SIRT6 protects against palmitate-induced pancreatic β-cell dysfunction and apoptosis

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Abstract

Chronic exposure of pancreatic β-cells to abnormally elevated levels of free fatty acids can lead to β-cell dysfunction and even apoptosis, contributing to type 2 diabetes pathogenesis. In pancreatic β-cells, sirtuin 6 (SIRT6) has been shown to regulate insulin secretion in response to glucose stimulation. However, the roles played by SIRT6 in β-cells in response to lipotoxicity remain poorly understood. Our data indicated that SIRT6 protein and mRNA levels were reduced in islets from diabetic and aged mice. High concentrations of palmitate (PA) also led to a decrease in SIRT6 expression in MIN6 β-cells and resulted in cell dysfunction and apoptosis. Knockdown of Sirt6 caused an increase in cell apoptosis and impairment in insulin secretion in response to glucose in MIN6 cells even in the absence of PA exposure. Furthermore, overexpression of SIRT6 alleviated the palmitate-induced lipotoxicity with improved cell viability and increased glucose-stimulated insulin secretion. In summary, our data suggest that SIRT6 can protect against palmitate-induced β-cell dysfunction and apoptosis.

Introduction

The prevalence of type 2 diabetes mellitus (T2DM), a chronic metabolic disorder, has been increasing steadily all over the world (Kahn et al. 2006, Doria et al. 2008). The pathogenesis of T2DM is multifactorial, but insulin secretory deficiency due to reduced pancreatic β-cell mass or function is a major pathogenic factor (Prenki & Nolan 2006, Muoio & Newgard 2008). In the prediabetic stage, because of insulin resistance, islets respond to increased insulin demand with enhanced insulin secretion and expanded β-cell mass in order to maintain euglycemia by compensatory hyperinsulinemia. However, as T2DM ensues, β-cells exhibit decompensation, a defect that has been attributed to both β-cell dysfunction and β-cell death (Weir & Bonner-Weir 2004). A common feature of the prediabetic stage is an excess of circulating lipids, especially saturated fatty acids, which impairs not only peripheral insulin sensitivity but also islet β-cell function (Weir & Bonner-Weir 2004). Acute exposure to elevated free fatty acids (FFA) increases β-cell proliferation and insulin secretion, whereas prolonged exposure to FFA inhibits insulin secretion and induces β-cell apoptosis (Shimabukuro et al. 1998, Biden et al. 2004). However, the underlying mechanisms responsible for the β-cell lipotoxicity remain incompletely understood.

The sirtuin protein family has seven members in mammals (SIRT1–7) (Dong 2012). SIRT6 is a
SIRT6 protects β-cells from lipotoxicity

In recent years, SIRT6 has been identified to modulate many important cellular processes, such as DNA repair, tumor suppression, anti-inflammation and metabolism. Sirt6-deficient mice display severe hypoglycemia and a multisystemic aging phenotype and died around 4 weeks after birth (Mostoslavsky et al. 2006, Xiao et al. 2010). Interestingly, SIRT6 transgenic mice treated with high-fat diet secrete more insulin in response to glucose compared with their wild-type littermates (Kanfi et al. 2010). In our recent study, we have shown that deletion of Sirt6 in pancreatic β-cells in mice leads to impaired glucose-stimulated insulin secretion (GSIS). We have further identified that SIRT6 regulates insulin secretion by maintaining mitochondrial function and modulating intracellular Ca\(^{2+}\) dynamics (Xiong et al. 2016). However, whether SIRT6 is involved in β-cell lipotoxicity remains unclear. The aim of this study was to illustrate the role of SIRT6 in palmitate (PA)-induced β-cell dysfunction and apoptosis.

Materials and methods

Cell culture and treatment

MIN6 cells between passages 15 and 20 were cultured in Dulbecco’s modified Eagle’s medium with 25 mM glucose supplemented with 15% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 50 mM β-mercaptoethanol. Palmitic acid (Sigma-Aldrich) was conjugated with fatty-acid-free BSA before addition to cell culture. PA was dissolved in 99% ethanol to a concentration of 100 mM, and then mixed with 10% BSA in serum-free high-glucose DMEM to make a 4 mM PA stock solution. The BSA-conjugated PA was added to MIN6 cells at a final concentration of 0.4 mM.

Adenovirus transduction

Adenoviruses carrying SIRT6 or GFP were generated using pAdEasy system (Agilent) while adeno viral Sirt6 (mouse) or GFP shRNAs were generated using BLOCK-it system (Invitrogen). Adenoviruses were amplified in HEK293A cells and purified by CsCl gradient centrifugation. The viruses were titered using QuickTiter Adenovirus Titer Immunoassay Kit (Cell Biolabs) according to the manufacturer’s protocol. Generally, we used 25–50 multiplicity of infection (MOI) for overexpression and 50–100 MOI for shRNA knockdown experiments.

Real-time RT PCR

Total islet RNA samples were prepared by using TRIzol reagent (Invitrogen) and converted into cDNA using a cDNA synthesis kit (Applied Biosystems). Real-time PCR analysis was performed using SYBR Green Master Mix (Promega) in an Eppendorf Realplex real-time PCR system.

Western blotting

Protein extracts from cells were made in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl\(_2\), 1 mM EGTA) and an additional protease cocktail tablet from Roche at one tablet/10 mL final buffer volume. Protein extracts were resolved on an SDS-PAGE gel and transferred to nitrocellulose membrane (Santa Cruz Biotechnology). The membrane was incubated with the following antibodies: Sirt6 (Sigma-Aldrich), Actinin (Santa Cruz Biotechnology), Ac-H3K9, Cleaved Caspase 3 (Cell Signaling Technology). Detection of proteins was carried out by incubation with...
HRP-conjugated secondary antibodies, followed by ECL detection reagents (Thermo Fisher Scientific).

**Insulin secretion assay**

On the day of experiment, MIN6 cells were preincubated with secretion assay buffer (SAB; 114 mM NaCl, 4.7 mM KCl, 1.2 mM KH$_2$PO$_4$, 1.16 mM MgSO$_4$, 20 mM HEPES, 2.5 mM CaCl$_2$, 25.5 mM NaHCO$_3$, 0.2% BSA, pH 7.2) containing 2.5 mM glucose for 1 h. After removal of the incubation medium, cells were then incubated for 1 h in SAB containing the indicated glucose concentrations. Incubation medium was then collected and the amount of secreted insulin was analyzed using a mouse insulin ELISA kit (ALPCO). Protein concentration was determined by BCA protein assay kit (Thermo Fisher Scientific) and used to normalize insulin levels.

**MTT assay**

MIN6 cells were seeded in 96-well plates with $5 \times 10^4$ cells per well. The cells were exposed to 0.4 mM PA treatment or transduced with indicated adenoviruses for the indicated periods of time. Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) kit (Sigma-Aldrich) according to the manufacturer’s protocol.

**Statistical analysis**

All data are presented as mean ± s.e.m. Analysis was performed using 2-tailed unpaired Student’s $t$-test for two-group comparisons and one-way ANOVA for multiple-group comparisons, and $P<0.05$ was considered as significant.

**Results**

**SIRT6 is decreased in both diabetic and aged pancreatic islets**

Our recent study has shown that $\text{Sirt6}$ deletion in β-cells results in β-cell dysfunction (Xiong et al. 2016), so we...
were curious about the SIRT6 status under diabetic and aging conditions. The leptin receptor mutant db/db mouse is a widely used type 2 diabetic mouse model. We analyzed SIRT6 protein levels in isolated islets from the db/db and control wild-type mice, and the data showed that SIRT6 protein levels were decreased in the db/db islets compared with controls (Fig. 1A). As a substrate of SIRT6, acetylated H3K9 was elevated in the db/db islets (Fig. 1A). Since SIRT6 has been recently identified as an important regulator of aging, we also checked whether SIRT6 is altered in the aged pancreatic islets. Indeed, SIRT6 protein levels were decreased in aged mouse islets compared with young controls (Fig. 1B).

Consistently, acetylated H3K9 was also elevated in the aged islets as expected (Fig. 1B).

Palmitate decreases Sirt6 expression in mouse islets and MIN6 cells

As shown in Fig. 1, Sirt6 expression is downregulated in islets from diabetic or aged mice. Since hyperlipidemia is one of the common features of type 2 diabetes, we also examined Sirt6 expression in mouse islets exposed to PA (0.4 mM) for 72 h. SIRT6 protein levels in the mouse islets were decreased after 72 h of PA treatment relative to the controls (Fig. 2A). To further assess the effect of PA on Sirt6 gene expression in pancreatic β-cells, MIN6 cells were treated with 0.4 mM PA. As shown in Fig. 2B, Sirt6 mRNA levels were significantly reduced in MIN6 cells exposed to PA for 48 h. The Western blot data also confirmed that PA treatment decreased SIRT6 protein in a time-dependent manner (Fig. 2C and D). As expected, acetylation of the H3K9 residue was elevated after the PA treatment (Fig. 2C and D). It is known that lipotoxicity can ultimately lead to β-cell dysfunction and apoptosis (Sharma & Alonso 2014, Janikiewicz et al. 2015). To investigate the lipotoxic effect of PA on MIN6 cells, we further examined glucose-stimulated insulin secretion (GSIS) and cell viability in MIN6 cells in the presence or absence of PA. As shown in Fig. 2E, MIN6 cell viability was significantly decreased after exposure to PA for 48 h. As expected, a significant induction of cleaved caspase 3 by PA was observed. Consistent with the previous studies, although PA treatment did not reduce the basal insulin secretion in MIN6 cells, high-glucose-stimulated insulin secretion was remarkably attenuated. As shown in Fig. 2E, PA-treated MIN6 cells secreted ~30% less insulin compared with BSA-treated MIN6 cells when stimulated with 16.7 mM glucose.

Sirt6 knockdown in MIN6 cells leads to cell apoptosis and insulin secretory impairment

To verify whether Sirt6 deficiency can lead to apoptosis in MIN6 cells, we performed Sirt6 knockdown by transduction with Sirt6 shRNA adenoviruses. Sirt6 knockdown was confirmed by Western blot analysis (Fig. 3A and B). As expected, Sirt6 knockdown in MIN6 cells increased the levels of Ac-H3K9 and cleaved caspase 3 (Fig. 3A and B). Moreover, PA treatment further exaggerated the elevation of Ac-H3K9 and cleaved...
caspase 3 induced by Sirt6 knockdown (Fig. 3A and B). Consistently, Sirt6-deficient MIN6 cells exhibited decreased cell viability and impaired GSIS even without PA treatment (Fig. 3C and D).

**SIRT6 protects MIN6 cells against PA-induced cell apoptosis and insulin secretory defects**

To examine whether SIRT6 can protect MIN6 cells from lipotoxicity, we overexpressed SIRT6 in MIN6 cells by adenoviral transduction. Overexpression of SIRT6 in MIN6 cells under regular culture conditions exhibited no effect on cell survival and insulin secretory capacity (Fig. 4A, B, C, D). However, after 48-h PA treatment, SIRT6 overexpression resulted in reduced caspase 3 activation and enhanced cell viability (Fig. 4A, B, C). Moreover, overexpression of SIRT6 improved insulin secretory capacity in response to glucose stimulation in PA-treated MIN6 cells compared with control GFP (Fig. 4D).

**Discussion**

Hyperlipidemia is known to cause β-cell dysfunction, which is characterized by impaired GSIS and increased apoptosis (Sharma & Alonso 2014, Janikiewicz et al. 2015). Individuals with T2DM have elevated levels of PA, which is one of the most prevalent saturated fatty acids in the circulation and has been linked to functional impairment of pancreatic β-cells (Kharroubi et al. 2004). Several molecular processes associated with lipotoxicity in β-cells have been reported, including endoplasmic reticulum (ER) stress, mitochondrial dysfunction, increased reactive oxygen species (ROS), elevated ceramide and impaired autophagy (Sharma & Alonso 2014, Janikiewicz et al. 2015).
SIRT6 is primarily characterized as a nuclear NAD+-dependent deacetylase of histone H3K9, H3K18 and H3K56 (Michishita et al. 2008, 2009, Yang et al. 2009, Tasselli et al. 2016). SIRT6 has been implicated in diverse cellular functions including anti-inflammation, metabolic homeostasis, stress resistance and tumor suppression (Kugel & Mostoslavsky 2014). In this study, we have examined the role of SIRT6 in protection against PA-induced β-cell lipotoxicity. Our data reveal that SIRT6 expression is significantly decreased not only in PA-treated MIN6 cells but also in diabetic and aged mouse islets. Knockdown of Sirt6 in MIN6 cells increases cell apoptosis and impairs GSIS, whereas overexpression of SIRT6 protects β-cells from apoptosis and improves insulin secretory capacity in response to glucose.

We have demonstrated in our previous work that Sirt6 knockout in pancreatic β-cells reduces ATP production and increases mitochondrial damage (Xiong et al. 2016). Mitochondrial oxygen consumption rates (OCR) are significantly decreased in Sirt6-knockdown MIN6 cells as well (Xiong et al. 2016). Consistent with our findings, mitochondrial defects have also been observed in Sirt6-knockout mouse ES cells and Sirt6-knockout breast cancer cells (Zhong et al. 2010, Choe et al. 2015). Chronic PA exposure induced β-cell dysfunction, and apoptosis is partly mediated by mitochondrial dysfunction (Janikiewicz et al. 2015). Thus, it is likely that SIRT6 may exert its protective effect against the PA-induced β-cell dysfunction through regulation of mitochondrial function.

Oxidative stress generated by fatty acid oxidation has been considered to be involved in the pathogenesis of PA-induced β-cell dysfunction and apoptosis (Janikiewicz et al. 2015). A recent report has shown that SIRT6 can protect human mesenchymal stem cells from oxidative stress through regulation of nuclear factor erythroid 2-related factor 2 (NRF2) (Pan et al. 2016). SIRT6−/− hMSCs exhibit elevated ROS, dysregulated redox metabolism and increased sensitivity to oxidative stress. Mechanistically, SIRT6 acts as a transactivator of transcription factor NRF2 to modulate the expression of genes involved in antioxidant pathway (Pan et al. 2016). However, whether SIRT6 also plays an anti-oxidative stress role in pancreatic β-cells is unclear.

Autophagy, a self-degradative process, has been shown to play a protective role in the PA-induced death of β-cells (Lee 2014, Watada & Fujitani 2015). Interestingly, several studies have demonstrated that SIRT6 functions as a positive regulator of autophagy in some cell types, such as bronchial epithelial cells, cardiomyocytes and neurons. In these cells, SIRT6 regulates the autophagy process via inhibiting the AKT activity (Takasaka et al. 2014, Lu et al. 2016, Shao et al. 2016). Considering a critical role of autophagy in PA-induced β-cell dysfunction, it is reasonable to postulate that SIRT6 may exert a protective effect against the PA-induced β-cell dysfunction and apoptosis through modulation of autophagy.

In summary, our data suggest that SIRT6 plays a critical role in the protection of pancreatic β-cells from lipotoxicity-induced cellular dysfunction or even cell death. These findings are significant in T2DM as dyslipidemia is often associated with the pathogenesis of this type of diabetes. A decrease in SIRT6 protein or activity in β-cells may contribute to the T2DM development. Targeting SIRT6 may be useful for therapeutic development in the treatment of T2DM.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
X X designed and carried out the study, interpreted data, analyzed data and wrote the manuscript. X S, Q W, X Q, Y Z and X P contributed to data collection. X C D conceived the hypothesis, designed the experiments, analyzed and interpreted the data, and wrote the manuscript. All authors approved the manuscript.

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