

Vitamin D induces autophagy of pancreatic β -cells and enhances insulin secretion

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Abstract. Epidemiological evidence indicates that vitamin D is involved in defense against diabetes; however, the precise underlying mechanism remains to be elucidated. In the present study, the effect of vitamin D on the pathogenesis of diabetes was investigated, with an emphasis on its direct effect on pancreatic β -cells. A streptozotocin (STZ)-induced type 1 diabetes mellitus (T1DM) mouse model and MIN6 mouse insulinoma β -cells were subjected to vitamin D treatment. Histopathological analysis of pancreatic islets was performed to investigate insulinitis, and reverse transcription-quantitative polymerase chain reaction and western blotting were used to determine the mRNA and protein expression levels of markers of autophagy [microtubule-associated protein 1A/1B-light chain 3 (LC3) and Beclin 1] and regulation of apoptosis [B-cell lymphoma 2 (Bcl-2)]. Apoptosis of MIN6 cells was examined by flow cytometry following annexin V/propidium iodide labeling. The secretion of insulin was measured by ELISA. The results revealed that vitamin D reduced the incidence of T1DM, enhanced insulin secretion and relieved pancreatic inflammation in STZ-treated mice. Furthermore, vitamin D increased the mRNA expression levels of *LC3* and *Beclin 1*, and increased Bcl-2 protein expression levels in STZ-treated MIN6 cells, while decreasing the apoptosis rate. The results of the present study demonstrated, for the first time to the best of our knowledge, that vitamin D induces autophagy and suppresses apoptosis of pancreatic β -cells, as well as preventing insulinitis. These findings regarding vitamin D provide insights into its involvement in diabetes, and suggest a potential novel strategy for the treatment of diabetes via agents enhancing autophagy in pancreatic β -cells.

Introduction

Diabetes mellitus is a worldwide health problem, with global incidence estimated at >380 million cases and rising (1). The International Diabetes Federation estimates that by 2035, 592 million people will suffer from diabetes (1). Among diabetic patients, ~10% suffer from type 1 diabetes mellitus (T1DM). T1DM, which primarily affects younger people, results in lifelong dependency on exogenous insulin treatment for survival and has the potential for serious complications, including cardiovascular disease, chronic renal failure and eye damage (2). Although the etiology and pathogenesis of T1DM remain to be fully elucidated, there is a consensus that the autoimmune destruction of insulin-producing pancreatic islet β -cells, as a result of environmental and genetic factors, is critical for its development (3).

Vitamin D has been well-characterized as a regulator of calcium-phosphorus metabolism and bone mineralization (4). While vitamin D exerts other biological effects, its extra-skeletal activities have been extensively investigated. Numerous epidemiological studies have suggested that vitamin D may have a role in defense against diabetes (5,6). Vitamin D deficiency is prevalent among the diabetic population; early and long-term vitamin D supplementation is associated with a decrease in the risk of developing diabetes, and the incidence of T1DM is greater in areas with fewer days of sunlight per year (7,8). However, the underlying mechanisms of the involvement of vitamin D in T1DM remain to be elucidated. The present study aimed to investigate the role of vitamin D in the development of diabetes and the underlying mechanisms.

Autophagy ('self-eating') is a catabolic process of the lysosomal degradation pathway that enables metabolic turnover and homeostasis (9). It is characterized by the sequestration of cytoplasm and organelles to form autophagosomes, which fuse with lysosomes to form autophagolysosomes, resulting in the proteolysis of sequestered material (10). It has been reported that autophagy may influence diverse physiological processes and affect the occurrence and outcome of numerous diseases, including diabetes (11,12). Previous studies have revealed that baseline autophagy is crucial for the maintenance of the normal architecture of pancreatic islets and intracellular insulin content (13,14). In the present study, the role of vitamin D in the pathogenesis of diabetes was investigated, in particular the direct effects of vitamin D on pancreatic β -cells. The results revealed, for the first time to the best of our knowledge,

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that vitamin D enhances autophagy while inhibiting apoptosis of streptozotocin (STZ)-treated β -cells, increases insulin secretion and increases resistance of β -cells to cellular stress encountered during diabetes.

Materials and methods

Animals and experimental protocol. A total of 40 C57BL/6J male mice (age, 10 weeks) were provided by Shanghai Laboratory Animal Center, Chinese Academy of Sciences (Shanghai, China). All mice were maintained at $23\pm 2^\circ\text{C}$ in $50\pm 5\%$ relative humidity under a 12-h light/dark cycle, and had free access to food and water. The T1DM mouse model was induced by multiple intraperitoneal injections of low-dose STZ (Sigma-Aldrich, St. Louis, MO, USA) as described previously (15). Briefly, the experimental mice received intraperitoneal injections of freshly prepared 40 mg/kg STZ [dissolved in 0.1 M citrate buffer (Sigma-Aldrich)] for 5 consecutive days. The first day of STZ administration was designated as day 1 of the study. Body weight, food and water intake, and plasma glucose were monitored weekly for 21 days. Blood was collected from the tip of the murine tail vein, and non-fasting blood glucose concentrations were measured by the glucose oxidase method as described previously (16). Mice were considered diabetic when their glucose levels were >16.7 mmol/l. To investigate the effect of vitamin D on the course of the STZ-induced T1DM mouse model, $1,25(\text{OH})_2\text{D}_3$ (Sigma-Aldrich), the physiologically active metabolite of vitamin D, was administered intraperitoneally 1 h prior to STZ injection at a dose of $5 \mu\text{g/kg}$ (dissolved in peanut oil), and every second day until sacrifice. Mice in the control group were left untreated. Mice were sacrificed by cervical dislocation on day 21; pancreases were removed for histological examination and plasma separated from blood by centrifugation at $1,500 \times g$, 4°C for 10 min was stored at -80°C for the insulin assay. The protocol for the present study was in accordance with the Principles of Laboratory Animal Care and approved by the Administration Committee of Laboratory Animals of Soochow University (Suzhou, China).

Histopathological analysis of pancreatic islets. The pancreases were dissected out, fixed in 10% formalin for 2 h and embedded in paraffin. Sections ($5\text{--}10 \mu\text{m}$) were stained with hematoxylin and eosin as previously described (17). Histopathological evaluation was performed in a blinded fashion. The severity of insulinitis was determined by the extent of cellular infiltration and the presence of islet atrophy was analyzed under light microscopy (magnification, $\times 400$).

Cell culture and analysis of insulin secretion. MIN6 mouse insulinoma β -cells (Shanghai Bioleaf Biotech Co., Ltd., Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich) supplemented with 15% fetal bovine serum (Sigma-Aldrich), 50 mg/l streptomycin and 75 mg/l penicillin at 37°C in an incubator containing 5% CO_2 . Preliminary experiments revealed that treatment with 5 mM STZ for 30 min resulted in significant destruction of MIN6 cells. To evaluate the direct effect of vitamin D on pancreatic β -cells, MIN6 cells were treated

with 0.01 nM $1,25(\text{OH})_2\text{D}_3$ in DMEM for 24 h prior to STZ administration. Control untreated MIN6 cells, MIN6 cells treated with 5 mM STZ for 30 min, and MIN6 cells treated with $1,25(\text{OH})_2\text{D}_3$ prior to STZ treatment were collected and incubated in low (2.8 mM) or high (20 mM) glucose concentrations for 1 h. Insulin secretion was subsequently measured using an enzyme-linked immunosorbent assay (ELISA) kit (BioExpress; VWR International, Radnor, PA, USA) according to the manufacturer's instructions.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). To evaluate the mRNA expression levels of the autophagy markers, *Beclin 1* and microtubule-associated protein 1A/1B-light chain 3 (*LC3*) in MIN6 cells, RT-qPCR was performed as previously described (18). Total RNA was extracted from MIN6 cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), and cDNA was synthesized with the RevertAid[™] First Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. The mRNA expression levels of *Beclin 1* and *LC3* were measured using the Platinum SYBR Green qPCR SuperMix-UDG 92 (Invitrogen; Thermo Fisher Scientific, Inc.) and an ABI7500 Real-Time system (Applied Biosystems; Thermo Fisher Scientific, Inc.). A total of 40 cycles were performed, under the following conditions: denaturation at 95°C for 30 sec, annealing at 60°C for 34 sec and extension at 72°C for 30 sec. The primer sequences are listed in Table I. Each reaction was repeated three times and the mRNA expression levels of *Beclin 1* and *LC3* were normalized to *GADPH* quantification cycle (C_q) values using the comparative quantification cycle $2^{-\Delta\Delta C_q}$ method (19).

Western blotting. To determine the protein expression levels of Beclin 1 and B-cell lymphoma 2 (*Bcl-2*), markers of autophagy and apoptosis regulation, respectively, cells were treated with lysis solution (Promega Corporation, Madison, WI, USA), sonicated and centrifuged at $8,000 \times g$, 4°C for 10 min. The supernatant was collected and protein concentration determined using a bicinchoninic acid assay (Pierce; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Proteins ($45 \mu\text{g}$) were loaded onto 10% SDS-PAGE gels, subjected to electrophoresis (120 V for 65 min) and transferred onto polyvinylidene difluoride membranes. Following blocking in 5% non-fat milk for 1 h at room temperature, membranes were probed with mouse anti- β -actin antibody (1:5,000; catalog no. AM1021B), rabbit anti-Bcl-2 antibody (1:600; catalog no. AP1303A) or rabbit anti-Beclin 1 antibody (1:500; catalog no. AP1818b) at 4°C overnight. Subsequently, the membrane was incubated at room temperature for a further 2 h, washed in Tris-buffered saline with Tween 20 three times and treated with horseradish peroxidase-conjugated goat anti-mouse IgG (1:5,000; catalog no. ASS1021) or goat anti-rabbit IgG (1:5,000; catalog no. ASR1038) at room temperature for 90 min. All antibodies were obtained from Abgent, Inc., San Diego, CA, USA. Protein bands were visualized with the Enhanced Chemiluminescence Plus Western Blotting Detection system (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA). The protein expression levels of Beclin 1 and Bcl-2 were normalized to β -actin.

Table I. Primer sequences.

Gene	Sequence, 5'-3'	
	Forward	Reverse
<i>Beclin 1</i>	TGCTGACGAATCTCAAGTGG	GCTATACATGGCGTGCTGTG
<i>LC3</i>	CATGCCGTCCGAGAAGACCT	TGAGCCGGACATCTTCCACT
<i>GAPDH</i>	CCTTCATTGACCTCAACTACATG	CTTCTCCATGGTGGTGAAGAC

LC3, microtubule-associated protein 1A/1B-light chain 3.

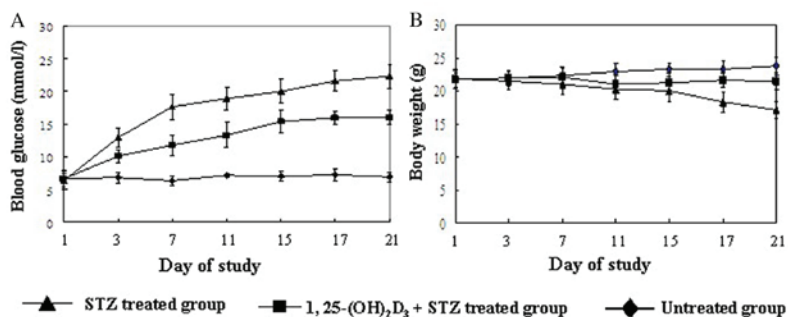


Figure 1. Effect of vitamin D on blood glucose and body weight of diabetic mice. Treatment with 1,25(OH)₂D₃ (A) gradually decreased blood glucose and (B) increased body weight of diabetic mice, compared with mice treated with STZ alone. STZ, streptozotocin.

Apoptosis assay. Annexin V-Fluorescein Isothiocyanate Apoptosis Detection Kit I (BD Biosciences, Franklin Lakes, NJ, USA) was used for assessment of apoptosis. According to the manufacturer's instructions, three groups of MIN6 cells were pelleted by centrifugation at 600 x g for 5 min at room temperature, washed once with ice-cold phosphate-buffered saline and resuspended in binding buffer. Cell suspensions were then incubated with annexin V and propidium iodide at 25°C for 5-15 min in the dark. Analysis was performed using a flow cytometer (FC500; Beckman Coulter, Brea, CA, USA) and Kaluza software version 1.2 (Beckman Coulter).

Statistical analysis. Data are expressed as the mean ± standard deviation from at least three experiments. Statistical analyses were performed in SPSS version 17.0 (SPSS, Inc., Chicago, IL, USA). Comparisons between groups were conducted using one-way analyses of variance and chi-square tests, followed by Tukey's *post hoc* test. *P* < 0.05 was considered to indicate a statistically significant difference.

Results

Vitamin D reduces the incidence of T1DM and increases insulin secretion in the mouse model. To investigate the role of vitamin D in diabetes, its effect on the incidence of diabetes and mouse phenotypes was investigated. Treatment with 1,25(OH)₂D₃ markedly improved diabetes in mice, suggested by gradually decreased blood glucose and increased body weight (Fig. 1). Mice presented with progressive hyperglycemia following STZ treatment, and by 1 week following the initial injection of STZ their plasma glucose levels were all >16.7 mmol/l and diabetes was therefore defined.

Table II. Effect of vitamin D on the incidence of STZ-induced diabetes in mice on specific days following the initiation of STZ treatment.

Group	Incidence of diabetes, %		
	Day 7	Day 14	Day 21
STZ-treated	100	100	100
1,25-(OH) ₂ D ₃ - and STZ-treated -treatment	0	20	40
P-value	0.007	0.039	0.045

n=10 mice per group. STZ, streptozotocin.

Administration of 1,25(OH)₂D₃ significantly reduced the incidence of diabetes (Table II). To evaluate pancreatic β-cell function with regard to insulin secretion, mouse plasma insulin levels were measured on day 21. As presented in Fig. 2, plasma insulin levels were significantly decreased in STZ-treated mice, and 1,25(OH)₂D₃ inhibited the STZ-induced insulin reduction (*P*=0.033), suggesting a possible protective effect against β-cell damage. Administration of 1,25(OH)₂D₃ alone had no effect on insulin secretion compared with the untreated control group.

Vitamin D relieves insulinitis of diabetic mice. Histological analysis of the pancreas of STZ-induced diabetic mice revealed cellular infiltration in and around islets, distorted islets and β-cell degeneration. In accordance with the declined incidence

Table III. Effect of vitamin D on the insulin secretion of STZ-treated MIN6 cells.

Group	Insulin secretion (ng/ml)		
	Low glucose	High glucose	High/low ratio
Untreated control	60.17 \pm 11.85	95.38 \pm 19.11	1.59 \pm 0.44
STZ-treated	39.95 \pm 8.13	42.66 \pm 9.05	1.07 \pm 0.19
1,25-(OH) $_2$ D $_3$ - and STZ-treated	49.82 \pm 5.99	60.37 \pm 13.32	1.21 \pm 0.21
P-value ^a	0.044	0.033	0.041

Cells from the three groups were incubated with low (2.8 mM) and high (20 mM) glucose concentrations for 1 h, and insulin secretion was measured. Data are presented as the mean \pm standard deviation (n=6 per group). ^a1,25-(OH) $_2$ D $_3$ - and STZ-treated vs. STZ-treated. STZ, streptozotocin.

Table IV. Effect of vitamin D on the apoptosis of STZ-treated MIN6 cells.

Group	Apoptosis rate (%)	P-value
Untreated control	6.89 \pm 1.26	0.022 ^a
STZ-treated	37.96 \pm 7.51	0.026 ^b
1,25-(OH) $_2$ D $_3$ - and STZ-treated	20.77 \pm 3.18	0.033 ^c

Apoptosis rate was determined by annexin V and propidium iodide staining. Data are presented as the mean \pm standard deviation (n=6 per group). ^avs 1,25-(OH) $_2$ D $_3$ - and STZ-treated group; ^bvs. untreated control group; and ^cvs. STZ-treated group. STZ, streptozotocin.

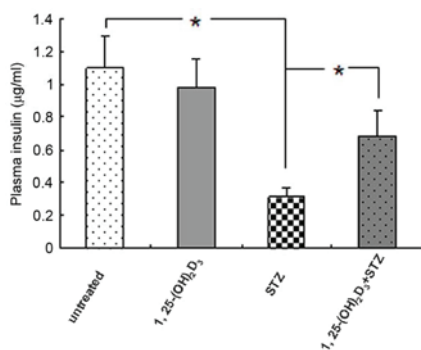


Figure 2. Effect of vitamin D on plasma insulin levels. The insulin level of STZ-treated mice was significantly decreased compared with the untreated control group. The effect was attenuated by treatment with 1,25(OH) $_2$ D $_3$. Administration of 1,25(OH) $_2$ D $_3$ alone had no effect on insulin level. Data are presented as the mean \pm standard deviation (n=10 mice per group). *P<0.05. STZ, streptozotocin.

of diabetes, the administration of 1,25(OH) $_2$ D $_3$ prior to STZ treatment markedly reduced the infiltration of inflammatory cells and other histopathological changes in pancreatic islets (Fig. 3). These results indicate that vitamin D ameliorated islet lesions and β -cell destruction.

Vitamin D enhances insulin secretion of STZ-treated MIN6 cells. To further investigate the *in vivo* data, the effect of vitamin D on insulin secretion by MIN6 pancreatic β -cell lines was assessed *in vitro*. Insulin secretion was significantly

decreased in STZ-treated MIN6 cells compared with the untreated control group, and 1,25(OH) $_2$ D $_3$ enhanced insulin secretion of STZ-treated cells in conditions of low and high glucose concentrations (Table III).

Vitamin D induces the expression of Beclin 1, LC3 and Bcl-2. Relative mRNA expression levels of *Beclin 1* (Fig. 4A) and *LC3* (Fig. 4B) in MIN6 cells were determined by RT-qPCR. The mRNA content of untreated MIN6 cells was designated as 1.0. The results revealed a significant decrease in *Beclin 1* (0.5 \pm 0.08; P=0.032) and *LC3* (0.6 \pm 0.1; P=0.047) mRNA expression levels in the STZ-treated group compared with the untreated control group, while 1,25(OH) $_2$ D $_3$ significantly increased mRNA expression levels of *Beclin 1* (4.5 \pm 0.7; P=0.009) and *LC3* (3.2 \pm 0.5; P=0.007). 1,25(OH) $_2$ D $_3$ treatment alone slightly increased *Beclin 1* and *LC3* mRNA expression levels compared with the untreated control group (Fig. 4). The results indicate an autophagy deficiency in cells following STZ treatment and induction of autophagy activity by vitamin D.

Western blotting revealed that the protein expression levels of the autophagy and regulation of apoptosis markers, Beclin 1 and Bcl-2, respectively, were markedly downregulated in STZ-treated MIN6 cells compared with untreated cells. This effect was attenuated by treatment with 1,25(OH) $_2$ D $_3$ (Fig. 5).

Vitamin D inhibits apoptosis of STZ-treated MIN6 cells. As presented in Table IV and Fig. 6, and consistent with the western blotting results, apoptosis of STZ-treated MIN6 cells was markedly increased, and intervention with 1,25(OH) $_2$ D $_3$ decreased the apoptosis rate significantly.

Discussion

T1DM is a global disease of increasing incidence; however, preventive measures and causal treatments remain lacking. It is widely accepted that genetic predisposition and environmental factors contribute to the development of diabetes. Despite the evidence that vitamin D deficiency is one of the environmental risk factors linked to the development of T1DM (20-23), the precise underlying mechanisms remain to be fully elucidated. The present study used a well-established T1DM animal model of STZ-induced diabetes and the insulinoma cell line, MIN6 to investigate the potential involvement of vitamin D

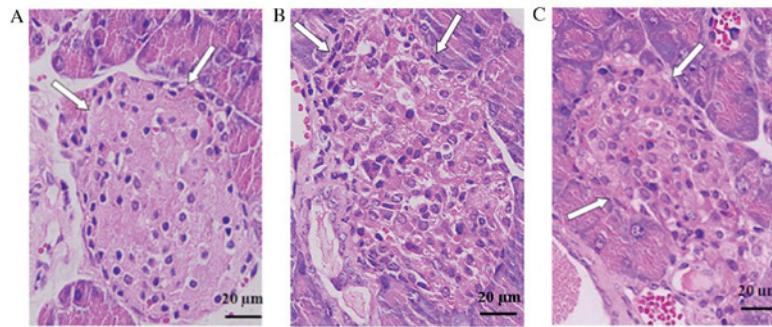


Figure 3. Effect of vitamin D on insulinitis development in diabetic mice. Hematoxylin and eosin staining was performed on pancreatic tissue from (A) untreated, (B) STZ-treated and (C) 1,25(OH)₂D₃- and STZ-treated mice. White arrows indicate pancreatic islets and cellular infiltration was observed in the islets of STZ-treated mice.

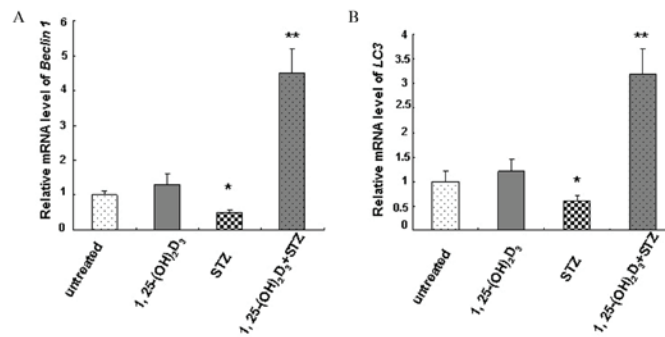


Figure 4. Effect of vitamin D on mRNA expression levels. mRNA expression levels of (A) *Beclin 1* and (B) *LC3* in MIN6 mouse insulinoma β -cells were detected by reverse transcription-quantitative polymerase chain reaction. mRNA expression levels of *Beclin 1* and *LC3* were significantly decreased following STZ treatment, and significantly increased by 1,25(OH)₂D₃ and STZ treatment. *P<0.05 vs. untreated; **P<0.01 vs. STZ alone. STZ, streptozotocin.

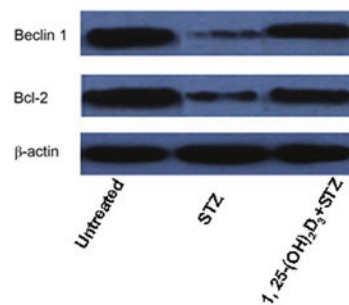


Figure 5. Effect of vitamin D on protein expression levels. Protein expression levels were measured in untreated control, STZ-treated and 1,25(OH)₂D₃- and STZ-treated MIN6 mouse insulinoma β -cells. Protein expression levels of the autophagy and regulation of apoptosis markers, Beclin 1 and Bcl-2, respectively, were markedly downregulated in STZ-treated MIN6 cells compared with untreated cells. This effect was attenuated by treatment with 1,25(OH)₂D₃. Bcl-2, B-cell lymphoma 2; STZ, streptozotocin.

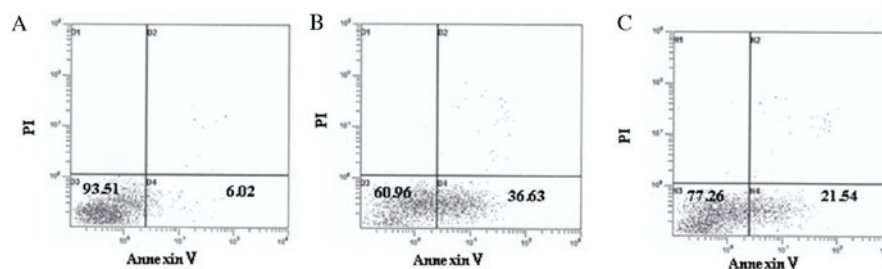


Figure 6. Effect of vitamin D on the apoptosis of STZ-treated MIN6 cells. Cells within the lower left quadrant (Ann V- PI-) were defined as viable and cells within the lower right quadrant (Ann V+ PI+) were defined as early apoptotic. Representative dot plots are presented, with the percentage of cells within the lower quadrants, from (A) untreated control, (B) STZ-treated and (C) 1,25(OH)₂D₃- and STZ-treated groups. The results are representative of two separate experiments (n=6). STZ, streptozotocin; Ann V, annexin V; PI, propidium iodide.

in the pathogenesis of diabetes, with an emphasis on its direct effects on pancreatic islet β -cells.

During T1DM development, β -cell injury is induced by infiltrating immune cells, leading to a progressive impairment of insulin production and ultimately resulting in apoptosis (24). In the present study, insulin secretion by STZ-treated β -cells was decreased and pre-treatment with $1,25(\text{OH})_2\text{D}_3$ promoted insulin secretion. STZ-treated mice exhibited cellular infiltration in pancreatic islets and β -cell apoptosis, while vitamin D significantly reversed insulinitis and protected β -cells against apoptosis. These results demonstrated that vitamin D directly affects β -cells and increases their resistance to cellular stress encountered during diabetes.

Autophagy is an important mechanism underlying cell stability and acts as a defense against cellular stress. The degradation of unnecessary and impaired cellular components by autophagy is essential for the maintenance of normal cellular architecture (9,10). It has been verified that autophagy is important for the survival and function of β -cells (12,14). In the present study, the mRNA expression levels of *LC3* and *Beclin 1* in STZ-treated MIN6 cells were reduced compared with untreated cells, while $1,25(\text{OH})_2\text{D}_3$ enhanced expression levels. *LC3* and *Beclin 1* are essential proteins involved in autophagy, and *Beclin 1* is regarded as a marker of autophagy initiation (25). The results of the present study suggested an autophagy deficit of β -cells in this diabetes model, and demonstrated that vitamin D may induce autophagy and therefore accelerate the renewal of organelles under hyperglycemic conditions. Autophagy activation may serve as a compensatory response in protection from apoptosis (26). MIN6 cell damage induced by STZ was a dynamic process, and injured cells may release certain pro-apoptotic factors that may be removed via vitamin D-induced autophagy. In addition, expression levels of the anti-apoptotic protein *Bcl-2* in STZ-treated MIN6 cells was increased by vitamin D treatment, suggesting the suppression of apoptosis by vitamin D. It has been reported that *Beclin 1*, a critical mediator of autophagy, may be regulated by *Bcl-2*. The formation of a *Beclin 1/Bcl-2* complex affects autophagy and apoptosis (27,28). Autophagy is a dynamic process; preliminary data from our laboratory using the inhibitors of autophagy, 3-methyladenine or bafilomycin A_1 and the autophagy agonist, rapamycin *in vitro* and *in vivo*, has revealed a complex association between vitamin D-mediated autophagy and its protective effects. This association is currently being further investigated in our laboratory, and will be reported in a future publication.

In conclusion, the results of the present study demonstrate, for the first time to the best of our knowledge, that vitamin D induces autophagy and suppresses apoptosis of pancreatic β -cells, as well as preventing insulinitis. These results suggest a potential novel strategy for the treatment of diabetes via agents enhancing autophagy in pancreatic β -cells. Future studies are required to investigate the associations between autophagy, apoptosis and inflammation, and provide novel insights into the involvement of vitamin D in diabetes.

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