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Glucotoxic and diabetic conditions induce caspase 6-mediated degradation of nuclear lamin A in human islets, rodent islets and INS-1 832/13 cells

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Abstract

Nuclear lamins form the lamina on the interior surface of the nuclear envelope, and regulate nuclear metabolic events, including DNA replication and organization of chromatin. The current study is aimed at understanding the role of executioner caspase 6 on lamin A integrity in islet β -cells under duress of glucotoxic (20 mM glucose; 24 hrs) and diabetic conditions. Under glucotoxic conditions, glucose-stimulated insulin secretion (GSIS) and metabolic cell viability were significantly attenuated in INS-1 832/13 cells. Further, exposure of normal human islets, rat islets and INS-1 832/13 cells to glucotoxic conditions leads to caspase 6 activation and lamin A degradation, which is also observed in islets from the Zucker diabetic fatty rat, a model for type 2 diabetes (T2D), and in islets from a human donor with T2D. Z-Val-Glu-Ile-Asp-fluoromethylketone, a specific inhibitor of caspase 6, markedly attenuated high glucose-induced caspase 6 activation and lamin A degradation, confirming that caspase 6 mediates lamin A degradation under high glucose exposure conditions. Moreover, Z-Asp-Glu-Val-Asp-fluoromethylketone, a known caspase 3 inhibitor, significantly inhibited high glucose-induced caspase 6 activation and lamin A degradation, suggesting that activation of caspase 3 might be upstream to caspase 6 activation in the islet β -cell under glucotoxic conditions. Lastly, we report expression of ZMPSTE24, a zinc metallopeptidase involved in the processing of prelamin A to mature lamin A, in INS-1 832/13 cells and human islets; was unaffected by high glucose. We conclude that caspases 3 and 6 could contribute to alterations in the integrity of nuclear lamins leading to metabolic dysregulation and failure of the islet β -cell.

Keywords

Pancreatic islet; glucotoxicity; caspase 3; caspase 6; lamin A; diabetes

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Conflict of interests

The authors declare no conflict of interests.

Author contributions

Conceived, designed experiments, and wrote the manuscript: AK. Performed the experiments and analyzed the data and reviewed the manuscript: KS, RV and VS.

Introduction

The nucleus is a complex subcellular organelle and represents the center of cellular events during the cell cycle progression and development. It undergoes dynamic processing of a number of constituent macromolecules, including DNA and lamins. The nuclear lamina is a fibrillar meshwork of proteins lining the interior of the nuclear envelope. In mammalian cells, the nuclear lamina consists mainly of three proteins, namely, lamins A, B and C. These intermediate filament proteins subserve several cellular functions, including DNA replication, chromatin organization, differentiation, nuclear structural support, and nuclear envelope assembly [1–4]. Evidence in multiple cell types suggests significant alterations in nuclear structure and organization during apoptosis [5–9]. Some of these include accelerated degradation of nuclear lamins A, B and C by executioner caspases 3 and 6 leading to defective nuclear assembly and mistargeting of constituents of nuclear lamina to improper subcellular compartments [e.g., cytosol], thus triggering nuclear collapse and apoptotic cell death [7,10,11]. This has been an active area of investigation recently since very little is understood with regard to these cellular events in the pathology of diseases, including neurological and metabolic diseases, such as Alzheimer's disease and diabetes mellitus.

Several lines of evidence suggest that exposure of pancreatic β -cells to hyperglycemic conditions leads to the onset of metabolic stress, loss in glucose-stimulated insulin secretion (GSIS) and cell demise. While the underlying mechanisms involved in high glucose-induced β -cell death remain elusive, available evidence in other cell types implicates endoplasmic reticulum (ER) and oxidative stress appear to play key roles in the pathology of β -cell failure in diabetes [12–14]. Evidence is also available to indicate that both ER and oxidative stress promote mitochondrial dysregulation, including loss of membrane potential, release of cytochrome C and activation of pro-apoptotic caspases 2, 3, and 6, which, in turn, promote proteolysis of key survival proteins, such as nuclear lamins [15–17]. Despite compelling evidence little is known in the context of the islet β -cell, on potential detrimental effects of glucotoxic conditions on caspase activation and associated degradation of their respective substrate proteins. In this context, we have recently reported significant activation of caspase 3 in clonal β -cells, normal rat islets and human islets exposed to glucotoxic conditions; such effects were mimicked by thapsigargin, a known inducer of ER stress. Our findings also demonstrated a marked increase in the degradation of nuclear lamin B under conditions of caspase 3 activation. Lastly, nifedipine, a known calcium channel blocker, significantly reduced high glucose-induced caspase 3 activation and lamin degradation suggesting that calcium overload in high glucose-challenged β -cell could contribute to caspase activation and lamin B degradation [11]. Published evidence suggests sequential activation of executioner caspases in cells destined for apoptosis [18, 19]. Therefore, as a logical extension of our ongoing investigations, we undertook the current investigation to investigate if glucotoxic conditions also promote activation of other executioner caspases, such as caspase 6 in the islet β -cell. Further, we asked if activation of these caspases leads to proteolysis of other nuclear proteins, including lamin A. We accomplished these goals by investigating the degree of activation of caspase 6 and lamin A degradation in clonal β (INS-1 832/13) cells, normal rat islets and human islets. Our findings provide the first evidence to suggest that glucotoxic conditions promote sequential activation of caspase 3

and 6 to trigger proteolysis of lamins A and B culminating in the demise of the pancreatic β -cell.

Materials and Methods

Materials

Antisera directed against cleaved lamin A and cleaved caspase 6 were from Cell Signaling (Danvers, MA) and antibody for ZMPSTE24 was obtained from Abcam (Cambridge, MA). Anti-mouse IgG and anti-rabbit IgG conjugated to horseradish peroxidase were from GE Healthcare UK. The rat insulin ELISA kit was purchased from American Laboratory Products Co (Windham, NH). Inhibitors of caspase 3 (Z-DEVD-FMK) and caspase 6 (Z-VEID-FMK) were from Enzo Lifesciences (Farmingdale, NY). Anti- β actin, metabolic cell viability assay kit (MTT) and all other reagents employed in the current studies were from Sigma Aldrich (St. Louis, MO).

Insulin-secreting INS-1 832/13 cells

INS-1 832/13 cells were cultured in RPMI-1640 medium containing 10% heat-inactivated FBS supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol and 10 mM HEPES (pH 7.4). The cultured cells were subcloned twice weekly following trypsinization and passages 53–61 were used for the study. INS-1 832/13 cells were treated with low (2.5 mM) and high glucose (20 mM) for 24 hr as indicated in the text. For the studies involving inhibitors, cells were pre-incubated for 1 hr, in the presence and absence of caspase inhibitors, Z-DEVD-FMK (Caspase 3 inhibitor; 3 μ M) and Z-VEID-FMK (Caspase 6 inhibitor; 3 μ M) and then further treated with low (2.5 mM) and high glucose (20 mM) in the continuous presence or absence of inhibitors for additional 6 hr. At the end of the incubation period the cells were harvested and lysed in RIPA buffer containing protease inhibitor cocktail, 1 mM NaF, 1 mM PMSF and 1 mM Na_3VO_4 .

Normal and diabetic rat islets

All animal protocols were reviewed and approved by our Institutional Animal Care and Use Committees at Wayne State University and John D. Dingell VA Medical Center. Islets from normal 6 week-old male Sprague–Dawley rats (Harlan Laboratories, Oxford, MI) were isolated by the collagenase digestion method as described previously [20]. After isolation, islets were cultured overnight in RPMI-1640 medium containing 10% heat-inactivated FBS supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate and 10 mM HEPES (pH 7.4) and further incubated in the low (2.5 mM) and high glucose (20 mM). At the end of the incubation period (24 hr) the islets were harvested and lysed in RIPA buffer.

Male Zucker Diabetic Fatty (ZDF) and Zucker Lean Control (ZLC) rats (9–11 weeks; Charles River Laboratories, Wilmington, MA) were maintained in a 12 hr light/dark cycle with free access to water and food (Purina Diet 5008, Charles River Laboratories, Wilmington, MA). Hyperglycemia in diabetic rats was confirmed by blood glucose measurements by tail vein puncture using Freestyle lite glucometer (Abott Diabetes Care,

Inc., Alameda, CA). The body weights of the ZLC and ZDF rats at the time of sacrifice were 275 ± 8 and 365 ± 7 g, respectively. The blood glucose levels for the ZLC and the ZDF rats were 80 ± 12 and 276 ± 37 mg/dl, respectively. Islets from these animals were isolated as described above, and lysed in RIPA buffer and processed for Western blotting.

Normal and diabetic human islets

Human islets (~90–95% purity) from two normal (38-year-old female and 64-year-old male) and one diabetic (44-year-old male) donors and islet culture medium were procured from Prodo Laboratories, Inc. (Irvine, CA). Following incubations in the presence of low (5.8 mM) or high (30 mM) glucose, normal human islets and T2D human islets were homogenized in RIPA buffer and used for Western blotting.

Insulin release studies

INS-1 832/13 cells were incubated in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose. After 24 hr, the cells were incubated with KRB buffer for 1 hr, prior to stimulation with low or high glucose for 45 min at 37°C. Insulin released into the medium was quantified by ELISA [21].

Metabolic cell viability assay

INS-1 832/13 cells were cultured for 24 and 48 hr in 96-well plates and treated with low or high glucose as above. Metabolic cell viability was assessed by using a colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) which measures the reduction of MTT into the blue formazan product by metabolically active cells at 570 nm.

Western blotting

Cellular lysate proteins (30–50 µg/lane) were separated by SDS-PAGE and electrotransferred to a nitrocellulose membrane. The membranes were blocked with 5% non-fat dry milk in 10 mM Tris-HCl, pH 7.6, 1.5 M NaCl and 0.1% Tween 20 (TBS-T) followed by incubation with primary antibodies (cleaved lamin A-1:200, cleaved caspase 6- 1:200, ZMPSTE24 1:400) in TBS-T containing 5% BSA at room temperature for 1 hr and washed 5X for 5 min each with TBS-T. The membrane was then incubated with corresponding secondary antibodies conjugated to horseradish peroxidase (1:1000) in 5% non-fat dry milk in TBS-T at room temperature for 1 hr. After washing, the protein signal was enhanced by chemiluminescence system and developed using Kodak Pro Image 400 R (New Haven, CT) and Carestream Molecular Imaging Software was used to measure the band density. The same blots were stripped and used to probe for β actin to ensure equal loading and transfer of proteins.

Statistical analysis

The statistical significance of the differences between the experimental conditions was determined by t-test or ANOVA where appropriate. *P* value < 0.05 was considered significant.

Results

High glucose exposure significantly reduces GSIS and metabolic cell viability in INS-1 832/13 cells

At the outset, we quantified effects of high glucose exposure (20 mM; 24 hr; referred to as glucotoxic conditions throughout) on GSIS in INS-1 832/13 cells. Data in Figure 1 indicate a significant increase (~ 2 fold) in basal secretion from these cells following exposure to glucotoxic conditions; (bar 1 vs. 3). In addition, insulin secretion elicited by stimulatory glucose concentrations decreased significantly in these cells exposed to glucotoxic conditions (bar 2 vs. 4). In this context, we recently reported near complete inhibition of GSIS in INS-1 832/13 cells after 48 hr incubation with high glucose [21]. Additional studies have suggested a 13 and 19 percent reduction in metabolic cell viability in these cells following exposure to glucotoxic conditions at 24 and 48 hr, respectively (n=2 independent studies; additional data not shown). Together, these data indicate significant impairment in GSIS even at 24 hr of incubation. Based on these observations and our recent findings on caspase 3 activation and lamin B degradation under glucotoxic conditions [11], we undertook the present study to determine effects of glucotoxic conditions on caspase 6 activation and lamin A degradation in a variety of insulin-secreting cells, including INS-1 832/13 cells and normal rodent and human islets.

High glucose induces caspase 6 activation and cleavage of lamin A in INS-1 832-13 cells, normal rat and human islets and diabetic human islets

We determined if exposure of INS-1 832/13 cells to glucotoxic conditions results in activation of caspase 6 and associated degradation of lamin A. Data in Figure 2 (Panel a) represents a Western blot from one of these experiments, which indicates a significant increase in caspase 6 activity in high glucose-treated cells as evidenced by emergence of a cleaved 18 kDa biologically active peptide of caspase 6. Furthermore, we noticed a corresponding increase in the abundance of a 28 kDa lamin A degradation product in lysates derived from cells exposed to high glucose. Pooled data from multiple experiments are provided in Panels b and c. Subsequent studies in normal rat islets (Figure 3; Panels a–c), human islets (Figure 4; Panel a) and in islets from a human donor with T2D (Figure 4; Panel b) confirmed our observations in INS-1 832/13 cells. Together, these findings (Figures 2–4) suggest that glucotoxic and diabetic conditions promote activation of caspase 6 and lamin A degradation in a variety of insulin secreting cells (human islets, rodent islets and INS-1 832/13 cells).

Increased activation of caspase 6 and associated degradation of lamin A are also demonstrable in diabetic rat islets

As a logical extension of the above studies, we quantified caspase 6 activity and lamin A degradation in islets derived from the ZDF rat, a known model for T2D. Islets from age-matched ZLC rats were used as controls in these studies. Data in Figure 5 (Panel a) represent a Western blot demonstrating a significant increase in the activation of caspase 6 in islets from the ZDF rat compared to the ZLC rat islets. Compatible with our findings above, we also noticed a marked increase in lamin A degradation in the ZDF islets compared to their counterparts in ZLC islets (Panel c). Pooled data in islets from multiple ZLC and ZDF rats

are given in Panels b (caspase 6) and d (lamin A). Collectively, data shown in Figures 2–5 provide compelling evidence for increased caspase 6 activation and lamin A degradation in pancreatic β -cells exposed to glucotoxic conditions and T2D islets from rodents and humans.

Z-VEID-FMK, a known inhibitor of caspase 6, markedly attenuates glucose-induced caspase 6 activation and breakdown of lamin A in INS-1 832/13 cells

In order to confirm that caspase 6 mediates lamin A degradation, we investigated the effects of Z-VEID-FMK, a known inhibitor of caspase 6, on high glucose-induced caspase 6 activation and lamin A degradation in INS-1 832/13 cells. Data in Figure 6 (Panel a) represents a Western blot demonstrating significant inhibition of high glucose-induced activation of caspase 6 by its inhibitor. It should be noted that we consistently observed a modest increase in the caspase 6 activation in cells exposed to its inhibitor under low glucose conditions (Figure 6; Panel a). However, data from multiple experiments indicated that this increase was not significant (Figure 6; Panel b). Furthermore, Z-VEID-FMK inhibited high glucose-induced degradation of lamin A under the conditions it inhibited caspase 6 (Figure 6; Panel c); these findings provide support to the notion that caspase 6 mediates cleavage of lamin A in pancreatic β -cells under glucotoxic and diabetic conditions. Pooled data from multiple experiments are provided in Figure 6; Panel d. It should be noted that as in the case of caspase 6 activation, we noticed increase in lamin A degradation in INS-1 832/13 cells exposed to caspase 6 inhibitor under low glucose treatment conditions. Collectively, our findings implicate caspase 6 in lamin A degradation under glucotoxic conditions.

Z-DEVD-FMK, a known inhibitor of caspase 3, significantly reduces high glucose-induced caspase 6 activation and lamin A degradation in INS-1 832/13 cells

We recently reported that glucotoxic conditions induce ER stress to promote caspase 3 activation and lamin B degradation in INS-1 832/13 cells, normal rodent islets and human islets [11]. Published evidence in other cell types indicates that caspase 3 activation step is upstream to activation of caspase 6 [22, 23], and/or both caspase 3 and caspase 6 are activated under apoptotic conditions induced by specific stimuli [24, 25]. Therefore, in the next set of experiments, we examined if caspase 3 activation precedes caspase 6 activation in β -cells under the duress of glucotoxicity. To address this, we quantified caspase 6 activation in INS-1 832/13 cells under high glucose-treatment conditions in the absence or presence of caspase 3 inhibitor (Z-DEVD-FMK). Data shown in Figure 7 (Panel a), demonstrate significant inhibition of glucose-induced caspase 6 activation following inhibition of caspase 3. Pooled data from multiple experiments are included in Panel b. It is noteworthy that as in the case of caspase 6 inhibitor (Z-VEID-FMK), caspase 3 inhibitor also activated caspase 6 under low glucose incubation conditions. Furthermore, Z-DEVD-FMK significantly attenuated high glucose-induced lamin A degradation (Figure 7; Panel c), further supporting the notion that glucose-induced caspase 3 activation might lie upstream to caspase 6 activation and lamin A degradation. Collectively, at least, based on our current and recently published findings [11, 26, 27], we conclude that executioner caspases (caspase 3 and 6) mediate nuclear lamin (A and B) degradation and chromatin condensation and collapse of nuclear envelope under conditions of glucotoxicity culminating in cell apoptosis.

Immunological evidence for expression of ZMPSTE24, a zinc metallopeptidase, which is involved in processing of pre-lamin A to mature lamin A in INS-1 832/13 cells and human islets

Mature lamin A is synthesized as prelamin A, the precursor form, which subsequently undergoes post-translational modifications, including farnesylation and carboxylmethylation. The modified form of prelamin A is subsequently cleaved by the enzyme ZMPSTE24, a zinc metallopeptidase, which detaches 15 amino acids from the carboxyl end, thereby releasing mature lamin A [28–30]. Earlier studies from our laboratory have demonstrated carboxylmethylation of lamins in islet β -cells [31]. Therefore, in the last set of studies we asked if ZMPSTE24 is expressed in the islet β -cells, and whether glucotoxic conditions affect the expression of this peptidase. Data depicted in Figure 8 provide evidence for the expression of ZMPSTE24 in INS-1 832/13 cells (Panel a) and human islets (Panel c). Furthermore, we noticed no significant effects of glucotoxic conditions on the expression of this protein in the two cell types studied (Panel a–c), suggesting that proteolytic processing of lamin A may not be affected under glucotoxic conditions. Future studies will validate this model (see Discussion).

Discussion

One of the objectives of the current study was to investigate regulatory roles of caspase 6 on nuclear lamin A metabolism in the pancreatic islet under the duress of chronic hyperglycemia and diabetes. Our findings demonstrate that: [i] glucotoxic conditions promote loss in metabolic cell viability and impair GSIS; [ii] glucotoxic conditions promote activation of caspase 6 and degradation of lamin A in a variety of insulin-secreting cells, including INS-1 832/13 cells, normal rodent islets and human islets; [iii] increased activation of caspase 6 and lamin A degradation are also seen in islets from the ZDF rat, a model for T2D and islets from T2D human donors; [iv] a specific inhibitor of caspase 6 inhibits high glucose-induced caspase 6 activation and lamin A degradation; [v] a specific inhibitor of caspase 3 inhibits high glucose-induced caspase 6 activation and proteolysis of lamin A; and [vi] ZMPSTE24 is expressed in clonal β -cells and human islets, and high glucose exposure conditions exert no significant effects on the expression of this enzyme, which is involved in processing of prelamin A to mature lamin A. Collectively, our findings provide support to our hypothesis that glucotoxicity promotes mitochondrial dysfunction culminating in caspase activation leading to abnormalities in nuclear lamin structure (i.e., degradation) culminating in cell demise.

A growing body of evidence suggests that exposure of pancreatic islet β -cells to glucotoxic and lipotoxic conditions as well as proinflammatory cytokines (IL-1 β , TNF α and IFN γ) results in metabolic dysfunction, which may, in part, be due to increased oxidative and ER stress [32–36]. It is also well established that both oxidative and ER stress, singly, or in combination, elicit direct regulatory effects on subcellular structures, including mitochondria, leading to loss of mitochondrial membrane permeability pore transition and leakage of mitochondrial proteins (cytochrome C) into the cytosolic compartment [17,37,38]. Such conditions favor activation of executioner caspases (caspase 3 and caspase 6) thereby promoting the hydrolysis/breakdown of cell survival proteins resulting in cell

death *via* apoptosis. In this context, we reported earlier treatment of insulin-secreting β -cells to IL-1 β resulted in significant alterations in the redistribution of lamin B, specifically between the nuclear membrane and matrix fractions of the nucleus. A significant increase in the relative abundance of lamin B degradation products was also observed in the nuclear fraction from the IL-1 β -treated cells [26].

Our current findings further support our recent observations demonstrating a significant activation of caspase 3 and lamin B degradation in INS-1 832/13 cells, normal rat islets or human islets exposed to high glucose conditions [11]. Glucotoxic conditions also promoted significant alterations in the subcellular distribution of lamin B and its degradation products. We also demonstrated that forced induction of ER stress in these cells, using thapsigargin, also resulted in caspase 3 activation and lamin B degradation; and effects of glucotoxic conditions were prevented by 4-phenyl butyric acid, a known inhibitor of ER stress. Nifedipine, a known blocker of calcium channel activation, inhibited high glucose-induced caspase 3 activation and lamin B degradation in these cells, suggesting that glucotoxicity-induced calcium overload promotes caspase 3 activation and lamin B degradation. Based on these findings we concluded that glucotoxic conditions promote caspase 3 activation and lamin B degradation, which may, in part, be due to increased ER stress under these conditions. It is also noteworthy that our findings also provided supporting evidence to support beneficial effects of calcium channel blockers against metabolic dysfunction of the islet β -cell induced by hyperglycemic conditions. Based on these data, we concluded that high glucose treatment results in significant increase in lamin degradation mediated by caspases, possibly leading to dissolution of the nuclear envelope, culminating in the demise of the β -cell.

Previous studies have demonstrated that *in vitro* exposure of islets from non-diabetic organ donors to high glucose levels resulted in increased production and release of IL-1 β , followed by NF κ B activation, Fas upregulation, caspases 8 and -3 activation, DNA fragmentation, and impaired β -cell function [39–41]. The preferential recognition of different caspases for specific amino acid sequences on the substrate proteins have been taken advantage of, in the development of specific peptide inhibitors. These caspase inhibitors are cell permeable and bind to the active site of the proteases and thereby obstruct the progression of apoptotic pathway downstream of caspase activation. *In vitro* studies on β -cell lines utilizing caspase inhibitors have demonstrated that the caspase 2 inhibitor is effective in protecting the HIT-T15 β -cell line against an experimental model of cell death [42]. Furthermore, murine β TC-1 cell lines transfected with human Fas were shown to be protected against Fas-induced β -cell apoptosis by the caspase 3 inhibitor Z-Asp-Glu-Val-Asp-fluoromethyl ketone [43] and palmitate-induced β -cell apoptosis, could be prevented by executioner caspase 6 inhibitors [44]. We recognize potential caveats in our studies involving the use of inhibitors of caspase 3 and caspase 6. While we were able to demonstrate their inhibitory effects on caspase activity and lamin degradation under high glucose conditions, thus confirming our hypothesis, we also observed that they exerted untoward effects on β -cells under low glucose conditions. Future studies, including siRNA-mediated knockdown of individual caspases will validate this model further.

Several lines of evidence in other cell types implicates that inhibition of requisite post-translational modifications of lamins (A and B), including farnesylation and geranylgeranylation results in alterations in cell metabolism leading to cell death *via* apoptosis. For example, using TAD-2 thyroid cell line Matola and associates reported that lovastatin, an inhibitor of protein farnesylation and geranylgeranylation, induces cell death by inducing mitochondrial dysfunction, cytochrome C release, caspase 3 activation and lamin B degradation [45]. Likewise, more recent studies by Chang and associates have demonstrated abnormalities in lamin A processing following inhibition of its farnesylation using FTIs. They reported significant accumulation of unprocessed lamin A intracellularly [46]. This remains to be verified in the islet under various pathological conditions, including glucolipotoxicity and exposure to proinflammatory cytokines. Our current findings suggest no significant effects of high glucose exposure on the expression of ZMPSTE24, the lamin A processing protease in INS-1 832/13 cells and human islets. However, prenylation status of these proteins under conditions of glucolipotoxicity, ER stress and diabetes need to be verified in the islet in a methodical fashion. In conclusion, based on current and our recently published evidence in INS-1 832/13 cells, normal rat islets and human islets, we conclude that executioner caspases (caspase 3 and 6) could contribute to alterations in the structure and functions of lamin A and B leading to metabolic dysregulation and failure/demise of the islet β -cell.

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Abbreviations used

ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
FTI	Farnesyl transferase inhibitor
GSIS	Glucose stimulated insulin secretion
IFNγ	Interferon gamma
IL-1β	Interleukin-1beta
iNOS	Inducible nitric oxide synthase
LMNA	Lamin A gene
NO	Nitric oxide
T2D	Type 2 Diabetes
TNF α	Tumor necrosis factor alpha
Z-DEVD-FMK	Caspase 3 inhibitor (Z-Asp-Glu-Val-Asp-fluoromethylketone)

ZDF	Zucker diabetic fatty
ZLC	Zucker lean control
ZMPSTE24	Zinc metalloproteinase (STE24 homolog)
Z-VEID-FMK	Caspase 6 inhibitor (Z-Val-Glu-Ile-Asp-fluoromethylketone)

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Figure 1. Glucotoxic conditions attenuate GSIS in INS-1 832/13 β -cells

INS-1 832/13 cells were cultured in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 24 hr following which they were stimulated with low (2.5 mM) or high (20 mM) glucose for 45 min. Insulin released into the medium was quantified by ELISA [see Methods for additional details]. The data are expressed as insulin release (ng/ml) and are means $\pm$ SEM from three independent experiments. * $P < 0.05$ vs. LG under 24 hr low glucose treatment; ** $P < 0.05$ vs. HG under 24 hr low glucose treatment.

Figure 2. High glucose treatment induces caspase 6 activation and lamin A cleavage in INS-1 832/13 cells

INS-1 832/13 cells were incubated in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 24 hr. Caspase 6 activation and lamin A cleavage were determined by Western blotting. Protein lysates (40 μ g) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved caspase 6 and cleaved lamin A and immune complexes were identified using ECL detection kit (Panel a). To ensure equal protein loading, the membranes were stripped and probed for β actin. Intensity of protein bands was quantified by densitometry. Data represent mean $\pm$ SEM from three independent experiments and expressed as fold change in caspase 6 (Panel b) and lamin A cleavage (Panel c). * $P < 0.05$ vs. LG.

Figure 3. High glucose treatment results in caspase 6 activation and lamin A cleavage in normal rat islets

Rat islets were incubated in the presence of low (2.5 mM; LG) and high glucose (20 mM; HG) for 24 hr. Approximately 40 μ g protein lysate was resolved by SDS-PAGE and the degree of abundance of cleaved caspase 6 and lamin A were determined by Western blot analysis. Immune complexes were identified using ECL detection kit (Panel a). β actin was used as a loading control. Quantitation of caspase 6 activation and lamin A degradation were carried out by densitometry. Data represent mean $\pm$ SEM from three independent experiments and are expressed as fold change in caspase 6 (Panel b) and lamin A (Panel c) cleavage. * $P < 0.05$ vs. LG.

Figure 4. Glucotoxic conditions promote caspase 6 activation and lamin A cleavage in normal human islets treated with high glucose and in diabetic human islets

Normal human islets were incubated in the presence of low (5.8 mM; LG) and high glucose (30 mM; HG) for 24 hr as described in the text (Panel a). Islets obtained from normal and diabetic individuals were lysed using RIPA buffer (Panel b). Lysate proteins (25 μ g) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved caspase 6, and lamin A and immune complexes were identified using ECL detection kit. To check equal protein loading, the membrane was stripped and probed for β actin. Human islet data from normal and diabetic individuals were accrued from a single batch of islet preparation.

Figure 5. Activation of caspase 6 and lamin A degradation are observed in ZDF rat islets
Islets isolated from ZDF and ZLC rats by collagenase digestion method were lysed using RIPA buffer. Lysate proteins (40μg) were resolved by SDS-PAGE and the abundance of cleaved caspase 6 and lamin A was determined by Western blotting. Immune complexes were identified using ECL detection kit. To assess equal protein loading, the membrane was probed for β actin. Representative blots from three ZLC and ZDF islet preparations are shown in Panel a and c. Intensity of protein bands was quantified by densitometry and data represent mean ± SEM from three islet preparations and are expressed as fold change in caspase 6 (Panel b) and lamin A cleavage (Panel d). * $P < 0.05$ vs. ZLC

Figure 6. High glucose-induced caspase 6 activation and breakdown of lamin A cleavage were attenuated by Z-VEID-FMK, a specific inhibitor of caspase 6, in INS-1 832/13 cells
 INS-1 832/13 cells were preincubated with Z-VEID-FMK (3 μ M) for 1 hr and further treated with low (2.5 mM; LG) or high (20 mM; HG) glucose in the presence and absence of Z-VEID-FMK for 6 hr. Caspase 6 activation and lamin A cleavage were determined by Western blotting. Protein lysates (40 μ g) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved caspase 6 (Panel a) and cleaved lamin A (Panel c), and immune complexes were identified using ECL detection kit. To ensure equal protein loading, the membranes were probed for β actin. Intensity of protein bands was quantified by densitometry. Data represent mean $\pm$ SEM from three independent experiments and expressed as fold change in caspase 6 (Panel b) and lamin A cleavage (Panel d). * P < 0.05 vs. LG. NS: Not significant vs. LG

Figure 7. Z-DEVD-FMK, a known inhibitor of Caspase 3, prevented high glucose induced caspase 6 activation and lamin A breakdown in INS-1 832/13 cells

INS-1 832/13 cells were preincubated with Z-DEVD-FMK (3 μ M) for 1 hr and further treated with low (2.5 mM; LG) or high (20 mM; HG) glucose in the presence and absence of Z-DEVD-FMK for 6 hr. Caspase 6 activation (Panel a) and lamin A cleavage (Panel c) were determined by Western blotting. To check equal protein loading, the membranes were probed for β actin. Intensity of protein bands was quantified by densitometry. Data represent mean $\pm$ SEM from three independent experiments and expressed as fold change in caspase 6 (Panel b) and lamin A cleavage (Panel d). * P < 0.05 vs. LG; # P < 0.05 vs. HG.

Figure 8. Immunological evidence to indicate expression of lamin A processing protease (ZMPSTE 24) in INS-1 832/13 cells and normal human islets: Lack of effects of high glucose exposure on the expression of ZMPSTE 24

INS-1 832/13 cells were incubated in the presence of low (2.5 mM; LG) or high (20m M; HG) glucose for 24 hr (as in Figure 2). Protein lysates (40 μ g) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. A representative blot from three studies is shown in Panel a. Intensity of protein bands was quantified by densitometry and data represent mean $\pm$ SEM from three independent experiments and are expressed as fold change in ZMPSTE 24 (Panel b). NS: Not significant vs. LG. Normal human islets were incubated in the presence of low (5.8mM; LG) and high glucose (30mM; HG) for 24 hr (as in Figure 4; Panel a). Twenty five μ g lysate proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were probed for ZMPSTE 24 and immune complexes were identified using ECL detection kit. To check equal protein loading, the membranes were probed for β actin. A blot from a single human islet preparation is shown in Panel c.