Effect of aging on islet beta-cell function and its mechanisms in Wistar rats

Zhaoyan Gu · Yingzhen Du · Yu Liu · Lichao Ma ·
Lin Li · Yanping Gong · Hui Tian · Chunlin Li

Received: 23 March 2011 / Accepted: 24 August 2011 / Published online: 7 September 2011
© American Aging Association 2011

Abstract Type 2 diabetes mellitus is characterized by islet β -cell dysfunction and its incidence increases with age. However, the mechanisms underlying the effect of aging on islet β -cell function are not fully understood. We characterized β -cell function in 4-month-old (young), 14-month-old (adult), and 24-month-old (old) male Wistar rats, and found that islet β -cell function decreased gradually with age. Old rats displayed oral glucose intolerance and exhibited a decrease in glucose-stimulated insulin release (GSIR) and palmitic acid-stimulated insulin release (PSIR). Furthermore, total superoxide dismutase (T-SOD), CuZn superoxide dismutase (CuZn-SOD), and glutathione peroxidase (GSH-Px) activity decreased, whereas serum malondialdehyde (MDA) levels increased in the older rats. Moreover, we detected a significant reduction in β -cell proliferation and an increase in the frequency of apoptotic β -cells in the islets of rats in the old group. Finally,

Anx1 expression in the islets of old rats was significantly upregulated. These data provide new insights into the development of age-related β -cell dysfunction in rats.

Keywords Aging · Insulin resistance · Islet secretion function · Rats

Abbreviations

GSIR	Glucose-stimulated insulin release
PSIR	Palmitic acid-stimulated insulin release
GIR	Glucose infusion rate
CV _{BG}	Blood glucose coefficient of variation
CV _{GIR}	GIR coefficient of variation
OGTT	Oral glucose tolerance test
IRT	Insulin release test
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
PCNA	Proliferating cell nuclear antigen
FBG	Fasting blood glucose
BG peak time	Time when blood glucose level was at peak
BG peak	Value of blood glucose level at peak
OGTT2hPG	Plasma glucose at 120 min post-oral glucose
AUCg	Area under the curve for glucose throughout the 120 min period

Z. Gu and Y. Du made equal contributions to this study.

Z. Gu · Y. Du · Y. Liu (✉) · L. Ma · L. Li · Y. Gong ·
H. Tian · C. Li (✉)
Department of Geriatric Endocrinology,
PLA General Hospital,
Beijing, China
e-mail: liuyu1227@yahoo.com.cn
e-mail: lcl301@yahoo.com.cn

FINS	Fasting blood insulin
INS peak time	Time when blood insulin level was at peak
INS peak	Value of blood insulin level at peak
AUCi	Area under the curve for insulin throughout the 120-min period
AUCi/g	Area ratio under the curve for insulin/glucose
$\Delta I_{10}/\Delta G_{10}$	$\Delta I_{10}/\Delta G_{10} = (10 \text{ min insulin value} - 0 \text{ min insulin value}) / (10 \text{ min glucose value} - 0 \text{ min glucose value})$ (Alvarsson et al. 2005)

Introduction

Glucose tolerance progressively declines with age, and the incidence of impaired glucose tolerance (IGT) and type 2 diabetes mellitus (T2DM) is increased in the elderly (DeFronzo 2004; Alvarsson et al. 2005; Tfayli and Arslanian 2009). As a result of economic development and improvements in life expectancy, the prevalence of diabetes is rapidly growing in China (Yang et al. 2010). For example, an epidemiological study demonstrated that the peak prevalence of T2DM and IGT occurred in people 60–64 years of age (Pan et al. 1996). Aging is associated with an increased incidence of diabetes and IGT (Harris et al. 1998; Resnick et al. 2000). The increase in insulin resistance and decrease in insulin secretion that occurs during the aging process is believed to underlie age-related hyperglycemia (DeFronzo 1981; Chang and Halter 2003). However, it is unclear how these dynamic changes in β -cell function, glucose tolerance, and the development of insulin resistance occur during the progression of aging in a relatively “healthy” population.

Blood glucose metabolism is regulated primarily by insulin, which is secreted by the pancreatic islet β -cells, and the development of IGT is associated with β -cell dysfunction. Previous studies have shown that impaired insulin secretion underlies the increase in insulin resistance in the elderly (Chang and Halter 2003; Scheen 2005; Basu et al. 2003). However, the mechanisms underlying the age-related decline in β -cell glucose-stimulated insulin release (GSIR) are unknown. Previous studies have suggested that oxidative stress-related β -cell apoptosis and the

inhibition of proliferation are associated with β -cell dysfunction and the development of T2DM (Kiraly et al. 2010; Harmon et al. 2009). Recent studies have shown a reduction in β -cell proliferation and increased sensitivity to apoptosis triggers in elderly individuals (Rankin and Kushner 2009; Maedler et al. 2006; Tschen et al. 2009; Krishnamurthy et al. 2006). Additionally, alterations in gene regulation and expression are related to the aging process (Ihm et al. 2007; Lavrovsky et al. 2000). The goal of the present study was to characterize the function of islet β -cells during the natural aging process with the aim of elucidating the factors that underlie the development of age-related β -cell dysfunction. We investigated systemic oxidative stress, proliferation, and apoptosis in the islet β -cells, and gene expression in the islets in rats of different ages.

Materials and methods

Animals, maintenance and blood sampling

Male Wistar rats were purchased from Vital River laboratory animals (Beijing, China) and maintained in a specific-pathogen free facility in the animal center of the PLA general hospital, Beijing. All rats were fed standard laboratory chow (containing 5% fat). The experimental protocol was approved by the Animal Research Protection Committee of the PLA General Hospital.

Rats at 4, 14, or 24 months of age were placed in the young, adult, or old group, respectively. The rats were fasted overnight and blood samples were obtained from the inner canthus under ether anesthesia. Individual blood samples were conglutinated overnight at 4°C and centrifuged at 1,000 rev/min for 10 min for preparation of sera. The concentrations of serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-CH), low-density lipoprotein-cholesterol (LDL-CH), and free fatty acids (FFA) in individual sera were measured using a routine oxidase method.

Hyperinsulinemic–euglycemic clamp

Individual insulin sensitivity was determined using the hyperinsulinemic–euglycemic clamp (EHC) assay during a 30-min basal and then a 120-min EHC

period (Kraegen et al. 1983). Briefly, rats were fasted overnight and administered intravenous insulin ($12 \text{ mU kg}^{-1} \text{ min}^{-1}$) to decrease blood glucose to 0.5 mmol/l during the basal period. Subsequently, the animals received a continuous infusion of intravenous insulin ($4 \text{ mU kg}^{-1} \text{ min}^{-1}$) while they were infused with an increasing rate of 5% glucose during the EHC period. The blood glucose concentration was monitored every 5 min to adjust the glucose infusion rate (GIR) to maintain blood glucose at $4.4\text{--}5.5 \text{ mmol/l}$. The GIR, which reflects the sensitivity of individual rats to insulin, blood glucose coefficient of variation (CV_{BG}), and GIR coefficient of variation (CV_{GIR}) were calculated between 60 and 120 min.

Oral glucose tolerance test and insulin release test

Rats were fasted overnight and orally administrated glucose (2 g/kg body weight). Blood samples were collected from the angular vein of individual rats, and the concentrations of blood glucose and insulin were measured at approximately 0, 10, 30, 60, and 120 min post-glucose challenge.

Glucose- and palmitic acid-stimulated insulin release in vitro

Rats were fasted overnight and their pancreatic islets were isolated using the collagenase digestion method (Maedler et al. 2001; Ihm et al. 2007; Gotoh et al. 1985). Briefly, individual rats were anesthetized, and their common bile duct was clamped near the major duodenal papilla and the end of the common hepatic duct. Subsequently, approximately 10 ml of cold Hanks' solution containing 0.5 mg/ml of collagenase V (Sigma, St. Louis, MO, USA) was injected using a 25-gauge hypodermic needle near the hepatopancreatic ampulla, which allowed the Hanks' solution to perfuse the entire pancreas through the pancreatic duct. The distended pancreas was then gently dissected out and digested with collagenase at 4°C for 10 min with gentle shaking. Digestion was stopped using fetal bovine serum solution (FBS, 10 ml; Gibco, Grand Island, NY, USA) and Hank's solution (30 ml), and the tissue products were passed through a $400\text{-}\mu\text{m}$ filter and centrifuged on a Ficoll 400 gradient (Sigma). The resolved islets at the interface were collected carefully and washed with Hank's

solution. Individual islets were then handpicked using micropipettes under a dissection microscope.

The isolated islets were cultured in RPMI 1640 medium with 10% FBS overnight, and the aliquots (50/well) of islets were incubated in triplicate in RPMI 1640 medium containing 2.8 mM glucose at 37°C for 30 min. The islets were then incubated in RPMI 1640 medium containing 2.8 mM glucose, 16.7 mM glucose, or $200 \mu\text{M}$ palmitic acid at 37°C for 1 h. Supernatants were harvested for the measurement of immunoreactive insulin (IRI) using an enzyme immunoassay (Linco Research Inc., St. Charles, MO, USA). The values of released insulin were normalized to islet protein contents and expressed as $\text{mU IRI/ng protein/h}$. The contents of the islet proteins were measured using a BCA kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturers' instructions. The mean amount of insulin released in response to the high concentration of glucose (16.7 mM) was divided by the mean amount of insulin released by the low concentration of glucose (2.8 mM) to yield the GSIR index (Ihm et al. 2007).

Measurement of oxidative stress indicators

The concentrations of serum total superoxide dismutase (T-SOD), CuZn superoxide dismutase (CuZn-SOD), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were measured using specific kits (NJBI, Nanjing, China,) on a 721 spectrophotometer (The Third Analytical Instrument Factory, Shanghai, China), according to the manufacturers' instructions.

Immunohistochemistry

The pancreatic tissue was fixed in formaldehyde for 4 h, dehydrated, and embedded in paraffin. One portion was cut into $4\text{-}\mu\text{m}$ -thick sections and co-stained with mouse monoclonal antibody against insulin ($1:200$ dilution; Thermo Scientific, Foster City, CA, USA) and anti-glucagon antibody ($1:2,000$ dilution; AbCam, Cambridge, UK). To identify apoptotic β -cells, another subset of pancreatic tissue sections were co-stained with anti-insulin and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) using the In Situ Cell Death Detection Kit (Boehringer, Mannheim, Germany) according to the manufacturer's instructions. For the

β -cell proliferation studies, two consecutive sections from each block were stained with mouse polyclonal antibodies against proliferating cell nuclear antigen (PCNA, 1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or with anti-insulin. The percentage of apoptotic β -cells was determined by dividing the number of TUNEL-positive cells in the islets by the number of insulin-positive cells (Harb and Korbitt 2006). The percentage of proliferative β -cells was determined by dividing the number of PCNA-positive cells in the islets by the number of insulin-positive cells. At least 60 islets from each group of more than six rats were analyzed. The images were analyzed for each factor using Image-Pro Plus software (version 5.0.1; Media Cybernetics, Silver Spring, MD, USA).

Microarray assay

To determine the potential mechanisms underlying aging-related β -cell dysfunction, we isolated islets from the three age groups and extracted their total RNA. After reverse transcription into cDNA, the differentially expressed genes in the islets were characterized using GeneChips microarray assays (Affymetrix, Beijing, China) containing 31,100 probe sets, according to the manufacturer's instructions. GoMiner software (<http://discover.nci.nih.gov/gominer/>) was used to analyze gene function. Genes related to the pancreatic β -cells function or aging, particularly for genes associated with cell proliferation and apoptosis, were selected for analysis.

Real-time RT-PCR

The GeneChips results revealed that the *Anx1* gene was differentially expressed in the three age groups. *Anx1* is a crucial regulator of cell apoptosis and proliferation, inflammatory responses, and lipid metabolism (Chen et al. 2010; Pupjalis et al. 2010; McArthur et al. 2010; Paschalidis et al. 2010). Accordingly, it is possible that *Anx1* contributes to aging-related β -cell dysfunction; thus, we verified the expression of *Anx1* gene using real-time reverse transcription-polymerase chain reaction (RT-PCR). Briefly, total RNA was extracted from the freshly isolated islets of each age group using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), according to manufacturer's instructions, and reverse transcribed into cDNA using M-MLV reverse transcriptase. RT-

PCR was conducted using the Bio-Rad IQ5 system (Invitrogen, Carlsbad, CA, USA). The primers were designed from the full-length *Anx1* mRNA sequence (GenBank Accession no. NM_012904) and synthesized by SBS Biotechnology (Beijing, China). Primer sequences were as follows: forward 5'-TGCTGGAAGCTCGCCATAAGA-3'; and reverse 5'-CAGGATGGCTTGGCAGAGA-3'. The rat β -actin gene was amplified as an internal control using the following primers: forward 5'-GGCCATCTCTTGCTCGAAGT-3'; and reverse, 5'-CTCATGCATCCTGCGTCT-3'. The relative levels of *Anx1* mRNA transcripts to control β -actin were analyzed using the $\Delta\Delta$ Ct method.

Western blot analysis

Freshly isolated islets from the three age groups were lysed and the lysate proteins (100 μ g/lane) were separated by 10% SDS polyacrylamide gels, followed by electrical transfer to nitrocellulose membranes. After blocking with 5% fat free milk, the membranes were incubated with rabbit polyclonal antibodies against Annexin A1 (1:250, AbCam) or rabbit anti- β -actin (1:400; Biosynthesis Biotechnology, Beijing, China). The bound antibodies were detected with HRP-goat anti-rabbit IgG (1:4,000; Scirest, Beijing, China). Immune complexes were visualized using the cECL Western blot kit (Cwbio, Beijing, China).

Statistical analysis

All data are expressed as mean $\pm$ SD. An analysis of variance (ANOVA) was used to determine differences

Table 1 Levels of serum lipid indicators in young, adult, and old rats

Indicator (mmol/l)	Young group	Adult group	Old group
TG	0.76 $\pm$ 0.11	2.04 $\pm$ 0.11*	3.85 $\pm$ 0.60*. [#]
TC	1.25 $\pm$ 0.53	1.50 $\pm$ 0.29	2.84 $\pm$ 0.63*. [#]
HDL-CH	1.43 $\pm$ 0.34	0.90 $\pm$ 0.11*	0.56 $\pm$ 0.03*
LDL-CH	0.14 $\pm$ 0.03	0.26 $\pm$ 0.05	0.52 $\pm$ 0.06*. [#]
FFA	0.76 $\pm$ 0.25	0.96 $\pm$ 0.24	1.51 $\pm$ 0.07*

Data are expressed as mean $\pm$ SD of each group ($n=6$)

* $P<0.05$ vs. young group

[#] $P<0.05$ vs. adult group

Fig. 1 Glucose infusion rate (GIR). The GIR of each group of rats was determined using a hyperinsulinemic–euglycemic clamp test. During the test, the rats were maintained at a CV_{BG} of 3.85–4.95%, CV_{GIR} of 0.52–1.71%, and a steady-state blood glucose level of 5.03–5.06 mmol/l. Data are expressed as the mean $\pm$ SD of each group ($n=6$ per group) of rats from three separate experiments. * $P<0.05$ vs. young rats; # $P<0.05$ vs. adult rats

among groups followed by a Bonferroni correction. Between-group comparisons were conducted using the Student's *t*-test. All statistical tests were conducted using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA, USA). A *P* value <0.05 was deemed as statistically significant.

Results

General condition and insulin sensitivity during aging

The blood lipid analysis revealed that serum TG, TC, LDL-CH, and FFA concentrations in the adult and old

rat groups were significantly higher than that in the young rats, whereas the level of serum HDL-CH was significantly lower in the adult and old groups as compared with the young rats ($P<0.05$; Table 1). Furthermore, serum TG, TC, and LDL-CH concentrations were significantly higher in the old rats than in the adult rats ($P<0.05$). Thus, the concentrations of risk lipids increased and the beneficial lipids decreased with age. The CV_{BG} and CV_{GIR} values were within the normal range during the glucose clamp, indicating that the results of the clamp experiment were accurate and reliable. The GIR gradually decreased with age, such that the GIR value in the old group was significantly lower than that of the young and adult rat groups ($P<0.05$; Fig. 1).

Changes in islet β -cell function with age

The analysis of glucose metabolism-related parameters (Table 2) revealed no significant difference in the FBG levels among groups; however, the BG peak, 2hPG, and AUCg values were significantly greater in the old group as compared with the young rats. Moreover, the BG peak and AUCg of the old rats were greater than those of the adult rats, and were accompanied by a significantly delayed peak time. The BG peak, 2hPG, and AUCg values of the adult rats were higher than those of the young rats. (Table 2, Fig. 2a). The insulin secretion analysis revealed that the FINS increased with age. The FINS concentration was significantly higher in the old group as compared with the adult and young rats, and higher in the adult as compared with the young rats. Similarly, the AUCi

Table 2 Blood glucose and insulin levels in the oral glucose tolerance test

Blood insulin concentrations were measured by radioimmunoassay, with the coefficient of variation $<10\%$. Data are expressed as mean $\pm$ SD of each group ($n=6$, 7, and 6 for young, adult, and old, respectively)

* $P<0.05$ vs. young group

$P<0.05$ vs. adult group

Indicator	Young group	Adult group	Old group
FBG (mmol/l)	4.76 $\pm$ 0.29	4.8 $\pm$ 0.11	5.03 $\pm$ 0.55
BG peak (mmol/l)	8.68 $\pm$ 0.28	9.96 $\pm$ 0.76	13.10 $\pm$ 0.42*#
BG peak time (min)	10	30*	30*
OGTT2hPG	5.14 $\pm$ 0.07	7.50 $\pm$ 0.51*	8.90 $\pm$ 1.65*
AUCg (mmol l ⁻¹ min ⁻¹)	25.85 $\pm$ 0.46	31.99 $\pm$ 1.67*	41.13 $\pm$ 2.70*#
FINS (ng/ml)	0.59 $\pm$ 0.14	1.60 $\pm$ 0.15*	2.37 $\pm$ 0.06*#
INS peak (ng/ml)	5.69 $\pm$ 0.44	4.18 $\pm$ 0.35	5.24 $\pm$ 0.05
INS peak time (min)	10	10	30*
AUCi (ng ml ⁻¹ min ⁻¹)	12.05 $\pm$ 0.57	12.62 $\pm$ 0.95	16.08 $\pm$ 0.26*#
AUCi/g	0.47 $\pm$ 0.02	0.40 $\pm$ 0.03	0.39 $\pm$ 0.02
$\Delta I_{10}/\Delta G_{10}$	1.43 $\pm$ 0.25	1.16 $\pm$ 0.13	0.51 $\pm$ 0.07*#

Fig. 2 Oral glucose tolerance. Individual rats were challenged with oral glucose and the concentrations of blood glucose and plasma insulin were measured longitudinally. **a** Dynamic changes in blood glucose levels. **b** Dynamic changes in plasma insulin concentrations. Data are expressed as the mean $\pm$ SD of each group of rats (young, $n=6$; adult, $n=7$; and old, $n=6$) from three separate experiments

values and $\Delta I_{10}/\Delta G_{10}$ ratio in the old group were greater than that in young and adult rats, and was accompanied by a significantly delayed INS peak time ($P<0.05$). However, no significant difference in the INS peak value or AUCi/g ratio was found among groups (Table 2, Fig. 2b).

The in vitro study of freshly isolated islets indicated that the GSIR index and PSIR decreased in an age-dependent fashion. The GSIR index and

PSIR values were significantly lower in the old rats as compared with the young and adult groups, and the values in the adult group were significantly lower than those of the young rats (Table 3).

Oxidative stress levels

As shown in Table 4, activity levels of the antioxidants T-SOD, CuZn-SOD, and GSH-Px gradually

Table 3 In vitro glucose-stimulated insulin release (GSIR) index and palmitic acid-stimulated insulin release (PSIR) in the islets of young, adult, and old rats

Indicator	Young group	Adult group	Old group
GSIR index	2.53±0.03	1.93±0.05*	1.32±0.05*. [#]
PSIR	3.99±0.05	2.86±0.56*	1.73±0.26*. [#]

Data are expressed as mean ± SD of each group (*n*=6 per group)

**P*<0.05 vs. young group

[#]*P*<0.05 vs. adult group

decreased, while the levels of serum MDA increased with age. The concentrations of T-SOD and CuZn-SOD were significantly lower in the old group than in the young and adult groups (*P*<0.05). Similarly, GSH-Px levels in adult and old rats were significantly lower than that found in young rats. In contrast, MDA levels in old rats were significantly higher as compared with adult and young rats (*P*<0.05; Table 4).

β-Cell apoptosis and proliferation

The area of anti-glucagon staining in the pancreatic islets of old rats was greater than that of young rats, indicating an increase in the relative frequency of α-cells in the islets during the aging process (Fig. 3a). As shown in Fig. 3b and e, the percentage of TUNEL-positive islet cells in the old group was high as compared with that of the young rats, suggesting that more islet cells underwent spontaneous apoptosis in the old rats. Conversely, the percentage of PCNA-positive islet cells in the old rats was lower than that of the young rats (Fig. 3c, d, f), indicating that spontaneous islet cell proliferation gradually declined with age.

Anx1 expression

Using microarray screening, we identified 372 genes potentially related to aging; expression was down-

regulated in 77 genes in response to aging and upregulated in 295 genes, including Anx1. Given that Anx1 belongs to the Ca²⁺-dependent phospholipid binding protein family and is a crucial regulator of cell apoptosis and proliferation, inflammatory responses, and lipid metabolism, upregulation of Anx1 expression may contribute to age-related β-cell dysfunction. Further analysis revealed that the relative levels of Anx1 mRNA transcripts in the islets from the old rat group were higher than those in the young and adult rats (Fig. 4). A similar pattern of Anx1 protein expression was observed in the islets of different age groups (Fig. 5). Thus, the results clearly indicated that the islets of older rats expressed higher levels of Anx1 as compared with the other groups.

Discussion

The interaction of several factors associated with aging are likely to contribute to age-related changes in glucose tolerance in the elderly population (Chang and Halter 2003; Patti and Corvera 2010). However, a better understanding of the mechanisms underlying age-related changes in metabolic function is necessary to develop preventive and therapeutic strategies for this high-risk population. The present study examined the pathophysiological characteristics in a naturally aging rat model. We studied rats aged 4 months (equivalent to 14 years of age in humans), 14 months (approximately 50 years of age in humans), and 24 months (similar to 84 years of age in humans). We found that aging rats displayed blood lipid disorders. Some researchers (Phielix et al. 2010) have suggested that insulin resistance occurs independently of age and is primarily the result of an unhealthy lifestyle. However, we found that GIR increased in an age-dependent manner in rats fed a normal fat diet, suggesting that the decrease in insulin sensitivity and increase in insulin resistance was a function of

Table 4 Levels of serum antioxidants and MDA in young, adult, and old rats

Data are expressed as mean ± SD of each group (*n*=6 per group)

**P*<0.05 vs. young group

Indicator	Young group	Adult group	Old group
T-SOD (U/ml)	56.13±0.72	48.07±0.37*	39.82±2.35*. [#]
CuZn-SOD (U/ml)	55.15±0.97	47.85±3.97*	38.12±1.29*. [#]
GSH-Px (mmol/l)	756.5±31.09	675.88±34.37*	655.938±34.37*
MDA (mmol/l)	6.19±1.35	24.67±6.09	61.52±18.65*. [#]

Fig. 3 Immunohistochemical analysis of islets. Pancreatic tissue was harvested from the different age groups and subjected to an immunohistochemical analysis using specific antibodies. **a** Double immunostaining for insulin (brown) and glucagon (blue). **b** Double immunostaining of the islets with anti-insulin (brown) and DNA fragmentation using the TUNEL assay (blue). Black arrows positive cells for TUNEL staining. **c** Immunohistochemical staining of the pancreatic tissue sections with anti-proliferating cell nuclear antigen (PCNA, in brown). **d** Immunohistochemical staining of the pancreatic tissue sections with anti-insulin (brown) in the same islet as in c. **e** and **f** Quantitative analysis: data are expressed as the mean $\pm$ SD of the relative percentage of TUNEL-positive or PCNA-positive β -cells from at least 60 islets from each group of six rats. The frequency of TUNEL-positive or PCNA-positive β -cells in young rats was designated as 100% (2.3% TUNEL-positive β -cells and 17.6% PCNA-positive β -cells in the young group of rats). * P <0.05 vs. young rats. # P <0.05 vs. adult rats

age. This age-related decrease in the insulin sensitivity may be associated with an increase in body fat content, as abnormal blood lipid changes have been shown to be involved in insulin resistance (Slawik and Vidal-Puig 2006).

We evaluated pancreatic β -cell function in vivo and in vitro and found an age-related delay in insulin secretion induced by the oral glucose challenge, and our findings showed that rats in the old group had abnormal glucose tolerance, specifically, postprandial hyperglycemia. This finding is consistent with a previous report (Arioglu et al. 2000). Moreover, our results showed that the FINS increased with age,

Fig. 4 Quantitative RT-PCR analysis of mRNA transcripts in islets. The relative level of Anxa1 mRNA transcripts to control β -actin in the islets of the three age groups was determined using RT-PCR. Data are expressed as the mean $\pm$ SD of the relative levels of Anxa1 to β -actin in the islets of the three groups (n =6 per group) from three separate experiments. * P <0.05 vs. young rats. # P <0.05 vs. adult rats

Fig. 5 Western blot analysis of Anxa1 expression in islets. The relative level of Anxa1 to β -actin expression in the islets of young, adult, and old age groups was determined using Western blot assays with specific antibodies. Data shown are representative images or expressed as mean $\pm$ SD of the relative levels of the target protein in islets of the three age groups (n =6 per group) from three separate experiments. **a** Western blot analysis; **b** Quantitative analysis. * P <0.05 vs. young rats. # P <0.05 vs. adult rats

although the FBG values were within the normal range in all three age groups. These findings suggest that the adult and old rats increased insulin production to maintain normal levels of FBG; thus, hyperinsulinism was an indirect indication of insulin resistance. Furthermore, the results of the in vitro experiment showed that the GSIR index and PSIR were significantly lower in the isolated islets of the old rats as compared with the young and adult rats. This finding is in agreement with previous observations (Ihm et al. 2006, 2007; Perfetti et al. 1995). These results suggest that abnormalities in insulin secretion occurred independent of the aging-related increase in insulin resistance and decrease in the β -cell mass.

The generation and metabolism of free radicals are closely regulated under physiological conditions and dysregulation can lead to oxidative stress, which is

associated with the development of impaired β -cell function (Droge 2002). To understand the mechanisms underlying the impact of aging on β -cell function, we measured the levels of serum SOD, GSH-Px, and MDA. Our results indicated that antioxidant enzyme activity was significantly reduced in old rats as compared with the young and adult rats, suggesting a decrease in the capacity of the body to remove free radicals in old rats. An imbalance between oxidation and antioxidant status has been shown to cause β -cell dysfunction (Miyazawa et al. 2009). Increased free radical levels can damage the pancreatic islet β -cells directly or cause indirect damage by affecting the signal transduction pathway associated with insulin synthesis and secretion, thereby leading to β -cell dysfunction.

Age plays a role in regulating the proliferative and regenerative capacity of pancreatic β -cells (Tschen et al. 2009). In mice, adaptive β -cell proliferation is severely restricted at advanced age (Rankin and Kushner 2009), and the β -cells in aged and adult mice have extremely low rates of replication with minimal evidence of turnover (Teta et al. 2005). Characterization of islets from rats of different ages revealed pathological changes in old rats. Compared with the islets from young and adult rats, the relative frequency of α -cells increased while β -cells decreased in the islets of old rats, which may be associated with a reduction in insulin secretion. Physiologically, the dynamic balance between cell proliferation and apoptosis maintains a relatively stable number of cells (Henis-Korenblit et al. 2000). β -Cell apoptosis is crucial at several points during disease progression, initiating leukocyte invasion into the islets and terminating the production of insulin by the β -cells (Mathis et al. 2001). We observed that decreased proliferation was accompanied by increased apoptosis of β -cells in the islets of old rats, and that this imbalance could reduce the β -cell mass and insulin secretion from the islets, thus impairing the ability of old rats to regulate blood glucose.

Through a combination of microarray, PCR, and Western blot analyses, we showed that Anx1 expression was significantly upregulated in the islets of old rats. Given that Anx1 is a member of the Ca^{2+} -dependent phospholipid binding protein family and a crucial regulator of cell apoptosis and proliferation, inflammatory responses, and lipid metabolism, the dramatically upregulated Anx1 expression may

contribute to the age-related β -cell dysfunction in old rats. We plan to further investigate how Anx1 regulates β -cell function and whether Anx1 interacts with other factors, such as PDX-1 (Ihm et al. 2007), p16^{INK4a} (Krishnamurthy et al. 2006), cyclin D₂ (He et al. 2009), and insulin, to influence to age-related β -cell dysfunction.

In summary, our data indicated that islet β -cell function decreases with age and is accompanied by islet structure disorders in old rats. The development of β -cell dysfunction may be attributed to high levels of oxidative stress, imbalance of islet cell proliferation and apoptosis, and upregulation of regulatory genes, such as Anx1. Thus, our findings may provide new insights into the age-related development of β -cell dysfunction and damage.

Acknowledgements This work was supported by grants from the National Nature Science Foundation of China to C.L.L. (no. 38073412) and from the National Nature Science Foundation of China to Y.L. (no. 30801201).

References

- Alvarsson M, Wajngot A, Cerasi E, Efendic S (2005) K-value and low insulin secretion in a non-obese white population: predicted glucose tolerance after 25 years. *Diabetologia* 48 (11):2262–2268
- Arioglu E, Gottlieb NA, Koch CA, Doppman JL, Grey NJ, Gorden P (2000) Natural history of a proinsulin-secreting insulinoma: from symptomatic hypoglycemia to clinical diabetes. *J Clin Endocrinol Metab* 85(10):3628–3630
- Basu R, Breda E, Oberg AL, Powell CC, Dalla Man C, Basu A, Vittone JL, Klee GG, Arora P, Jensen MD, Toffolo G, Cobelli C, Rizza RA (2003) Mechanisms of the age-associated deterioration in glucose tolerance: contribution of alterations in insulin secretion, action, and clearance. *Diabetes* 52(7):1738–1748
- Chang AM, Halter JB (2003) Aging and insulin secretion. *Am J Physiol Endocrinol Metab* 284(1):E7–E12
- Chen Z, Yoshihara E, Son A, Matsuo Y, Masutani H, Sugie K, Maeda M, Yodoi J (2010) Differential roles of Annexin A1 (ANXA1/lipocortin-1/lipomodulin) and thioredoxin binding protein-2 (TBP-2/VDUP1/TXNIP) in glucocorticoid signaling of HTLV-I-transformed T cells. *Immunol Lett* 131(1):11–18
- DeFronzo RA (1981) Glucose intolerance and aging. *Diabetes Care* 4(4):493–501
- DeFronzo RA (2004) Pathogenesis of type 2 diabetes mellitus. *Med Clin North Am* 88(4):787–835, ix
- Droge W (2002) Free radicals in the physiological control of cell function. *Physiol Rev* 82(1):47–95
- Gotoh M, Maki T, Kiyozumi T, Satomi S, Monaco AP (1985) An improved method for isolation of mouse pancreatic islets. *Transplantation* 40(4):437–438

- Harb G, Korbitt GS (2006) Effect of prolonged in vitro exposure to high glucose on neonatal porcine pancreatic islets. *J Endocrinol* 191(1):37–44
- Harmon JS, Bogdani M, Parazzoli SD, Mak SS, Oseid EA, Berghmans M, Leboeuf RC, Robertson RP (2009) beta-Cell-specific overexpression of glutathione peroxidase preserves intranuclear MafA and reverses diabetes in db/db mice. *Endocrinology* 150(11):4855–4862
- Harris MI, Flegal KM, Cowie CC, Eberhardt MS, Goldstein DE, Little RR, Wiedmeyer HM, Byrd-Holt DD (1998) Prevalence of diabetes, impaired fasting glucose, and impaired glucose tolerance in U.S. adults. The Third National Health and Nutrition Examination Survey, 1988–1994. *Diabetes Care* 21(4):518–524
- He LM, Sartori DJ, Teta M, Opare-Addo LM, Rankin MM, Long SY, Diehl JA, Kushner JA (2009) Cyclin D₂ protein stability is regulated in pancreatic beta-cells. *Mol Endocrinol* 23(11):1865–1875
- Henis-Korenblit S, Strumpf NL, Goldstaub D, Kimchi A (2000) A novel form of DAP5 protein accumulates in apoptotic cells as a result of caspase cleavage and internal ribosome entry site-mediated translation. *Mol Cell Biol* 20(2):496–506
- Ihm S-H, Matsumoto I, Sawada T, Nakano M, Zhang HJ, Ansite JD, Sutherland DER, Hering BJ (2006) Effect of donor age on function of isolated human islets. *Diabetes* 55(5):1361–1368
- Ihm S-H, Moon HJ, Kang JG, Park C-Y, Oh KW, Jeong IK, Oh Y-S, Park SW (2007) Effect of aging on insulin secretory function and expression of beta cell function-related genes of islets. *Diabetes Res Clin Pract* 77(Suppl 1):S150–S154
- Kiraly MA, Campbell J, Park E, Bates HE, Yue JT, Rao V, Matthews SG, Bikopoulos G, Rozakis-Adcock M, Giacca A, Vranic M, Riddell MC (2010) Exercise maintains euglycemia in association with decreased activation of c-Jun NH₂-terminal kinase and serine phosphorylation of IRS-1 in the liver of ZDF rats. *Am J Physiol Endocrinol Metab* 298(3):E671–E682
- Kraegen EW, James DE, Bennett SP, Chisholm DJ (1983) In vivo insulin sensitivity in the rat determined by euglycemic clamp. *Am J Physiol* 245(1):E1–E7
- Krishnamurthy J, Ramsey MR, Ligon KL, Torrice C, Koh A, Bonner-Weir S, Sharpless NE (2006) p16INK4a induces an age-dependent decline in islet regenerative potential. *Nature* 443(7110):453–457
- Lavrovsky Y, Chatterjee B, Clark RA, Roy AK (2000) Role of redox-regulated transcription factors in inflammation, aging and age-related diseases. *Exp Gerontol* 35(5):521–532
- Maedler K, Spinas GA, Dyntar D, Moritz W, Kaiser N, Donath MY (2001) Distinct effects of saturated and monounsaturated fatty acids on beta-cell turnover and function. *Diabetes* 50(1):69–76
- Maedler K, Schumann DM, Schulthess F, Oberholzer J, Bosco D, Berney T, Donath MY (2006) Aging correlates with decreased beta-cell proliferative capacity and enhanced sensitivity to apoptosis: a potential role for Fas and pancreatic duodenal homeobox-1. *Diabetes* 55(9):2455–2462
- Mathis D, Vence L, Benoist C (2001) beta-Cell death during progression to diabetes. *Nature* 414(6865):792–798
- McArthur S, Cristante E, Paterno M, Christian H, Roncaroli F, Gillies GE, Solito E (2010) Annexin A1: a central player in the anti-inflammatory and neuroprotective role of microglia. *J Immunol* 185(10):6317–6328
- Miyazawa M, Ishii T, Yasuda K, Noda S, Onouchi H, Hartman PS, Ishii N (2009) The role of mitochondrial superoxide anion (O₂⁻) on physiological aging in C57BL/6J mice. *J Radiat Res (Tokyo)* 50(1):73–83
- Pan CY, Lu JM, Tian H, Kong XT, Lu XP, Yao C, Jiang CE, Deng XX, Wang SY, Zhang XL, Wang ZS, Cui L (1996) Study of the prevalence of diabetes mellitus in adults in the Shougang Corporation in Beijing. *Diabetes Med* 13(7):663–668
- Paschalidis N, Huggins A, Rowbotham NJ, Furmanski AL, Crompton T, Flower RJ, Perretti M, D'Acquisto F (2010) Role of endogenous annexin-A1 in the regulation of thymocyte positive and negative selection. *Cell Cycle* 9(4):784–793
- Patti ME, Corvera S (2010) The role of mitochondria in the pathogenesis of type 2 diabetes. *Endocr Rev* 31(3):364–395
- Perfetti R, Rafizadeh CM, Liotta AS, Egan JM (1995) Age-dependent reduction in insulin secretion and insulin mRNA in isolated islets from rats. *Am J Physiol* 269(6 Pt 1):E983–990
- Phielix E, Szendroedi J, Roden M (2010) Mitochondrial function and insulin resistance during aging—a mini-review. *Gerontology*
- Pupjalis D, Goetsch J, Kottas DJ, Gerke V, Rescher U (2010) Annexin A1 released from apoptotic cells acts through formyl peptide receptors to dampen inflammatory monocyte activation via JAK/STAT/SOCS signalling. *EMBO Mol Med*
- Rankin MM, Kushner JA (2009) Adaptive beta-cell proliferation is severely restricted with advanced age. *Diabetes* 58(6):1365–1372
- Resnick HE, Harris MI, Brock DB, Harris TB (2000) American Diabetes Association diabetes diagnostic criteria, advancing age, and cardiovascular disease risk profiles: results from the Third National Health and Nutrition Examination Survey. *Diabetes Care* 23(2):176–180
- Scheen AJ (2005) Diabetes mellitus in the elderly: insulin resistance and/or impaired insulin secretion? *Diabetes Metab* 31(Spec No 2):S257–S2534
- Slawik M, Vidal-Puig AJ (2006) Lipotoxicity, overnutrition and energy metabolism in aging. *Ageing Res Rev* 5(2):144–164
- Teta M, Long SY, Wartschow LM, Rankin MM, Kushner JA (2005) Very slow turnover of beta-cells in aged adult mice. *Diabetes* 54(9):2557–2567
- Tfayli H, Arslanian S (2009) Pathophysiology of type 2 diabetes mellitus in youth: the evolving chameleon. *Arq Bras Endocrinol Metabol* 53(2):165–174
- Tschen SI, Dhawan S, Gurlo T, Bhushan A (2009) Age-dependent decline in beta-cell proliferation restricts the capacity of beta-cell regeneration in mice. *Diabetes* 58(6):1312–1320
- Yang W, Lu J, Weng J, Jia W, Ji L, Xiao J, Shan Z, Liu J, Tian H, Ji Q, Zhu D, Ge J, Lin L, Chen L, Guo X, Zhao Z, Li Q, Zhou Z, Shan G, He J (2010) Prevalence of diabetes among men and women in China. *N Engl J Med* 362(12):1090–1101