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Pancreatic islet cell autophagy during aging in rats

Abstract

Purpose: Autophagy induces pancreatic β cell death. The purpose of the present study was to examine the hypothesis that the extent of pancreatic autophagy is associated with aging and age-related diabetes.

Methods: Pancreatic tissue and blood samples were collected from Sprague Dawley rats receiving a normal diet at 2 (the young group), 6 (the adult group), 12 (the middle-age group) and 20-24 (the aged group) months of age. Body weight and fasting blood glucose, serum lipid levels and serum insulin levels were determined. Pancreatic cell structure and autophagy were determined using transmission electron microscopy of rats at 6, 12 and 24 months of age. Lamp2 and LC3b protein expression levels were determined by both immunohistochemistry and Western blot analyses, and islet cell apoptosis was assessed using the TUNEL assay.

Results: Fasting blood glucose, triglyceride and FFA levels increased significantly with age ($p < 0.05$). Compared with levels seen in two-month-old rats, insulin secretion of islet cells *in vitro* was significantly reduced at 6, 12, and 20 months of age ($p < 0.05$). Autophagosomes were only observed in islet cells of 24 month-old rats. Increased expression of the autophagic markers, Lamp2 and LC3b, was observed with age. A significant increase in apoptotic index was observed between young rats (two-months-old) and older rats (six-, 12- and 24-months-old), but no differences were observed between rats six, 12 and 24 months of age.

Conclusion: Appearance of autophagosomes and increased Lamp2 and LC3b expression in pancreatic islet cells coincided with a significant decrease in insulin secretion and elevation of fasting blood glucose in aged rats.

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TABLE 1. Composition and caloric content of the rat diets.

	Normal	
	Total calories: 3.2 Kal/g	
	Content (%)	Calories (%)
Carbohydrate	53.77	66.5
Fat	3.67	10.21
Protein	18.83	23.29
Other	23.73	0

Aging is frequently associated with impaired glucose tolerance and increased incidence of type 2 diabetes [1]. Visceral adiposity, reduced physical activity, decreased insulin sensitivity and diminished β cell function are thought to contribute to the age-related increase in diabetes incidence [2].

Autophagy is a highly regulated process that governs the turnover of long-lived proteins, cytosolic components or damaged organelles [3]. With apoptosis, autophagy may regulate the proliferation and survival of islet β cells [4,5]. The increased presence of autophagosomes correlated with β cell death in chronic stress and hyperglycemia [6]. Human β cells isolated from a patient with type 2 diabetes showed accumulation of autophagic vacuoles and autophagosomes and increased levels of dead β cells with vacuole engulfment [6]. Thus, impairment of the autophagosomal-lysosomal network may predispose individuals to type 2 diabetes. In contrast, other reports suggest that autophagy has a positive role in the maintenance of the architecture and function of β cells [7].

Analyzing the extent of autophagy in aging may help identify its possible role in age-associated diabetes. Previous studies have reported a decline in β cell autophagy with age [8,9]; however, autophagy may increase with aging in rats, and the extent of pancreatic islet cell autophagy may be associated with age-related diabetes.

Materials and Methods

Animals

A total of 20 male Sprague Dawley (SD) rats ($n=5$ per group) were purchased from the animal center of Sichuan University. The rats received a normal chow diet (3.2 Kal/g; Table 1). Animals were sacrificed after 2 months (the young group), 6 months (the adult group), 12 months (the middle-age group) and 20-24 months (the aged group). Rats aged 20-24 months correspond to humans aged 60 y [10].

The animals fasted for 12-14 h prior to the sample collection after which they were anesthetized with intraperitoneal injection of 3% sodium pentobarbital (30 mg/kg). The blood

was collected from the heart and stored at -20°C . Pancreatic tissue was also collected, and a portion was immediately fixed for electron microscopy while other tissue was processed to obtain islet cell cultures or used for Western blot analysis.

Biochemical analysis of blood glucose, lipids, and FFA levels

Blood glucose was measured with ACCU-CHEK[®] Active blood glucose meter (Roche, Basel, Switzerland). Triglyceride levels were determined using the GPO-POD assay following the manufacturer's instruction (Roche). Cholesterol levels were assessed using an enzyme-coupled colorimetric assay (Zhejiang Dongou Biotech, Dongou, China). FFA levels were measured using a colorimetric assay (Beijing Jiuqiang, Beijing, China). Insulin concentrations were measured using a rat insulin radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO, USA).

Electron microscopy

Pancreatic tissues from two rats of each age group were isolated and fixed in 3% glutaraldehyde followed by 1% osmium tetroxide. Dehydration was performed in graded acetone, and tissues were embedded in Epon812 (Shanghai Yiwei Information Technology, Shanghai, China). Semi-thin sections were stained with toluidine blue (0.1%) for identifying the islets of Langerhans as well as optical alignment. Ultra-thin sections were then obtained using an ULTRACUT-E ultramicrotome (Reichert-Jung, city, Austria) and stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (H-600IV, Hitachi, city, Japan). An average of 20 islet cells (10 cells/rat) from two rats was examined for the presence of autophagosomes in each age group. Autophagy was characterized by the presence of vacuolar structures containing cytoplasm and organelles and degradation in the vacuolar structure by lysosomes as previously described [11].

Isolation and culture of islet cells

Islets of Langerhans were isolated as described previously [12]. Pancreatic tissue was washed twice with D-Hanks Balanced Salt Solutions (D-HBSS). After the major blood vessels, lymph nodes and connective tissue were removed, the tissue was cut and digested with Collagenase V (1 mg/L) at 37°C for 20 min; the enzymatic reaction was stopped with the addition of ice-cold D-HBSS. The digestion was repeated three times after which the tissue was centrifuged and the supernatant removed. The pellet was mixed with RPMI 1640, without D-glucose plus 15% of FBS.

Islet cells were collected manually using a 10 μ L pipette under a dissecting microscope. The isolated islet cell masses were washed with RPMI 1640 without D-glucose and resuspended in RPMI 1640 supplemented with 15% FBS and either 5.5 mmol/L D-glucose or 16.7 mmol/L D-glucose. The islet cell masses containing a total 30 islet cells were collected under an inverted microscope and transferred into 6-well cell culture plates after which 3 mL of culture medium was added per well. The cells were incubated at 37°C with 5% CO₂. A 5 μ L-sample of culture medium was collected from each well after two, four, six, 12, and 24 h. The samples were stored at -20°C for analysis of insulin levels. Insulin concentrations were measured using a rat insulin radioimmunoassay (RIA) kit purchased from Linco Research Inc. (St. Charles, MO, USA).

Immunohistochemistry analysis

Tissues sections (4 μ m) were mounted on a slide, dewaxed, rehydrated in graded alcohol solutions and incubated in 10 mM citrate (pH 6.0) at 92-95°C for 5 min for epitope retrieval. Endogenous peroxidase activity was neutralized using 3% H₂O₂ for 10 min and nonspecific binding was blocked using goat serum (Biovision, Mountain View, CA, USA) for 5 min at room temperature. Slides were then incubated with either rabbit anti-rat lysosomal-associated membrane protein 2 (Lamp2; Biovision) or rabbit anti-rat LC3b (Beyotime Institute Biotechnology, Jiangsu, China) primary antibodies at 4°C overnight. After washing twice with PBS (5 min/wash), slides were incubated with biotinylated goat-anti-rabbit IgG (Biovision) secondary antibody at 37°C for 40 min followed by HRP-conjugated streptavidin (Biovision) at 37°C for 40 min. After washing twice with PBS (5 min/wash), slides were incubated with 3% H₂O₂ plus 3,3-diaminobenzidine (DAB) chromogen (Biovision) for 5 min and counterstained with hematoxylin, dehydrated and mounted with Canada balsam mounting medium. Cells were visualized using an Olympus microscope and the mean optical density (MOD) was analyzed using image-pro plus 5.0 (Media Cybernetics, Rockville, MD, USA). Two slides were selected from each animal and five fields in each slide were randomly selected for calculating MOD.

Western blot analysis

Animals were sacrificed after 12 months or 20-24 months after which the pancreatic tissue was collected (n=5 per group). Tissues were cut into pieces that were then ground in liquid nitrogen. Homogenization was performed in cell lysis buffer (Beyotime Institute Biotechnology, Jiangsu, China) with protease inhibitors (Roche Applied Science, Penzberg, Germany) until

complete lysis after which cell proteins were collected by centrifugation at 10000 g for 10 min. After determination of protein concentration using the BCA method (BioTeke Co. Beijing, China), 40-60 μ g total cell protein was separated by SDS PAGE and transferred onto nitrocellulose membranes. The membranes were incubated overnight with the blocking buffer at 4°C followed by incubation with primary antibodies (rabbit anti-rat Lamp2, Biovision, Mountain View, CA, USA, 1 μ g/mL; LC3b, Beyotime Institute Biotechnology, 1 μ g/mL; or GAPDH, Boster Biological Technology, Fremont, CA, USA 1 μ g/mL) at room temperature for 2-3 h or overnight at 4°C. Subsequently, membranes were incubated in horseradish peroxidase (HRP)-conjugated secondary antibodies (goat-anti-rabbit IgG, Biovision) diluted in TBST at room temperature for 1 h or overnight at 4°C. Chemiluminescence was employed for visualization at room temperature for 5 min (Millipore, Billerica, MA, USA). The optical densities of the resultant bands were analyzed using ImagePro Plus 6.0 (IPP6.0; Media Cybernetics, Silver Springs, MD, USA) software. The relative expression level was determined as the ratio of the optical density between target protein and GAPDH.

TUNEL assay

Pancreatic tissues sections (4 μ m) were mounted on a slide, dewaxed and rehydrated in graded alcohol solutions. Immunohistochemical detection and quantification of terminal transferase dUTP nick end labeling (TUNEL) was performed using an *in situ* cell death detection kit (TUNEL-POD; Roche) following manufacturer's instructions. Apoptotic cells (brown color) were visualized using an Olympus microscope under 400x magnification. Two slides were selected from each animal and five islets in each slide were randomly selected for calculation. Apoptotic index was expressed as mean $\pm$ standard deviation (SD) for % positive cells.

Statistical analysis

Results for biochemical parameters are given as means and SD. Differences in body weight, fasting blood glucose levels, serum lipid levels and serum insulin levels at different time points were evaluated using ANOVA with Bonferroni correction. The difference of insulin secretion of islet cells at different time points were evaluated using ANOVA. Data analysis was performed using SAS 9.0 statistical software (SAS Institute Inc., Cary, NC USA.) and *p*-values < 0.05 were considered statistically significant.

FIGURE 1. The effects of aging on body weight, and serum levels of fasting blood glucose, lipid and insulin in SD rats. (A) body weight; (B) fasting blood glucose; (C) triglyceride; (D) total cholesterol; (E) free fatty acid; and, (F) insulin. Data were analyzed using ANOVA. Data represent the mean $\pm$ SD, $n=5$. $p<0.05$ compared with the *6- and †12-month time points.

TABLE 2. Insulin secretion by islet cells isolated from 2-, 6-, 12- and 20-month-old rats and cultured in medium containing either 5.5 or 16.7 mmol/L D-glucose. (n=5)

		Insulin secretion (mU/L 30 islets)			
		2-month	6-month	12-month	20-month
5.5 mmol/L	2 h	17.4±3.6	11.6±3.2*	7.7±1.8*	4.0±1.0*†
	4 h	24.3±6.1	12.5±2.5*	11.1±1.9*	5.3±1.1*†‡
	6 h	28.2±4.4	9.0±1.7*	6.8±2.3*	7.6±2.1*†
	24 h	29.1±3.8	9.3±2.0*	8.4±2.4*	6.9±1.6*
16.7 mmol/L	2 h	28.1±3.1	13.4±0.7*	13.6±0.6*	7.1±1.1*
	4 h	36.5±6.7	19.9±4.1*	13.4±4.9*	10.8±2.3*
	6 h	37.1±6.1	12.3±3.7*	8.4±0.6*	11.6±2.2*
	24 h	38.3±9.0	22.4±4.9*	12.6±2.6*	9.7±1.2*

Data were mean ± SD of five rats. Data were analyzed using ANOVA with Bonferroni correction.

* $P < 0.05$ compared to the two-month group after Bonferroni correction.

† $P < 0.05$ compared to the six-month group after Bonferroni correction.

‡ $P < 0.05$ compared to the 12-month group after Bonferroni correction.

Results

The effects of normal aging on body weight, fasting blood glucose, and lipid levels in SD rats

Age-related changes in body weight, and levels of fasting glucose, triglyceride, total cholesterol, FFA and insulin were analyzed (Figure 1A-E). No change in body weight was observed over time (Figure 1A); however, fasting glucose (Figure 1B), triglyceride (Figure 1C) and FFA (Figure 1E) levels were significantly increased at 20 months in comparison with the six-month time point ($p < 0.05$). Total cholesterol levels at the 12-month time point were significantly higher than observed at the six- and 20-months points ($p < 0.05$) (Figure 1D). Serum insulin levels were not significantly increased at 20-24-months (Figure 1F).

Effect of aging on the insulin secretion by the islet cells

Islet cells isolated from 2-, 6-, 12-, and 20-month-old rats were divided into two groups: one group was cultured in medium containing 5.5 mmol/L D-glucose and the other group in medium containing 16.7 mmol/L D-glucose. Compared to islet cells isolated from young rats (two months), those isolated from 6-, 12- and 20-month-old rats secreted significantly less insulin ($p < 0.05$; Table 2). This observation was consistent for islet cells cultured in both 5.5 and 16.7 mmol/L D-glucose. For the islet cells cultured in 5.5 mmol/L D-glucose, cells isolated from 20-month-old rats (the aged group) secreted significantly less insulin than the cells from 6-month-old rats (the adult group) ($p < 0.05$; Table 2).

Effects of aging on pancreatic islet cell autophagy

Using transmission electron microscopy, the subcellular structures of the islet cells were observed in rats of different ages (Figure 2). No autophagy was observed in islet cells of young SD rats (data not shown) and rats aged six months (Fig 2A). As shown in Figure 2A, the nuclei of islet cells in SD rats aged six months were oval and the chromatin was evenly distributed. The nuclear membrane, mitochondria, ribosomes and rough endoplasmic reticulum were clearly observed (Fig 2A). Although an increase in the number of primary lysosomes was observed in SD rats aged 12 months, no autophagic organelles were observed (Fig 2B). Autophagy characterized by the presence of vacuoles surrounding the cytoplasm and organelles as well as lysosomal degradation of substances within the vacuoles was identified in most of the islet cells from aged SD rats (20-24 months old) (Figure 2C).

Expression levels of Lamp2 and LC3b in pancreatic tissue with age

The level of mature autophagosomes was determined by analyzing the expression levels of Lamp2 and LC3b in pancreatic tissue using both immunohistochemistry and Western blot analyses. Immunohistochemical staining revealed increasing expression of both Lamp2 (Figure 3) and LC3b (Figure 4) in pancreatic islet cells with age. As shown in Table 3, semi-quantitative analysis (MOD) of Lamp2 and LC3b expression confirmed a significant increase in Lamp2 and LC3b expression with increased age (both $p < 0.0001$). The MOD of Lamp2 increased from 308 ± 11.2 at two months to 446.5 ± 24.5 at 20

FIGURE 2. Effects of aging on cell autophagy in pancreatic islets of SD rats. A: Morphology of a normal cell within an islet of Langerhans at six-months (10000 $\times$). B: The primary lysosome (red arrow) was observed in an islet cell at 12-months of age. No autophagic organelles were observed (17000 $\times$). C: Red arrow: the presence of an autophagosome in an islet cell at 24-months of age (17000 $\times$); red triangle: special granules in the cytosol of normal β cells; black arrowhead: oval-shaped nucleus with clear nuclear membrane; red arrowhead, mitochondria swelling and degeneration.

FIGURE 3. Changes in Lamp2 expression in Islets of Langerhans with age. Tissues isolated from rats (A) 2-, (B) 6-, (C) 12- and (D) 24-months of age were analyzed for Lamp2 expression (brown staining) using immunohistochemistry followed by counterstaining with hematoxylin (blue) ($\times 400$).

FIGURE 4. Changes in LC3b expression in Islets of Langerhans with age. Tissues isolated from rats (A) 2-, (B) 6-, (C) 12-, and (D) 24-months of age were analyzed for LC3b expression (brown staining) using immunohistochemistry followed by counterstaining with hematoxylin (blue) ($\times 400$).

TABLE 3. Aging experiment: Apoptosis, Lamp2 and LC3b

	2-month	6-month	12-month	20-month	<i>P</i> ^c
Apoptosis, % positive cells	0.50±0.21	2.21±0.77*	3.22±0.36*†	3.62±0.60*†	<0.0001
Lamp2, MOD value	308±11.2	353.2±21.7*	408.4±12.9*†	446.5±24.5*††	<0.0001
LC3b, MOD value	330.8±22.4	358±32.4	431.1±27.1*†	511.6±31.9*††	<0.0001

^a % positive cells in TUNEL assay.^b Mean Optical Density, MOD^c *P* value was derived from ANOVA for analyzing the time trend.**P*<0.05 comparing to two-month, after Bonferroni's correction† *P*<0.05 comparing to six-month, after Bonferroni's correction‡ *P*<0.05 comparing to 12-month, after Bonferroni's correction

FIGURE 5. Western blot analysis showing effects of aging on Lamp2 and LC3b protein expression. The band of M_r 110 kDa is Lamp2; the bands of M_r 16 kDa and 14kDa are LC3b-I (upper band) and LC3b-II (lower band; a cleavage product of LC3b-I), respectively.

months of age ($n=5$; $p<0.05$). LC3b expression significantly increased from 330.8 ± 22.4 at two months to 511.6 ± 31.9 at 20 months of age ($n=5$; $p<0.05$). This observation was also confirmed by Western blot analysis, which revealed that both Lamp2 and LC3b (LC3b-I and LC3b-II) expression increased with age (Figure 5). The relative expression levels of Lamp2 increased from 483.0 ± 73.0 at 12 months to 690.4 ± 81.1 at 20 months of age ($n=5$; $p<0.003$). LC3b expression significantly increased from 1506.6 ± 291.4 at 12 months to 2265.9 ± 259.1 at 20 months of age ($n=5$; $p<0.002$).

Effects of aging on pancreatic islet cell apoptosis

Pancreatic islet cell apoptosis was assessed using the TUNEL assay. As shown in Figure 6, no difference in the number of

FIGURE 6. Effects of aging on islet cell apoptosis. Apoptosis of cells within an islet of Langerhans was assessed in rats (A) 6-, (B) 12- and (C) 24-months of age using the TUNEL assay (400x).

apoptotic cells (brown color) were observed between rats at six and 24 months of age. In addition, no significant difference in the apoptotic index (% positive islet cells) was observed between rats six, 12 and 24 months of age (Table 3). A significant increase in the apoptotic index was observed between young rats (two-months-old; $0.50\pm0.21\%$) and older rats (six-, 12- and 24-months-old) (Table 3).

Discussion

The present study was undertaken with the aim of analyzing the relationship among autophagy, aging and diabetes. Results of the present study revealed that aging induced mitochondrial and endoplasmic reticulum swelling, vacuolar degeneration

and autophagy. In addition, insulin secretion by pancreatic islet cells *in vitro* was reduced in aged SD rats. Finally, increasing expression of both Lamp2 and LC3b in pancreatic islet cells was observed with age.

In the present study, Lamp2 and LC3b protein expression were evaluated as a reflection of autophagy. LC3b is the best-characterized autophagic marker to date. Autophagosome formation and maturation requires the activation of two ubiquitin-like molecules: Atg8p, also known as LC3 or microtubule-associated protein light chain 3 in mammals, and Atg12p, by the activating enzyme Atg7p [13]. LC3 levels are thought to correlate with the level of mature autophagic membranes present [14]. Lamp2 is another marker of the mature autophagosome; it is a ubiquitous lysosomal membrane protein that is highly expressed in normal human pancreatic tissue and is required for the proper fusion of lysosomes with autophagosomes in the late stage of the autophagic process [15]. In the present study, LC3 and Lamp2 expression in islet cells increased with age, which is suggestive of the presence of autophagy in the pancreatic tissue of aged SD rats (20-24 months old).

Marchetti *et al.* [5] and Masini *et al.* [6] have observed increased β cell death in diabetic islets as compared with controls. Approximately 50% of these cells showed signs of apoptotic cell death while the remaining underwent autophagy-associated cell death characterized by massive vacuole overload in the cytoplasm [5]. These observations led us to propose that autophagy might hamper the function of β cells (i.e., insulin secretion); thus, in this study, the activation of autophagy and subsequent appearance of autophagosomes within islet cells, including β cells, during aging may have resulted in reduced insulin secretion by β cells. Apoptosis of islet cells in response to aging was not observed.

The relationship between aging and autophagy in β cells remains controversial. Electron microscopic analysis also revealed an increased number of autophagosomes in β cells of diabetic *db/db* mice [16]. The same research group observed degeneration of islets and impaired glucose tolerance with reduced insulin secretion in the β cells of autophagy-deficient mutants with genetic ablation of Atg7 [7,17]. Instead of hampering the function of β cells, the degradation of cellular components by basal autophagy is essential for the maintenance of normal architecture and function of β cells [7,17]. Previous reports also showed an aging-associated decrease in β cell autophagy [8,9]. Based on the hypothesis proposed by Fujitani *et al.* [7], aging may result in degeneration of islets and reduced insulin secretion by β cells due to decreased autophagy.

Fujitani *et al.* [7] argued that the level of autophagosomes alone does not provide unambiguous evidence that autophagic flux has increased. Increased autophagosome levels in *db/db* mice may indicate decreased autophagy flux caused by impaired autophagosome/lysosome fusion. If this is true, the absence of autophagosomes in the young rats in our study could be due to rapid fusion between autophagosomes and lysosomes, and thereby maintenance of normal architecture and function of β cells. Consequently, the increased presence of autophagosomes in aged rats might be a result of reduced fusion between autophagosomes and lysosomes. Similarly, increased LC3b levels could either be due to formation of early autophagosomes or inhibition of autolysosomal functions. Further analysis is required to clarify these points by following the guidelines for monitoring autophagy in higher eukaryotes, such as measuring the insulin secretion by pancreatic islet cells in the presence and absence of lysosomal protease inhibitors (e.g., chloroquine or leupeptine) [17].

Significant increases in fasting glucose, triglyceride levels and FFA levels were detected in SD rats 20-24 months of age in the present study. In mice fed a high-fat diet, autophagy in β cells was markedly upregulated, and increased FFAs resulted in insulin resistance associated with diabetes and induced autophagy in β cells [7]. In a preliminary study conducted by our group, Wistar rats fed a high-fat diet for six months displayed increased FFA levels, and autophagosomes and apoptotic bodies were observed (data not shown). Thus, an increase in FFA in aged SD rats might induce autophagy in β cells. Again, whether FFA-induced autophagy in β cells correlated with the reduction in insulin secretion by pancreatic islet cells requires further analysis.

The present study has the limitation that islet cell autophagy was only evaluated using transmission electron microscopy without a quantitative assessment, and autophagy-associated islet cell death was not directly measured in aged rats. In addition, Lamp2 and LC3b protein expression are considered to be the indirect markers of autophagy maturation [14]. More direct evidence, such as autophagic flux assessments, may be required to monitor the dynamic change of autophagosome. Finally, although increased islet cell autophagy was observed with age in the present study, the analysis of autophagy markers was not β cell-specific. Further studies to quantitatively evaluate the effect of aging on β cell autophagy will also be the subject of future analysis.

In conclusion, *in vitro* insulin secretion by pancreatic islet cells was significantly reduced at both 12- and 24-months of age, and elevated of fasting blood glucose, triglyceride and FFA levels were observed with age in SD rats. Autophagosomes

were observed in aged rats, which were accompanied with increased islet cell expression of both LC3 and Lamp2. Whether there is a link between FFA levels, autophagy and reduction of insulin secretion by pancreatic islet cells remains to be determined in future studies.

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