Impaired autophagic function in rat islets with aging

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Abstract Type 2 diabetes is characterized by a deficit in β -cell function and mass, and its incidence increases with age. Autophagy is a highly regulated intracellular process for degrading cytoplasmic components, particularly protein aggregates and damaged organelles. Impaired or deficient autophagy is believed to cause or contribute to aging and age-related disease. Autophagy may be necessary to maintain structure, mass, and function of pancreatic β -cells. In this study, we investigated the effects of age on β -cell function and autophagy in pancreatic islets of 4-month-old (young), 14-month-old (adult), and 24-month-old (old) male Wistar rats. We found that islet β -cell function decreased gradually with age. Protein expression of the autophagy markers LC3/Atg8 and Atg7 exhibited a marked decline in aged islets. The

expression of Lamp-2, a good indicator of autophagic degradation rate, was significantly reduced in the islets of old rats, suggesting that autophagic degradation is decreased in the islets of aged rats. However, protein expression of beclin-1/Atg6, which plays an important role in the induction and formation of the pre-autophagosome structure by associating with a multimeric complex of autophagy regulatory proteins (Atg14, Vps34/class 3 PI3 kinase, and Vps15), was most prominent in the islets of adult rats, and was higher in 24-month-old islets than in 4-month-old islets. The levels of p62/SQSTM1 and polyubiquitin aggregates, representing the functions of autophagy and proteasomal degradation, were increased in aging islets. 8-Hydroxydeoxyguanosine, a marker of mitochondrial and nuclear DNA oxidative damage, exhibited strong immunostaining in old islets. Analysis by electron microscopy demonstrated swelling and disintegration of cristae in the mitochondria of aged islets. These results suggest that β -cell and autophagic function in islets decline simultaneously with increasing age in Wistar rats, and that impaired autophagy in the islets of older rats may cause accumulation of misfolded and aggregated proteins and reduce the removal of abnormal mitochondria in β -cells, leading to reduced β -cell function. Dysfunctional autophagy in islets during the aging process may be an important mechanism leading to the development of type 2 diabetes.

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Introduction

Macroautophagy (here referred to as autophagy) is a process of sequestration and removal of damaged organelles/proteins to recycle their constituents and nutrients. It also removes apoptotic cells and provides genomic stability (Levine and Kroemer 2008). Hence, autophagy is generally considered a protector of cells against various types of injury or continuous cellular degradation. Autophagy is expected to play a protective role in diverse types of cellular stress. This may be of particular importance during the aging process.

Aging denotes a post-maturational deterioration of cells and organisms with the passage of time, an increased vulnerability to challenges and age-associated diseases, and a decreased ability to survive (Marino and Lopez-Otin 2008; Rajawat et al. 2009). The pancreatic islet is a target organ of age-associated tissue damage, and the increased incidence of type 2 diabetes (T2D) in the elderly is a health problem worldwide (Cowie et al. 2006; Yang et al. 2010). The prevalence of T2D increases with age due to changes in or insufficient compensation of the β -cell functional mass in the face of increasing insulin resistance, but the underlying mechanism(s) within the dysfunctional β -cells have not been fully elucidated. Apoptosis, oxidative stress, mitochondrial dysfunction, and endoplasmic reticulum (ER) stress responses have been suggested as mechanisms of pancreatic β -cell dysfunction in T2D. However, mitochondria and ER, which play crucial roles in β -cell survival, death, and insulin secretion, primarily rely on autophagy for proper function (Laybutt et al. 2007; Petersen et al. 2004). Furthermore, several lines of evidence have shown that autophagy is involved in aging and is an essential part of the caloric restriction anti-aging mechanism (Rajawat and Bossis 2008; Rajawat et al. 2009; Wohlgemuth et al. 2007). Recently, it has been shown that pancreatic β -cell-specific Atg7-knockout mice develop hypoinsulinemia and hyperglycemia. β -Cell mass is reduced in autophagy-deficient mice because of increased apoptosis and decreased β -cell proliferation (Jung et al. 2008). These results suggest that autophagy is necessary to maintain the structure, mass, and function of pancreatic β -cells. Dysfunctional autophagy of islets may be an important mechanism behind the increasing prevalence of T2D in the elderly.

Many studies have demonstrated that age-related changes result from the accumulation of reactive oxygen species (ROS) and oxidative damage (Beckman

and Ames 1998; Sohal and Weindruch 1996). The increase in cytoplasmic ROS levels is a causative event of aging (Angelopoulou et al. 2009; Blagosklonny 2008), and mitochondria are a key source of ROS in many cell types, including β -cells (Lenaz 2001; Newsholme et al. 2007; Turrens 2003). Autophagy is the only intracellular degradative mechanism for removing damaged mitochondria. Growing evidence indicates that ROS play a pivotal role in β -cell dysfunction in T2D by reducing insulin secretory capability and enhancing β -cell apoptosis (Drews et al. 2010; Sakai et al. 2003; Sakuraba et al. 2002). Autophagy-deficient β -cells show increased ROS content and enhanced apoptosis (Marsh et al. 2007). Thus, we speculate that impaired autophagy with age may lead to mitochondrial dysfunction and increased ROS content in β -cells. However, it is unknown whether age-related changes in autophagy occur in the islets. In the present study, we investigated the effect of age on the expression of autophagy-related genes and 8-oxo-2'-deoxyguanosine (8-OHdG), a marker of mitochondrial and nuclear DNA injury and oxidative stress (Shigenaga et al. 1989), in the islets of 4-, 14-, and 24-month-old male Wistar rats.

Materials and methods

Animals

Male Wistar rats were purchased from Vital River Laboratory Animals (Beijing, China) and were maintained under specific pathogen-free conditions of 22 ± 1 °C, 40 % humidity, a 12/12-h light/dark cycle, five males per cage, and free access to food at the animal center of the General Hospital of the Chinese People's Liberation Army (PLA), Beijing. All rats were fed standard laboratory chow (containing 5 % fat). The experimental protocol was approved by the Animal Research Protection Committee of the General Hospital of the PLA. Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (40 mg/kg). Three age groups of rats were used: 4 months ($n=10$), 14 months ($n=10$), and 24 months old ($n=10$), corresponding to young, adult, and old rats, respectively.

Oral glucose tolerance test (OGTT)

Rats were fasted overnight and orally administered glucose at 2 g/kg body weight. Their blood

samples were collected from the angular vein, and blood glucose concentrations were measured at approximately 0, 10, 30, 60, and 120 min post-glucose challenge.

Isolation of pancreatic islets

Islets of Langerhans were isolated from the rats using *in situ* pancreatic collagenase infusion and were separated by density gradient centrifugation at $800\times g$, according to a modified protocol described previously (Kinasiewicz et al. 2004).

Glucose-stimulated insulin release

Isolated islets were cultured in RPMI 1640 medium with 10 % fetal bovine serum overnight, and aliquots (50/well) of islets were incubated in triplicate in RPMI medium containing 2.8 mM glucose at 37 °C for 30 min. Subsequently, the islets were incubated in RPMI medium containing 2.8 mM glucose and then 16.7 mM glucose at 37 °C for 1 h. The supernatants were harvested to measure immunoreactive insulin (IRI) by enzyme immunoassay (Linco, St. Charles, MO, USA). The values of released insulin were normalized to islet protein content and expressed as mU IRI/ng protein. The islet protein content was measured using a BCA kit, according to the manufacturer's instructions (Thermo Scientific, Rockford, IL, USA).

Senescence-associated β -galactosidase staining

The pancreatic tissues were removed from each rat and a sample was immersed in OCT compound (Tissue-Tek; Sakura Finetek, Torrance, CA, USA) and stored at -80 °C. Cryostat sections (4 μ m) were mounted onto glass slides and fixed in 0.2 % glutaraldehyde and 2 % formaldehyde at room temperature for 15 min. Sections were washed in phosphate-buffered saline (PBS) and incubated in freshly prepared senescence-associated β -galactosidase (SA- β -gal) staining solution (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate [pH 6.0], 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, and 2 mM $MgCl_2$) overnight at 37 °C without CO_2 . Tissue sections were counterstained with eosin and examined under a microscope.

Western blotting

Freshly isolated islets from the three groups of rats were lysed in RIPA buffer (50 mM Tris-Cl [pH 7.6], 150 mM NaCl, 1 % NP-40, 0.1 % SDS, 0.5 % deoxycholic acid, 1 μ g/ml leupeptin, 1 μ g/ml aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride) for 30 min on ice prior to centrifugation at 12,000 rpm for 30 min at 4 °C. Protein concentration was determined with the Pierce BCA Assay kit (lot JK126465; Thermo Fisher Scientific). Total protein (60–100 μ g) was separated by 10–16 % SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5 % skim milk for 1 h at room temperature, and probed with the following primary antibodies at 4 °C overnight: rabbit polyclonal anti-LC3 antibody (1:2,000; Sigma), mouse monoclonal anti-p16 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal anti-Atg7 (1:1,000; Beyotime Institute of Biotechnology, Beijing, China), rabbit polyclonal anti-beclin-1 (1:2,000; Abcam Ltd, Hong Kong, China), rabbit monoclonal anti-Lamp2 (1:1,000; Epitomics, Inc., CA, USA), mouse monoclonal anti-ubiquitin (1:1,000; MABtech, Nacka Strand, Sweden), and mouse monoclonal anti-p62 (1:1,000; Abcam Inc., Cambridge, MA, USA). Blots were subsequently probed with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Santa Cruz Biotechnology) (1:1,000). Immunoreactive bands were visualized by enhanced chemiluminescence, and densitometry was performed using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemistry staining

Pancreatic tissue samples were fixed in 10 % formaldehyde at 4 °C overnight and then embedded in paraffin according to standard procedures. Sections of 3- μ m thickness were prepared. For immunohistochemical analysis, tissue sections were subjected to antigen retrieval by trypsinization for 20 min in 10 mM sodium citrate buffer (pH 6.0). Endogenous peroxidase was blocked by incubation with 3 % hydrogen peroxide for 15 min. Sections were washed in PBS and incubated with 1.5 % normal goat serum for 20 min, followed by incubation with a 1:50 dilution of guinea pig polyclonal anti-insulin (Abcam) and mouse monoclonal anti-LC3 (MBL Ltd, Tokyo, Japan) for double immunohistochemistry staining, or

rabbit polyclonal anti-beclin-1 (1:1,000), mouse monoclonal anti-p62 (1:500), or mouse monoclonal anti-8-OHdG antibody (Santa Cruz Biotechnology) overnight at 4 °C. Sections were washed three times with PBS and incubated with biotin-conjugated goat anti-guinea pig IgG (Santa Cruz Biotechnology), anti-mouse IgG (Invitrogen, Carlsbad, CA, USA), or anti-rabbit IgG (Invitrogen) for 30 min at room temperature. Sections were washed again in PBS and incubated with labeled streptavidin-biotin horseradish peroxidase (Invitrogen) or labeled streptavidin-biotin alkaline phosphatase (Dako, Carpinteria, CA, USA) for 30 min at room temperature. Sections were washed a final time in PBS, incubated with DAB (brown) and NBT/BCIP (blue) sequentially for double staining of insulin and LC3, incubated again with DAB (brown) for beclin-1, p62, and 8-OHdG, and then examined by microscopy.

Electron microscopy

Pancreatic tissues were post-fixed with 2 % osmium tetroxide after fixation in 2.5 % glutaraldehyde in 0.01 mol/l phosphate buffer at 4 °C and washing in PBS. The tissues were dehydrated in a series of graded ethanol solutions. Propylene oxide was substituted for ethanol, and the tissues were embedded in epoxy resin. Ultrathin sections were double-stained with uranyl acetate and lead and examined under a JEM1200EX transmission electron microscope (JEOL, Tokyo, Japan) at 80 kV.

Statistical analysis

All data analyses were performed using SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean±SD. Comparisons among groups were made using analysis of variance. A *P* value <0.05 was considered statistically significant.

Results

Changes in islet β -cell function with aging

Although no significant differences were observed in the levels of fasting blood glucose among the rat groups, the results from OGTT revealed significant glucose intolerance in 24-month-old rats. Plasma glucose at 120 min post-oral glucose challenge

(2hPG) and the area under the glucose curve throughout the 120-min period (AUCg) were significantly greater, and the plasma glucose peak significantly delayed, in old rats than in young and adult rats. The 2hPG and AUCg values were higher in adult rats than in young rats (Fig. 1a,b).

We then conducted a functional analysis of pancreatic islets, measuring insulin secretion *ex vivo* using the same number of viable primary islets of similar size, and found that basal insulin secretion was decreased in the islets of old rats compared to young rats, whereas no difference was observed between adult and old rats (Fig. 1c). High glucose-stimulated insulin secretion from primary islets was also reduced significantly in old rats compared to young and adult rats (Fig. 1d). These results indicate that β -cell function declines with increasing age in Wistar rats.

Changes in senescence markers in rat pancreatic islets with aging

We measured the expression of p16, a robust biomarker and a possible effector of mammalian aging (Krishnamurthy et al. 2004), in the rats' pancreatic islets and found that it was significantly higher in 24-month-old rats (Fig. 2) than in the other age groups, and was higher in 14-month-old rats than in 4-month-old rats. We also determined the expression of another well-defined *in vivo* senescence marker, SA- β -gal, which has high-pH galactosidase activity in senescent cells and tissues (Dimri et al. 1995). As shown in Fig. 3, the positive rate of SA- β -gal staining was markedly elevated in the islets of 24-month-old rats compared to 4-month-old rats. Furthermore, it was observed predominantly in the senescent cells of islets and was hardly detected in aging exocrine pancreatic tissue. These results suggest that these aging-related changes were mainly limited to the pancreatic islets.

Examination of autophagy-related protein expression during the rat islet aging process

Autophagic function is usually measured by quantifying autophagy-related (Atg) proteins. Among the 31 Atg proteins identified, Atgs 1–10 are involved in autophagosome formation and are markers for isolation membrane formation. We evaluated changes in the expression of autophagy-related proteins (LC3/Atg8, beclin-1/Atg6, Atg7, Lamp-2, p62, and polyubiquitin

Fig. 1 Changes in β -cell islet function with aging. **a** Plasma glucose curve from oral glucose tolerance test (OGTT) in young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats ($n=6$, 7, and 6, respectively). Rats were fasted overnight, and blood glucose levels were measured before and after a glucose challenge. **b** Area under the glucose curve (AUCg) throughout the 120-min OGTT in the three groups. **c**,

d Insulin secretion ex vivo. Pancreatic islets from rats of different ages were incubated in RPMI 1640 medium containing 2.8 mM glucose for 1 h and then in RPMI 1640 medium containing 16.7 mM glucose for 1 h ($n=6$ /group). Data are expressed as the mean $\pm$ SD for each group. * $P<0.05$ vs. young rats; # $P<0.05$ vs. adult rats

aggregates) in autophagosome formation and lysosomal fusion (Fig. 4) during the aging of pancreatic islets.

LC3/Atg8

Microtubule-associated protein light-chain 3 (LC3; also known as Atg8) is the only Atg protein that remains associated with the completed autophagosome; hence, it serves as one of the few autophagy markers (Kabeya et al. 2000). Pre-LC3 is cleaved into its cytosolic form LC3-I by Atg4. LC3-I is then activated by Atg7 and converted into its membrane-bound form, LC3-II, localized in pre-autophagosomes and autophagosomes (Maiuri et al. 2007). An increased amount of LC3-II and the LC3II/I ratio correlates with increased autophagy, serving as a good indicator of autophagosome formation (Kadowaki and Karim 2009). Furthermore, changes in the intracellular localization of LC3 provide the best method for detecting autophagy. When autophagy is activated, LC3-I is converted into LC3-II, which translocates to autophagosomes to form LC3-II puncta structures. We first

observed LC3 expression in the rat pancreas by immunohistochemistry. The navy blue punctae reflected aggregation of LC3-II dots in the histochemical images (Fig. 5a). We found that the number of LC3-II punctae formed in the rat islets decreased gradually with aging. LC3-II puncta formation was significantly reduced in 24-month-old rats compared to 4- and 14-month-old rats (Fig. 5a). Western blot analyses revealed that LC3-I expression did not change remarkably, and that LC3-II expression decreased significantly in 24-month-old rats compared to the other groups, leading to a significantly decreased LC3II/I ratio in the oldest rats. These results indicate that autophagy declines in the islets of aged rats (Fig. 5b,c).

Atg7

Atg7 is required for the formation and expansion of autophagosomes, which it triggers by initiating the conjugation of Atg12 to Atg5 and of LC3 to phosphatidylethanolamine. Overexpression of Atg effectively induces basal autophagy with no detrimental effects on

Fig. 2 Expression of the p16 senescent biomarker in the pancreatic islets of young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats. **a** Western blotting results for p16 protein. **b** Quantitative analysis of p16 band densities. Protein expression data are presented as the mean±SD ($n=6$). * $P<0.05$ vs. young rats, # $P<0.05$ vs. adult rats

cell survival, suggesting that it can activate autophagy (Juhász et al. 2007; Pattison et al. 2011). We examined Atg7 protein expression and found that it was significantly lower in the oldest rats (Fig. 6b,d), suggesting that autophagy is reduced in the islets of aged rats.

Lamp-2

Lysosomal-associated membrane protein 2 (Lamp-2) is a ubiquitous lysosomal membrane protein that is

required for the proper fusion of lysosomes with autophagosomes in the late stages of autophagy. Lamp-2 depletion results in the inhibition of autophagic degradation secondary to the failure of lysosomes and autophagosomes to fuse to each other (Eskelinen 2006; Eskelinen et al. 2002). Therefore, Lamp-2 is a good indicator of the autophagic degradation rate. We found that Lamp-2 expression was significantly reduced in the islets of old rats, suggesting that the autophagic degradation rate and autophagic flux were decreased in the islets of aged rats (Fig. 6b,e).

Beclin-1/Atg6

Beclin-1, the mammalian homolog of yeast Atg6, is part of an autophagy regulatory protein complex (Atg14, Vps34/class 3 PI3 kinase, and Vps15) (He and Klionsky 2009) that promotes the formation of autophagosomes in mammalian cells (Itakura et al. 2008). Beclin-1 is correlated with diverse biological processes, including aging, development, tumor suppression, and protection against certain cardiac and neurodegenerative diseases (Levine and Kroemer 2008). Previous studies have found that beclin-1 has a significant increase in the aged rat heart (Wohlgemuth et al. 2007) and skeletal muscle (Wohlgemuth et al. 2010), but a significant decrease in the liver of old rats (Wohlgemuth et al. 2007). Changes in beclin-1 are tissue and cell context dependent. We observed beclin-1 expression in pancreas tissues by immunohistochemical staining and found that its expression is most prominent in the islets of adult rats and least prominent in that of young rats (Fig. 7a). Western blot analyses revealed that beclin-1 expression in the islets was higher in old rats than in young rats but lower compared to adult rats (Fig. 7b,c). These results suggest that beclin-1 may function through direct interactions

Fig. 3 Senescence-associated β-galactosidase staining results in pancreatic tissues from young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats. Magnification:

×400. Blue precipitation in the cytoplasm was observed in the senescent islet with aging but was hardly detectable in pancreatic exocrine tissues. Scale bars: 100 μm

Fig. 4 Autophagy-related proteins (LC3/Atg8, beclin-1/Atg6, Atg7, Lamp-2, p62, and polyubiquitin aggregates) in autophagosome formation and lysosomal fusion: (1) Beclin 1-Vps34/class 3 PI3 kinase complex regulate autophagy initiation. (2) Expansion of the membrane to form early autophagosomes is modulated by LC3/Atg8 and Atg7, which control the size of the autophagosomes and the amplitude of autophagy. (3) p62 mediates the specific recognition of polyubiquitin aggregates

(an intracellular protein aggregate) by binding to LC3, and is then scavenged by autophagy, (4) Lamp-2, a lysosomal trans-membrane protein, is required for the proper fusion of lysosomes with autophagosomes in the late stage of the autophagic process. (5) Once autophagosomes fuse with lysosomes to form autolysosomes, autophagosomal contents are degraded by lysosomal enzymes to regenerate nutrients

with proteins that can either promote or inhibit autophagy, rather than a change in its expression during the aging process. Moreover, beclin-1 expression changes with age may be in line with its various functions in the complex and interdependent regulation of autophagy, tumorigenesis and apoptosis, all of which undergo age-related changes.

SQSTM1/p62 and polyubiquitin aggregates

Sequestosome 1 (SQSTM1, also known as p62) mediates the specific recognition of ubiquitinated protein aggregates by binding to LC3, and is then scavenged by autophagy. Therefore, its accumulation is a good indicator of suppressed autophagy and can be used to monitor fluctuations in autophagy under certain conditions (Bjorkoy et al. 2005). Polyubiquitin, an intracellular protein aggregate, is a signal for degradation by the ubiquitin–proteasome

system. The accumulation of polyubiquitin aggregates can be induced by dysfunction in protein proteasome degradation pathways and by autophagy (Bjorkoy et al. 2006). SQSTM1/p62 plays a major role in the degradation of polyubiquitin substrates (Seibenhener et al. 2004). Thus, we also investigated its expression in the islets by immunohistochemistry and found that it significantly accumulated in the islets of old rats, but was hardly detectable in young and adult rats (Fig. 6a). It probably accumulated because its degradation decreased as autophagic function declined with age (Komatsu et al. 2006). Western blot analyses revealed that the expression of SQSTM1/p62 and polyubiquitin aggregates was significantly increased in 24-month-old rat islets (Fig. 6b,c,f). These results suggest that impaired autophagy with age leads to the accumulation of polyubiquitin aggregates associated with SQSTM1/p62.

Fig. 5 **a** Dual immunohistochemistry staining results for the LC3 protein (blue) and insulin (brown), in the pancreatic islets of young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats. LC3 levels decreased gradually with aging. Scale bars: 100 μ m. **b** LC3-I and LC3-II protein

expression in the pancreatic islets of young, adult and old Wistar rats was quantified by Western blotting. **c** Quantitative analysis of LC3-II band densities. **d** Ratio of the LC3-II band to the LC3-I band. Protein expression data are presented as mean $\pm$ SD ($n=6$). * P <0.05 vs. young rats, # P <0.05 vs. adult rats

Analysis of mitochondrial oxidative damage in the islets of older rats

Impaired autophagy may result in the accumulation of oxidative damage, ultimately leading to aging. Therefore, oxidative damage in pancreatic islets was evaluated by analyzing 8-OHdG expression. We found strong 8-OHdG reactions in the islet cell cytoplasm of old rats. In contrast, islets in young rats did not show positive reactions, whereas those in the adult rats showed conspicuous staining of many endocrine cells (Fig. 8), indicating that oxidative damage in rat islets increased significantly during the aging process. We further observed changes in the

mitochondrial structures using electron microscopy. The results showed that β -cells of 14-month-old rats showed little mitochondrial oxidative damage, whereas the mitochondria in the β -cells of the 24-month-old rats exhibited swelling and disintegration or disruption of mitochondrial cristae (Fig. 9).

Discussion

The incidence of and susceptibility to T2D increases with age, but the underlying mechanism(s) of this phenomenon are not yet clearly understood. It has been hypothesized that insulin resistance increases

Fig. 6 **a** Immunohistochemistry results for SQSTM1/p62 (brown) in the pancreatic islets of young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats. Scale bars: 100 μ m. **b, c** Expression of Atg7, Lamp-2, p62/SQSTM1, and polyubiquitin aggregate proteins in the pancreatic islets of

young, adult, and old Wistar rats was quantified by Western blotting. **d, e, f** Quantitative analysis of Atg7, Lamp-2, and p62/SQSTM1 band densities. Protein expression data are presented as mean $\pm$ SD ($n=6$). * $P<0.05$ vs. young rats, # $P<0.05$ vs. adult rats

with age due to increased adiposity, decreased lean muscle mass, changes in dietary habits, and reduced physical activity (Scheen 2005). However, these factors alone do not account for age-related glucose intolerance. Studies of the effects of age on β -cells have shown that aging is negatively correlated with baseline β -cell proliferative activity and positively correlated with enhanced sensitivity to glucose-induced apoptosis (Gu et al. 2011; Maedler et al. 2006). The limited adaptive capacity of aging β -cells may contribute to the risk of developing T2D. A retrospective analysis of the European Group for the

Study of Insulin Resistance database revealed a 25 % decline in the insulin delivery rate (calculated as the sum of the clamp-derived posthepatic insulin clearance rate and fasting plasma insulin concentration) from age 18 to 85 years (Ioizzo et al. 1999). That study controlled for body mass index, fasting plasma glucose, and insulin sensitivity in both men and women. These results suggest that β -cell function declines with age. However, the underlying mechanism(s) of this decline have not been fully elucidated.

Pancreatic β -cell-specific Atg7-knockout mice show increased apoptosis and decreased proliferation

Fig. 7 **a** Immunohistochemistry results for beclin-1 (brown) in the pancreatic islets of young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats. Scale bars: 100 μm. **b** Beclin-1 protein expression in the pancreatic islets of young,

adult, and old Wistar rats was quantified by Western blotting. **c** Quantitative analysis of beclin-1 band densities. Protein expression data are presented as mean ± SD ($n=6$). * $P<0.05$ vs. young rats, # $P<0.05$ vs. adult rats

of β-cells, leading to impaired glucose tolerance and decreased levels of serum insulin (Jung et al. 2008), suggesting that autophagy is a protective mechanism in β-cells. Interestingly, a high-fat diet causes profound deterioration of glucose tolerance in Atg7-deficient mice (Ebato et al. 2008). These findings support the hypothesis that autophagy is essential for maintenance of normal β-cell mass and function and that it plays a protective role in diabetes pathogenesis. Dysfunctional autophagy in islets during the aging process may be an important mechanism leading to the development of T2D. Although autophagy decreases with age in the liver (Wohlgemuth et al. 2007) and kidney (Cui et al. 2011), the effects of aging

on autophagy in the mammalian pancreatic islets have not yet been elucidated.

In the present study, we used 4-, 14-, and 24-month-old rats (equivalent to 14-, 50-, and 84-year-old humans, respectively), to depict a natural aging model and reflect the pathophysiological characteristics of aging in animals. We evaluated pancreatic β-cell function in vivo and in vitro, and found that old rats had abnormal glucose tolerance, particularly in the case of postprandial hyperglycemia (the values of 2hPG and 2hOGTT AUCg were significantly increased in older rats), although fasting glucose levels were not significantly different in young and older rats, consistent with previous reports (Ihm et al.

Fig. 8 Immunoreactive 8-hydroxy-2'-deoxyguanosine (8-OHdG) reactions (brown) in the pancreatic islets of young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats. Scale bars: 100 μm. Islets in young rats did not

show positive reactions (a). In contrast, many islet cells in adult rats showed conspicuous staining (b), and the islets of old rats showed strong 8-OHdG reactions (c)

Fig. 9 Electron microscopic analysis of ultrastructural changes in β -cells in young (4-month-old), adult (14-month-old) and old (24-month-old) Wistar rats. *Arrowhead* regular mitochondria; *white arrows* swelling of mitochondria and broken mitochondrial crests in the β -cells of old rats. Scale bars: 200 μ m

2006, 2007; Perfetti et al. 1995). Furthermore, there was significantly less *in vitro* basal insulin secretion and high-glucose-stimulated insulin secretion in the primary islets of old rats. These results indicate that β -cell function declines with increasing age in rats.

We examined changes in the expression of the autophagy-related proteins LC3/Atg8, beclin-1/Atg6, Atg7, Lamp-2 and SQSTM1/p62, as well as polyubiquitin aggregates, in aging islets. The expansion of the membrane to form early autophagosomes is modulated by Atg7, LC3/Atg8, and Atg9, which control the size of the autophagosomes and the amplitude of autophagy. LC3/Atg8 is crucial for sequestration of large mitochondria and protein aggregates. The intracellular localization of LC3-II punctae and the LC3-II/I ratio have been used to evaluate the extent of autophagy. The results showed that the expression of LC3-II and the LC3II/I ratio decreased gradually with age, possibly as a result of decreased conversion of LC3-I to LC3-II or faster LC3-II degradation. We further detected the expression of Atg7 and Lamp-2 in the islets of rats. Atg7 can activate LC3-I and convert it into LC3-II. The reduced Atg7 expression in the islets of aged rats indicated decreased LC3 conversion. Lamp-2 is required for the proper fusion of lysosomes with autophagosomes during the late stages of autophagy and is a good indicator of the autophagic degradation rate. We found that Lamp-2 expression was significantly reduced in the islets of aged rats, suggesting that autophagic degradation of LC3-II declined with increasing age. The decreased LC3II/I ratio and decreased expression of LC3-II, Atg 7, and Lamp-2 in the islets of old rats indicate that autophagic influx declines in islet cells during the aging process. In addition, we found that beclin-1 was predominantly produced in the islet cell cytoplasm but was hardly detectable in exocrine tissues. Beclin-1 protein expression was most prominent in adult rats, and was higher

in 24-month-old rats than in 4-month-old rats. Previous studies have found that beclin-1 levels increase significantly with age in the rat heart (Wohlgemuth et al. 2007) and in skeletal muscle (Wohlgemuth et al. 2009). Beclin-1 plays a central role in autophagy, and promotes crosstalk between apoptosis and autophagy. It also interacts with several cofactors (Atg14L, UVRAG, Bif-1, Rubicon, Ambra1, and survivin) to regulate the lipid kinase Vps-34 and promote the formation of beclin-1–Vps34–Vps15 core complexes, thereby inducing autophagy. In contrast, binding of the beclin-1 BH3 domain to Bcl-2 or Bcl-XL inhibits autophagy (Cao and Klionsky 2007; Kang et al. 2011; Maiuri et al. 2010). Beclin-1 dysfunction has been implicated in many disorders, including cancer and neurodegeneration. It may function through temporally modulated or spatially modulated interactions with its positive and negative regulators in autophagy, rather than a change in its expression.

Many studies have shown that the protein p62 recognizes toxic cellular waste, which is then scavenged by autophagy. Lack of autophagy leads to accumulation of p62-containing aggregates of ubiquitinated proteins, which is detrimental to liver cells as it induces cellular oxidative stress; however, unexpectedly, removal of p62 reverses oxidative stress in autophagy-deficient liver cells (Rusten and Stenmark 2010). Interestingly, autophagy-deficient pancreatic β -cells have inclusion bodies containing large polyubiquitin aggregates and SQSTM1/p62 in their cytoplasm (Ebato et al. 2008; Jung et al. 2008). We found that SQSTM1/p62 accumulated in the islet cells of 24-month-old rats, but was only weakly expressed in the islets of 4- and 14-month-old rats. Accumulation of SQSTM1/p62 is also observed in pancreatic islet cells of Zucker diabetic fatty rats and in insulinoma cells treated with high concentrations of glucose (Kaniuk et al. 2007). We also observed greater levels of SQSTM1/p62 and polyubiquitin aggregates in the islet cells of older rats by Western blotting. Accumulating evidence has indicated that aging tissues exhibit an age-dependent decline in the turnover rate of long-lived proteins, leading to accumulation of damaged proteins, consistent with a defect in autophagy (Marino and Lopez-Otin 2008; Rajawat and Bossis 2008). Increases in the intracellular accumulation of lipofuscin bodies (LBs) and lipid-storing vesicles in human and non-human β -cells with age have been described (Masini et al. 2009a, b). As waste storage organelles, LBs represent the end stage of lysosomal

degradation. They are formed through the incorporation of senescent/damaged mitochondria and secretory granules into lysosomes by autophagy (Kurz et al. 2007). Our data suggest that impairment of autophagy in islet cells with age may be an important mechanism leading to the accumulation of misfolded and aggregated proteins in LBs and lipid and polyubiquitin aggregates associated with SQSTM1/p62, which may contribute to decreased β -cell function in the autophagy-impaired islets of old rats and may be related to the increase in the risk of T2D with increasing age.

Mitochondria play a central role in the regulation of aging. They are the primary sites of ROS generation, and enhanced production of ROS and oxidative damage have been found in aging cells, which may be a reason why they are more affected by age than other organelles. If the impaired mitochondria are not removed (due to deficient autophagy), they can generate additional ROS, which further aggravate oxidative damage, forming a vicious circle (Cadenas and Davies 2000). In a model of autophagy deficiency in pancreatic β -cells, impairment of autophagy led to the accumulation of damaged and dysfunctional mitochondria and a corresponding increase in intracellular ROS levels (Hur et al. 2010; Wu et al. 2009). Mice lacking Atg7 in their β -cells exhibited a marked decrease in basal respiration by mitochondria and a significant decrease in mitochondrial oxidative capacity in isolated islets; treatment with the antioxidant *N*-acetylcysteine (NAC) ameliorated these metabolic defects (Wu et al. 2009). These results demonstrate the potential role of mitochondrial dysfunction and oxidative stress in autophagy-deficient β -cells. 8-OHdG has been used as a marker of oxidative stress-related DNA damage in mitochondria and nuclei (Toyokuni et al. 1997). It is a sensitive biomarker of mitochondrial DNA (mtDNA) oxidative damage as mtDNA is more vulnerable to oxidative damage than nuclear DNA (most of which is protected by histones). We found strong 8-OHdG staining in the islets of old rats, whereas the islets of young rats did not show positive reactions. Our results also reveal swelling of mitochondria in aged rat islets. The increased number of 8-OHdG-positive islet cells and mitochondrial swelling in older rats suggest that declining autophagy with age is associated with increased oxidative stress in rat islets, which contributes to impaired β -cell function.

In conclusion, we clearly demonstrated that insulin secretion by β -cells and autophagic function of islets simultaneously decline with increasing age in rats.

Impairment or dysregulation of autophagy with age may cause accumulation of misfolded and aggregated proteins and reduce removal of abnormal mitochondria in β -cells, leading to reduced β -cell function. Apoptotic cell death may not be the only mechanism responsible for the decrease in β -cell function and mass. Prolonged inhibition of autophagy may also contribute to β -cell dysfunction. Our results help to elucidate the role of autophagy in the onset and development of T2D with age and may be useful for exploring new therapies for diabetes prevention and treatment.

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