1,25(OH)\textsubscript{2}D\textsubscript{3} Activates Autophagy to Protect against Oxidative Damage of INS-1 Pancreatic Beta Cells

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Diabetes mellitus is a serious disease endangering human health worldwide. Vitamin D (Vit D) is a well-characterized regulator of calcium-phosphorus metabolism that also exerts other biological effects extending far beyond mineral homeostasis. Some epidemiological studies have suggested that Vit D has a role in defense against diabetes, although the mechanism remains unclear. Autophagy, an intracellular catabolic process, is necessary to maintain the normal structure and function of host cells. In our previous study, we found that Vit D could induce autophagy of pancreatic beta cells and prevent insulitis, although the underlying mechanisms remain to be fully elucidated. In this study, the protective effect of 1,25(OH)\textsubscript{2}D\textsubscript{3}, the physiologically active metabolite of Vit D, against streptozotocin-induced cytotoxicity in rat insulinoma cell line (INS-1) cells was explored. Cell viability and insulin secretion of INS-1 cells in response to different treatments were measured with a cell counting kit and enzyme-linked immune absorbent assay (ELISA), respectively. In addition, malondialdehyde (MDA) content and total antioxidant capacity (T-AOC) were measured by ELISA. RT-PCR and Western blot analyses were used to detect autophagy levels, reactive oxygen species (ROS) was assessed by fluorescence microscope, ultrastructure analysis was performed using transmission electron microscopy. The results demonstrated that 1,25(OH)\textsubscript{2}D\textsubscript{3} could increase cell viability and insulin secretion of INS-1 cells, and protected cells from oxidative damage induced by streptozotocin (STZ) through autophagy activation. These findings shed light on mechanisms underlying the ameliorative effects of Vit D on diabetes mellitus.

Key words 1,25(OH)\textsubscript{2}D\textsubscript{3}; rat insulinoma cell line (INS-1) cell; oxidative damage; autophagy

INTRODUCTION

Diabetes mellitus (DM), commonly referred to as diabetes, is a major public healthcare concern worldwide. It was estimated that as of 2016, 422 million people had diabetes throughout the world, increased from an estimated 382 million people in 2013; prevalence is 8.5% among adults, nearly double the rate of 4.7% in 1980.\textsuperscript{1,2} Diabetes can cause many complications, such as cardiovascular disease, chronic renal failure, stroke, foot ulcers, and eye damage, which result in a major disease burden globally.

Vitamin D (Vit D), also known as cholecalciferol, was first discovered in 1922. It is a well-characterized regulator of calcium and phosphorus metabolism that maintains bone homeostasis, but it also exerts other important biological effects. Vit D deficiency is closely related to the development of many pathologies, including bone fractures, cardiovascular disease, diabetes, cancer, and more.\textsuperscript{3–5} In recent years, the impact of Vit D on the incidence of diabetes has become a hot topic in biomedical research. Indeed, a number of \textit{in vitro} and \textit{in vivo} experiments have suggested potential roles for Vit D in the development of diabetes. For example, low Vit D levels have been shown to increase the risk for type 2 diabetes (T2DM) in older adults.\textsuperscript{6} However, the mechanism by which Vit D influences diabetes has not been fully elucidated thus far.

In our previous study, we found that Vit D could induce autophagy of pancreatic beta cells and prevent insulitis, thus affecting the development of diabetes.\textsuperscript{7} Autophagy, literally ‘self-eating,’ is a catabolic process characterized by lysosomal degradation of materials or organelles to maintain cellular energy balance and organelle function. Moreover, enhancement of this process is vital for the active reallocation of nutrients from unnecessary processes to more pivotal processes required for survival. Recent studies have provided evidence for roles of autophagy in human health and disease. A low level of autophagy has been shown to be essential for proper cell function. Furthermore, increasing evidence suggests that autophagy plays an important role not only in insulin release and insulin sensitivity, but also in maintaining the mass, structure, and function of pancreatic beta cells.\textsuperscript{8,9} To our knowledge, few reports have addressed the involvement of Vit D in cell autophagy and subsequent effects on islet cell function.

Hyperglycemia-mediated oxidative stress plays a major role in the development of diabetic complications,\textsuperscript{10} and Vit D can protect cells against oxidative damage.\textsuperscript{11} This inspired and motivated us to study the effect of Vit D on diabetes development through its functions of anti-oxidation and autophagy activation. In the present study, INS-1 cells, a rat insulinoma cell line that stably secretes insulin and is ideal for studying pancreatic beta cell function,\textsuperscript{12} and 1,25(OH)\textsubscript{2}D\textsubscript{3}, the physiologically active metabolite of Vit D, were used to explore underlying mechanisms of Vit D in amelioration of DM.

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MATERIALS AND METHODS

Cell Culture INS-1 cells purchased from Shanghai Bioleaf Biotech Company (China) were cultured in RPMI 1640 media (Gibco, U.S.A.) supplemented with 10% fetal bovine serum (Gibco), 2 mM L-glutamine (Sigma, U.S.A.), 50 µM β-mercaptoethanol (Sigma), 1 × 10^5 IU/L penicillin, and 100 µg/mL streptomycin. Cells were maintained at 37°C in a humidified incubator containing 5% CO2 and 95% air. To investigate the effects of Vit D on INS-1 cells damaged by streptozotocin (STZ), INS-1 cells were incubated with 5 mM STZ (Sigma) for 3 h (labeled as STZ group) before adding 1,25(OH)2D3 (Sigma) for an additional 24 h (labeled as STZ + VitD3 group). In another group, INS-1 cells were pre-cultured with 10−7 M 1,25(OH)2D3, followed by incubation with STZ for 3 h (labeled as VitD3 + STZ group).

Cell Viability Assay Cell viability was determined with a cell counting kit CCK8 (Dojindo, Japan). Briefly, cells were treated with 10 µL CCK8 for 30 min after incubation with different concentrations of 1,25(OH)2D3 or STZ. Absorbance was measured at 450 nm using a microplate reader.

Glucose-Stimulated Insulin Secretion (GSIS) To test glucose-stimulated insulin release, INS-1 cells were washed twice with phosphate-buffered saline (PBS, Gibco) and then pre-incubated with Krebs–Ringer buffer (137 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4–7H2O, 2.5 mM CaCl2–2H2O, 25 mM NaHCO3, and 0.25% bovine serum albumin) containing 2.8 mM glucose for 1 h at 37°C. Afterward, this buffer was replaced with the same buffer containing 20 mM glucose and cells were incubated for an additional 1 h. The supernatant was collected, and insulin concentration in the supernatant was measured using a rat insulin enzyme-linked immune absorbent assay (ELISA) kit (Bio Express, U.S.A.) according to the manufacturer’s protocol. The GSIS index was calculated as the ratio of GSIS with high glucose (20 mM) to low glucose (2.8 mM).

Detection of Malondialdehyde (MDA) Content and Total Antioxidant Capacity (T-AOC) To evaluate the protective and reparative effects of Vit D on INS-1 cells damaged by STZ, MDA content and T-AOC were analyzed using ELISA kits (Bio Express) according to the manufacturer’s instructions.

Reactive Oxygen Species (ROS) Assessment by Fluorescence Microscope INS-1 cells were seeded into 6-well plates (2.5 × 10^4 cells/well) and cultured at 37°C for 24 h. Then, cells were exposed to different reagents, and stained with 10 µM 2,7-dichlorodihydrofluorescein-diacetate (DCFA) for 30 min at 37°C. Stained Cells were then washed twice with PBS and observed under fluorescence microscope.

RNA Isolation and Quantitative Real Time PCR (qRT-PCR) To evaluate autophagy levels in INS-1 cells, expression of autophagy-related genes was examined at the mRNA level. Total RNA was extracted using Trizol reagent (Invitrogen, U.S.A.) and cDNA was synthesized with RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific, U.S.A.) according to the manufacturer’s instructions. Primer sequences are listed in Table 1. An iTaq™ Universal SYBR Green Supermix (BioRad, U.S.A.) and cDNA templates were used to perform qRT-PCR. Gene expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GADPH). Each reaction was repeated three times and the relative expression level of each target gene was then calculated using the 2^ΔΔCt method.[13]

Western Blot Analysis Cells were lysed in RIPA buffer on ice for 20 min, then whole proteins were extracted by centrifugation at 14000 × g for 10 min at 4°C. Equivalent amounts of protein samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transferred to a polyvinylidene fluoride (PVDF) membrane, which was blocked in 5% skim milk in tris-buffered saline with Tween for 1 h before probing with rat monoclonal antibodies against β-actin (1:5000), Beclin1 (1:500), LC3 (1:600), Nuclear factor, erythroid 2-like 2 (Nrf2) (1:1000) and Keap1 (1:1000) overnight at 4°C. The following day, the membrane was incubated with horseradish peroxidase-conjugated anti-goat or anti-mouse secondary antibodies (1:2000) for 60 min at 37°C. Immune-reactive bands were visualized with a Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Scientific).

Table 1. Sequence of Primers

| Gene          | 5’−3’            | Primers                                                                 |
|---------------|------------------|------------------------------------------------------------------------|
| GAPDH         | Forward          | CCTTCATTGACCTCAAATACATG                                                |
|               | Reverse          | CTTTCTCCATCGTGTTGAAGAC                                                 |
| Beclin1       | Forward          | TGGTGAAGTCTCAAATCTGA                                                    |
|               | Reverse          | GCTATACATGGCGTGCTGTT                                                  |
| LC3           | Forward          | CATGCCGTCGGAAGACCT                                                     |
|               | Reverse          | GATGAGCCCGACATCTCCACT                                                  |

Fig. 1. Effect of Different Concentrations of Vit D on INS-1 Cells
A. The glucose-stimulated insulin secretion in INS-1 cells measured by ELISA.
B. The insulin secretion index calculated by the ratio of insulin secretion at high glucose to low glucose. C. Cell viability analyzed by CCK-8. **p < 0.01.
**Electron Microscopy Evaluation** INS-1 cells were collected after treatment and fixed with 2.5% glutaraldehyde in 0.1M PBS (pH 7.4) at 4°C for 2 h. Subsequent procedures for transmission electron microscopy (TEM) evaluation were performed as previously described. 

**Statistical Analyses** Statistical analyses were performed using Prism version 5.0 (GraphPad, U.S.A.). Comparisons were made using a two-tailed Student’s t test or one-way ANOVA for experiments involving more than two groups. \( p < 0.05 \) was considered statistically significant. Experiments were repeated in total three times for each group.

**RESULTS**

**Effect of Different Concentrations of 1,25(OH)\(_2\)D\(_3\) on INS-1 Cells** The best working concentration of 1,25(OH)\(_2\)D\(_3\) was first determined by examining the effects of four different concentrations (10\(^{-6}\), 10\(^{-7}\), 10\(^{-8}\), and 10\(^{-9}\)mM) of 1,25(OH)\(_2\)D\(_3\) on INS-1 cells after 24 h of incubation. We found that 10\(^{-7}\)mM 1,25(OH)\(_2\)D\(_3\) plus exposure to high glucose could significantly promote insulin secretion, as this yielded the maximum GSIS index (Figs. 1A, B). Furthermore, cell viability was significantly increased with this treatment compared with that of control cells.

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Fig. 2. Effect of Vit D on INS-1 Cells Damaged by STZ

A&B. The high and low glucose-stimulated insulin secretion in STZ-damaged INS-1 cells measured by ELISA, respectively; C. The calculation of insulin secretion index. D. Cell viability analyzed by CCK-8. E. Effect of Vit D on the MDA of INS-1 cells. F. Effect of Vit D on the T-AOC of INS-1 cells. *\( p < 0.05 \), **\( p < 0.01 \), ***\( p < 0.001 \) (\( n = 6 \)).
with the control group (Fig. 1C). Thus, $10^{-7}$ mM 1,25(OH)$_2$D$_3$ was used in subsequent experiments.

**Effects of 1,25(OH)$_2$D$_3$ on INS-1 Cells Damaged by STZ.** Our results demonstrated that cells in the STZ group were seriously damaged, as insulin secretion was significantly decreased in response to both low and high levels of glucose stimulation (Figs. 2A, B). Insulin secretion and cell viability were also decreased in both STZ + VitD$_3$ and VitD$_3$ + STZ groups compared with the control group, although significantly increased compared with the STZ group (Figs. 2C, D). These results indicated that 1,25(OH)$_2$D$_3$ played a role in protection and/or repair of STZ-damaged INS-1 cells.

**Protective Role of 1,25(OH)$_2$D$_3$ against Oxidative Damage of INS-1 Cells** MDA and T-AOC levels were detected to evaluate the antioxidant capacity of INS-1 cells with different treatments. The results showed that MDA contents in STZ + VitD$_3$ and VitD$_3$ + STZ groups were lower than in the control group, but higher than in the STZ group (Fig. 2E). Meanwhile, T-AOC levels in both STZ + VitD$_3$ and VitD$_3$ + STZ groups were higher than in the STZ group (Fig. 2F). These results demonstrated that Vit D could protect against STZ-induced oxidative damage of INS-1 cells.

**1,25(OH)$_2$D$_3$ Stimulates Autophagosome Accumulation in INS-1 Cells** Ultrastructural alterations of INS-1 cells were observed by TEM. Beta particles and normal organelles, such as endoplasmic reticulum, were present in control group cells (Fig. 3A). INS-1 cells treated with $10^{-7}$ mM Vit D for 24h, C. INS-1 cells pretreated with $10^{-7}$ mM Vit D for 24h before adding STZ in the media (Vit D + STZ). D. INS-1 cells with STZ treatment followed by adding $10^{-7}$ mM Vit D (STZ + Vit D). In A, B, C, D, boxed areas were magnified in panels below. Black arrows, autophagosomes; white arrows, beta particles. E. INS-1 cells with STZ treatment.

**1,25(OH)$_2$D$_3$ Increases Expression of Autophagy-Related Proteins** Relative mRNA expression levels of Beclin1 and LC3 in INS-1 cells were determined by qRT-PCR. mRNA levels of control cells were designated as 1.0. The results revealed significantly increased levels of Beclin1 and LC3 in cells treated with 1,25(OH)$_2$D$_3$, while STZ significantly decreased these levels (Fig. 4A). The results of Western blot analysis demonstrated the same trend (Fig. 4B).

**1,25(OH)$_2$D$_3$ Activates Autophagy to Protect against...**
**Oxidative Damage of INS-1 Cells** To further confirm the functional relationship between antioxidant effect of Vit D and autophagy, we used 3-methyladenine (3-MA), a specific inhibitor of autophagy, to block autophagy that was induced by Vit D. Compared with treatment with $10^{-7}$ mM Vit D for 12 h alone, INS-1 cells that were pretreated with 5 mM 3-MA for 12 h and then cultured with $10^{-7}$ mM Vit D for another 12 h, showed a significant decrease in the level of LC3-II (Figs. 5B, C). We further detected the ROS after pretreatment with 3-MA to elucidate the relationship between oxidative stress and autophagy in STZ-induced toxicity. Compared with 3-MA group, the intracellular ROS content in Vit D group decreased significantly (Fig. 5A). Meanwhile, we also detected the changes in the expression of Nrf2-Keap1 proteins. Consistent with these results, the expression of Nrf2 was attenuated in the 3-MA group compared with the Vit D group, whereas the level of the Nrf2 repressor Keap1 was enhanced in the 3-MA group compared with the Vit D group (Figs. 5B, D, E).

**DISCUSSION**

Vit D plays an important role in maintaining physical fitness and can contribute to diverse processes essential for life in higher animals that extend far beyond mineral homeostasis. However, Vit D deficiency is highly prevalent throughout the world due to a lack of appropriate sunlight exposure and reduced Vit D consumption in modern diets. In recent decades, an increasing number of studies have demonstrated that Vit D is a modulating environmental factor for the development of DM. The occurrence of type 1 diabetes mellitus (T1DM), an autoimmune disease, is dependent on a mutual influence between genetic and environmental factors; 1,25(OH)D$_3$ plays an immune modulatory role in the prevention of T1DM. Moreover, it is possible that Vit D deficiency during childhood predisposes one to the onset T1DM, as Vit D is involved in the complicated mechanisms mitigating beta cell destruction. Several studies have also suggested that Vit D status is closely linked to the risk of T2DM, which is characterized by both insulin resistance and pancreatic beta cell dysfunction. Notably, the biologically active form of Vit D, 1,25(OH)$_2$D$_3$, promotes insulin production and secretion. Although many in vitro and in vivo experiments have elucidated a relationship between Vit D and DM, the underlying mechanisms remain unclear.

Pancreatic beta cells are crucial for the control of glucose homeostasis, such that abnormalities in these cells impairs insulin production, leading to severe diabetes. In this study, we examined whether Vit D had a protective effect on STZ-induced damage of INS-1 pancreatic beta cells and analyzed potential mechanisms. As STZ can selectively destroy pancreatic beta cells through apoptosis or necrosis, it is widely used to induce models of DM. We found that INS-1 cells treated with STZ exhibited obvious cell dysfunction, but 1,25(OH)$_2$D$_3$ intervention could significantly alleviate this damage. Further investigation suggested that 1,25(OH)$_2$D$_3$ could increase levels of Beclin1 and LC3 of INS-1 cells. Beclin1 has a well-established role in regulating autophagy by promoting vesicle nucleation. LC3 is a microtubule-associated protein, and its cytosolic form (LC3-I) converts to LC3-phosphatidylethanolamine (LC3-II) during autophagosome formation. Detection of LC3 has become a reliable method for surveying autophagy. In the present study, the results showed that LC3-II levels in INS-1 cells treated with 1,25(OH)$_2$D$_3$ were significantly increased, and autophagosome accumulation was observed by TEM in cells treated with 1,25(OH)$_2$D$_3$. Our results demonstrated that Vit D could activate autophagy of INS-1 pancreatic beta cells, consistent with our previous study. At the onset of diabetes, the occurrence of oxidative stress is considered a major causative factor. Excessive production of ROS is closely related to the pathogenesis of diabetes. ROS can directly injure islet beta cells, promote beta cell apoptosis and induce inflammatory reaction of beta cells through indirect activation of nuclear factor-kappaB (NF-κB) pathways.

**Fig. 4. Vit D Increases the Level of Beclin 1 and LC3 in INS-1 Cells**

A. Relative mRNA levels of Beclin 1 and LC3 in INS-1 cells determined by qRT-PCR. B. Protein expression levels of Beclin 1 and LC3 in INS-1 cells by Western blot analysis. *$p<0.05$, **$p<0.01$, ***$p<0.001$. In the present study, the results showed that LC3-II levels in INS-1 cells treated with 1,25(OH)$_2$D$_3$ were significantly increased, and autophagosome accumulation was observed by TEM in cells treated with 1,25(OH)$_2$D$_3$. Our results demonstrated that Vit D could activate autophagy of INS-1 pancreatic beta cells, consistent with our previous study. At the onset of diabetes, the occurrence of oxidative stress is considered a major causative factor. Excessive production of ROS is closely related to the pathogenesis of diabetes. ROS can directly injure islet beta cells, promote beta cell apoptosis and induce inflammatory reaction of beta cells through indirect activation of nuclear factor-kappaB (NF-κB) pathways.
Keap1–Nrf2 pathway is one of the most important defense mechanisms against oxidative stress, and decoupling of Nrf2 and Keap1 is an important part of Nrf2 activation. Therefore, Keap1 is regarded as a sensor of oxidative stress. Similarly, oxidative stress plays a major role in the cytotoxic effect of STZ on pancreatic beta cells. Vit D plays an important role against oxidative stress. In this study, we detected MDA contents and T-AOC, and found that 1,25(OH)2D3 could decrease MDA content while increasing T-AOC. To further confirm the functional relationship between antioxidant effect of Vit D and autophagy, we applied autophagy inhibitor 3-MA, and detected the markers associated with antioxidant stress. Autophagy level was lowered by 3-MA. Furthermore, we also found that 3-MA almost completely blocked the inhibitory effect of 1,25(OH)2D3 on STZ-induced oxidative stress. The results displayed that ROS content increased, Nrf2 expression decreased and Keap1 expression enhanced. Our results further clarify the antioxidant property of Vit D, as our findings about the effect of Vit D on pancreatic beta cell autophagy led us to conclude that Vit D has a pivotal role against STZ-induced oxidative damage in INS-1 cells through autophagy activation. To date, there have been only a few studies investigating the link between Vit D and oxidative stress in mechanisms underlying DM. Here, the effects of Vit D against STZ-induced cytotoxicity of INS-1 cells were assessed. Our results indicated that Vit D protected against oxidative stress.
in INS-1 cells through autophagy activation, thus illuminating the process by which Vit D can affect the development of DM. However, as the correlation between oxidative stress and autophagy is very complex, more studies are needed to better clarify the underlying mechanisms of the ameliorative effects of Vit D on DM.

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Conflict of Interest  The authors declare no conflict of interest.

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