57–68, 1993
21. Kalman R, Lazarovici G, Bar-On H, Ziv E: The sand rat *(Psammomys obesus)*: morphologic, physiologic and biochemical characteristics of a model for type II diabetes mellitus. *Contemp Topics Lab Anim Sci* 35:67–70, 1996
22. Gadot M, Leibowitz G, Shafrir E, Cerasi E, Gross D, Kaiser N: Hyperproinsulinemia and insulin deficiency in the diabetic *Psammomys obesus*. *Endocrinology* 135:610–616, 1994
23. Gadot M, Ariav Y, Cerasi E, Kaiser N, Gross D: Hyperproinsulinemia in the diabetic *Psammomys obesus* is a result of increased secretory demand on the B-cell. *Endocrinology* 136:4218–4223, 1995
24. Ziv E, Kalman R, Hershkop K, Barash V, Shafrir E, Bar-On H: Insulin resistance in the NIDDM model *Psammomys obesus* in the normoglycaemic, normoinsulinaemic state. *Diabetologia* 39:1269–1275, 1996
25. Gross DJ, Leibowitz G, Cerasi E, Kaiser N: Increased susceptibility of islets from diabetes-prone *Psammomys obesus* to the deleterious effects of chronic glucose exposure. *Endocrinology* 137:5610–5615, 1996
26. Kaiser N, Corcos AP, Tur-Sinai A, Ariav Y, Cerasi E: Monolayer culture of adult pancreatic islets on extracellular matrix: long-term maintenance of differentiated B-cell function. *Endocrinology* 123:834–840, 1988
27. Gerdes J, Schwab U, Lemke H, Stein H: Production of mouse monoclonal antibody reactive with a human nuclear antigen associated with cell proliferation. *Int J Cancer* 31:13–20, 1983
28. Campana D, Coustan-Smith E, Janossy G: Double and triple staining methods for studying the proliferative activity of human B and T lymphoid cells. *J Immunol Methods* 107:79–88, 1988
29. Bouwens L, Lu WG, De Krijger R: Proliferation and differentiation in the human fetal endocrine pancreas. *Diabetologia* 40:398–404, 1997
30. Gavrieli Y, Sherman Y, Ben Sasson SA: Identification of programmed cell death in vitro via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 199:493–501, 1992
31. Kaneto H, Fujii J, Seo HG, Suzuki K, Matsuoka T, Nakamura M, Tatsumi H, Yamasaki Y, Kamada T, Taniguchi N: Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. *Diabetes* 44:733–738, 1995
32. van Lookeren Campagne M, Lucassen PJ, Vermeulen JP, Balazs R: NMDA and kainate induce internucleosomal DNA cleavage associated with both apoptotic and necrotic cell death in the neonatal rat brain. *Eur J Neurosci* 7:1627–1640, 1995
33. Corbett JA, Tilton RG, Chang K, Hasan KS, Ido Y, Wang JL, Sweetland MA, Lancaster JRJ, Williamson JR, McDaniel ML: Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes* 41:552–556, 1992
34. Brownlee M, Vlassara H, Kooney A, Ulrich P, Cerami A: Aminoguanidine prevents diabetes-induced arterial wall protein cross-linking. *Science* 232:1629–1632, 1986
35. Tajiri Y, Moller C, Grill V: Long-term effects of aminoguanidine on insulin release and biosynthesis: evidence that the formation of advanced glycosylation end products inhibits B-cell function. *Endocrinology* 138:273–280, 1997
36. Takaya K, Ogawa Y, Isse N, Okazaki T, Satoh N, Masuzaki H, Tamura N, Hosoda K, Nakao K: Molecular cloning of rat leptin receptor isoform complementary DNAs: identification of missense mutation in Zucker fatty *(fa/fa)* rats. *Biochem Biophys Res Commun* 225:75–83, 1996
37. Phillips MS, Liu Q, Hammond HA, Dugan V, Hey PJ, Caskey CT, Hess JF: Leptin receptor missense mutation in the fatty Zucker rat. *Nat Genet* 13:18–19, 1996
38. Pick A, Clark J, Kubstrup C, Levisetti M, Pugh W, Bonner-Weir S, Polonsky KS: Role of apoptosis in failure of β-cell mass compensation for insulin resistance and β-cell defects in the male Zucker diabetic fatty rat. *Diabetes* 47:358–364, 1998
39. Shimabukuro M, Zhou Y-T, Levi M, Unger RH: Fatty acid-induced β-cell apoptosis: a link between obesity and diabetes. *Proc Natl Acad Sci U S A* 95:2498–2502, 1998
40. Lee Y, Hirose H, Ohneda M, Johnson JH, McGarry JD, Unger RH: Beta-cell lipotoxicity in the pathogenesis of non-insulin-dependent diabetes mellitus of obese rats: impairment in adipocyte-beta-cell relationships. *Proc Natl Acad Sci U S A* 91:10878–10882, 1994
41. Lee Y, Hirose H, Zhou Y-T, Esser V, McGarry JD, Unger RH: Increased lipogenic capacity of the islets of obese rats: a role in the pathogenesis of NIDDM. *Diabetes* 46:408–413, 1997
42. Hoorens A, Van de Casteele M, Kloppel G, Pipeleers D: Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *J Clin Invest* 98:1568–1574, 1996
43. Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG, Eizirik DL: Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* 138:2610–2614, 1997
44. Kaneto H, Fujii J, Myint T, Miyazawa N, Islam KN, Kawasaki Y, Suzuki K, Nakamura M, Tatsumi H, Yamasaki Y, Taniguchi N: Reducing sugars trigger oxidative modification and apoptosis in pancreatic β-cells by provoking oxidative stress through the glycation reaction. *Biochem J* 320:855–863, 1996
45. Wyllie AH: Apoptosis: an overview. *Br Med Bull* 53:451–465, 1997