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Age-dependent reduction in GLUT-2 levels is correlated with the impairment of the insulin secretory response in isolated islets of Sprague–Dawley rats

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Abstract

In this study we have investigated the insulin secretory response to glucose and other secretagogues (2-ketoisocaproate, 3-isobutyl-1-methyl-xanthine and arginine) of pancreatic islets isolated from Sprague–Dawley rats of various ages (from 2 to 28 months). Our results showed a significant decline in the glucose-stimulated insulin secretion, starting at 12 months of age. On the other hand, the response to non-glucose secretagogues (and mainly to 2-ketoisocaproate) was less impaired with advancing age than that to glucose. We also observed a progressive age-related decline of protein levels of the glucose transporter GLUT-2 in pancreatic islets, which was temporally concomitant and quantitatively comparable with the β-cell alteration in glucose responsiveness (−40/50%). Finally, we observed a significant increase of the islets insulin content in older rats with respect to younger animals. We conclude that in the islet of older rats the impaired capability to respond to glucose could be dependent, at least in part, on the age-dependent reduction in GLUT-2 and could be compensated by mechanisms including a preserved responsiveness to non-glucose secretagogues and/or the development of islet hypertrophy.

Keywords: Aging; Insulin secretion; GLUT-2; Isolated islets

1. Introduction

Normal aging is usually associated with a progressive deterioration in most endocrine functions that may be responsible of serious disturbances of metabolic homeostasis.

Actually, an impairment in carbohydrate tolerance is a well known feature of aging in

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both humans and experimental animals (Berger et al., 1978; De Fronzo, 1984; Furnsinn et al., 1991), although the underlying mechanisms have not been completely elucidated.

Due to the key role played by pancreatic hormones in the maintenance of glucose homeostasis, the influence of aging mainly on insulin secretion has been extensively investigated. However, most of the in vivo studies have provided conflicting results, due to both difficulties in measuring beta cell insulin secretion directly, and interference of concomitant confounding factors, such as chronic diseases, obesity and physical activity (Coordt et al., 1995). On the other hand, several in vitro studies using islets isolated from animals of different ages have shown that the amount of insulin secreted, mainly in response to glucose, decreased with advancing age (Reaven et al., 1979; Tsuchiyama et al., 1991; Perfetti et al., 1995), but some other investigators showed no age-dependent changes (McDonald, 1990; Starnes et al., 1991; Ruhe et al., 1992).

The aim of the present study was to expand knowledge about this relevant topic by investigating the effects of a number of insulin-releasing substances, known to act through different mechanisms, on the secretory capabilities of pancreatic islets isolated from Sprague–Dawley rats of various ages from 2 up to 28 months, which is the median life span of this strain. Thus, the influence of both development and senescence on insulin secretory capabilities could be evaluated in correlation with a number of physiological parameters, such as body and pancreas weight, circulating glucose and insulin levels, pancreatic and islet insulin content. Finally, we measured the age-related variations of the levels of the glucose transporter GLUT-2 in pancreatic islets, to test a major possible mechanism of the impairment of islet function observed during aging, which has not been investigated so far.

2. Materials and methods

2.1. Animals

Male Sprague–Dawley rats were purchased from Harlan–Nossan, Milan, at 8 weeks of age and housed thereafter in the animal facility of our Department under constant conditions of temperature (24–25°C) and artificial lighting (12 h light-dark cycle), until used for the study. Tap water and standard laboratory chow were given ad lib. All experiments were conducted on non-fasting animals and started between 9:00 to 11:00 a.m. The "Principles of laboratory animal care" (NIH publication No. 83-25, revised 1985) were followed, as well as all the recommendations of the Italian law for the use of experimental animals (DL No. 116, 1992).

2.2. Isolation and incubation of islets

Rats of various ages were anaesthetized with an intraperitoneal (i.p.) injection of 50 mg/kg b.w. pentobarbital sodium. For each age-group, the pancreases of three to four animals were carefully removed, trimmed free of adipose tissue, blotted on filter paper and individually weighed. Pancreases were then pooled and minced in Hank's solution to obtain the isolated islets used for a single incubation experiment. Two separate incubation experiments were carried out for rats of 2, 12, 18, and 24 months of age. Islets were

isolated by a modification of the method ofLacy and Kostianovsky (1967), according to the suggestions of Trueheart Burch et al. (1984), who warned against the utilization of too harsh procedures for isolation of islets from older rats, to prevent damage. In particular, the pancreatic minces from both young and old rats were subjected to collagenase digestion (2 mg/ml), carried out in test tubes by vigorous manual shaking at 37°C for 6 or 7 min depending on the degree of digestion as judged by inspection. Occasionally, the digests of pancreases from old donors required a few additional shakings. The digests were washed four times with cold Hank's solution and islets were hand-picked under stereomicroscope observation.

Batches of 7 to 10 islets were placed in each incubation well, very small or very large islets being discarded as were those surrounded by undigested material, found more frequently in digests from old animals. The overall time required for the isolation was slightly more than one hour, on average. After a 60-min preincubation period in modified Krebs–Ringer bicarbonate (KRB) buffer containing 0.5% BSA, 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (Hepes) and 2.8 mM glucose (pH 7.4), islets were incubated for 60 min at 37°C in a humidified atmosphere of 5% CO_2 in air, in 1 ml KRB-Hepes buffer, in the presence of 2.8 mM glucose ^ or 20 mM 2-ketoisocaproate (2-KIC), or 16.7 mM glucose ^ 20 mM arginine or 1 mM 3-isobutyl-1-methyl-xanthine (IBMX) or 20 mM 2-ketoisocaproate. At the end of this incubation, the medium was collected for insulin determination. Finally, 1 ml of cold acidified ethanol (150:47:3 v/v absolute ethanol: H_2O: concentrate HCl) was added to the islets to extract their insulin content.

2.3. Measurement of GLUT-2 content in pancreatic islets

To obtain the high number of islets required for GLUT-2 quantitation from a single animal, a different procedure was followed. After cannulation of the pancreatic duct, collagenase was injected at a concentration of 1.5 mg/ml in 10 ml Hank's–Hepes buffered saline (HHB) containing 0.55 g/l of glucose. The dilated pancreas was removed and incubated at 37°C for 15 min, in 10 ml HHB in water bath. At the end of this period, the pancreas was disrupted by passing it through syringe 6×. Sequential washes with HHB with BSA and filtration through a strainer were performed, essentially as described by Sutton et al. (1986). Islets were purified by a density gradient procedure, the digest from one rat was resuspended in 10 ml of cold Hystopaque 1077; overlayered with 10 ml HHB-BSA and centrifuged at 900× g for 10 min at 10°C. The islets were collected from the interface and washed in HHB-BSA. At this point, the islets were handpicked under a dissecting microscope.

Five to six hundred isolated islets were sonicated in 200 μl buffer (20 mM Hepes, 1 mM EDTA, 250 mM sucrose, pH 7.4). Aliquots containing the same amount of protein were solubilized in Laemmli buffer (Laemmli, 1970) containing 2% SDS and electrophoresed on a 10% polyacrylamide resolving gel. Separated proteins were electrophoretically transferred to nitrocellulose sheets (0.45 μm), which subsequently were blocked overnight at 4°C with 5% powdered dry milk in PBS (pH 7.4) containing 0.02% sodium azide. Nitrocellulose sheets were incubated with a saturating concentration of a polyclonal anti-GLUT-2 antibody (Biogenesis, UK) at a dilution 1:200 in PBS containing 1% powdered

milk for 60 min at 37°C. After three washes in PBS containing 1% Triton X-100 for 20 min at 22°C, nitrocellulose sheets were incubated with ^{125}I-labeled protein A (0.25 μCi/ml) in PBS containing 1% powdered milk for 60 min at 37°C. Then, the sheets were washed again, air dried and autoradiographed with Kodak X-Omat AR films and DuPont intensifying screens at-70°C for 48 h. To determine the amount of ^{125}I associated with GLUT-2, immunolabeled bands from the nitrocellulose sheets were excised and counted in a gamma counter. Results were corrected for background radiation.

2.4. Total insulin content of the pancreas

Fragments of the pancreas removed before islet isolation were weighed and homogenized in cold acid-ethanol mixture for extraction of insulin, as detailed elsewhere (Bergamini et al., 1991).

2.5. Assays

Plasma glucose was measured by the glucose-oxidase method using commercially available kits (Glucinet, Sclavo Diagnostici, Siena, Italy).

Plasma insulin, that released into the buffer and that extracted from the islets at the end of the incubation period(s), were measured by radioimmunoassay according to Herbert et al. (1965), using rat insulin as a standard. The minimum detectable insulin level was 0.13 ng/ml in the assay, and the intra-assay and inter-assay variations were 3.0% and 10.5%, respectively.

Protein content of sonicated islets samples was quantitated with Bradford's method (Bio–Rad, Richmond, CA, USA) using BSA as standard (Bradford, 1976).

2.6. Chemical reagents

Arginine, 2-KIC and IBMX were purchased from Sigma, St. Louis, MO, USA. Collagenase used for islet digestion was obtained from Serva Feinbiochemica GMBH, Heidelberg, FRG. ^{125}I-insulin was kindly provided by professor R. Navalesi, Cattedra di Malattie del Ricambio, University of Pisa. All reagents were of analytical grade.

2.7. Expression of results and statistical analysis

Data are given as means ^ SEM. Statistical significance was evaluated by ANOVA, followed by the two-tailed unpaired Student's *t*-test as a method of post-hoc analysis to assess two-by-two differences, when appropriate. A P value of <0.05, at least, was considered as significant.

3. Results

Body weight and pancreas weight showed parallel age-dependent changes, increasing rapidly from 2 to 6 months of age and slowly between 6 and 18 months when maximum weights were achieved, with a subsequent decrease in senescent animals (Table 1). Thus, the ratio of pancreatic to body weight was approximately the same at different ages, from 2

Table 1
Effect of age on body and pancreas weights and on plasma glucose and insulin levels in Sprague–Dawley rats of various ages (Results are expressed as means ^ SEM of 4–12 observations according to the number of rats indicated for each age subgroup. Statistical analysis (one-way ANOVA): the effect of age was significant for body weight ($P < 0.01$), pancreas weight ($P < 0.01$), plasma insulin ($P < 0.05$), not significant for plasma glucose.)

Age (months)	body weight (g)	Pancreas weight (mg)	Plasma glucose (mg/dl)	Plasma insulin (ng/ml)
2 (n = 12)	263 ^ 9	576 ^ 38	128 ^ 2.2	3.6 ^ 1.0
6 (n = 4)	545 ^ 14	1190 ^ 50	122 ^ 10	4.5 ^ 0.7
12 (n = 7)	626 ^ 18	1321 ^ 54	120 ^ 4	4.9 ^ 0.8
18 (n = 6)	739 ^ 16	1684 ^ 70	125 ^ 4	5.3 ^ 0.5
24 (n = 10)	590 ^ 14	1196 ^ 92	125 ^ 9	8.3 ^ 0.9
28 (n = 4)	600 ^ 29	1076 ^ 47	128 ^ 10	5.9 ^ 1.3

to 24 month-old rats, slightly decreasing in 28-month-old animals. As also reported in Table 1, no age-related differences were observed for plasma glucose concentrations in these animals throughout life, whereas plasma insulin levels showed a significant change with age ($F = 4.52$, $P < 0.01$), progressively increasing from 2 to 24 months of age and partially declining from 24 to 28 months.

Fig. 1 shows insulin secretion from islets isolated from rats of different ages in the

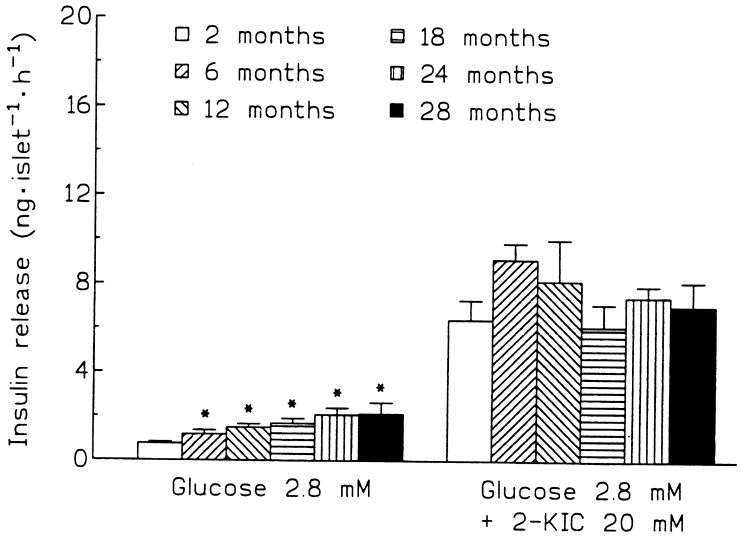

Fig. 1. Insulin release from pancreatic islets isolated from Sprague–Dawley rats of various ages and incubated in the presence of 2.8 mM glucose and 20 mM 2-ketoisocaproate (2-KIC). Two separate incubation experiments were carried out for 2-, 12-, 18-, and 24-month-old subgroups. Islets were isolated from three to four pooled pancreases for each age and experiment. Means ^ SEM of 6 to 12 observations are given. Statistical analysis: one-way ANOVA showed that the effect of age on insulin release was significant ($F = 5.05$, $P < 0.01$) for 2.8 mM glucose; not significant ($F = 0.85$, NS) for 2.8 mM glucose + 2-KIC. *$P < 0.05$ versus 2-month-old rats (Student's *t*-test).

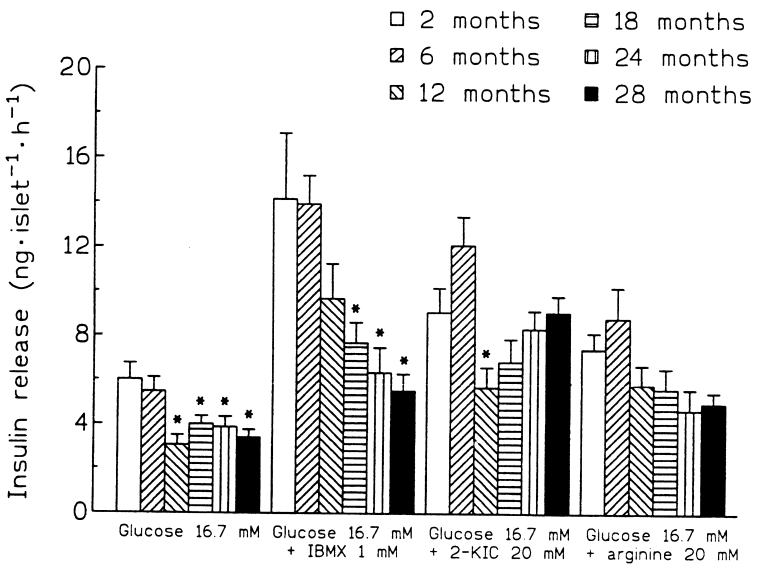

Fig. 2. Insulin release from pancreatic islets isolated from Sprague–Dawley rats of various ages and incubated in the presence of the indicated secretagogues. Two separate incubation experiments were carried out for 2-, 12-, 18-, and 24-month-old subgroups. Islets were isolated from 3–4 pooled pancreases for each age and experiment. Means ^ SEM of 6 to 12 observations are given. Statistical analysis: one-way ANOVA showed that the effect of age on insulin release was significant for 16.7 mM glucose alone ($F = 4.88$, $P < 0.01$) or in combination with IBMX ($F = 6.30$, $P < 0.01$) or 2-KIC ($F = 3.89$, $P < 0.01$). *$P < 0.05$ versus 2-month-old rats (Student's *t*-test).

presence of non-stimulating (2.8 mM) glucose concentrations with or without 2-ketoisocaproate (2-KIC), the deamination product of leucine, which enhances mitochondrial metabolism. Basal insulin release progressively increased with increasing age ($F = 5.05$, $P < 0.01$). Upon stimulation with 2-KIC, a clear-cut increase of insulin secretion was observed at all ages, with no significant difference among age-groups ($F = 0.85$, NS). However, the incremental increase over basal secretion was remarkable (7–8-fold) in 2- and 6-month-old rats, and limited (2–3-fold) in older animals, due to the higher basal secretion in the latter.

Fig. 2 shows the effects of high glucose concentrations alone or combined with 2-KIC, IBMX (which increases intracellular cAMP) or arginine (which probably acts through membrane depolarization due to its cationic charge). In the islets of 2- and 6-month-old animals insulin release was stimulated by 16.7 mM glucose (6–7-fold increase over basal), and further increased by 1 mM IBMX, 20 mM 2-KIC or 20 mM arginine, as expected (Fig. 2). In the islets of 12-, 18-, 24-, and 28-month-old rats, glucose-stimulated insulin release moderately increased over basal values (2-fold in the average), attaining significantly lower values than in 2-month-old controls ($P < 0.05$, post-hoc Student's *t*-test). In these aging animals, both IBMX and 2-KIC were able to potentiate significantly the glucose-stimulated insulin release (as shown by two-way ANOVA), with the difference that the responsiveness to IBMX and that to 2-KIC showed a tendency to decrease and increase, respectively, with advancing age. Actually, upon stimulation with 2-KIC at

Table 2
Pancreatic and islet insulin content in Sprague–Dawley rats of various ages (Results are expressed as mean ^ SEM of the number of observations indicated in parentheses. Statistical analysis (one-way ANOVA): the effect of age was significant ($P < 0.01$) for islet insulin content, not significant for pancreas insulin content.)

Age (months)	Pancreas insulin content (μg/mg)	Islet insulin content (ng/islet)
2	220 ^ 30 (12)	108 ^ 3.0 (70)
6	270 ^ 38 (4)	147 ^ 5.0 (50)
12	212 ^ 40 (7)	225 ^ 6.0 (35)
18	220 ^ 30 (5)	242 ^ 6.2 (40)
24	205 ^ 36 (10)	225 ^ 14.3 (30)
28	290 ^ 39 (4)	285 ^ 3.8 (30)

high glucose, insulin release from islets of 18-, 24-, and 28-month-old rats was not significantly different from that of 2-month-old animals (post-hoc Student's *t*-test).

It should also be noticed that the pancreatic insulin concentrations remained substantially stable through lifespan ($F = 1.54$, NS), whereas the insulin content of isolated islets approximately doubled in 12-, 18-, 24-, and 28-month-old rats with respect to 2-month-old animals ($F = 8.83$, $P < 0.01$) (Table 2).

Immunoblotting of islet homogenates, performed using a polyclonal anti-GLUT-2 antibody, detected a protein of the expected apparent molecular weight (55 000 M_r); no corresponding signal was observed using 10 μg of skeletal muscle homogenate as

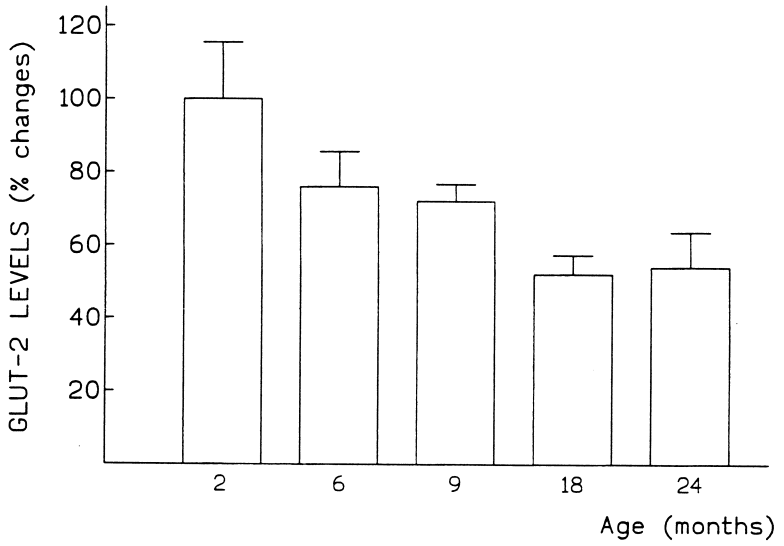

Fig. 3. Changes in GLUT-2 protein levels in pancreatic islets isolated from Sprague–Dawley rats of various ages. Data are expressed as % changes with respect to the levels found in 2-month-old rats, assumed as 100%. Mean ^ SEM of 4 to 5 observations are given. Statistical analysis: one-way ANOVA showed a significant effect of age on islet GLUT-2 protein levels ($F = 7.88$, $P < 0.01$).

negative control (data not shown). Quantitative analysis of immunoblotting transfers showed that GLUT-2 protein levels decreased approximately by 25% in islets of 6- and 12-month-old rats and by 50% in those of 18- and 24-month-old animals with respect to 2 month-old rats (Fig. 3).

4. Discussion

This study was aimed at exploring the age-related changes in insulin secretion of pancreatic islets isolated from Sprague–Dawley rats in a large range of ages (from 2 to 28 months). Our results show that, at least in this particular rat strain, aging is actually associated with significant alterations of the secretory performance of isolated islets, mainly in response to glucose stimulation.

Firstly, it was observed that the islets of Langerhans from aging rats have progressively higher basal insulin secretory rates than those of younger rats, which could be related to a possible increase in constitutive release at non-stimulating glucose concentrations or to metabolic abnormalities such as glycogen-derived endogenous glucose production by β-cells (Malaisse et al., 1977).

Secondly, a significant decline in the insulin secretion stimulated by 16.7 mM glucose occurs in the isolated islets of aging rats, starting at 12 months of age, with little additional changes, if any, at older ages. Therefore, our data seem to point that the onset of the age-related impairment of pancreatic endocrine function should be timed by the age of 12 months, in agreement with previous reports on insulin action (Dardevet et al., 1994), insulin secretion (Bombara et al., 1995), and glucose transport (Gulve et al., 1993).

It should also be reminded that results on this topic are not consonant in the literature. Indeed, it has been reported that total glucose-stimulated insulin secretion was unchanged (McDonald, 1990; Starnes et al., 1991; Ruhe et al., 1992), impaired (Reaven et al., 1980;Chaudhuri et al., 1983; Bergamini et al., 1991; Tsuchiyama et al., 1991; Perfetti et al., 1995), or even increased (Trueheart Burch et al., 1984; Leiter et al., 1988) with increasing age of the animals. This inconsistency of the results obtained so far may be attributed to many factors, like differences in the species or strain of the animals, in the ages studied or in the technique used (isolated islets or perfused pancreas).

Interestingly, our data demonstrate that in isolated islets, whereas an early defect of glucose-stimulated insulin secretion occurs, the response to non-glucose secretagogues and mainly to 2-KIC, which is also effective in the absence of glucose, seems less impaired with advancing age. This could help to maintain glucose homeostasis in the aging animals.

Several hypotheses have been raised to explain the observed defect of glucose-induced insulin release in the islets of aging rats, such as e.g. a reduced glucose diffusion (Molina et al., 1985) and/or oxidation (Reaven and Reaven, 1980) as well as the impairment of a more distal step in the stimulus-secretion coupling (Lipson et al., 1981; Goodman et al., 1986). Because the insulinotropic effect of 2-KIC, which is solely metabolized into mitochondria (Lembert and Idahl, 1998) and thus should bypass glycolytic events, is quite well preserved in aging, it is likely that the main defect could be localized at the level of glucose sensing mechanisms. We hypothesize that the observed deficiency might be related to changes in the levels of GLUT-2 protein, the high-capacity and low-affinity

facilitative glucose transport system present in the β cell. Indeed, we found that GLUT-2 protein levels were progressively reduced during aging and this reduction seemed to be temporally and quantitatively related to the impairment in glucose-stimulated insulin secretion. As far as we know, there is no previous report on age-related changes in islet GLUT-2 protein levels, whereas GLUT-2 mRNA levels have been previously explored during aging and found decreased in mice (Perfetti et al., 1996) and Wistar rats (Wang et al., 1997) and increased in Fischer 344 rats (Giddings et al., 1995). A reduced expression of islet GLUT-2 has been observed in animal models of experimental diabetes and suggested to be an etiological factor in diabetes by impairing glucose uptake and metabolism (Unger, 1990; Efrat et al., 1994). Furthermore, transgenic mice expressing GLUT-2 antisense RNA in β-cells, where GLUT-2 levels were reduced by 80%, became diabetic (Valera et al., 1994). On the basis of some experimental evidence (Ferber et al., 1994), it has also been suggested that GLUT-2 might have functions other than glucose transport, being implicated in the induction of genes encoding metabolic enzymes such as glucokinase (Prentki, 1996).

Our data on islet insulin content in aging rats are in agreement with those obtained by Gold et al. (1981) and by Draznin et al. (1985) and are also consistent with the changes in islet volume reported by Adelman (1989). The increase of islet insulin content, usually considered an index of β-cell hypertrophy and/or hyperplasia (Jahr et al., 1978), may represent a major mechanism by which islets compensate their loss in secretory effectiveness during aging, remaining substantially able to match insulin release with the metabolic requirements in basal conditions (Nacher et al., 1998).

As an additional comment, we may note that from the comparison of insulin content in the whole pancreas (unchanged with age) and in isolated islets (increased with age) it could be also argued that the total number of islets is probably decreased during aging. A significant hyperinsulinemia occurs in 24-month-old rats in the presence of normal concentrations of blood glucose, suggesting some reduction in peripheral insulin sensitivity. Although we did not perform a glucose tolerance test in the present study, we have previously demonstrated a decreased tolerance to a glucose oral challenge in aging rats of this strain (De Tata et al., 1993).

In conclusion, our results confirm that in Sprague–Dawley rats aging is characterized by a functional alteration of pancreatic beta cells that mainly regards their responsiveness to glucose and is associated with reduction in islet GLUT-2 protein levels. However, this impaired capacity to respond to the main physiological stimulus for insulin secretion may be compensated by several mechanisms such as preserved responsiveness to other secretagogues and/or development of islet hypertrophy.

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