Mechanisms involved in the β-cell mass increase induced by chronic sucrose feeding to normal rats

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Abstract

The aim of the present study was to clarify the mechanisms by which a sucrose-rich diet (SRD) produces an increase in the pancreatic β-cell mass in the rat. Normal Wistar rats were fed for 30 weeks either an SRD (SRD rats; 63% wt/wt), or the same diet but with starch instead of sucrose in the same proportion (CD rats). We studied body weight, serum glucose and triacylglycerol levels, endocrine tissue and β-cell mass, β-cell replication rate (proliferating cell nuclear antigen; PCNA), islet neogenesis (cytokeratin immunostaining) and β-cell apoptosis (propidium iodide). Body weight (g) recorded in the SRD rats was significantly ($P<0\cdot05$) larger than that of the CD group ($556\cdot0 \pm 8\cdot3$ vs $470\cdot0 \pm 13\cdot1$). Both serum glucose and triacylglycerol levels (mmol/l) were also significantly higher ($P<0\cdot05$) in SRD than in CD rats (serum glucose, $8\cdot11 \pm 0\cdot14$ vs $6\cdot62 \pm 0\cdot17$; triacylglycerol, $1\cdot57 \pm 0\cdot18$ vs $0\cdot47 \pm 0\cdot04$). The number of pancreatic islets per unit area increased significantly ($P<0\cdot05$) in SRD rats ($3\cdot29 \pm 0\cdot1$ vs $2\cdot01 \pm 0\cdot2$). A significant increment ($2\cdot6$ times) in the mass of endocrine tissue was detected in SRD animals, mainly due to an increase in the β-cell mass ($P=0\cdot0025$). The islet cell replication rate, measured as the percentage of PCNA-labelled β cells increased $6\cdot8$ times in SRD rats ($P<0\cdot03$). The number of apoptotic cells in the endocrine pancreas decreased significantly (three times) in the SRD animals ($P=0\cdot03$). The cytokeratin-positive area did not show significant differences between CD and SRD rats. The increase of β-cell mass induced by SRD was accomplished by an enhanced replication of β cells together with a decrease in the rate of β-cell apoptosis, without any evident participation of islet neogenesis. This pancreatic reaction was unable to maintain serum glucose levels of these rats at the level measured in CD animals.

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Introduction

Prolonged administration of either a high fructose or sucrose diet to genetically selected rats induces a metabolic syndrome characterised by insulin resistance and decreased glucose tolerance, as well as increased plasma triglyceride and cholesterol levels (Cohen *et al.* 1977, Cohen 1978). We have previously reported that administration of a sucrose-rich diet (SRD) to normal rats for 6 months induced such metabolic abnormalities, together with a significant increase in both pancreatic islet number and β-cell area (Lombardo *et al.* 1996). These changes were accompanied by normal insulin plasma levels and pancreatic insulin content. However, the mechanisms by which the sucrose feeding produced the pancreatic morphological changes were not clear.

We have also found that sucrose administration to young normal hamsters results in time-dependent pancreatic changes, with increased β-cell mass caused by incremental β-cell replication and islet neogenesis, but keeping blood glucose levels within the normal range (Del Zotto *et al.* 1999). Administration of this diet to pregnant normal hamsters induced similar pancreatic changes in the newborn animals. However, these changes were of greater magnitude and were accompanied by a significant decrease in β-cell apoptosis together with a significant decrease in blood glucose levels (Gagliardino *et al.* 2000).

Normal pancreatic β-cell mass results from the final rate between two opposite processes: growth and destruction. Many experimental procedures lead to the stimulation of β-cell growth (Rosenberg *et al.* 1983, Bonner–Weir *et al.* 1989, Parsons *et al.* 1992, Wang *et al.* 1995, Lombardo *et al.* 1996). Among them, those obtained by modifying food intake are of especial interest for studying the pathophysiology of type 2 diabetes.

In order to clarify the mechanisms by which a high sucrose diet produces the above-mentioned pancreatic changes in the rat, we have studied β-cell replication rate, islet neogenesis and β-cell apoptosis in normal Wistar rats

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fed an SRD for 6 months. Our data showed that increased β-cell replication and decreased β-cell apoptosis could account for the increase in β cells induced by SRD at this period.

Materials and Methods

Animals and diets

Normal male Wistar rats (180–200 g body weight; National Institute of Pharmacology, Buenos Aires, Argentina) were maintained in a temperature-controlled room (23 °C) with a fixed 12 h light:12 h darkness cycle. They were initially fed standard rat laboratory chow (Ralston Purina, St Louis, MO, USA) to standardise the nutritional status. After 1 week, the rats were randomly divided into two groups: the experimental group received a semi-synthetic SRD (63% wt/wt), while the control rats received the same semi-synthetic diet but with starch instead of sucrose in the same proportion (CD; 63% wt/wt). Details of this procedure have been reported previously (Chicco *et al*. 1994). Both diets provide approximately 15·28 kj/g chow. The animals had free access to food and water and were maintained on their respective diets for a period of 30 weeks.

The weight of each animal was recorded twice per week, and the individual caloric intake and weight gain of at least ten animals in each group were assessed twice per week during the experimental period. On the day of the experiment, food was removed at 0900 h, and experiments were performed between 0900 and 1200 h. The experimental protocol was approved by the Human and Animal Research Committee of the School of Biochemistry, Universidad Nacional del Litoral, Santa Fe, Argentina.

Blood parameters

Rats were anaesthetised with an intraperitoneal injection of pentobarbital (60 mg/kg body weight), and blood samples were drawn from the jugular vein and centrifuged at 4 °C. The serum samples obtained were assayed either immediately or within the next 3 days after having been stored at − 20 °C. Serum glucose (Bergmeyer 1974) and triacylglycerol (Laurell 1966) levels were determined by spectrophotometric methods.

Immunohistochemical and morphometrical study of islets

After removal of the whole pancreas, the fat tissue was carefully dissected away and the wet weight of the total gland recorded. Samples of the tail of the pancreas were then fixed in Bouin's fluid and embedded in paraffin, and serial sections (5 μm) were obtained from different levels of the blocks. Haematoxylin–eosin staining was used for general structural observation. Each section from a given series was mounted on separate slides to stain adjacent sections for immunocytochemical identification of (1) insulin-secreting cells (β cells) and (2) glucagon-, somatostatin- and pancreatic polypeptide-secreting cells (non-β cells). For this purpose, specimens were incubated with appropriate dilutions of (1) our own guinea pig anti-insulin serum (1:20 000) and (2) a mixture of the other three rabbit antisera: antiglucagon (1:400), anti-pancreatic polypeptide (1:10 000) (both kindly provided by Dr Lise Heding, Novo Nordisk, Copenhagen, Denmark) and anti-somatostatin (1:6000) (a gift from Dr Suad Efendic, Department of Endocrinology, Karolinska Institute, Copenhagen, Denmark). The reaction was completed using the streptavidin–biotin complex method, with either peroxidase or alkaline phosphatase together with carbazole and fast blue respectively as chromogens. Sections were counterstained with haematoxylin. Controls for serologic specificity were made by preincubating a given antiserum with an excess of the corresponding hormone for 24 h at 4 °C. Morphometrical analysis was made by the point-counting method using an 8×8 mm grid (256 squares and 289 intersections) mounted on the eyepiece of the microscope. To obtain the values of different parameters for β and non-β cells in the same section, we first processed the tissue for detection of β cells by using anti-insulin serum, and then made the morphometrical measurements. In a second step, and on the top of the same section, we added the immunostain for detecting non-β cells and made the measurement again. Values for non-β cells resulted from the difference between both evaluations. Using this procedure, 11 639 and 20 314 insular cells were surveyed for the CD and SRD groups respectively.

With the data recorded, we calculated a unit area of total pancreas (15 872 intersections of the grid=10 μm) excluding connective tissues. We were then able to obtain the area occupied by endocrine pancreas, exocrine pancreas, total pancreas, β cells and non-β cells, and several ratios and relationships as described in the Results. We also estimated the number of islets per unit area (mm^2). In addition, the ratio between islet cell area and the number of islet cells (β and non-β) were calculated to obtain cell sizes. To estimate the islet and β-cell mass, we multiplied the respective volume density by the total weight of the pancreas (Bonner-Weir *et al*. 1989).

Every islet or small group of endocrine cells was recorded in each section, thus obtaining the number and areas of both β and non-β cells.

β-cell apoptotic rate

To identify apoptotic images, we used the propidium iodine technique (Scaglia *et al*. 1997). Deparaffinised and dehydrated sections were washed in phosphate-buffered saline (PBS) before incubation for 30 min in a dark humidified chamber with a solution of propidium iodine

Table 1 Morphometrical analysis of pancreases from rats fed a sucrose-rich (SRD) or a control diet (CD) for 30 weeks. Values are the means ± S.E.M. of four rat pancreases included in each group

	No. of islets/mm^2	β-cell mass (mg)	PCNA index rate (%)	CK index rate (%)	Apoptotic index rate (%)
Diet					
CD	$2·01 ± 0·2^c$	$6·0 ± 0·27^a$	$0·04 ± 0·03^b$	$11·6 ± 3·1$	$4·3 ± 1·1$
SRD	$3·29 ± 0·1$	$15·5 ± 0·38$	$0·27 ± 0·09$	$13·9 ± 2·5$	$1·4 ± 0·5$

[a]$P<0·0025$; [b]$P<0·03$; [c]$P<0·05$.

(4 µg/ml; Sigma, St Louis, MO, USA) and ribonuclease A (100 µg/ml; Sigma). A Zeiss Axiolab epifluorescence microscope equipped with an HBO50 mercury lamp, together with two different filters, was used for visualisation of autofluorescent labelling. For the quantitative evaluation of immunofluorescence, positively labelled apoptotic endocrine cells were counted under a × 40 objective lens in sections obtained from different levels of the blocks. The number of apoptotic cells was expressed as the percentage of the total islet cells counted.

Cytokeratin immunostaining

To reveal the presence of cytokeratin (CK)-positive cells, we used a specific monoclonal antibody for CK 19 (anti-CK clone 4·62; 1:40; Sigma) and the streptavidin–biotin complex method, with peroxidase and carbazole as chromogens. Before performing the staining, we treated deparaffinised sections with 250 ml antigen–retrieval solution (Vector Laboratories Inc., Burlingame, CA, USA) for 10 min in a 500 W microwave oven (Madsen *et al.* 1997). The number of CK-positive cells is expressed as the percentage of the total islet cells counted.

Islet cell replication rate: double-immunolabelling studies

Islet cell replication rate was estimated by detecting proliferating cell nuclear antigen (PCNA; 1:4000; Sigma), using a modified avidin–biotin peroxidase method (Hsu *et al.* 1981). We quantified and expressed the replication rate as the percentage of PCNA-labelled cells among the total islet cells.

We followed two different procedures. (1) We performed double staining of the following pairs: β (insulin antibody) and PCNA (PCNA antibody), and no β (glucagon, somatostatin and pancreatic polypeptide pool) and PCNA. We then used the streptavidin–biotin complex method, with peroxidase and alkaline phosphatase together with carbazole and fast blue respectively as chromogens. Incubations with primary antibodies were overnight whereas those with the secondary biotinylated antibodies were for 30 min. (2) For serial double-immunofluorescence labelling, sections pretreated with non-immune sera from rabbit diluted in Tris-buffered

saline (pH 7·4) were incubated in a moist chamber at room temperature for 30 min with a solution of propidium iodine (4 µg/ml; Sigma) and ribonuclease A (100 µg/ml; Sigma) and for 1 h with glucagon antibody. After washing with PBS, fluorescence labelling of primary antibody was accomplished through a second incubation at room temperature for 45 min in the dark with the IgG-specific fluorescein–conjugated affinity-purified goat antibody against IgG (heavy plus light chains; Jackson Immuno Research Laboratories, Inc., Baltimore, MD, USA). After another washing with PBS, the sections were mounted in Tris–glycerol (pH 8·4) for analysis by fluorescence microscopy.

Statistical analysis

Data are presented as means ± S.E.M. Statistical analysis was performed using paired and unpaired Student's *t*-test. A *P* value of <0·05 was considered significant.

Results

Body weight recorded in animals given the SRD (SRD rats) at the time of death was significantly higher than that of the CD group (556 ± 8 vs 470 ± 13 g; *P*<0·05). The pancreas weight did not show significant differences between CD and SRD rats (1·56 ± 0·07 mg vs 1·62 ± 0·04 mg respectively). Both serum glucose and triacylglycerol levels (mmol/l) were significantly higher (*P*<0·05) in SRD rats: glucose, 6·62 ± 0·17 for CD and 8·11 ± 0·14 for SRD; triacylglycerol, 0·47 ± 0·04 for CD and 1·57 ± 0·18 for SRD.

The sections stained with haematoxylin–eosin revealed the general histological pancreatic normal appearance both in CD and SRD rats. Immunohistochemistry showed the usual topographic distribution of the cells within the islets in both groups of animals, with the β cells occupying the central zone and the non-β cells located at the islet periphery.

Morphometrical analysis of the pancreases is shown in Table 1. The number of pancreatic islets per unit area increased significantly (*P*<0·05) in SRD rats compared with CD rats. Moreover, a significant increase was
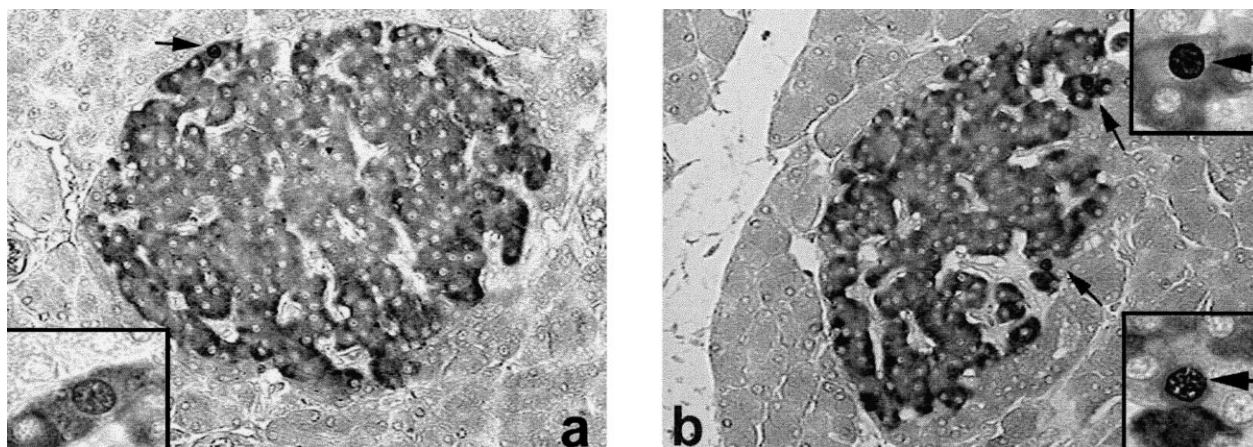
Figure 1 Double-immunolabelling pancreatic islets: nuclear proliferating cell nuclear antigen (PCNA) and cytoplasmic (insulin) stainings. The arrows point to positive-reacting cells, which are also seen at higher magnification (inserts). (a) Control (CD) and (b) sucrose-rich diet (SRD) rats (× 200; × 1000 (inserts)).

observed in the mass of endocrine tissue of SRD animals. This increment was mainly due to an increase in the β–cell mass (2·6 times). The islet cell replication rate (Fig. 1), measured as the percentage of PCNA-labelled β cells, increased 6·8 times in SRD rats. In addition, although the number of apoptotic cells was scarce in the endocrine pancreas, this number decreased significantly (three times) in the SRD animals (Fig. 2).

The CK immunostaining revealed positive–reacting endocrine cells always located at the islet periphery. The CK–positive area did not show significant differences between CD and SRD rats (Fig. 3). Other signs of neogenesis, such as a higher number of insulin–reacting cells in ductal epithelium together with an increase in their PCNA index, as well as a higher proportion of both small islets and islets in close contact with ducts were not found. The non–β-cell mass was also larger in the SRD group,

although to a lesser extent with respect to the β–cell increase. On the other hand, the ratio between the islet cell area and the number of islet cells, which expresses the endocrine cell size, did not reveal significant differences; the values (μm^2) were as follows (CD vs SRD): β cells, $121·48 \pm 9·73$ vs $118·35 \pm 6·46$; non-β cells, $97·55 \pm 6·24$ vs $95·59 \pm 7·05$.

Discussion

Our current results showed and confirmed those of Lombardo *et al.* (1996) that normal rats fed for 30 weeks with an SRD have a significant increase of both pancreatic islet and β-cell mass.

Chronic administration of glucose induces an insulin–resistant state which represents an increased demand for
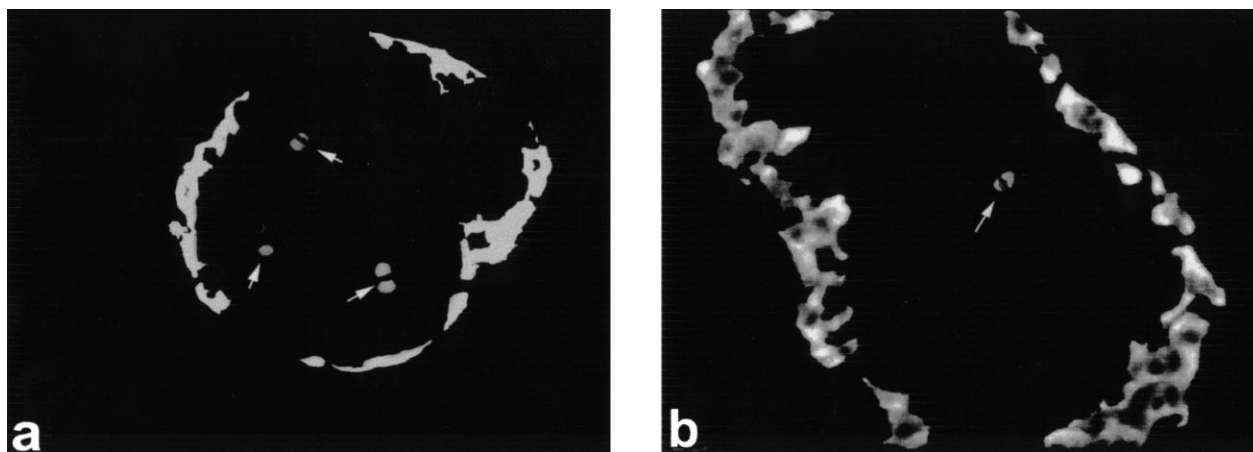
Figure 2 Pancreatic islets showing glucagon-positive cells in the periphery (immunofluorescence). Apoptotic nuclei (propidium iodine) corresponding to B cells are seen in the central zone (arrows). (a) CD rats and (b) SRD rats (× 200).
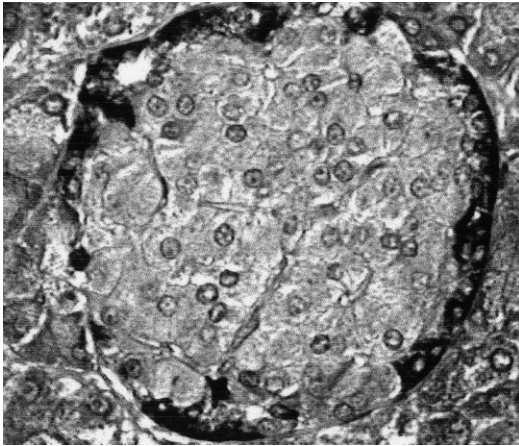
Figure 3 CK19-immunolabelled pancreatic islet from a CD rat. Positive-reacting non-β cells are seen in the periphery; counterstained with haematoxylin (× 200).

insulin secretion that triggers changes in β-cell function (Leahy 1996). Pancreatic β-cell mass has to cope with insulin demand to keep serum glucose levels within normal range. Long-term adaptation of the β-cell mass to an increased demand results from an increase in the β-cell number through hyperplasia and neogenesis (Polonsky *et al.* 1998, Bonner-Weir 2000). However, β-cell expansion can be offset by concomitant apoptosis (Pick *et al.* 1998, Donath *et al.* 1999). In the present study, the increase of β-cell mass induced by SRD was accomplished by an enhanced replication of B cells (increased PCNA index rate) together with a decrease in the rate of β-cell apoptosis, without any evident signs (participation) of islet neogenesis. This pancreatic reaction was, however, unable to cope with the increased peripheral demand of insulin, since SRD animals exhibited higher serum glucose levels than did CD animals.

The moderate hyperglycaemia portrayed by our animals represents an additional challenge for the pancreatic β cells since glucose can increase the replication of these cells, as occurs in both rats (Bonner-Weir 1989) and *Psammomis obesus* (Donath *et al.* 1999). However, hyperglycaemia can also adversely affect β-cell turnover in human islets (Maedler *et al.* 2001*b*).

Replication of pre-existing β cells represents the main mechanism by which the normal adult increases the insulin-secreting active mass, although such a replication progressively decreases with ageing (Bonner Weir 2000, Montanya *et al.* 2000). Even when, during foetal and neonatal life, islets are newly formed from precursor cells that bud off from the pancreatic ducts (Swenne 1982, Hellerström 1984), only a low degree of neogenesis exists in the adult, this mechanism being enhanced under strong stimuli (Bonner-Weir 2000). CKs are considered good markers of the neogenetic area from which cells, originating in the ductal epithelium (protodifferentiated stem

cells), are added to the islet mass (Bouwens *et al.* 1994, Wang *et al.* 1995). In the present study, CKs immuno-reacted only with peripheral islet cells, probably non-β cells, as was previously reported (Bouwens *et al.* 1994). Other signs of islet neogenesis, such as an increase in the replication rate and in the number of insulin-labelled cells at the ductal compartment, as well as an increment in the proportion of small islets and islets closely associated with ductal epithelium, were not evident in the pancreas of the SRD animals. Altogether, these observations suggest that neogenesis is not an important mechanism related to the islet mass increase currently observed.

The number of apoptotic cells significantly decreased when the rats received an SRD. Apoptosis is a morphologically identifiable form of cell death, triggered by a variety of metabolic stimuli, that plays an important role in remodelling the β-cell mass as the counterpart of proliferation (Wyllie *et al.* 1980, Steller 1995, Scaglia *et al.* 1997). Hoorens *et al.* (1996) postulated that the rat β cells have a constitutive apoptotic programme that is blocked by proteins whose synthesis is stimulated by glucose in a dose–response manner. Thus, stimulation of such proteins triggered by SRD could account, at least partly, for the decreased apoptotic rate found in our experiments.

It has been reported that elevated glucose concentrations directly induce β-cell apoptosis in cultured islets from diabetes-prone *P. obesus* (Donath *et al.* 1999) and from normal humans (Federici *et al.* 2001), but an increase in glucose concentration to 11 mM in rat islets promotes β-cell survival (Hoorens *et al.* 1996, Efanova *et al.* 1998, Donath *et al.* 1999, Maedler *et al.* 2001*a*). To explain this uneven behaviour, it has been argued that apoptosis induced by high glucose might involve Fas and upregulation of the Fas receptor (Maedler *et al.* 2001*b*), and that human islets constitutively express FasL, while islets from 2- to 3-month-old rats do not (Hanke 2000). Thus, differences in sensitivity to glucose may exist among species and also among individuals of the same species.

Sucrose administration to young hamsters for 5 weeks induces an increase in islet- and β-cell mass caused by incremented β-cell replication rate and islet neogenesis (Del Zotto *et al.* 1999). Even when the animals were kept on this diet for up to 24 weeks, they maintained their blood glucose levels within the normal range. A simultaneous increase of islet neogenesis-associated protein-positive cell mass was also observed in these animals (Del Zotto *et al.* 2000), suggesting the participation of this peptide in the control of islet neogenesis.

Although sucrose administration to normal hamsters and rats triggered in both animals an increased β-cell mass, we found differences in the mechanisms leading to such an increase: neogenesis plus increased β-cell replication in the former and increased replication together with decreased apoptosis in the latter. When feeding hamsters for 24 weeks and rats for 30 weeks with an SRD, we found that the hamsters remained normoglycaemic while the rats

showed a significant increase in serum glucose, as a result of the lack of a neogenic reaction. Thus, this reaction may play a key role in islets to satisfactorily adapt their β-cell mass to an increased demand of insulin. Additionally, we found that the ratio of β-cell mass:body weight is three times larger in hamsters than in rats (Del Zotto *et al.* 2000). This characteristic might also contribute to the more efficient pancreas reaction found in hamsters to the increased demand of insulin induced by SRD.

On the basis of the different capacity of pancreatic β-cell populations to cope with nutritionally increased insulin demand, Shafrir *et al.* (1999) have established two groups among models resembling type 2 diabetes. Those animals having a sturdy pancreas exhibit persistent hyperinsulinaemia, with an insulin resistance being compensated for by a good response of β cells (anti-diabetic adaptation), while other animals have a brittle pancreas which cannot respond efficiently to the demands for a compensatory insulin release, and consequently develop a diabetic syndrome. According to our results, hamsters would belong to the first and rats to the second group postulated by Shafrir *et al.* (1999).

Our study shows, coincidentally with that previously reported (Leahy 1996), that diverse animal models can respond in a different manner under similar conditions to either β-cell function overload or hyperglycaemia. The pancreatic response to hyperglycaemia seems to be genetically determined; thus, the efficacy of the mechanisms that attempt to cope with similar experimental conditions will vary, rendering either a compensatory hyperinsulinaemic-normoglycaemic state or a diabetic state. Further studies using models similar to the one presently employed may help to find the underlying mechanisms that condition the uneven pancreatic compensatory response.

Acknowledgements

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