

Islet neogenesis: an apparent key component of long-term pancreas adaptation to increased insulin demand

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Abstract

This study aimed to determine the relative importance of different functional and morphological pancreatic changes induced by the chronic administration of a sucrose-rich diet (SRD) to maintain normal glucose homeostasis. Male Wistar rats were fed either sucrose (SRD) or starch (CD) for 6 and 12 months. At both periods, serum glucose and triacylglycerol levels were significantly higher ($P < 0.05$; paired and unpaired Student's *t*-test) in SRD rats. Serum insulin levels were significantly lower in SRD only at 12 months. At 6 months, the insulin secretion dose-response curve in SRD rats showed a shift to the left that was no longer observed at 12 months, when SRD islets decreased their response to 16 mM glucose. At 6 months, SRD rats showed a significant increase in β -cell volume density (V_{vi}) and islet cell replication rate, together with a

decrease in β -cell apoptotic rate. Changes were not detected in the percentage of PDX-1- and islet neogenesis associated protein (INGAP)-positive cells. Conversely, at 12 months, there was a significant decrease in β -cell V_{vi} and in the percentage of PDX-1-positive cells; the islet cell replication rate was not modified, and the number of apoptotic β -cells increased significantly. No signs of increased neogenesis or INGAP-positive cells were recorded at any period in SRD rats. Our results show that SRD rats are unable to develop functional and morphological pancreatic reactive changes sufficient to maintain normal glucose and triacylglycerol levels for a long period. Such failure could be ascribed to their inability to increase the rate of neogenesis and of INGAP production.

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Introduction

The chronic administration of a sucrose-rich diet (SRD) to normal rats induces an insulin-resistant state characterized by moderate fasting hyperglycemia, increased triglyceride and free fatty acid (FFA) levels, normoinsulinemia and a modest increase in body weight (Cohen *et al.* 1977, Lombardo *et al.* 1996, Chicco *et al.* 2003). Islets isolated from these rats have a normal insulin content (Lombardo *et al.* 1996, Del Zotto *et al.* 2002), but their secretion pattern is characterized by the absence of the first peak and the enhancement of the second phase of insulin release in response to glucose (Chicco *et al.* 2003, Pighin *et al.* 2003). Islets from rats fed SRD for 9 months also had an increased content of triglyceride and reduced pyruvate dehydrogenase complex activity (PDHc) (Pighin *et al.* 2003). All these characteristics resemble those found in people with type 2 diabetes (Polonsky *et al.* 1996, Clark *et al.* 2001, Weir *et al.* 2001, Deng *et al.* 2004). After 6 months of SRD administration, the pancreas of these rats showed a significant increase in both number of islets

and β -cell area (Lombardo *et al.* 1996, Del Zotto *et al.* 2002).

The effect of SRD on glucose homeostasis and on pancreatic function and morphology has also been studied in hamsters (Del Zotto *et al.* 1999, 2000, Massa *et al.* 2001). In hamsters fed this diet for 12 months, serum glucose and triacylglycerol levels remained within normal range. Insulin secretion was enhanced *in vivo* and *in vitro*, and β -cell mass increased due to an increment in replication rate and islet neogenesis, while there were no changes in the rate of β -cell apoptosis. It is not clear as yet why rats and hamsters present such an uneven response to the increased insulin demand induced by sustained SRD administration.

In adult mammals, the mass of pancreatic β -cells undergoes dynamic changes to maintain serum glucose levels within normal range (Montanya *et al.* 2000), even under extremely different conditions such as pregnancy and obesity (Parsons *et al.* 1992, Milburn *et al.* 1995). The resultant β -cell mass depends on a subtle balance between cell growth and differentiation, and cell death

(Shafir *et al.* 1999, Bonner-Weir 2000). These processes are controlled by several transcription and humoral factors (Bonner-Weir & Smith 1994, Edlund 1998, Gradwohl *et al.* 2000, Jensen *et al.* 2000, Perfetti *et al.* 2000, McKinnon & Docherty 2001), probably including islet neogenesis-associated protein (INGAP) (Rosenberg *et al.* 1983, Flores *et al.* 2003, Gagliardino *et al.* 2003). Disruption of this balance may lead to an impairment of glucose homeostasis, such as the glucose intolerance developed with ageing, with a reduction in β -cell replication rate (Bonner-Weir 2000, Montanya *et al.* 2000). So far, it has not been established whether all the processes involved in this balance have either a similar or different importance in gaining a functional β -cell mass sufficient to maintain glucose homeostasis within the normal range.

In an attempt to answer this question, which is critical to understand the pathogenesis of type 2 diabetes and to develop appropriate strategies for the prevention and treatment of the disease, we have currently studied insulin secretion, volume density (Vvi), several indicators of islet neogenesis, and replication and apoptotic rate of β -cells, as well as the percentage of PDX-1- and INGAP-positive cells in normal Wistar rats fed SRD for 6 (SRD6) and 12 (SRD12) months. These results were then compared with those obtained previously in normal hamsters submitted to a similar dietary manipulation.

Our results show that SRD6 rats presented increased insulin secretion *in vitro* and β -cell Vvi, and this was ascribable to both an increase in β -cell replication rate and a decrease in apoptosis. However, all these functional and morphological changes were not observed in SRD12. The absence of increase in neogenesis rate and INGAP response might explain the limited capacity of the rat pancreas to cope with a sustained increased demand of insulin. The increased levels of glucose and triacylglycerol observed in SRD rats could also play a role in the mechanism (glucolipototoxicity) limiting the long-term reactive pancreas response.

Materials and Methods

Animals and diets

Normal male Wistar rats obtained from the National Institute of Pharmacology, Buenos Aires, Argentina (180–200 g body weight) were used. They were maintained in a temperature-controlled room at 23 °C, with a fixed 12-h light:12-h darkness cycle, and 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 semisynthetic SRD (63% w/w), while the control rats (CD) received the same semisynthetic diet but with starch instead of sucrose in the same proportion (63% w/w). Details of this procedure have been previously reported (Chicco *et al.* 1994). Both diets provided

approximately 15.28 kJ/g chow. The animals had free access to food and water and were maintained on their respective diets for 6 and 12 months.

The weight of each animal was recorded twice per week, while the individual caloric intake and weight gain of at least 10 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. Insulin levels were determined by radioimmunoassay (RIA) (Herbert *et al.* 1965), using an antibody against rat insulin, a rat-insulin standard (Linco Research, St Charles, MI, USA) and highly purified porcine insulin labelled with ¹²⁵I (Linde *et al.* 1980).

Insulin secretion in vitro

Groups of five islets isolated from pancreases of each experimental group by collagenase digestion (Lacy & Kostianovsky 1967) were incubated for 60 min at 37 °C in 0.6 ml of Krebs–Ringer bicarbonate (KRB) buffer, pH 7.4, previously gassed with a mixture of CO₂/O₂ (5%/95%) and containing 0.1% (w/v) bovine serum albumin and different glucose concentrations (0, 2, 4, 6, 8 and 16 mmol/l). At the end of the incubation period, insulin was measured in the medium by RIA (Herbert *et al.* 1965).

Immunohistochemical studies After removal of the whole pancreas, the fat tissue was carefully dissected away. Samples of the tail of the pancreas were then fixed in Bouin's fluid and embedded in paraffin wax; serial sections (5 μ m) were obtained from different levels of the blocks. Haematoxylin–eosin staining was used to assess the general structure of the pancreas. Each section from a given series was mounted on separate slides to stain adjacent sections for immunocytochemical identification of insulin-secreting cells (β -cells) and glucagon-, somatostatin- and pancreatic polypeptide-secreting cells (non- β cells). For this purpose, specimens were incubated with appropriate dilutions of our own guinea pig anti-insulin serum (1:20 000) and a mixture of the other

three rabbit antisera: antiglucagon (1:400), antipancreatic polypeptide (1:10 000) (both kindly provided by Novo Nordisk, Copenhagen, Denmark), and antisomatostatin (1:6000) (a gift from Dr S. Efendic, Department of Endocrinology, Karolinska Institute, Copenhagen, Denmark). The reaction was completed by the streptavidin–biotin complex method, with either peroxidase or alkaline phosphatase, together with carbazole and fast blue respectively as chromogens. Controls for serological specificity were made by preincubating a given antiserum with an excess of the corresponding hormone for 24 h at 4 °C.

Islet cell replication rate: double-immunolabelling studies

Islet cell replication rate was estimated by detecting proliferating cell nuclear antigen (PCNA; 1:4000, Sigma) by 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 performed double staining of the following pairs: a) β cells (insulin antibody) and PCNA (PCNA antibody), and b) non- β cells (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.

Indicators of islet neogenesis

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 a panspecific cocktail of antibodies against human CK clone AE1–AE3 (DAKO), 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, Burlingame, CA, USA) for 10 min in a 500 W microwave oven (Madsen *et al.* 1997). The number of CK-positive cells was expressed as the percentage of the total islet cells counted. We also estimated the relationship between the islets and duct cells, measuring the percentage of the total number of islets in close contact with the ducts (Bertelli *et al.* 2001).

Detection of PDX-1- and INGAP-positive cells

Sequential double staining for PDX-1 and INGAP detection in pancreatic cells was as follows. We first stained PDX-1 cells with the PDX-1 antibody (1:1200; kindly provided by Dr C. Wright, Department of Cell Biology, Vanderbilt University, Nashville, TN, USA), and revealed

them as described above, using carbazole as chromogen; the same section was then immunostained with the INGAP antibody (1:250), except that alkaline phosphatase and fast blue (Sigma) were used as chromogens. Then, the percentage of cells expressing separately or co-expressing these two factors was quantified within each subsector of the pancreas, that is, islet, extrainsular, and duct cells (no fewer than 1000 each). Furthermore, glucagon (fluorescein) and somatostatin (Texas red) were used to reveal co-expression of these hormones with PDX-1 and INGAP.

β -cell apoptotic rate: double-labelling studies

To identify apoptotic cells, we used the propidium iodide technique (Scaglia *et al.* 1997). Deparaffinised and hydrated sections were washed in PBS before incubation for 30 min in a dark, humidified chamber with a solution of propidium iodide (4 μ g/ml; Sigma) and ribonuclease A (100 μ g/ml; Sigma). Then, the sections pretreated with non-immune sera from rabbit diluted in Tris-buffered saline (pH 7.4) were incubated for 1 h with the 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 heavy and light IgG chains; Jackson Immuno Research Laboratories, Baltimore, MD, USA). After another washing with PBS, the sections were mounted in Tris–glycerol (pH 8.4) for analysis by fluorescence microscopy. Using this double labelling, we obtained β -cells surrounded by an immunofluorescent ring of α cells. A Zeiss Axiolab epifluorescence microscope equipped with an HBO50 mercury lamp, together with two different filters, was used to visualise autofluorescent labelling. For the quantitative evaluation of immunofluorescence, positively labelled apoptotic endocrine cells were counted under a $\times 40$ objective lens in sections obtained from different levels of the blocks. The number of apoptotic cells was expressed as the percentage of the total number of islet cells counted.

Morphometrical analysis The morphometrical analysis was performed by videomicroscopy with a Jenamed 2 Carl Zeiss light microscope and an RGB CCD Sony camera in combination with OPTIMAS software (Bioscan, Edmons, WA, USA). We were then able to obtain the area occupied by the endocrine pancreas, the exocrine pancreas, the total pancreas, β and non- β cells, and several ratios and relationships, as described in the Results section. We also estimated the number of islets per unit area (mm^2) and β -cell Vvi. In addition, the ratio of islet cell area to number of islet cells (β and non- β) was calculated to obtain cell size. 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.

Table 1 Clinical and serological changes induced by SRD feeding

Group	Body weight (g)	Glycaemia (mmol/l)	Triacylglycerol (mmol/l)	Insulin (μ U/ml)
CD6	470.0 \pm 13.0 ●	6.62 \pm 0.17 ●	0.47 \pm 0.04 ●	55.0 \pm 4.9
SRD6	556.0 \pm 8.0 ◆	8.11 \pm 0.14	1.57 \pm 0.18 ◆	50.8 \pm 5.8 ◆
CD12	491.0 \pm 9.0	6.50 \pm 0.20 ●	0.50 \pm 0.02 ●	58.8 \pm 0.2 ●
SRD12	520.0 \pm 11.0	8.30 \pm 0.20	2.10 \pm 0.02	23.0 \pm 2.0

Values represent the mean of four rats in each group \pm S.E.M.

● CD vs SRD (at either 6 or 12 months); ◆ SRD6 vs SRD12; ●◆ $P < 0.05$.

CD6 vs CD12, NS.

Statistical analysis

Data are presented as means \pm S.E.M. The statistical analysis was performed with paired and unpaired Student's *t*-test. A *P* value of < 0.05 was considered significant.

Results

At the time of death, body weight in SRD6 was significantly higher than in the corresponding CD group ($P < 0.05$), whereas such difference was not significant in SRD12 (Table 1).

Both serum glucose and triacylglycerol levels were also significantly higher in both SRD6 and SRD12 ($P < 0.05$), triacylglycerol levels being even higher in SRD12 (Table 1). Serum insulin levels were comparable in SRD6 and CD6, whereas they were significantly lower in SRD12 than in either SRD6 or CD12 rats ($P < 0.05$; Table 1).

Insulin secretion elicited *in vitro* by different glucose concentrations is shown in Fig. 1. The dose-response curve obtained with islets from SRD6 shows a shift to the left (Fig. 1a), thus suggesting a decreased β -cell glucose threshold for the glucose stimulus. Such an effect was no longer observed in islets isolated from SRD12 animals (Fig. 1b), excepting glucose at 8 mM; these islets also released less insulin in response to 16 mM glucose.

Sections from CD6 and SRD6 stained with haematoxylin-eosin revealed the general, normal histological pancreatic appearance. Furthermore, in both groups of animals, immunohistochemistry showed the usual topographic distribution of cells within the islets, with β -cells occupying the central zone and non- β cells located at the islet periphery. Conversely, pancreases from SRD12 rats showed striking changes in morphology, with islets disrupted by fibrous tissue.

SRD6 animals showed a significant increment in the number of pancreatic islets per unit area and in β -cell Vvi ($P < 0.02$), together with a 6.8-fold increase in β -cell replication rate (Table 2 and Fig. 2a and b); although to a lesser extent, non- β -cell Vvi was also larger in SRD than in CD animals (0.19 ± 0.03 vs 0.09 ± 0.01 ; $P < 0.02$).

Comparable values, however, were recorded in endocrine cell size (in μm^2 , CD vs SRD): β -cells, 121.48 ± 9.73 vs 118.35 ± 6.46 ; non- β cells, 97.55 ± 6.24 vs 95.59 ± 7.05 .

The number of apoptotic cells in the endocrine pancreas of SRD6 animals (Fig. 2k; Table 2) was three times lower than that counted in CD rats (Fig. 2j) ($P < 0.02$).

As an index of islet neogenesis, we measured the islet diameter, the percentage of CK-positive cells (Fig. 2g and h; Table 3), the number of islets in close contact with the ducts, the number of insulin-reacting ductal

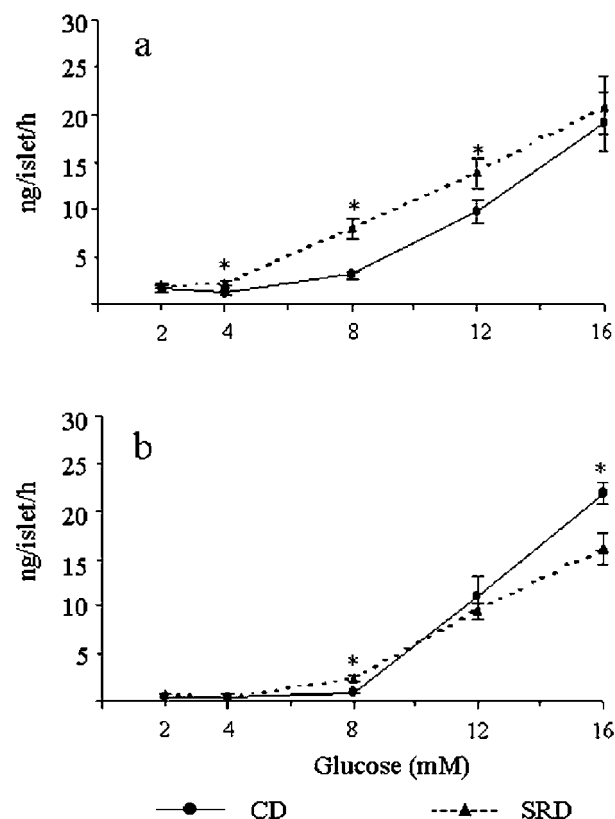


Figure 1 Insulin secretion: (a) CD6 vs SDR6 rats; (b) CD12 vs SDR12 rats. Each point in the line represents the mean of 15 cases from three different experiments \pm S.E.M. * $P < 0.05$.

Table 2 Morphometrical analysis of pancreases from 6- and 12-month SRD or CD rats

Group	Number of islets (mm ²)	β -cell Vvi (%)	PCNA index rate (%)	Apoptotic index rate (%)
CD6	2.0 \pm 0.2 ● ♣	0.4 \pm 0.2 ●	0.04 \pm 0.03 ●	4.3 \pm 1.1 ●
SRD6	3.3 \pm 0.1 ◆	1.0 \pm 0.1 ◆	0.30 \pm 0.10	1.4 \pm 0.5 ◆
CD12	3.0 \pm 0.1 ●	0.8 \pm 0.1 ●	0.10 \pm 0.08	4.9 \pm 0.5 ●
SRD12	1.8 \pm 0.1	0.5 \pm 0.07	0.40 \pm 0.20	13.5 \pm 0.5

Values represent the mean of four rat pancreases and three section levels \pm S.E.M.

●CD vs SRD (either at 6 or 12 months); ♣CD6 vs CD12; ◆SRD6 vs SRD12. In all cases, $P < 0.02$.

epithelium cells, and the percentage of islet PDX-1-positive cells (Fig. 2d and e; Table 3). None of these indicators showed significant changes in pancreases from SRD6 rats. The percentage of INGAP-positive cells in the islets was comparable in both groups of animals at this period (Fig. 2d and e; Table 3). We did not record either β -cells or PDX-1- and INGAP-positive cells at duct level at either 6 or 12 months. PDX-1-positive cells were also undetectable among acinar cells.

Data recorded in SRD12 animals were markedly different: there was a significant decrease in the number of islets ($P < 0.02$) and in β -cell Vvi ($P < 0.02$), attaining values close to those recorded in CD6 rats (Table 2). We also observed a non-significant increase in PCNA index rate (Fig. 2c and Table 2).

Comparable values were recorded in both groups of animals in non- β -cell Vvi (%) – CD vs SRD: 0.10 ± 0.01 vs 0.20 ± 0.03 ; $P < 0.02$. Similarly, islet cell-size values were also comparable in CD and SRD rats: β -cells, 129.48 ± 10.00 vs 121.35 ± 7.00 ; non- β cells, 98.00 ± 6.20 vs $96.00 \pm 7.10 \mu\text{m}^2$.

In contrast to what we found at 6 months, a significant increase in β -cell apoptotic rate was obtained in SRD12 rats ($P < 0.02$). This increase was more marked when values were compared with those recorded in SRD6 rats (Fig. 2l and Table 2).

In SRD12, at 6 months, the islet neogenesis indicators did not show significant changes, except for a marked reduction in the percentage of islet PDX-1-positive cells recorded in SRD rats ($P < 0.005$) (Fig. 2f and Table 3).

The number of INGAP-positive cells decreased significantly in SRD12 ($P < 0.02$) (Fig. 2f and Table 3). This decrease was striking, considering that the number of INGAP-positive cells was significantly higher in CD12 than CD6. PDX-1- and INGAP-positive cells were not present at acinar and ductal level (data not shown).

Discussion

Our current results show that the chronic administration of SRD to normal rats induces uneven sequential changes

both in insulin secretion and in the morphology of the endocrine pancreas.

It has been previously shown that the increased demand of insulin induced by different experimental procedures triggers a shift to the left in the *in vitro* dose–response curve profile of glucose-induced insulin release, in both rats and hamsters (Leahy 1996, Massa *et al.* 1997, 2001, Del Zotto *et al.* 1999). This effect could be partly ascribed to a change in the hexokinase:glucokinase ratio of islet cells (Leahy 1996, Massa *et al.* 2001), resulting in increased islet glucose metabolism (Massa *et al.* 2001). These results agree with the significantly higher second phase of insulin released by perfused islets in response to glucose reported in this animal model, as reported by Pighin *et al.* (2003). As described in that report, while insulin secretion *in vitro* was higher in SRD6 rats than in CD rats, their serum insulin levels were not. This apparent discrepancy between *in vitro* and *in vivo* results could be ascribed to the inhibitory effect of high levels of circulating fatty acids reported by our group (Pighin *et al.* 2003) and other authors as well (Zhou & Grill 1995, Chen & Reaven 1999). Consequently, the circulating levels of insulin in SRD6 rats were insufficient to cope with the increased peripheral demand of insulin, since these animals had significantly higher serum glucose and triacylglycerol levels than CD rats. These reactive changes in insulin secretion were no longer evident and even reversed at 12 months.

The fact that blood glucose levels are comparable in SRD6 and SRD12, and that insulin levels are lower in the former, would suggest that peripheral tissues have modified the sensitivity of SRD12 to insulin.

Our results indicate that normal rat islets can sustain the secretory overload elicited by the dietary-induced insulin-resistant state only for a limited period of time. Such limitation could be genetically determined, since normal hamsters submitted to a similar chronic SRD treatment can sustain this response for a significantly longer period (Del Zotto *et al.* 1999, 2000). On account of the enhancing effect of endogenous insulin upon insulin and glucokinase transcription (Leibiger *et al.* 2002), glucose metabolism in the islets and insulin secretion of normal

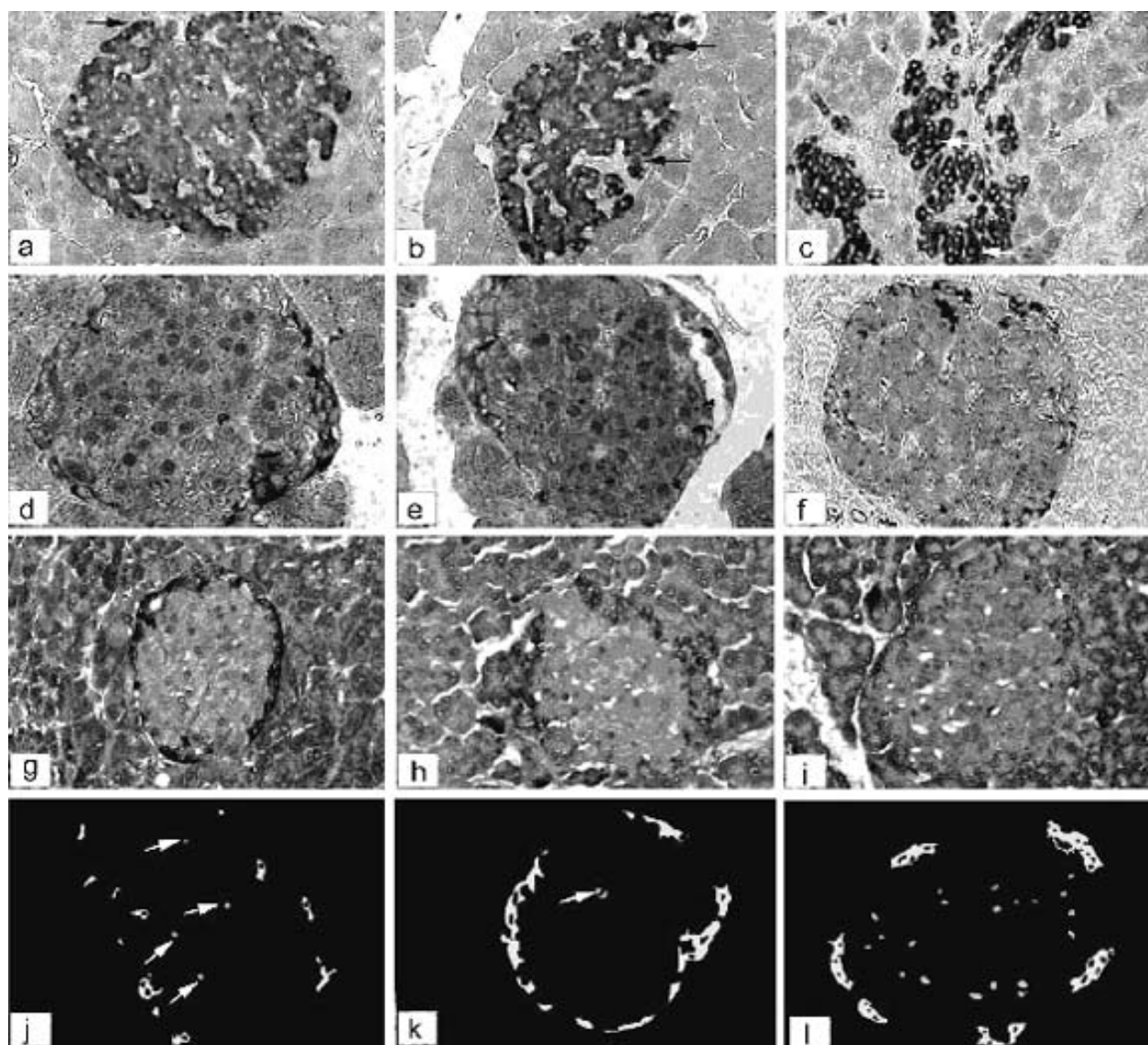


Figure 2 Pancreatic islets stained with different antibodies to show β and replication rate, neogenesis and apoptosis. Double immunolabelling: nuclei are stained with proliferating cell nuclear antibody (PCNA) (arrows), while the cytoplasm shows insulin immunostaining (a, CD6; b, SRD6; c, SRD12; $\times 100$); β -cell nuclei are stained with PDX-1 (carbazole) and the periphery with INGAP (fast blue) (d, CD6; e, SRD6; f, SRD12; $\times 200$); CK19-immunolabelled pancreatic islet; positive-reacting non- β -cells are seen in the periphery; counterstaining with haematoxylin (g, CD6; h, CD12; i, SRD12; $\times 100$). Pancreatic cells with glucagon-positive cells in the periphery (immunofluorescence); apoptotic nuclei (propidium iodide) corresponding to β cells are seen in the central zone of the islets (arrows) (j, CD6; k, SRD6; l, SRD12; $\times 100$).

hamsters (Borelli *et al.* 2003), the decreased release of insulin observed in SRD12 rats could establish a vicious negative circle contributing to the lower capacity of rats to cope with the increased demand of insulin.

The limited time-capacity of the rat pancreas to cope with a prolonged period of sustained insulin resistance is an important issue when we consider the implementation of prevention strategies. We and other authors have previously shown that replacement of sucrose by starch

(Chicco *et al.* 1999), administration of fish oil (Storlien *et al.* 1987, Lombardo *et al.* 1996, Soria *et al.* 2002) and troglitazone (Chicco *et al.* 2000) in the diet restored the altered pattern of serum lipids, insulin sensitivity, and glucose-induced insulin secretion in rats chronically fed a SRD. These results indicate that all these impaired functions are reversible and can be recovered when the animals receive the treatment in an appropriately timely manner. It remains to be demonstrated whether – and to

Table 3 Changes induced by SRD feeding upon indicators of islet neogenesis

Group	Islet diameter (μ m)	Cytokeratin islet index rate (%)	Islet in contact with ducts (%)	PDX-1+ islet cells (%)	INGAP+ islet cells (%)
CD6	160 \pm 5	11.6 \pm 3.1	40 \pm 5	58.3 \pm 1.3♣	0.12 \pm 0.01♣
SRD6	173 \pm 12	13.9 \pm 2.5	43 \pm 6	69.3 \pm 12.0♦	0.14 \pm 0.02
CD12	161 \pm 8	15.0 \pm 4.0	42 \pm 4	45.7 \pm 2.9●	0.20 \pm 0.03●
SRD12	169 \pm 4	12.8 \pm 5.0	41 \pm 5	32.4 \pm 3.2	0.10 \pm 0.01

Values represent the mean of four rat pancreases and three section levels \pm S.E.M.

●CD vs SRD (either at 6 or 12 months); ♣CD6 vs CD12; ♦SRD6 vs SRD12. In all cases, $P < 0.02$.

what extent – the same happens with the morphological SRD-induced islet cell alterations.

The pancreatic β -cell mass was also modified by the challenge of the increased demand of insulin induced by SRD administration (Lombardo *et al.* 1996, Del Zotto *et al.* 2002). We have currently reproduced the increase in β -cell Vvi observed in SRD6 rats reported by Lombardo *et al.* (1996). As occurred previously, this effect was due to an increase in the replication rate of these cells together with a marked decrease in their apoptotic rate (Del Zotto *et al.* 2002). In SRD12, however, the rate of PCNA was no longer increased and the apoptotic rate was significantly increased rather than decreased.

Apoptosis is a morphologically identifiable form of cell death triggered by a variety of metabolic stimuli, and it plays an important role in remodelling β -cell mass as the counterpart of proliferation (Wyllie *et al.* 1980, Steller 1995, Scaglia *et al.* 1997, Shafir *et al.* 1999). Thus, it has been shown that β -cell expansion can be offset by concomitant apoptosis (Pick *et al.* 1998). Hoorens *et al.* (1996) postulated that rat β -cell apoptosis is blocked by proteins whose synthesis is stimulated by glucose in a dose-dependent manner, while Donath *et al.* (1999) and Federici *et al.* (2001) showed that glucose increases this rate by triggering an 'on and off' expression of specific genes. The decreased rate of β -cell apoptosis recorded in SRD6 and its increase in SRD12 could be ascribed to these two opposite mechanisms triggered by glucose. These results suggest that changes in apoptosis could explain the increased/decreased β -cell Vvi measured at the two periods, but these changes could not provide β -cells functionally sufficient to maintain normal levels of serum glucose and triacylglycerol.

We assessed the possible contribution of islet neogenesis to increasing β -cell Vvi by the use of several indicators such as the presence of cytokeratins (CKs) (Bowens *et al.* 1994, Wang *et al.* 1995) and the increase in replication rate, number of insulin-labelled cells at the ductal compartment (Bowens & Klöppel 1996), proportion of small islets and of islets closely associated with the ductal epithelium (Bertelli *et al.* 2001), and percentage of PDX-1-positive cells (Sharma *et al.* 1999, Perfetti *et al.* 2000, Stoffers *et al.* 2000). None of these indicators showed

significant changes in SRD animals, thus suggesting that, in our rat experimental model, islet neogenesis was not involved in the mechanism responsible for the increment of β -cell Vvi.

Altogether, the reactive morphological changes, as well as insulin secretion, were time-limited and failed to overcome the sustained increased demand of insulin elicited by the chronic administration of SRD to normal rats.

We could observe substantial differences when we compared these data with those previously obtained in normal hamsters fed a SRD (Del Zotto *et al.* 1999, 2000, Massa *et al.* 2001). In these animals, both the shift in the dose-response curve (glucose stimulus) and the increase in β -cell Vvi and mass were sustained for up to 12 months. Another conspicuous difference was that hamsters showed an initial increase in islet neogenesis and in the number of INGAP-positive cells. In contrast to those observed in rats, the reactive changes detected in pancreas function and morphology in hamsters were sufficient to maintain fasting serum glucose and triacylglycerol levels within the normal range. We also found that the ratio β -cell mass/body weight was three times larger in hamsters than in rats (Del Zotto *et al.* 2000). We therefore assumed that in order to maintain normal serum glucose and triacylglycerol levels for a long period after SRD administration, islet neogenesis must be present to ensure a sufficiently functional β -cell mass. Accordingly, SRD rats cannot maintain those parameters within a normal range due to their inability to reassume islet neogenesis in adult life.

It has been claimed that INGAP stimulates islet neogenesis (Rosenberg *et al.* 1983, Rafaeloff *et al.* 1997). In addition, we have previously shown an increase in the number of INGAP-positive cells accompanying the increased neogenesis rate induced by the chronic administration of SRD to normal hamsters (Del Zotto *et al.* 2000). Furthermore, in newborn hamsters fed a SRD during pregnancy, we identified the presence of islet and ductal cells with high replication rate co-expressing PDX-1 and INGAP (Gagliardino *et al.* 2003). In the rat, therefore, the absence of islet neogenesis signs, accompanied by either no changes (SRD6) or a decrease (SRD12) in the percentage of INGAP-positive cells,

clearly contrasts with observations in the hamster, indicating a cause-effect relation rather than a simple coincidence. Although fully within the field of speculation, we might assume that the significant increase of INGAP-positive cells depicted in CD12 as compared with CD6 rats represents an effort of the rat pancreas to compensate for the low – or absent – neogenetic response to the INGAP stimulus (INGAP resistance?).

The moderate hyperglycaemia present in our rats and the altered glucose oxidation (decreased activity of the PDHc complex) recently reported in the same experimental model (Pighin *et al.* 2003) could represent an additional factor limiting the functional and morphological pancreas reaction. In fact, hyperglycaemia can impair the secretion of insulin (Leahy *et al.* 1986, Rossetti *et al.* 1987, Portha *et al.* 1988, Leahy 1996, Deng *et al.* 2004), the rate of β -cell replication and the degree of islet neogenesis (Maedler *et al.* 2001). Furthermore, it can also increase the rate of β -cell apoptosis (Donath *et al.* 1999, Federici *et al.* 2001, Maedler *et al.* 2001). The deleterious effect of glucose involves an impaired expression of genes related to glucose utilisation, β -cell replication and islet neogenesis (Weir *et al.* 2001, Laybutt *et al.* 2002). Hyperglycaemia, together with the increased triacylglycerol serum levels and the content of islet triglycerides (Pighin *et al.* 2003) – gluco-lipotoxicity – could affect all these processes by increasing the rate of oxidative stress (Sakuraba *et al.* 2002, Robertson *et al.* 2004).

In brief, our current results and those previously reported by our group (Del Zotto *et al.* 1999, 2000, Gagliardino *et al.* 2003) show that the response of two animal models to a similar β -cell function overload is different, and that this response would be genetically determined. Thus, the efficacy of the mechanisms that attempt to control an increased demand of insulin induced by similar experimental procedures can vary, and these mechanisms may cause either a compensatory hyperinsulinaemic–normoglycaemic state or a diabetic state. In this context, the development of neogenesis would play a key role in obtaining a successful sustained adaptation to the increased demand of insulin. The concomitant absence of increase in the number of INGAP-positive cells and lack of a neogenetic reaction would support the idea of a stimulatory effect of this peptide upon neogenesis (Rosenberg *et al.* 1983, Rafaeloff *et al.* 1997, Del Zotto *et al.* 2000, Gagliardino *et al.* 2003). When neogenesis is absent, at least in our model, the other pancreatic reactive mechanisms cannot ensure such adaptation. At present, therefore, either removing or decreasing the insulin demand would be the most reasonable strategy to prevent diabetes manifestations in cases where neogenesis is not present. The fact that administration of fish oil to SRD rats improves pancreatic damage and metabolic homeostasis (Pighin *et al.* 2003) lends further support to this assumption. Further studies using models similar to the one currently used may help to determine the molecular

mechanisms conditioning either the success or the failure of the pancreatic compensatory response to a functional overload.

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