1-1-2015

Islet Dysfunction In Diabetes

Syeda Khadija
Wayne State University,

ISLET DYSFUNCTION IN DIABETES

by

KHADIJA G. SYEDA

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

2015

MAJOR: PHARMACEUTICAL SCIENCES

Approved by:

_____

Advisor Date

_____

_____

_____

_____

DEDICATION

This work is dedicated to my mother, whose love and prayers have been a constant source of strength for me.

ACKNOWLEDGEMENTS

At the outset, I would like to express my sincere gratitude to my advisor, Dr. Anjan Kowluru, for first of all accepting to be a mentor for my graduate studies and research work, and then, for being a constant source of inspiration, guidance and motivation throughout my learning process. I have been privileged to have got the opportunity to be trained under the mentorship of Dr. Kowluru so early in my career and it is a matter of pride for me to graduate from one of the most productive and well run labs in the university.

I would like to extend my appreciation to all the previous and current members of Dr. Kowluru's lab especially, Abiy, Dr. Veluthakal, Vaibhav, Dr. Arora, Dr. Matti and Baker for their support and encouragement during various phases of my research work.

I am deeply grateful to Dr. Chen, Dr. Hadden and Dr. Commissaris for being members of my dissertation committee and for the time and constructive suggestions given by them during the committee meetings. Their inputs and advice have been extremely valuable for making progress in my project.

I would like to take this opportunity to extend my gratitude to Dr. Corcoran and the members of the Department of Pharmaceutical Sciences for their continued support and encouragement.

Special thanks to all my friends, old and new, for their tremendous support in times when I needed it the most.

Finally, I would like to express my heartfelt appreciation towards each member of my family for their steadfast care and encouragement during the tough times, their pride and happiness during the good times and for their unconditional love throughout.

TABLE OF CONTENTS

Dedication……..…….. ii

Acknowledgements……. iii

List of Tables……..….. vi

List of Figures…….……... vii

List of Abbreviations……... xi

Chapter 1: Introduction……. 1

Chapter 2: Materials and Methods…….. 30

Chapter 3: Mitochondrial dysfunction induced by glucotoxic and diabetic conditions results in caspase mediated lamin degradation in pancreatic beta cells……. 37

Chapter 4: Identification of downstream signaling events involved in glucotoxicity induced endoplasmic reticulum [ER] stress leading to dysfunction in pancreatic β-cells……...…… 55

Chapter 5: Consequences of inhibition of requisite post translational prenylation on the pancreatic β-cell…….…….. 73

Chapter 6: Discussion…… 86

Chapter 7: Conclusion and Future directions…….……. 99

Appendix A Syeda *et al.* 2013…….…. 103

Appendix B Syeda *et al.* 2014……. 112

References…… 123

Abstract……..... 139

Autobiographical Statement……. 141

LIST OF TABLES

Table 1-1: International Diabetes federation estimates of worldwide prevalence of Diabetes [2014]…….……. 2

Table 1-2: Key features of most common animal models of T2DM……. 8

Table 1-3: Clinical features of laminopathies…….. 22

Table 3-1: Body weights and blood glucose levels of ZLC and ZDF rats measured at the time of sacrifice…….……. 51

LIST OF FIGURES

Figure 1-1: Worldwide incidence of Diabetes and projected increase of the disease by 2035, International Diabetes federation estimates 2014……..……. 3

Figure 1-2: Pancreatic islets obtained from normal male Sprague Dawley rats observed under light microscope……..…… 4

Figure 1-3: Pathogenesis of T2DM.……..... 7

Figure 1-4: Protein structure of lamins……... 12

Figure 1-5: PTM steps involved in processing of nuclear lamins……..... 15

Figure 1-6: Assembly and disassembly of the nuclear lamina……..... 19

Figure 1-7: Acute and chronic effects of various glucose concentrations on the beta cell……. 27

Figure 1-8: Proposed working model for ER-stress induced, caspase-mediated degradation of nuclear lamins under glucotoxic conditions in pancreatic beta cells…….. 29

Figure 3-1: Exposure of INS-1 832/13 cells to glucotoxic conditions results in caspase 3 activation and lamin B degradation…….. 41

Figure 3-2: Caspase 6 activation and lamin A cleavage in INS-1 832/13 cells treated with high glucose…… 42

Figure 3-3: Glucotoxic conditions attenuate GSIS in INS-1 832/13 beta cells……..…….. 43

Figure 3-4: Treatment of normal rat islets with high glucose results in caspase 3 and 6 activation and hydrolytic cleavage of downstream substrates: lamin A and B…… 45

Figure 3-5: Glucotoxic conditions promote caspase 3 and 6 activation and nuclear lamina breakdown in normal human islets treated with high glucose…….. 46

Figure 3-6: ZMPSTE 24 is expressed in INS-1 832/13 cells and normal human islets. Lack of effects of high glucose exposure on the expression of ZMPSTE24…….. 48

Figure 3-7: Exposure to high glucose results in altered subcellular distribution of caspase 3 and degraded product of lamin B in INS-1 832/13 cells……. 50

Figure 3-8: Activation of caspase and lamin degradation are observed in islets obtained from ZDF rats…… 52

Figure 3-9: Caspase 3 and 6 activation and lamin A and B degradation in diabetic human islets…….. 53

Figure 4-1: Chemical Structure of Thapsigargin……..… 55

Figure 4-2: Thapsigargin, a known inducer of ER stress, also promotes caspase 3 activation and lamin B degradation in INS-1 832/13 cells…… 58

Figure 4-3: *Akita* mouse cells show noticeable increase in caspase 3 activation and lamin B degradation……... 60

Figure 4-4: Chemical Structure of PBA…….. 60

Figure 4-5: PBA, a known inhibitor of ER stress, markedly attenuates glucose-induced CHOP expression, caspase 3 activation and lamin B degradation in INS-1 832/13 cells……..… 61

Figure 4-6: Chemical Structure of Nifedipine…… 62

Figure 4-7: Nifedipine, a calcium channel blocker, inhibits glucose-induced caspase 3 activation and lamin B degradation in INS-1 832/13 cells…… 64

Figure 4-8: Z-DEVD-FMK, a known inhibitor of caspase 3, prevented high glucose induced caspase 3 activation and lamin B breakdown in INS-1 832/13 cells……. 66

Figure 4-9: 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……... 68

Figure 4-10: 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……. 70

Figure 4-11: Rottlerin had no effect on high glucose induced activation of caspase 3 and lamin B degradation…… 72

Figure 5-1: The cholesterol biosynthesis pathway…… 74

Figure 5-2: Exposure of INS-1 832/13 cells, rat pancreatic islets to simvastatin results in caspase 3 activation and degradation of lamin B along with changes in cell morphology…… 76

Figure 5-3: Simvastatin treatment causes a marked increase in phosphorylation of p38 MAP Kinase and significantly decreases phosphorylation of phospho-p44/42 ERK1/2 and a reduction in cell viability……. 79

Figure 5-4: FTI-277 treatment results in caspase 3 mediated degradation of lamin B along with alterations in its subcellular distribution and reduction in cell viability…… 82

Figure 5-5: GGTI-2147 has minimal effects on the integrity of nuclear lamin B and caspase 3 …….. 84

Figure 6-1: Working model for high glucose-mediated, ER-stress-induced β-cell damage/dysfunction via caspase 3 mediated degradation of lamin B in pancreatic beta cells…….. 87

Figure 6-2: Chemical structures of inhibitors of protein prenylation……. 94

LIST OF ABBREVIATIONS

CHOP: C/EBP homologous protein

ECL: Electrochemiluminescence

ELISA: Enzyme-linked immunosorbent assay

ER: Endoplasmic reticulum

ERK 1/2: extracellular mitogen regulated kinase ½

FBS: Fetal bovine serum

FPP: Farnesyl pyrophosphate

FTase: farnesyl transferase

FTI: farnesyl transferase inhibitor

GGPP: Geranylgeranyl pyrophosphate

GGTase: Geranylgeranyl transferase

GGTI: Geranylgeranyl transferase inhibitor

GSIS: Glucose stimulated insulin secretion

ICMT: Isoprenylcysteine carboxyl methyltransferase

IF: Intermediate filament

IFNγ: Interferon gamma

IL-1β: Interleukin-1beta

LMNA: Lamin A gene

LMNB1: Lamin B1 gene

NE: Nuclear envelope

PACS-2: phosphor-acidic cluster sorting protein 2

PBA: 4-phenylbutyric acid

PTM: Post translational modification

T1DM: Type 1 Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

Tg: Thapsigargin

TNFα: 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 metallopeptidase [STE24 homolog]

Z-VEID-FMK: Caspase 6 inhibitor [Z-Val-Glu-Ile-Asp-fluoromethylketone

CHAPTER 1

INTRODUCTION

Diabetes

Diabetes Mellitus is a chronic condition in which the pancreatic beta cells are rendered incapable of generating adequate insulin to meet the metabolic demands or when the tissues are unable to efficiently utilize the synthesized insulin due to increasing insulin resistance **[1].** The overall outcome of either of these conditions is a systemic increase in the levels of glucose circulating in the bloodstream, leading to hyperglycemia. Diabetes is associated with major complications such as, nephropathy, retinopathy, neuropathy, cardiac diseases and even premature death. According to the International Diabetes Federation [IDF] the worldwide incidence of diabetes in 2012 was 371 million and has since then risen unabatedly to 387 million in 2014 [with 46% undiagnosed cases]. The reports predict a drastic escalation of this number to 592 million by 2035. Growing along with the diabetes prevalence is the healthcare expenditure on diabetes, which was $612 billion for the year 2014 alone. **Table 1-1** and **Figure 1-1** depict the prevalence of diabetes in various geographical regions across the globe along with the respective percentage of undiagnosed cases.

International Diabetes Federation 2014 estimates of worldwide prevalence of Diabetes			
REGION	**PEOPLE LIVING WITH DIABETES**	**PERCENT PREVALENCE**	**UNDIAGNOSED CASES**
North America and Caribbean (NAC)	39 Million	11.4%	27.1%
South and Central America (SACA)	25 Million	8.1%	27.4%
Europe (EUR)	52 Million	7.9%	33.1%
Middle East and North Africa (MENA)	37 Million	9.7%	48.6%
Africa (AFR)	22 Million	5.1%	62.5%
South-East Asia (SEA)	75 Million	8.3%	52.8%
Western Pacific (WP)	138 Million	8.5%	53.6%

Table 1-1: International Diabetes Federation estimates of worldwide prevalence of Diabetes [2014]. [*http://www.idf.org/diabetesatlas*]

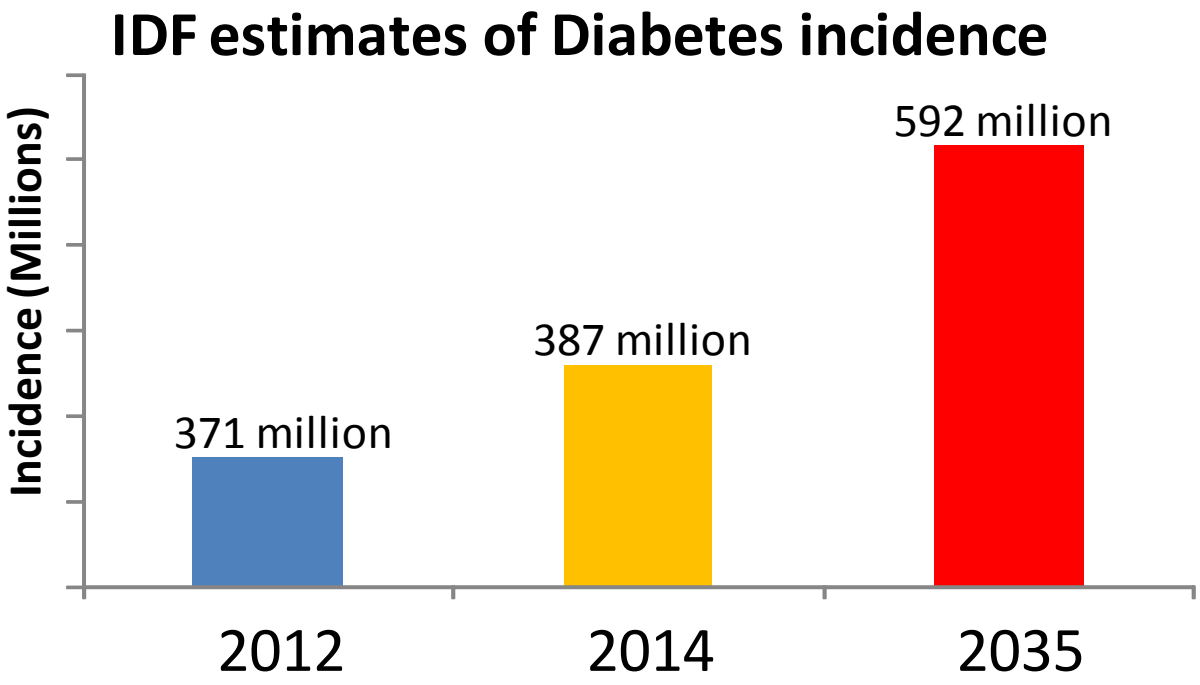


Figure 1-1: Worldwide incidence of Diabetes and projected increase of the disease by 2035 International Diabetes Federation estimates 2014. [*http://www.idf.org/diabetesatlas*]

Type 1 Diabetes Mellitus [T1DM] and Type 2 Diabetes Mellitus [T2DM] are the 2 major forms of diabetes.

T1DM: Previously called as juvenile onset diabetes, T1DM occurs early on in the developmental stages during childhood or adolescence, and is a result of autoimmune-mediated death of the beta cells of the islets of Langerhans. This results in a severe deficiency in the synthesis and secretion of insulin by the pancreas. Genetic predisposition as well as environmental factors could trigger the immune system to cause beta cell death in this form of the disease. Patients with T1DM require daily doses of insulin for glucose homeostasis.

T2DM: T2DM is now a global health crisis and approximately 90% of cases of diabetes fall under this class making it the most common form of diabetes. It is characterized by insulin resistance manifested by target tissues [adipose, muscle], which the beta cells initially try to

compensate for by synthesizing additional amounts of insulin [hyperinsulinemia]. However, as the disease progresses, the beta cells are unable to cope with the rapidly rising blood glucose levels and in due course, a decline in the secretion of insulin by the islet beta cells is observed, followed by absolute insulin deficiency, hyperglycemia and onset of diabetes.

Pancreatic islets

The islets of Langerhans derive their name from the German pathologist Dr. Paul Langerhans, who discovered this hormone-producing cluster of cells in the year 1869. The islets are found in the pancreas, located in the abdominal cavity posterior to the stomach. Typically a normal adult pancreas contains approximately a million islets. Each islet shows a well organized structure made up of 3 main types of cells: alpha [15-20%], beta [~65–80%] and delta [~3-10%] cells which secrete glucagon, insulin and somatostatin, respectively. Besides these, the islets also contain small number of pancreatic polypeptide [PP] cells which produce pancreatic polypeptide and Epsilon cells that secrete ghrelin **[2, 3].**

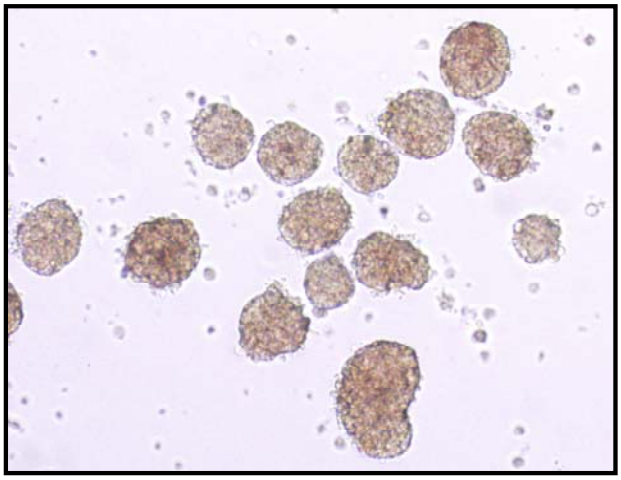


Figure 1-2: Pancreatic islets obtained from normal male, 8 week old, Sprague Dawley rats observed under light microscope. [The above image is a representative preparation that were used in my studies]

Fundamental role of beta cells in insulin secretion and maintenance of glucose homeostasis

Under physiological conditions, a balance between the levels of glucose circulating in the blood and the amount of glucose that the cells need for energy is tightly regulated, thus maintaining glucose homeostasis. A balanced production and secretion of hormones by the islet cells is essential towards maintaining optimum levels of glucose in the blood. After a meal, as the level of glucose in blood circulation ascends, the beta cells respond by secreting insulin. This facilitates the uptake of glucose by the muscle and adipose cells, thereby bringing the blood glucose levels back to a normal level. On the other hand, when blood glucose levels are low, the alpha cells release glucagon. This hormone exerts its stimulatory action on the liver, inducing the release of stored glucose from glycogen, and in doing so; glucose homeostasis is re-established **[4].**

The process of insulin secretion is very tightly regulated and involves a few key steps.

1. An increase in the blood glucose levels causes the uptake of glucose molecules by the GLUT-2 transporters located on the beta cells.

2. The internalized glucose undergoes metabolism generating high amounts of ATP in the process.

3. As the ATP/ADP ratio in the cell increases, it causes the closing of the ATP sensitive K^+ channels, resulting in membrane depolarization.

4. Following this, the voltage dependent Ca^{++} channels open up leading to influx of Ca^{++} ions into the cell.

5. Ca^{++} promotes the movement of insulin-laden secretory granules towards the plasma membrane where they fuse and release the insulin **[5].**

Factors that impair insulin release into the bloodstream cause a reduction in glucose uptake by the insulin-sensitive muscle and adipose tissues, which leads to increased levels of glucose circulating in the blood or hyperglycemia, a hallmark of T2DM.

Pathogenesis of T2DM

Beta cell dysfunction and insulin resistance are the 2 major contributing factors to the pathogenesis of T2DM. However, a lot remains to be understood in regard to the exact mechanisms that the beta cells undergo during these events. Insulin secretion by the beta cell occurs in a biphasic fashion; with the first phase burst occurring within 5-10 minutes of glucose ingestion, with the release of insulin from the granules docked at the membrane, followed by a steady second phase, which involves the mobilization of the insulin granules from the reserve pool in the cell towards the plasma membrane for exocytosis **[6].** Insulin release is considered as a direct measure of beta cell function and any impairment in either phase of insulin secretion, chiefly the first phase, may be the earliest evidence of an individual's progression towards T2DM. Assessment of insulin secretion as a function for beta cell performance is most commonly done by measurement of fasting plasma insulin concentrations, HOMA-B index [fasting insulin to fasting glucose ratios], plasma C-peptide concentrations, acute insulin response **[7, 8].** Insulin resistance is manifested by impairment in uptake of glucose by the insulin sensitive tissues [muscle and adipose] and could be a result of genetic and environmental factors, diet-induced obesity, inflammatory cytokines or cellular stress signaling. There has been considerable deliberation over the specific contribution of beta cell failure and insulin resistance and sequence of occurrence of either of these events towards the progression of T2DM. However, the two events are not mutually exclusive and there exists a feedback communication between them **[7].** The beta cell tries to play a compensatory role under conditions of insulin resistance, and as T2DM progresses the exhausted beta cell is unable to

cope with the increasing metabolic demands, culminating in beta cell dysfunction/damage. Therefore, most of the therapeutic modalities for T2DM involve treatments to improve insulin resistance and/or beta cell survival and function.

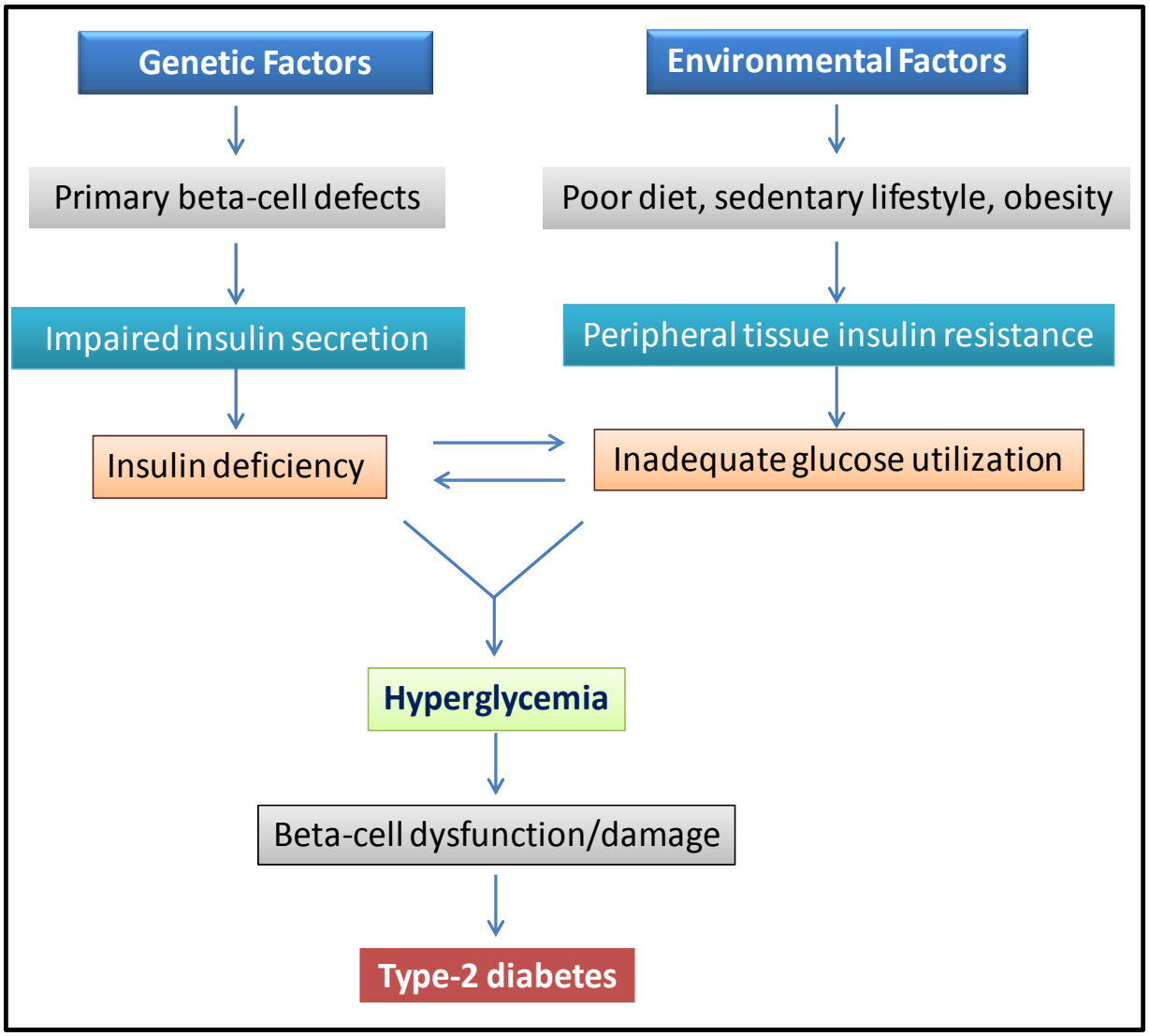


Figure 1-3: Pathogenesis of T2DM. [*http://t2ddb.ibab.ac.in/t2ddb_desc.shtml*]

Animal models of T2DM

Generally, animal models of T2DM display obesity and manifest insulin resistance and/or beta cell dysfunction. **Table 1-2** enlists the most common animal models used in T2DM research along with the method of induction and basic features **[9, 10 and 11].**

Model	T2DM induction method	Features
Lep $^{ob/ob}$ mice [Obese]	Spontaneous mutation in *ob* gene coding for the protein leptin	Leptin deficiency Exhibit rapid weight gain ~2 weeks Obesity induced hyperglycemia Develop hyperinsulinemia and insulin resistance in 3-4 weeks
Lepr $^{db/db}$ mice [Obese]	Autosomal recessive mutation in Leptin receptor gene- *Lepr*	Leptin receptor deficient Hyperinsulinemia and insulin resistant [~2 weeks] Obesity [~3-4 weeks] Hyperglycemia [~4-8 weeks] due to beta cell failure Short life span

Zucker Diabetic Fatty [ZDF] rat [Obese]	Point mutation in Leptin receptor gene- *Lepr*	Obesity [~4 weeks]induced hyperglycemia [~7 weeks] Hyperinsulinemia followed by decrease in insulin levels Diabetes develops at ~8-10 weeks
Kuo Kondo [KK] mice	Mildly obese polygenic model of T2DM and obesity	Obesity induced hyperglycemia Severe hyperinsulinemia and insulin resistance in muscle and adipose tissue
High fat feeding [C57BL/6] mice	Diet/Nutrition induced obesity	Obesity induced hyperglycemia Hyperinsulinemia Weight gain linked to insulin resistance Glucose intolerance due to beta cell insufficiency
Goto-Kakizaki [GK] rat	Non-obese	Defective glucose induced insulin secretion

		Hyperglycemia caused by loss of beta cell function
***Akita* mice [*Ins2*^{Akita}]**	Mutation in insulin gene-*Ins2*,causing misfolding of insulin in the ER resulting in beta cell damage/death	Pancreatic beta cell damage due to ER stress

Table 1-2: Key features of most common animal models of T2DM

Nuclear lamins

The nucleus of a cell is surrounded by a protective bilayer; comprised of the inner nuclear membrane [INM] and the outer nuclear membrane [ONM], which are collectively called the nuclear envelope [NE]. The globular form of the nucleus is held together by a network of filaments on the inner surface of the INM that provide mechanical support and maintain nuclear integrity **[12].** This network of filaments is known as the nuclear lamina and the major proteins of the lamina are the nuclear lamins which are type V intermediate filaments [IF]. The lamin protein network in the nucleus is situated on the inner [nucleoplasm facing] side of the INM, in close proximity to the outer fringes of chromatin **[13].** In addition to being important elements of the nuclear architecture, the nuclear lamins play an essential role in critical functions of the nucleus, such as nucleoplasmic organization of chromatin, DNA replication and damage repair **[14, 15, and 16].**

The lamin protein family is classified on the basis of the gene responsible for encoding of that particular lamin. Therefore, in humans, lamin A and C which are formed by alternative

splicing of the gene *LMNA* are placed in the same class [Type A] along with scarce isoforms, AΔ10 and C2, whereas lamin B1 and B2/B3, which are encoded by the genes *LMNB1* and *LMNB2* respectively, are Type B lamins. Type A lamins are expressed in differentiated cells, have neutral isoelectric points whereas, at least one of the type B lamins is expressed in every cell irrespective of differentiation status, have acidic isoelectric points, and during mitosis tend to remain associated with the nuclear membrane **[17-19].**

Depicted in **Figure 1-4** is the biochemical structure of the lamins proteins. All lamins have the typical structure of IF proteins, with a central α-helical “rod domain” having a non-α-helical N-terminal ‘‘head’’ and C-terminal ‘‘tail’ [called as immunoglobulin, Ig-like β-fold domain] on either ends. A nuclear localization sequence [NLS] segment, which facilitates nuclear targeting of lamins, consists of a lysine residue, followed by three other basic residues [K or R]; and lies in between the central rod domain and Ig-like β fold. Furthermore, all lamins except mammalian and drosophila lamin C **[Figure 1-4]** possess a C-terminal Ras-like CAAX-box motif composed of a cysteine [C], two aliphatic amino acid residues [AA], and any amino acid residue [X], and are subjected to a complex series of post-translational modifications, including prenylation [incorporation of C15 or C20 carbon metabolites derived from the cholesterol biosynthesis pathway], proteolytic cleaving and carboxylmethylation **[20].**

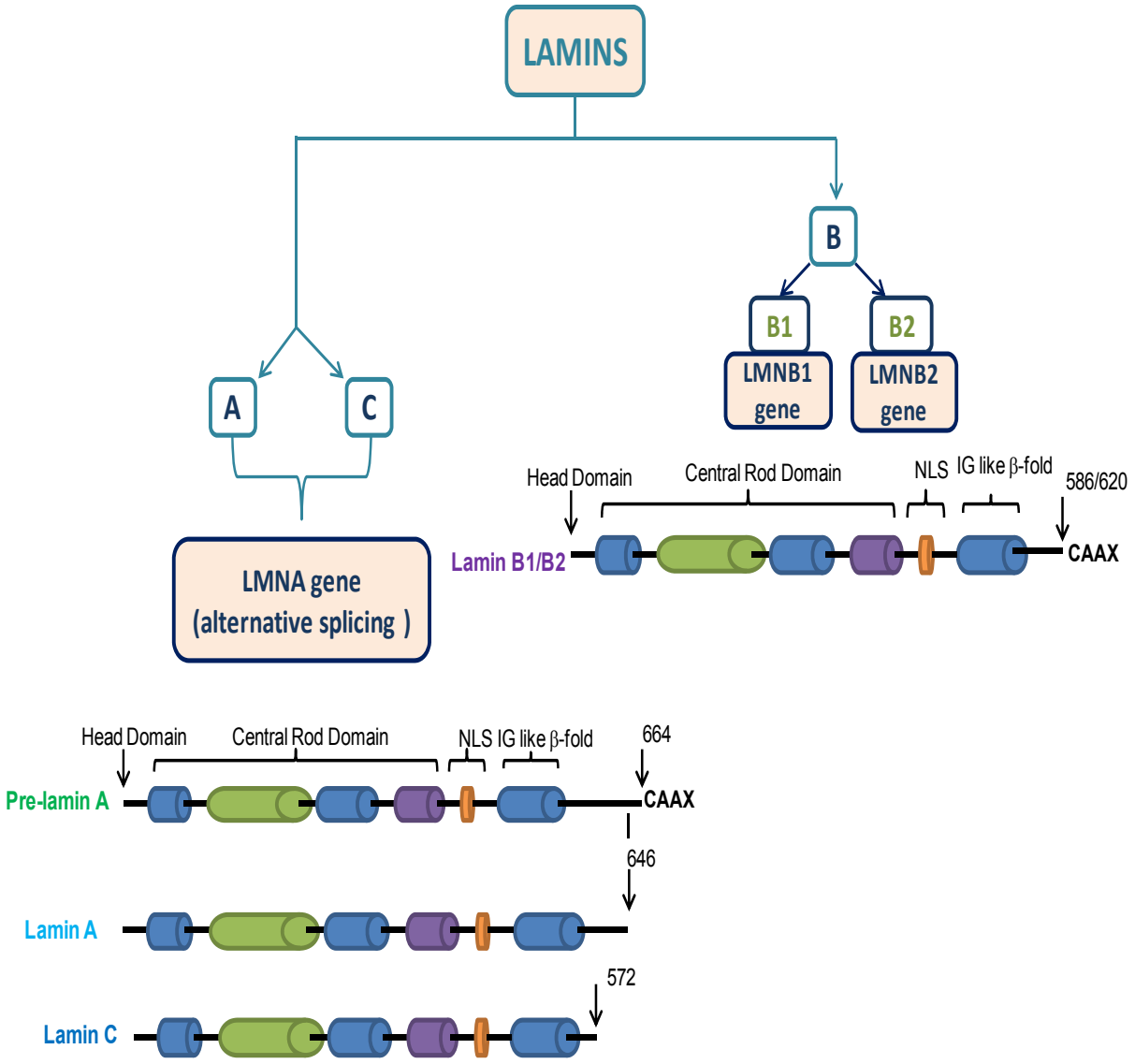


Figure 1-4: Protein structure of lamins. [Modified from Burke *et al.,* Ref. **21**]

Functions of nuclear lamina in cellular physiology and nuclear processes:

1) Mechanical Support:

A number of early studies have described lamins as structures comparable to IFs which are insoluble in non ionic detergents **[22-24].** Further studies involving mutational analyses of nuclear lamina proteins [lamins A, C and B1] by Lammerding and associates demonstrated a decrease in nuclear rigidity in cells devoid of lamins A and C exhibiting frailty of the nucleus,

which leads to increased cell death induced by mechanical stress. Lamin B1-deficient cells however, did not display aberrant nuclear mechanics, even though appearance of nuclear blebs increased significantly **[25].** Another rheological study conducted on lamin B 1 filaments demonstrates that these filaments exhibit tough elasticity when exposed to shear stress and the disintegration of the nuclear envelope would entail mechanical stress as well as biochemical alterations of lamin B1 **[26].** These studies highlight the importance of lamins A and C in maintaining the mechanical stiffness of nuclei, and lamin B1 towards preserving nuclear integrity.

2) Chromatin Organization:

At the molecular level, lamins bind to regions of the chromatin *via* chromatin binding proteins or by direct interaction. It has been shown that lamins form attachments with histones, DNA and chromatin-associated protein apart from directly associating with Lamin B receptors [LBR]. An earlier study conducted by Taniura and colleagues has identified chromatin binding sites on the tail region of lamins C, B1 and B2 **[27].** The transcriptionally silent heterochromatin is localized near the periphery of nuclei of most cells and lamins mediate the attachment of chromatin to the nuclear envelope. Studies conducted in *LMNA* knockout mice have shown that localization of the heterochromatin is completely lost from the nuclear periphery especially in fibroblasts and myocytes **[28, 29].** Similar phenotypical abnormalities have been noted in fibroblasts of patients with Hutchinson–Gilford progeria syndrome [HGPS] — a clinical disorder caused by mutations in the *LMNA* gene **[30].**

3) DNA replication:

An increasing body of evidence suggests that lamins play a key role in regulating the complex DNA replication process. One such study by Moir *et al.* demonstrated, in 3T3 cells exposed to BrDU and stained with proliferating cell nuclear antigen [PCNA], that lamin B1 localizes at the replication foci with BrDU and the replication factor PCNA in replication sites during “S phase”, suggesting the potential role of lamins in replication **[31].** Most of the evidence showing the association of lamin and replication process is based on studies carried out *in vitro* in Xenopus egg interphase nuclei extracts. Xenopus eggs when immunodepleted with XLB3, a major lamin in this species, or when transfected with a dominant-negative N-terminally deleted mutant of lamin B1, exhibited a reduction in DNA replication **[32-34].** Evidence for the importance of lamins in transcription is shown in studies with N-terminally deleted lamins, which disrupts the head-to-tail assembly of lamin dimers and the subsequent formation of lamin filament structures. This N-terminally deleted mutant of lamins, when inserted in Xenopus embryos, bring about dissociation of the lamina which then leads to a decrease in RNA polymerase II activity, which is directly involved in the process of transcription **[35].**

Processing of pre-lamins for the formation of mature lamin filaments

Post-Translational Modification

Post translational modifications [PTMs] are a series of steps implicated in the priming and processing of proteins and play a vital role in enhancing the functional range of the protein. They involve covalent addition of certain functional groups [phosphorylation, methylation, prenylation etc.], enzymatic cleavage of regulatory subunits or degradation of entire proteins [ubiquitination] and influence major facets of cell physiology and pathology. Identifying and understanding PTMs is critical in the study of cell biology and disease preclusion and therapy.

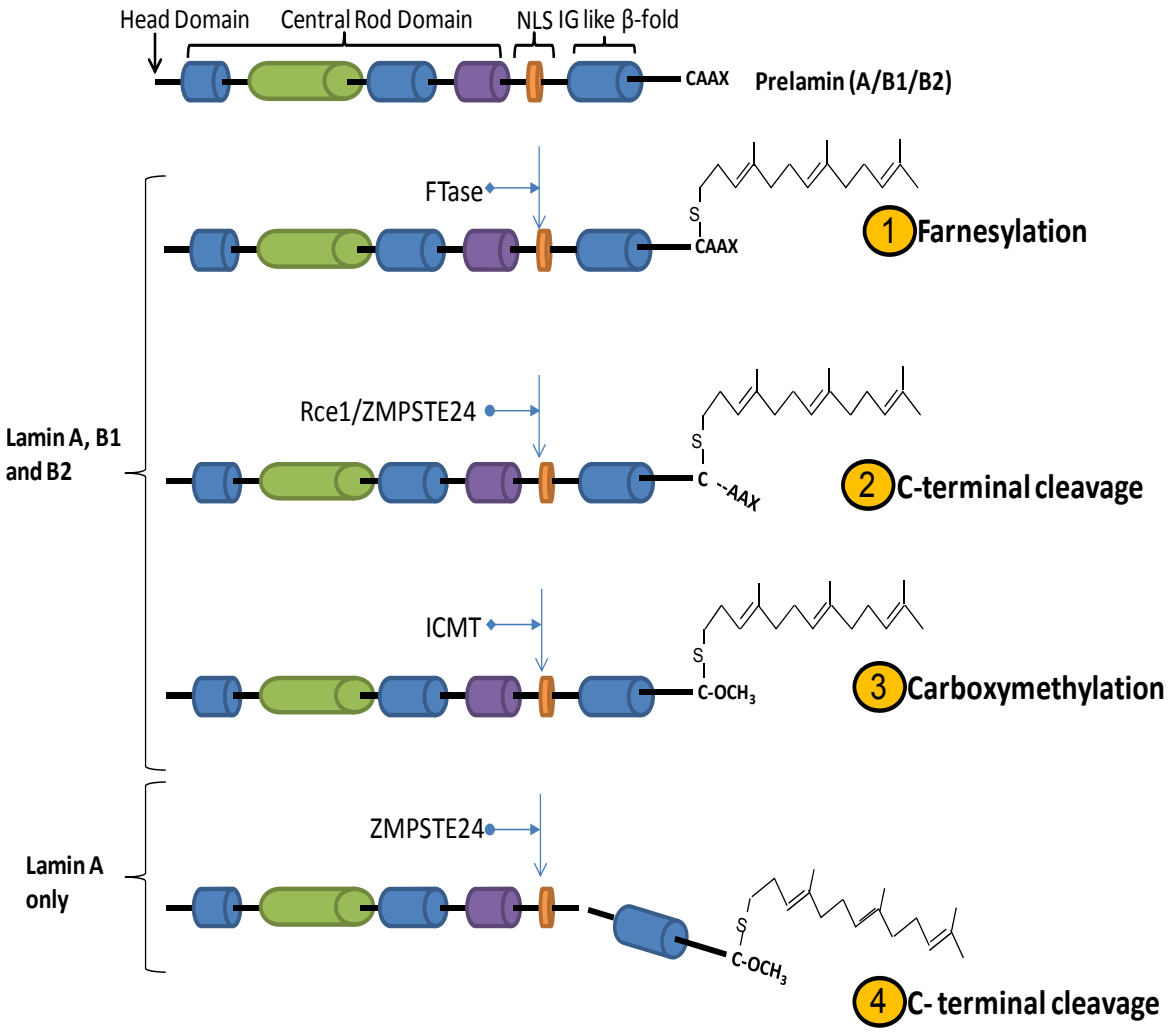


Figure 1-5: PTM steps involved in processing of nuclear lamins. [Modified from Burke *et al.*, Ref. **21**]

Small molecular weight G-proteins, the Ɣ subunits of trimeric G-proteins and nuclear lamins which possess the unique C-terminal amino acid sequences “CAAX motif” are ideal candidates for PTMs and serve as targets for isoprenylation and carboxymethylation. Isoprenylation is the enzyme-mediated addition of either 15 carbon farnesyl group or 20 carbon geranylgeranyl group at the C-terminal cysteine of the protein **[36, 37].**

Lamins A, B1 and B2, which are initially translated in the form of pre-lamins, possess the C-terminal CAAX motif which marks the proteins for farnesylation of the cysteine residue **[21].** Lamins require additional PTMs at CAAX box in order to be converted to their mature form. Soon after their synthesis, lamins are subjected to a multistep PTM process, in the following order: initial farnesylation by the enzyme farnesyltransferase [FTase], endoproteolytic cleavage of C-terminal -AAX amino acids by Ras converting enzyme 1 [Rce-1] or Zinc metallopeptidase [ZMPSTE24] and finally carboxymethylation by isoprenylcysteine carboxyl methyltransferase [ICMT] **[38, 39].** B type lamins are permanently farnesylated and carboxymethylated while prelamin A undergoes an additional step of removal of 15 amino acids at the C-terminal by ZMPSTE24 to generate mature lamin A which lacks the modifications at the C-terminal. This step is unique to lamin A. Lamin C on the other hand, does not possess the CAAX motif and hence is not farnesylated or modified **[21, 39, and 40].**

- **Farnesylation:**

The major purpose of these lipid post-translational modifications of lamins is directing them towards the membrane for protein-membrane interactions or protein-protein interactions for nuclear envelope assembly, which begins with the insertion of a 15 carbon farnesyl group at the C-terminal cysteine and is an irreversible modification **[36].** Farnesyl pyrophosphate is a metabolite derived during the synthesis of cholesterol from mevalonate. The presence of CAAX motif at C-terminal of lamins marks them as target proteins for FTase-catalyzed farnesylation. FTase incorporates this 15 carbon derivative of mevalonate into the C-terminal cysteine residue which has been shown to increase the hydrophobicity of the proteins and results in membrane targeting **[20].** A recent study by Jung and colleagues has highlighted the role of farnesylation of lamin B in neuronal development. They have developed knock-in mice expressing non-farnesylated versions of lamin B1 and lamin B2. In this study, the non-farnesylated lamin B1

expressing mice died due to neuronal abnormalities since the chromatin could not be retained within the nuclear lamina during the process of neuronal migration **[41].**

- **Endoproteolytic cleavage:**

Endoproteolytic cleavage involves the shortening of the C-terminal by severing the last 3 amino acids of the CAAX motif by either Rce-1 or ZMPSTE24, and the subsequent removal of the last 15 amino acids by ZMPSTE24 in the case of prelamin A producing mature lamin A **[42].** Rce-1 is a type II CAAX prenyl endopeptidase with a wide specificity, processing all farnesylated and geranylgeranylated CAAX proteins while ZMPSTE24 is a type I CAAX processing enzyme and has a specific role in processing of prelamin A **[43, 44].** These are integral membrane proteins that reside in the ER and INM. Recently, Manolaridis and associates have reported the crystal structure of an Rce-1 homologue from *Methanococcus maripaludis*, which has specificity for farnesylated peptides similar to that shown by eukaryotic Rce-1 **[38].**

- **Carboxymethylation:**

The next step in the PTM of lamins is the addition of carboxymethyl group to farnesyl cysteine of CAAX box by ICMT, a prenyl-protein specific methyltransferase of the ER. Initial studies from our laboratory have characterized the carboxylmethylation of lamin by vapor phase equilibration assay using either [^{3}H] S-adenosyl methionine in cell lysates or [^{3}H]-methionine in intact cells. The initial observation characterized a protein in the molecular range of 65-70 kDa was carboxymethylated. Further, studies were carried out by immunoprecipitating the lysates or intact beta cells after incubating with [^{3}H] S-adenosyl methionine or [^{3}H]-methionine using anti-lamin B antibody suggested that the methylated protein was lamin B **[45].**

Although farnesylation of lamins is involved in increasing their hydrophobicity and driving them towards the nuclear membrane, both endoproteolytic cleavage and carboxymethylation play an essential role in the proper formation of the nuclear lamina as shown in a recent study by Maske *et al.* They have demonstrated that absence of endoproteolysis or carboxymethylation in the C-terminal of lamins would lead to distorted nuclear lamina **[46].**

B type lamins are permanently farnesylated and carboxymethylated while prelamin A undergoes an additional endoproteolytic cleavage step and finally converts into mature lamin A **[21].** Recently we have also shown in INS-1 832/13 cells that ICMT produces an increase in activity of a small molecular weight GTP binding protein, Rac1, which is essential for the assembly of NOX2. NOX2 holoenzyme assembly mediates the production of reactive oxygen species [ROS], which is necessary for GSIS **[47].**

Nuclear lamina assembly and disassembly

Nuclear lamina assembly is a dynamic process that begins after the sister chromatids are formed during anaphase and in final stages of mitosis, especially telophase. The nuclear envelope [NE] and its associated structures start to reassemble around the separated sister chromosomes to form functional daughter nuclei. Nuclear reassembly is characterized at molecular level based on a tightly regulated sequence of protein interactions, which occur in the following order: [i] targeting of individual nucleoskeletal proteins to the chromosomal surface, [ii] membrane recruitment and fusion, [iii] assembly of nuclear pore complexes [NPCs], [iv] transport of the bulk of lamins into the nucleus through newly formed NPCs, and [v] formation of the nuclear lamina and, at the early G1 phase, the chromatin becomes fully enclosed by an intact NE. As the nucleus enlarges by import of nuclear proteins through NPCs, the NE expands and the chromatin becomes fully de-condensed. Being intermediate filament proteins, lamins

assemble together to form the nuclear lamina meshwork that remains connected during interphase, but disassembles during mitosis **[48, 49].**

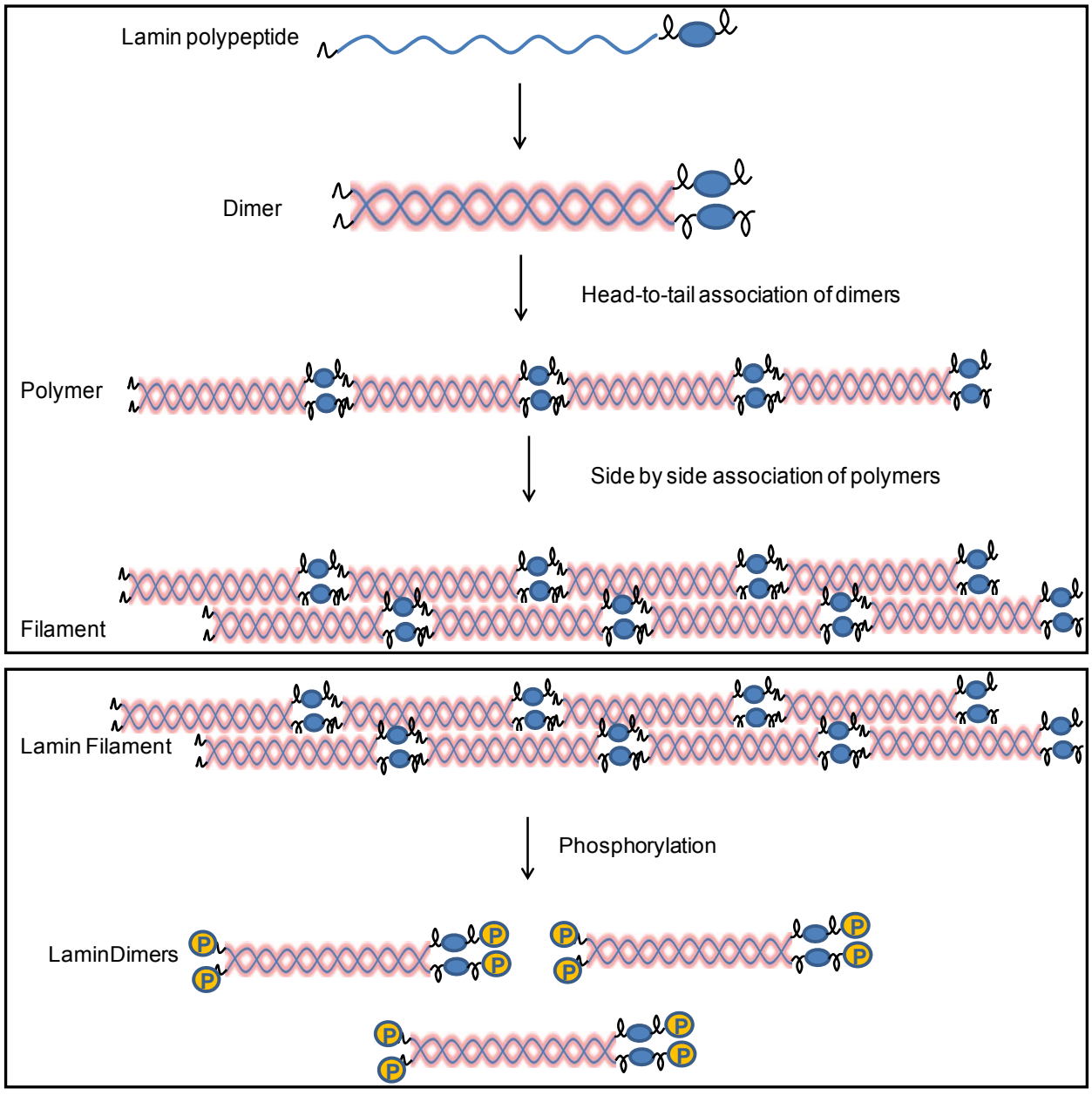


Figure 1-6: Assembly and disassembly of the nuclear lamina. [Modified from Davidson *et al.* Ref. **50** and THE CELL, Third Edition Ref. **51**].

Lamins [both A and B type] can self assemble into parallel coiled homodimers and further associate to form head-to-tail molecular strings that are formed by electrostatic interactions between the overlapping ends of successive coiled-coil domains. In the initial stage of lamina organization, two individual lamin monomers associate by winding around each other to form parallel coiled-coil structures. The structure of the central *α*-helical rod domain facilitates this dimer formation with the C-termini projecting out as globular heads. In conditions that favor lamina assembly, the dimers connect to form polymers by forming longitudinal head-to-tail links. These polymers subsequently form filaments by lateral association and finally emerge as paracrystals **[52, 53].**

Nuclear lamina disassembly occurs during mitosis as well as during apoptosis with several biochemical similarities in lamin filament dissociation occurring during both these processes **[54, 55].** A key feature of apoptosis is reduction in nuclear size and chromatin condensation followed by chromatin fragmentation; during this process lamin proteins are cleaved by the cysteine-dependent aspartate-directed proteases, namely; caspases **[56];** and this has been proposed as the main mechanism for lamina disassembly. For this to happen, prior phosphorylation by lamin kinases [e.g., protein kinases] is essential for the relaxation of lamin proteins for the access of caspases, followed by lamin disassembly. Activation of lamin kinases [protein kinase C-δ and protein Kinase C-βII] requires prior cleavage of the holoenzyme, to release C-terminal catalytic domain, by caspases **[57, 58].** Recently, Cross *et al.* demonstrated that protein kinase C-δ gets activated by caspase 3, and translocates to the nuclear compartment and colocalizes with lamin B during apoptosis and inhibition of PKC-δ delayed caspase 3-mediated proteolysis of lamin B **[59].** Furthermore, Eitel and associates have reported that kinase-negative PKC-δ mutant decreased free fatty acid-induced lamin B disassembly and cell death in pancreatic beta cells **[60].** The nuclear lamins were one of the first proteins identified as caspase targets. The aspartic acid 230 of lamin A located **[61]** in the

2B region of the central rod domain, a highly conserved region in all known IF proteins is critical for polymerization and is recognized as caspase cleavage site. Each caspase has a distinct type of lamin as a substrate, which possesses a unique recognition sequence for the protease, despite the fact that the aspartic acid 230 residue is the target site for caspase cleavage in human lamins A and B1. Rao and colleagues have demonstrated that HeLa cells which are transfected with caspase-uncleavable mutants of lamins, displayed an impediment in the advancement of shrinkage process of the nucleus, and a slowdown in chromatin condensation and cell death **[62]**.

Diseases due to genetic mutations and improper processing of lamins

Most of the disease related mutations in lamins originate in the *LMNA* gene [e.g., Emery-Dreifuss muscular dystrophy, dilated cardiomyopathy, Hutchinson-Gilford Progeria Syndrome *etc.*] along with few others [Adult onset autosomal dominant leukodystrophy and Pelger-Huet anomaly], occurring due to mutations in the lamin B1 [*LMNB1]* gene and lamin B receptor [*LBR]* gene **[63].** Enlisted in the table below are some of the major disorders associated with lamin protein family [also called 'laminopathies'], the mutated genes and the phenotypical characteristics of the diseases **[64-79].**

Disease	Lamin involved	Mutation gene	Clinical Phenotype
Emery-Dreifuss Muscular Dystrophy	Lamin A/C	*LMNA*	• Affects mainly skeletal and cardiac muscles • Phenotypical signs appear by

			age 10 • Joint deformities [contractures] at elbows and Achille's tendon • Rigidity in spine and neck • Increasing muscle weakness and wasting • Most patients have cardiac complications by adulthood
Restrictive Dermopathy	Accumulation of farnesylated prelamin A	*ZMPSTE24*	• Lethal congenital skin condition • Intrauterine growth retardation • Tightening/rigidity of skin [especially of face and around the mouth] • Respiratory insufficiency • Most infants do not live long due to pulmonary problems
Hutchinson-Gilford Progeria	Lamin A [accumulation of	*LMNA*	• Physical appearance is normal at birth

Syndrome.	aberrant form of prelamin A 'progerin']		• Premature and accelerated aging • Reduction/absence of subcutaneous fat • Alopecia • Hearing loss • Most do not live beyond 13-14 yrs • Death usually occurs due to cardiovascular diseases
Dunnigan-type familial partial lipodystrophy [FPLD]	Lamin A/C	*LMNA*	• Phenotypical appearance of the disease occurs during adolescence • Progressive loss of fat from limbs and trunk • Glucose intolerance • Hyperinsulinemia • Insulin resistance

			• T2DM
Adult Onset autosomal dominant leukodystrophy	Lamin B1 [Increased expression]	*LMNB1*	• Phenotype is similar to chronic progressive multiple sclerosis • Progressive neurological disorder • Autonomic dysfunction, e.g., low blood pressure, incontinence, blurred vision
Charcot-Marie tooth disease, axonal Type 2B1	Lamin A/C	*LMNA*	• Symptoms begin in late childhood/early adulthood • Progressive loss of muscle tissue and sensation • Mild sensory loss • Wasting and weakness of lower limbs
Pelger-Huet anomaly	Lamin B receptor	*LBR*	• Benign blood disorder [hyposegmentation of nucleus of WBCs/neutrophils]

			• Mild skeletal malformation
Dilated Cardiomyopathy	Lamin A/C	*LMNA*	• Affects mainly cardiac muscle, skeletal muscle is minimally affected • Progressive ventricular dilation • Impaired systolic function • Pulmonary edema

Table 1-3: Clinical features of laminopathies.

Nuclear Lamins in beta cell and T2DM

Beta cell damage is characterized by specific changes in the morphology of the cell. The cell undergoes shrinkage, blebbing [bulges in the plasma membrane], nuclear fragmentation and chromatin condensation. Degradation of lamins leads to the breakdown of nuclear lamina which is a preliminary stage of apoptosis as this is followed by DNA degradation and chromatin condensation **[80].** It is well established that beta cell dysfunction and failure are characteristic features of Type 1 and 2 diabetes. Therefore, it is necessary to study the intracellular events that the pancreatic beta cell undergoes, and understanding of such events will aid in searching for a possible drug target for the management and/or prevention of the disease. So far, not much is known regarding the role of lamins in the etiology of diabetes, apart from some genome-wide association studies [GWAS]. GWAS typically focus on associations between

single-nucleotide polymorphisms [SNPs] and major diseases by comparing the genetic materials from normal and diseased state individuals. *LMNA* gene mutations are responsible for multiple clinically relevant disorders, such as Dunnigan-type familial partial lipodystrophy [FPLD]. Individuals with this disease display a characteristic loss of subcutaneous fat, insulin resistance, glucose intolerance, hypertension in addition to other phenotypical changes observed in metabolic syndrome. Hegele and associates were the first research group to discover that a cause of Dunnigan-type lipodystrophy, a body fat disorder which leads to diabetes, is mutation in *LMNA* gene in the chromosome 1q21.22 **[81].**

Role of caspases in nuclear lamin degradation

The caspase family is one of the most important factors in the apoptotic pathway. Many proteins in the cell, including a large number of structural proteins, are cleaved by activated caspases which culminates in apoptosis of the cell. The caspase 8, 9 and 10 are initiator caspases whereas caspase 3, 6 and 7 are effector caspases. The initiator caspases cleave and activate the effector caspases leading to cell death **[82].** These caspases exist in the form of inactive zymogens [also referred to as pro-caspases], which are activated by the process of cleavage by other upstream proteases or by auto- or trans-activation **[83].** In the recent years a variety of substrates of caspases have been recognized [e.g., PARP, PKC-δ, nuclear lamins]. Caspase-dependent degradation of nuclear lamins has been identified as a precursor to nuclear lamina disassembly **[56].**

Glucotoxicity and the beta cell

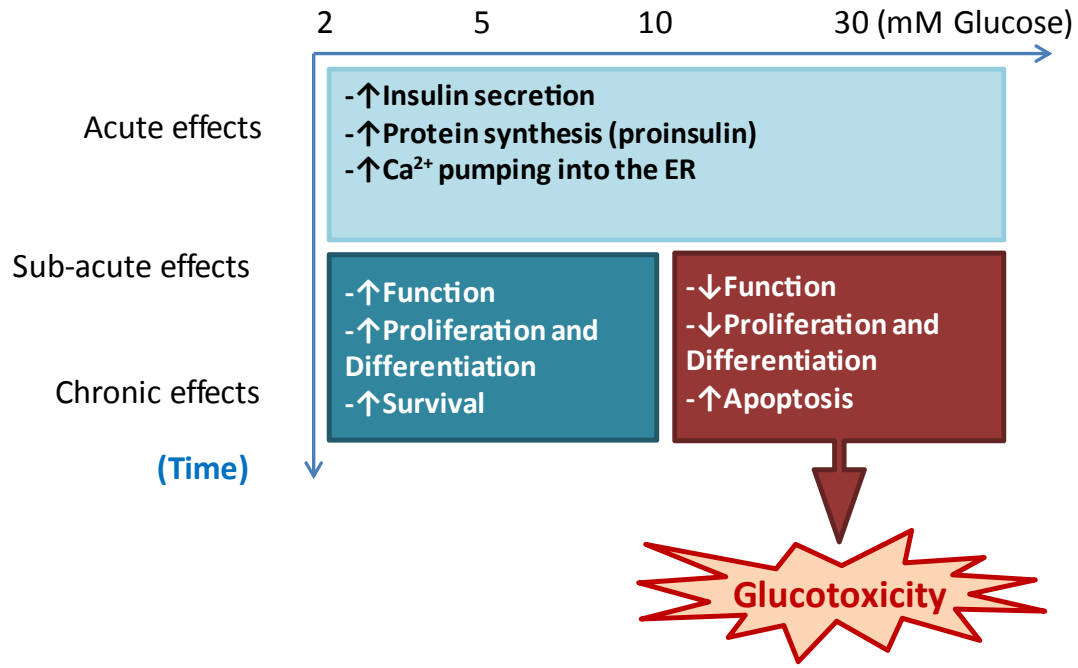


Figure 1-7: Acute and chronic effects of various glucose concentrations on the beta cell. [Modified from Bensallem *et al.* 2012, Ref. **84**]

A constant glucose overload on the beta cell causes reduced expression of *Glut2*, preproinsulin, *Pdx-1*, diminished glucose sensitivity and impairment in its basic function of insulin secretion and eventually beta cell damage **[84, 85]. Figure 1-8** shows the effect of glucotoxicity on beta cell function and survival. Several intracellular signaling events have been identified as causal to high glucose-induced metabolic dysregulation of the islet. The most commonly studied are endoplasmic reticulum [ER] and oxidative stress. It has been suggested that both oxidative and ER stress lead to mitochondrial dysfunction, cytochrome C release, and caspase activation **[86].** Recently, a multifunctional sorting protein, PACS-2 [phosphor-acidic cluster sorting protein 2], which controls the ER-mitochondria axis has been identified **[87].** Despite this growing body of evidence, very little is known in the context of the islet beta cells on potential detrimental effects of glucotoxic conditions on ER, mitochondria, caspase activation and associated degradation of their respective substrate proteins.

On the basis of the above literature review, the central objective of my thesis work is to study the mechanisms involved in beta cell dysfunction in diabetes and to elucidate the role of ER-mitochondrial axis in initiating the activation of executioner caspases which leads to nuclear lamina disassembly in insulin-secreting INS-1 832/13 cells exposed to acute and/or long-term incubation conditions. In addition, I propose to extend these *in vitro* studies to islets obtained from animal models of obesity and T2DM [e.g., Zucker Diabetic Fatty - ZDF rat].

Based on this objective, I proposed to test the hypotheses that: [i] Exposure of pancreatic beta cells to glucotoxic conditions induces ER stress which causes caspase-mediated lamin A & B degradation culminating in beta cell dysfunction; [ii] cross-talk between ER and mitochondria cause mitochondrial dysfunction leading to caspase activation and lamin degradation; and [iii] ER stress inhibitors and calcium channel blockers protect the pancreatic beta cell from glucotoxicity. A simplified working model based on these hypotheses has been depicted in **Figure 1-8.**

I worked towards testing these hypotheses by conducting experiments under the following three Specific Aims:

Specific Aim 1: To study the effect of glucotoxicity induced ER stress on pancreatic beta cells.

Specific Aim 2: To establish a role of ER-mitochondrial communication in beta cell dysfunction/death and to determine the effects ER stress inhibitors and calcium channel blockers on pancreatic beta cells.

Specific Aim 3: To investigate potential abnormalities involved in ER stress and mitochondrial dysfunction in animal models of impaired insulin secretion, obesity and diabetes.

Specific Aim 4: To study the consequences of inhibition of requisite post translational prenylation of lamins on the pancreatic beta cell.

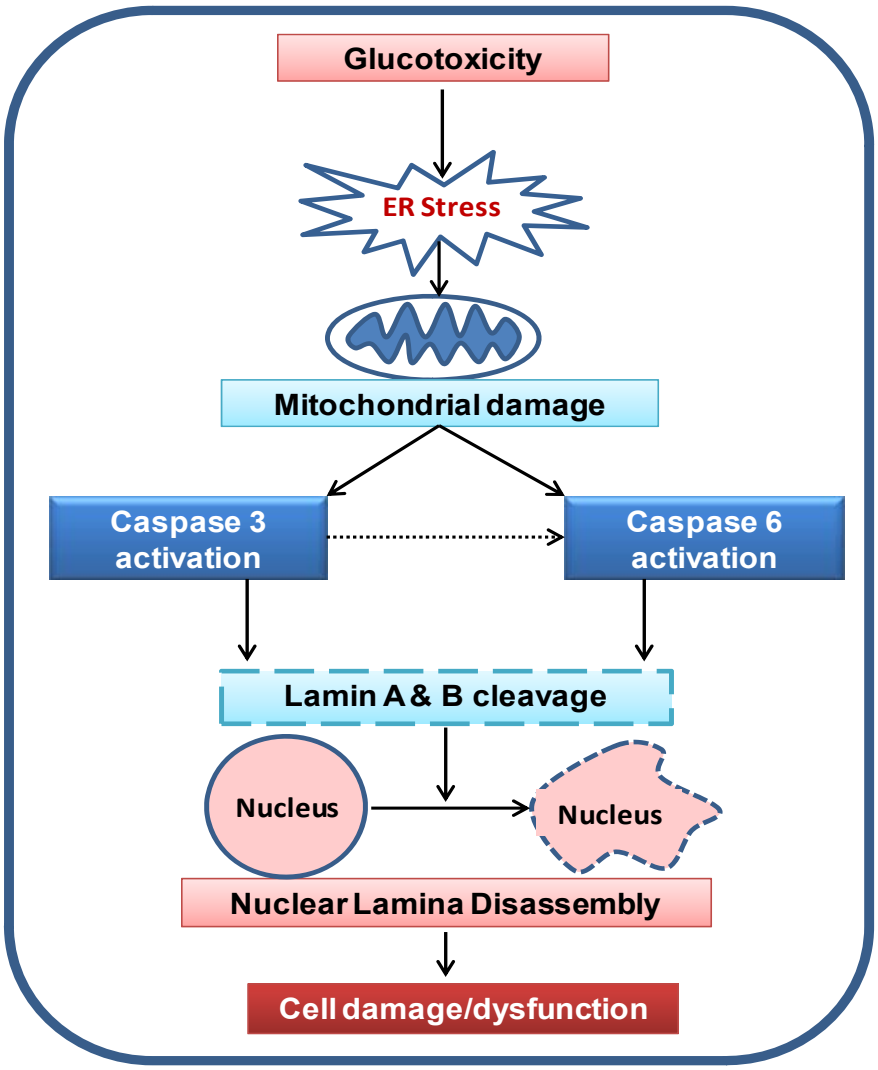


Figure 1-8: Proposed working model for ER-stress induced, caspase-mediated degradation of nuclear lamins under glucotoxic conditions in pancreatic beta cells. We propose that exposure of insulin-secreting cells to high glucose leads to increased ER stress leading to mitochondrial dysfunction **[86, 87].** As a consequence, cytosolic caspase 3 and 6 are activated by the released cytochrome C from the dysregulated mitochondria. Activation of caspases leads to degradation of lamin A and B culminating in nuclear damage and altered distribution of degraded lamins into various subcellular compartments, leading to nuclear lamina disassembly.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Chemicals: Glucose, nifedipine, thapsigargin, 4-phenylbutyric acid, simvastatin, triton X-114 were obtained from Sigma Aldrich [St. Louis, MO]. Inhibitors of caspase 3 [Z-DEVD-FMK] and caspase 6 [Z-VEID-FMK] were from Enzo Lifesciences [Farmingdale, NY]. FTI-277 was purchased from Tocris Biosciences [Minneapolis, MN]. GGTI-2147 was obtained from Calbiochem-EMD Millipore [Billerica, MA] and Rottlerin was procured from Cayman Chemical Company [Ann Arbor, MI].

Antibodies: Antisera directed against lamin A, caspase 3, cleaved caspase 3, cleaved caspase 6, CHOP, phospho-p44/42 ERK1/2 [Thr202/Tyr204] and total p44/42 ERK1/2 were obtained from Cell Signaling [Danvers, MA]. Antibody for lamin B, phospho-p38MAPK and total-p38MAPK was from Santa Cruz Biotechnology [Santa Cruz, CA]. Antibody for ZMPSTE24 was procured from Abcam [Cambridge, MA]. Anti-β actin was purchased from Sigma Aldrich [St. Louis, MO]. Anti-mouse IgG and Anti-rabbit IgG conjugated to horseradish peroxidase were from GE Healthcare [UK]. Anti-goat IgG conjugated to horseradish peroxidase was obtained from Santa Cruz Biotechnology [Santa Cruz, CA].

Assay kits: ELISA kit [Rat insulin] was procured from American Laboratory Products Co [Windham, NH]. Metabolic cell viability assay kit [MTT] was purchased from Sigma Aldrich [St. Louis, MO]. ProteoExtract - Subcellular Proteome Extraction Kit was obtained from EMD Millipore [Billerica, MA].

Animals: Male Sprague-Dawley rats were purchased from Harlan Laboratories [Oxford, MI]. Male Zucker Diabetic fatty [ZDF] and Zucker Lean Control [ZLC] rats were obtained from Charles River Laboratories [Wilmington, MA]. All animals were maintained in a 12 hrs light/dark cycle with free access to water and food. The ZDF and ZLC rats were fed the Purina Diet 5008. Blood glucose was measured by tail vein puncture using *Freestyle lite* glucometer [Abott Diabetes Care, Inc., Alameda, CA].

2.2 INS-1 832/13 cells, rat islets and human islets: Culture conditions

INS-1 832/13 cells: INS-1 832/13 cells were kindly made available by Dr. Chris Newgard, Duke University Medical Center, Durham, NC. The cells were cultured and maintained in RPMI-1640 medium prepared with 10% heat inactivated Fetal Bovine Serum [FBS]. The medium was supplemented with antibiotics [100 IU/ml penicillin and streptomycin], 10 mM HEPES, 1mM sodium pyruvate and 50 μM 2-mercaptoethanol [pH 7.4]. Passages 53-61 were used for the studies. Cells were exposed to low and high glucose [2.5 and 20 mM] or simvastatin/FTI-277/GGTI-2147 for time periods ranging from 0-48 hrs as indicated in each study. For the studies involving pharmacological inhibitors, cells were pre-incubated with the compounds for either 1 or 12 hrs as indicated for each study, and then further treated with low and high glucose in the continuous presence or absence of inhibitors. At the end of the incubation period, the cells were harvested and lysed in radio immunoprecipitation assay [RIPA] buffer containing protease inhibitor cocktail, 1 mM NaF, 1 mM PMSF and 1 mM Na_3VO_4.

Isolation of islets: All animal protocols were utilized after prior assessment and obtaining consent of the Institutional Animal Care and Use Committee at Wayne State University. Islets from normal 6-8 week-old male Sprague–Dawley rats were isolated by the collagenase digestion method, wherein collagenase was injected into the common bile duct of

the euthanized animal. The pancreata were then excised and digested at 37°C followed by density gradient purification using Histopaque 1077, in order to separate out islets from pancreatic acinar tissue. 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 islets were harvested and lysed in RIPA buffer.

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.

2.3 Isolation of subcellular fractions

Extraction of cytosolic, membrane/organelle and nucleic protein fraction was carried out as per the manufacturer's instructions using the ProteoExtract® Subcellular Proteome Extraction Kit. INS-1 832/13 cells were exposed to low and high glucose for 24 hrs. The cells were scraped and suspended in wash buffer and pelleted by centrifugation for 10 min at 200 *g* at 4°C. The pellet obtained was then resuspended in the extraction buffer-I and protease inhibitor cocktail provided in the kit. After incubation for 10 min at 4°C the cells were centrifuged for 10 min at 750 *g*, the supernatant obtained was the cytosolic fraction. The pellet was then resuspended in Extraction buffer-II and protease inhibitor cocktail and incubated for 30 min and then centrifuged for 10 min at 5500 *g*. The supernatant thus obtained was the membrane/organelle fraction. Finally the pellet was resuspended in extraction buffer-III, protease inhibitor cocktail and benzonase, incubated for 10 min

at 4°C and centrifuged at 6800 *g* for 10 min. The supernatant obtained was the nucleic protein fraction.

2.4 Subcellular phase partitioning using Triton X-114

INS-1 832/13 cells were incubated overnight in low glucose and 2.5% FBS and FTI-277 [10 μM]. Cells were then treated with media containing low glucose [2.5 mM] in the absence and presence of FTI-277 [10 μM] for 24 hrs. Cell lysates were homogenized in a homogenization buffer [20 mM Tris-HCl, pH 7.5, 0.5 mM EGTA, 2 mM $MgCl_2$ and protease inhibitor cocktail] and subjected to a single-step centrifugation at 100,000*g* for 60 min at 4°C in order to separate the cytosol [supernatant] and the membrane [pellet] fractions. The membrane fraction was then subjected to phase partitioning using Triton X-114, a non-ionic detergent. Triton X-114 exists as a homogenous solution at 0°C; however, as the temperature rises upto 20°C, it splits into aqueous and detergent phases. This property is taken advantage of during phase partitioning, by overlaying the sample on a 6% sucrose cushion containing 150 mM NaCl, 0.06% Triton X-114 in 10 mM Tris-Hcl buffer [pH 7.4] and centrifugation at 300*g* for 3 minutes at room temperature; wherein, the hydrophilic proteins emerge in the aqueous phase while the amphiphilic, integral membrane proteins are found in the detergent phase **[88].**

2.5 *Akita* cells

Akita cell line was provided by Prof. Sasanka Ramanadham, University of Alabama, Birmingham, AL. *Akita* and wild type cells were maintained in DMEM medium prepared with 17.5% heat-inactivated FBS, with antibiotic preparation [100 IU/ml penicillin and streptomycin] and 2-mercaptoethanol. The cultured cells were harvested and lysed in RIPA buffer containing protease inhibitor cocktail, 1 mM NaF, 1 mM PMSF and 1 mM Na_3VO_4.

2.6 Western Blotting

Cell lysate proteins [30-50 µg loaded per lane] were subjected to SDS-Polyacrylamide gel electrophoresis and electro transferred onto 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 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 blots in TBS-T again, the signal for proteins on the blots was enhanced using electrochemiluminescence [ECL] and the images of the blots were developed using Kodak Pro Image 400 R [New Haven, CT]. To ensure uniform protein loading and efficient transfer onto the membrane, the blots were stripped and re-probed with antibody raised against β actin. Intensity of protein bands was quantified by using Carestream Molecular Imaging Software to measure the band density. Ratios of band densities of proteins of interest with β actin were calculated and expressed as fold change values.

2.7 Measurement of lamin A and B degradation

Following treatment with low glucose [2.5 mM], high glucose [20 mM] or inhibitors [as indicated in the text], INS-1 832/13 cells, isolated rat or human islets were lysed in RIPA buffer supplemented with 1mM PMSF, 10 µg/mL leupeptin, 10 µg/mL aprotinin, 5 mM EGTA, 5 mM EDTA, 10 mM NaF and 1 mM sodium orthovanadate. The samples were clarified by incubating on ice for 15 min followed by centrifugation at 14000 rpm for 15 min. The supernatants were collected and subjected to a protein estimation assay using Pierce 660 nm protein assay reagent. Equal amounts of protein were loaded and separated by SDS-PAGE. Resolved

proteins were transferred onto nitrocellulose membrane and probed for lamin B or cleaved lamin A as indicated in text, followed by re-probing with β actin. Intensity of protein bands was quantified by using Carestream Molecular Imaging Software to measure the band density. Ratios of band densities of degraded lamin B and cleaved lamin A with β actin were calculated and expressed as fold change values in lamin A or B degradation.

2.8 Activation of caspase 3 and 6

Cells treated with low and high glucose or other stimulants/inhibitors [as indicated in the text]. Lysate proteins were subjected to Western blotting. Activation of caspases was estimated by the appearance of the bands of cleaved [active] hydrolytic product at 17 and 18 kDa for caspase 3 and 6 respectively.

2.9 Cell viability assay

INS-1 832/13 cells were cultured in 96-well plates and treated with low glucose [2.5 mM] in the absence or presence of Simvastatin or FTI-277 [10 μM]. After 24 hrs cell viability was assessed by using a colorimetric assay with MTT [tetrazolium salt] which measures the reduction of the tetrazolium salt into the purple formazan crystals at 570 nm.

2.10 Insulin release studies

Insulin secretion experiments were performed in static incubation conditions. INS-1 832/13 cells were incubated in the presence of low or high glucose. After 24 hrs, 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 secreted by the cells into the medium was detected and measured by ELISA.

2.11 Cell morphology studies

Observation of cell morphology was carried out using an Olympus IX71 microscope. INS-1 832/13 cells, incubated overnight in RPMI containing low glucose and 2.5% FBS, were treated the following day with media containing low glucose [2.5 mM] alone and in the presence of diluent [DMSO], FTI-277 [10 μM] or Simvastatin [15 and 30 μM]. Changes in cell morphology were visualized by light microscopy after 24 hrs.

2.12 Statistical analysis of experimental data

Data are represented as Mean ± SEM values. The statistical significance of the differences in values between the experimental conditions was established by either Student's t-test or ANOVA followed by SNK Post-Hoc test where appropriate. P value < 0.05 was considered significant.

CHAPTER 3

MITOCHONDRIAL DYSFUNCTION INDUCED BY GLUCOTOXIC AND DIABETIC CONDITIONS RESULTS IN CASPASE MEDIATED LAMIN DEGRADATION IN PANCREATIC BETA CELLS

Portions of this work have been published [copies of the published manuscripts are appended]

- **Khadija S**, Veluthakal R, Sidarala V, Kowluru A. Glucotoxic and diabetic conditions induce caspase 6-mediated degradation of nuclear lamin A in human islets, rodent islets and INS-1 832/13 cells. Apoptosis. 2014; 19[12]:1691-701.
- **Syeda K**, Mohammed AM, Arora DK, Kowluru A. Glucotoxic conditions induce endoplasmic reticulum stress to cause caspase 3 mediated lamin B degradation in pancreatic β-cells: protection by nifedipine. Biochem Pharmacol. 2013 1;86[9]:1338-46.

The interior of the nuclear envelope is lined by the nuclear lamina which is composed of three proteins; lamins A, B and C. The A type lamins, which include lamins A and C, are products of alternative splicing of the same gene *LMNA* whereas lamin B is encoded by the LMNB1 gene **[21].** These lamins are type V intermediate filament proteins and are lined up on the inner face of the inner nuclear membrane. The nuclear lamina is a fundamental part of major nuclear activities, namely mitosis, chromatin organization and in DNA replication. Lamins also play key functional roles in providing structural support, thereby contributing to the nuclear architecture **[89].** Evidence in multiple cell types suggests significant alterations in nuclear structure and organization during apoptosis **[62].** 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]. Degradation of lamins leads to the breakdown of nuclear lamina

which is a preliminary stage of apoptosis as this is followed by DNA degradation and chromatin condensation **[80].**

Previous results from our laboratory indicated that IL-1β treatment causes an increase in lamin B degradation mediated by caspases **[90].** In addition, a recent study highlighted the involvement of caspase 3 in the breakdown of the nuclear matrix by cleaving nuclear lamin B. It has been stated that this cleavage probably occurs by activation of caspase 3 directly or by other downstream proteases **[91].** Recent evidence in PC12 and rat cortical cells also implicates cell apoptosis *via* caspase 3 activation and cleavage of lamin B under lipotoxic conditions induced by saturated fatty acids, such as palmitic acid **[92].**

The overall objective of this study, therefore, is to investigate the role of caspases on the degradation of nuclear lamins, specifically lamin A and B in insulin secreting INS-1 832/13 cells, rodent and human islets under high glucose exposure.

Exposure to glucotoxic conditions cause caspase 3-mediated degradation of lamin B in INS-1 832/13 cells

At the outset, INS-1 832/13 cells were incubated with either low [2.5 mM] or high [20 mM] glucose for 12, 24 and 48 hrs, and caspase 3 activation, as evidenced by the emergence of caspase 3 degradation fragment, was monitored by Western blotting, and the data were then quantified by densitometry. Data depicted in **Figure 3-1** demonstrate a marked increase in caspase 3 activation as early as 12 hrs [1.8-fold; Panel A], which continued to increase as a function of time [2.2- and 2.6-fold increase at 24 and 48 hrs, respectively; Panels B and C]. Furthermore, we noticed a marked increase in the degradation of lamin B under these conditions [Figure 3-1]. For example the fold increase in lamin B degradation represented 1.6-fold at 12 hrs [Panel A], 1.8-fold at 24 hrs [Panel B] and 2.3-fold at 48 hrs [Panel C]. Panels D

and E depict combined data from multiple experiments. Together, data in Figure 3-1 suggest activation of caspase 3 and degradation of lamin B under glucotoxic conditions. It should be noted that the observed effects of glucose on caspase 3 activation and lamin B degradation are not due to osmotic effects of glucose since incubation of these cells with mannitol [20 mM], used as an osmotic control, did not elicit any effects on caspase 3 activation and lamin B degradation under these conditions [n = 2 experiments; additional data not shown].

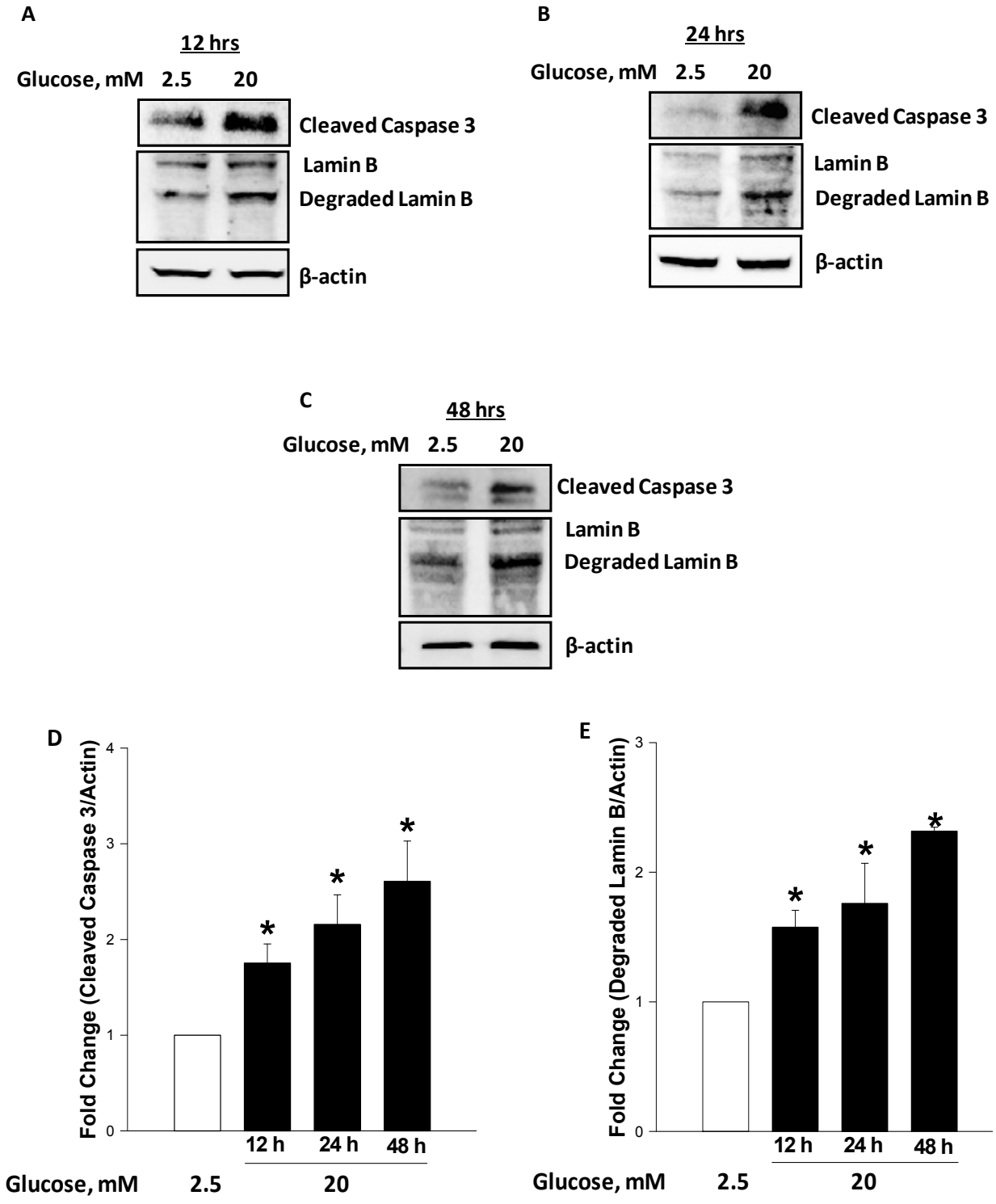

A
12 hrs
Glucose, mM 2.5 20
Cleaved Caspase 3
Lamin B
Degraded Lamin B
β-actin
B
24 hrs
Glucose, mM 2.5 20
Cleaved Caspase 3
Lamin B
Degraded Lamin B
β-actin
C
48 hrs
Glucose, mM 2.5 20
Cleaved Caspase 3
Lamin B
Degraded Lamin B
β-actin
D
Fold Change (Cleaved Caspase 3/Actin)
0
1
2
3
4
*
*
*
12 h
24 h
48 h
Glucose, mM 2.5 20
E
Fold Change (Degraded Lamin B/Actin)
0
1
2
3
*
*
*
12 h
24 h
48 h
Glucose, mM 2.5 20

Figure 3-1: Exposure of INS-1 832/13 cells to glucotoxic conditions results in caspase 3 activation and lamin B degradation. INS-1 832/13 cells were incubated in the presence of low [2.5 mM] or high [20 mM] glucose for 12 hrs [Panel A], 24 hrs [Panel B], and 48 hrs [Panel C], and protein lysates [~50 µg] were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Cleaved caspase 3 and lamin B antibodies were used to probe the membrane and immune complexes were identified using ECL. To ensure equal protein loading, the same membranes were reprobed with antisera against β-actin. Band-intensity of proteins was quantified by densitometry. T-test was used to measure the statistical significance of the differences in values between the control and experimental conditions. Data in Panel D and E represent mean ± SEM from three to four independent experiments and expressed as fold change in caspase 3 activation and lamin B degradation. *$P < 0.05$ *vs.* 2.5 mM glucose.

Caspase 6 activation and cleavage of lamin A in INS-1 832/13 cells treated with high glucose

Further, we wanted to examine if exposure of INS-1 832/13 cells to glucotoxic conditions would result in activation of caspase 6 and associated degradation of lamin A. Data in **Figure 3-2** [Panel A] represent 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. Panels B and C depict data accumulated from multiple experiments.

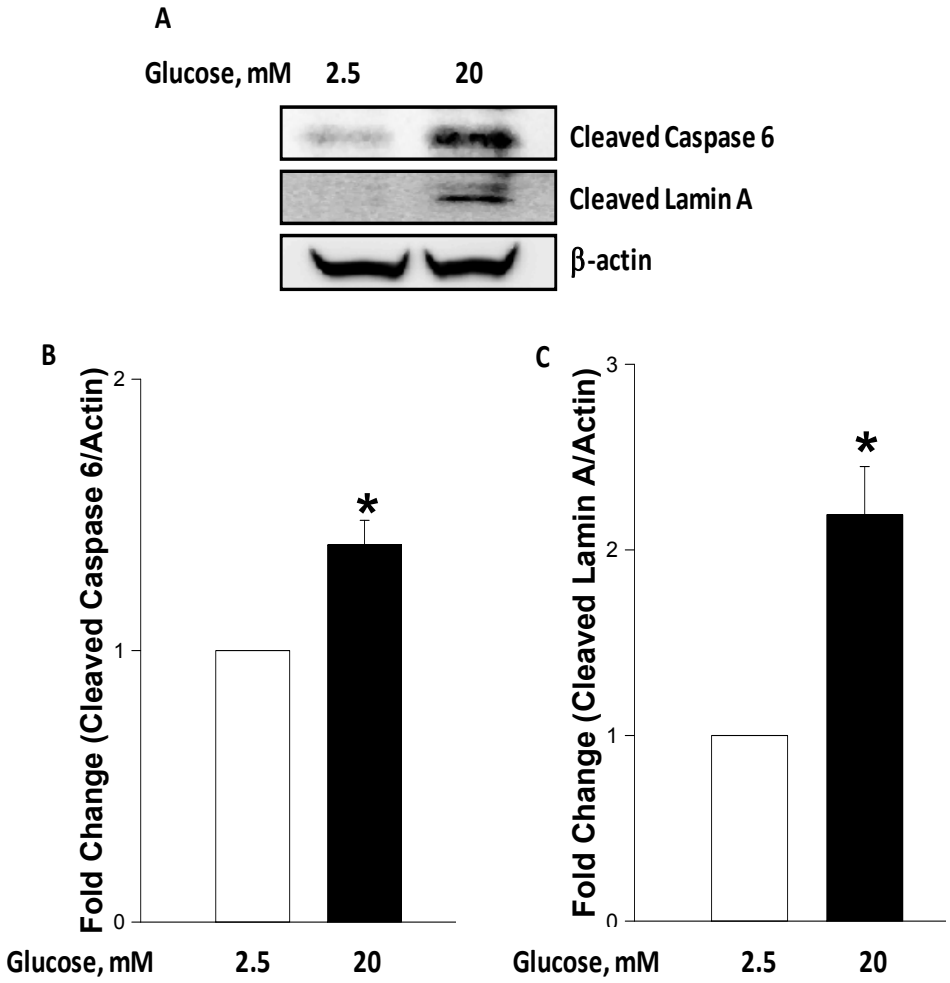


Figure 3-2: Caspase 6 activation and lamin A cleavage in INS-1 832/13 cells treated with high glucose. INS-1 832/13 cells were incubated in the presence of low or high glucose for 24 hrs. Caspase 6 activation and lamin A cleavage were determined by Western blotting. Protein lysates [~40 µg] were loaded onto SDS-Polyacrylamide gels and transferred onto a nitrocellulose membrane. Antibodies against cleaved caspase 6 and cleaved lamin A were utilized to probe the membrane and immune complexes were identified using ECL detection kit [Panel A]. After stripping the membranes, they were re-probed with β-actin antibody to ensure uniform loading of proteins. Band-intensity of proteins was evaluated by densitometry. Data represent mean ± 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.* 2.5 mM glucose.

Chronic glucose exposure significantly impairs GSIS in INS-1 832/13 cells

Several lines of evidence suggest that exposure of pancreatic beta cells to hyperglycemic conditions leads to the onset of metabolic stress, loss in glucose-stimulated insulin secretion [GSIS] and cell demise. Therefore, in the next set of studies, we quantified effects of high glucose exposure [20 mM; 24 hrs] on GSIS using the INS-1 832/13 cell line. Data in **Figure 3-3** 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]. These data indicate significant impairment in GSIS in INS-1 832/13 cells after 24 hrs of incubation in media containing high glucose.

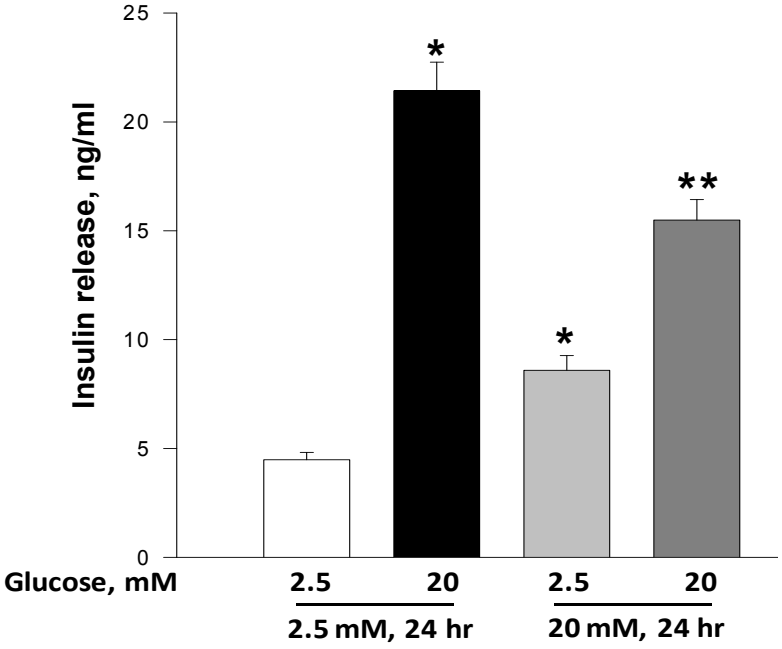
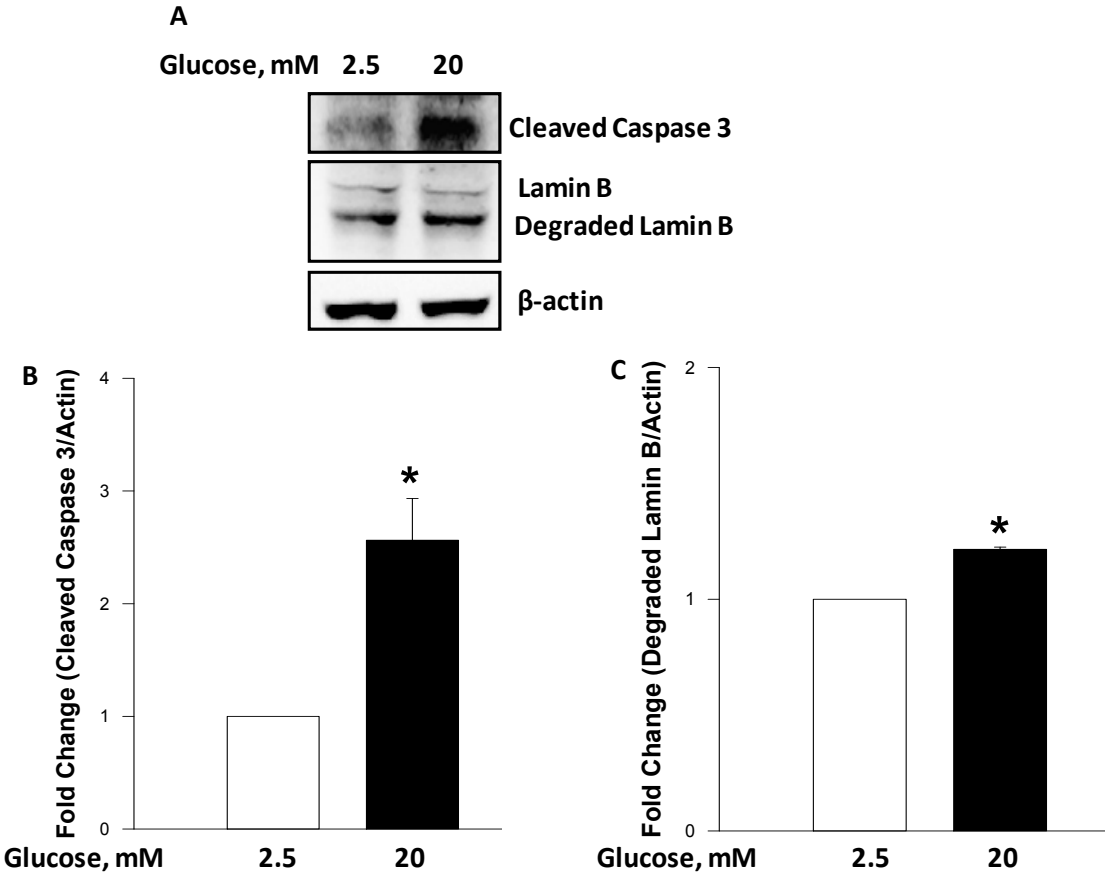


Figure 3-3: Glucotoxic conditions attenuate GSIS in INS-1 832/13 beta cells. INS-1 832/13 cells were cultured in the presence of low and high [2.5 and 20 mM] glucose. After 24 hrs they were stimulated with low or high glucose for 45 min. Amount of Insulin released into the culture medium was evaluated by ELISA [see Methods-section 2.7 for additional details]. The data are expressed as insulin release [ng/ml] and are means ± SEM from three independent experiments. *$P < 0.05$ *vs.* 2.5 mM glucose under 24 hrs low glucose treatment; **$P < 0.05$ *vs.* 20 mM glucose under 24 hrs low glucose treatment.

Glucotoxic conditions induce caspase 3 and 6 activation and subsequent degradation of lamin A and B in normal rat islets

Based on the above observations and our findings on caspase 3 and 6 activation and lamin A and B degradation under glucotoxic conditions, we repeated these studies in primary rat islets to further validate the observed effects of glucotoxicity on caspase activation and lamin degradation. Data depicted in **Figure 3-4** indicate an increase in caspase 3 and 6 activation followed by a corresponding increase in lamin A and B degradation under these conditions [Figure 3-4; Panels A and D]. Panels B,C and E,F display data combined from multiple experiments.



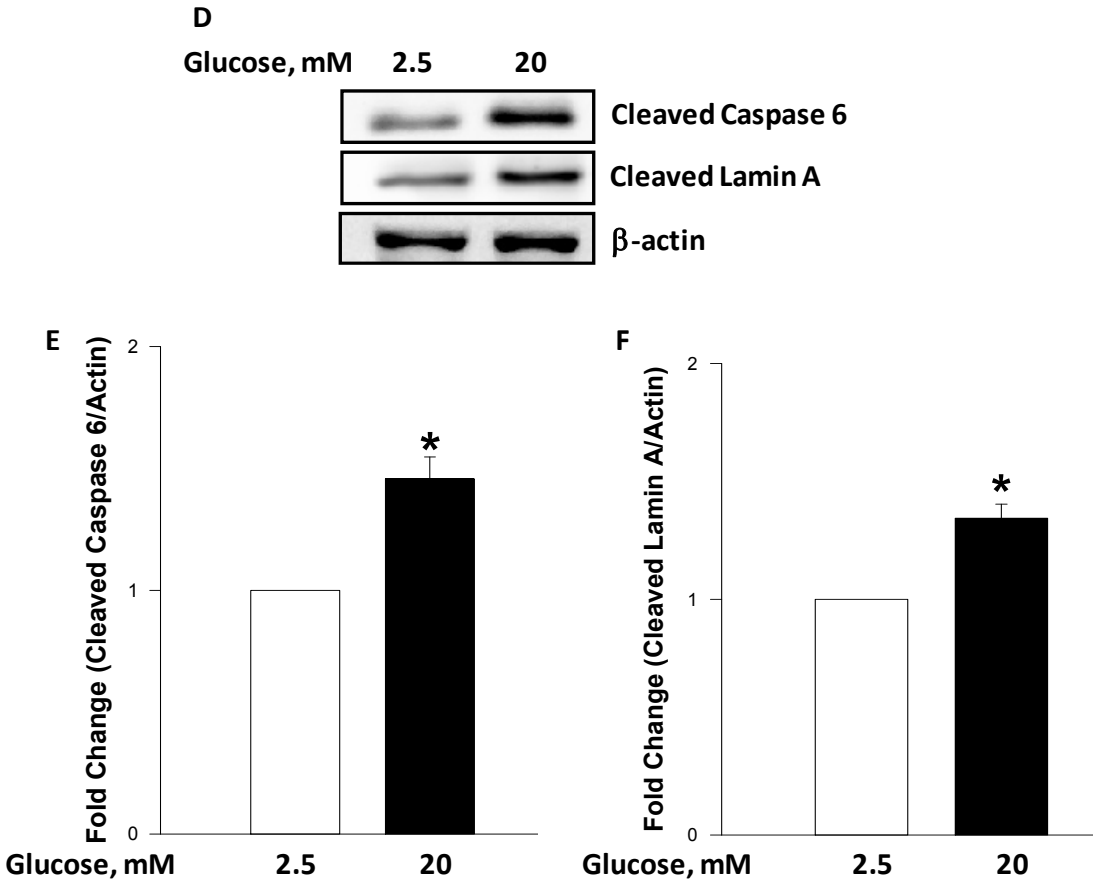
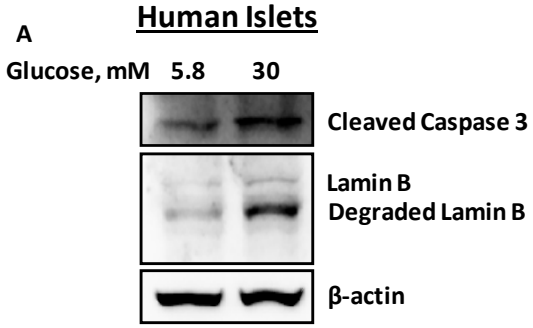


Figure 3-4: Treatment of normal rat islets with high glucose results in caspase 3 and 6 activation and hydrolytic cleavage of downstream substrates: lamins A and B. Rat islets were incubated in the presence of low [2.5 mM] and high glucose [20 mM] for 24 hrs. Approximately 40 µg protein lysates from each condition were resolved by SDS-PAGE and the degree of abundance of cleaved caspase 3 and lamin B [Panel A] and cleaved caspase 6 and lamin A [Panel D] were determined by Western blot analysis. The loading control used was β-actin. Quantitation of caspase activation and lamin degradation were carried out by densitometry. Data represent mean ± SEM from three independent experiments and are expressed as fold change in cleaved caspase 3 [Panel B], degraded lamin B [Panel C], cleaved caspase 6 [Panel E] and cleaved lamin A [Panel F]. *$P < 0.05$ *vs.* 2.5 mM glucose.

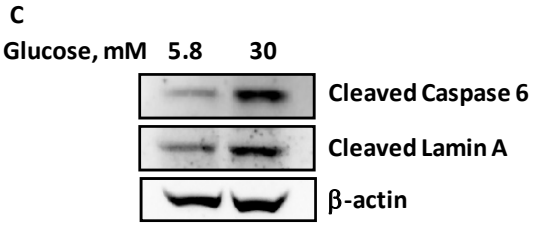
Increased activation of caspases and associated degradation of lamins are also demonstrable in human islets treated with high glucose

As a logical extension of the above studies, we quantified caspase activation and lamin degradation in islets obtained from human donors. We noticed a 1.9-fold increase in caspase 3 activation and 2-fold increase in lamin B degradation; likewise we also observed a 2.17-fold and a 1.69-fold increase in active caspase 6 and cleaved lamin A respectively in human islet preparations incubated with high glucose [30 mM; 24 hrs; **Figure 3-5**; Panels A-D]. These data further support our observations in INS-1 832/13 cells, suggesting that a similar mechanism of beta cell death may be operable in primary islets [rat and human] **[Figure 3-1 and 3-2].**



B

Fold Change(Cleaved Caspase 3/Actin)		Fold Change (Degraded Lamin B/Actin)	
LG	HG	LG	HG
1.0	1.9	1.0	2.0



D

Fold Change (Cleaved Caspase 6/Actin)		Fold Change (Cleaved Lamin A/Actin)	
LG	HG	LG	HG
1.0	2.17	1.0	1.69

Figure 3-5: Glucotoxic conditions promote caspase 3 and 6 activation and nuclear lamina breakdown in normal human islets treated with high glucose. Normal human islets were incubated in the presence of low [5.8 mM] and high glucose [30 mM] for 24 hrs as described in the text. 25 µg Lysate proteins were loaded onto SDS-Polyacrylamide gels and then transferred to nitrocellulose membranes. The membranes were probed for cleaved caspases 3 and 6, and lamin A and B and immune complexes were identified using ECL detection kit. To ensure equal protein loading, the same membrane was re-probed using antibody for β-actin. Human islet data were accrued from a single batch of islet preparation.

Evidence for expression of ZMPSTE24 [a zinc metallopeptidase responsible for endoproteolytic cleavage 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 **[39].** Earlier studies from our laboratory have demonstrated carboxylmethylation of lamins in islet beta cells **[45].** Therefore, in the last set of studies we asked if ZMPSTE24 is expressed in the islet beta cells, and whether glucotoxic conditions affect the expression of this peptidase. Data depicted in **Figure 3-6** 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.

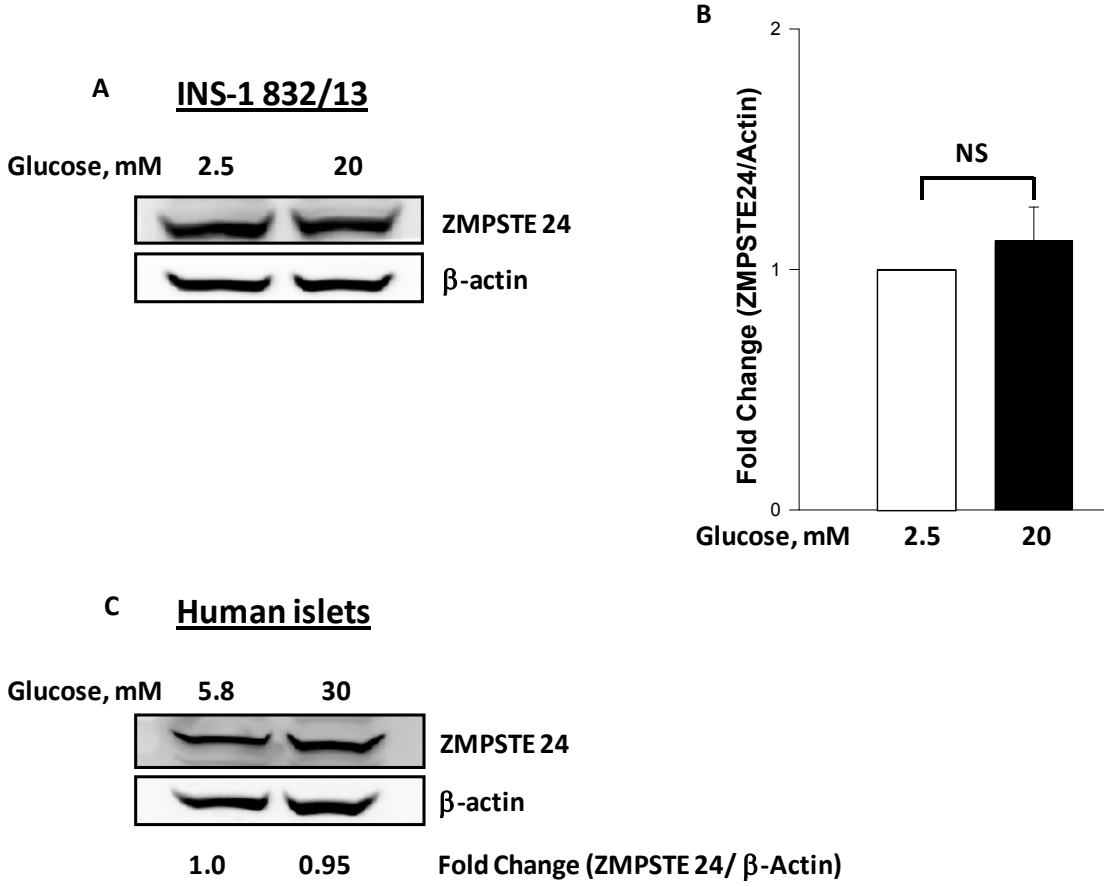


Figure 3-6: ZMPSTE 24 is expressed in INS-1 832/13 cells and normal human islets. Lack of effects of high glucose exposure on the expression of ZMPSTE 24. INS-1 832/13 cells were incubated in the presence of low [2.5 mM] or high [20 mM] glucose. After 24 hrs protein lysates were resolved by SDSPAGE and transferred to a nitrocellulose membrane. Panel A shows a representative blot from three studies. Intensity of protein bands was quantified by densitometry and data represent mean ± SEM from three independent experiments and are expressed as fold change in ZMPSTE 24 [Panel B]. NS not significant *vs.* 2.5 mM glucose. Normal human islets were incubated in the presence of low [5.8 mM] and high glucose [30 mM] for 24 hrs [as in Figure 3-5]. 25 µg of islet lysate proteins were loaded onto a SDS-Polyacrylamide gel and transferred to a nitrocellulose membrane. The membranes were probed with Antisera against ZMPSTE 24 and the protein was detected using ECL kit. To ensure equal protein loading, the membranes were probed for β actin. A blot from a single human islet preparation is shown in Panel C.

Alterations in the subcellular distribution of cleaved caspase 3 and lamin B in INS-1 832/13 cells exposed to glucotoxic conditions

In these studies, we determined potential alterations, if any, in the subcellular localization of active caspase 3 fragment and lamin B degradation products in INS-1 832/13 cells following exposure to glucotoxic conditions. To determine this, INS-1 832/13 cells were incubated for 24 hrs with low or high glucose [2.5 or 20 mM]. Individual subcellular fractions, namely the cytosolic fraction [fraction F1], membrane/organelle protein fraction [fraction F2] and the pure nucleic protein fraction [fraction F3] were isolated using a ProteoExtract subcellular proteome extraction kit [Materials and Methods- Section 2.3]. At the outset, we determined subcellular distribution of native caspase [pro-caspase] and lamin B in INS-1 832/13 lysates. Data depicted in **Figure 3-7** [Panel A], as expected, suggest that native lamin B and pro-caspase are localized predominantly in the nuclear [F3] and cytosolic [F1] fractions, respectively. However, glucotoxic conditions caused significant alterations in the subcellular distribution of biologically active [cleaved] caspase 3 and degraded lamin B [Figure 3-7; Panel B]. For example, we noticed significant accumulation of degraded lamin B in cytosolic [F1] and membrane/ organelle protein [F2] fractions under high glucose-treatment conditions. Interestingly, we also noticed significant accumulation of cleaved caspase 3 in these fractions. Together, these observations demonstrate abnormal distribution of lamin B under glucotoxic conditions further suggestive of disassembly of nuclear structure under these conditions.

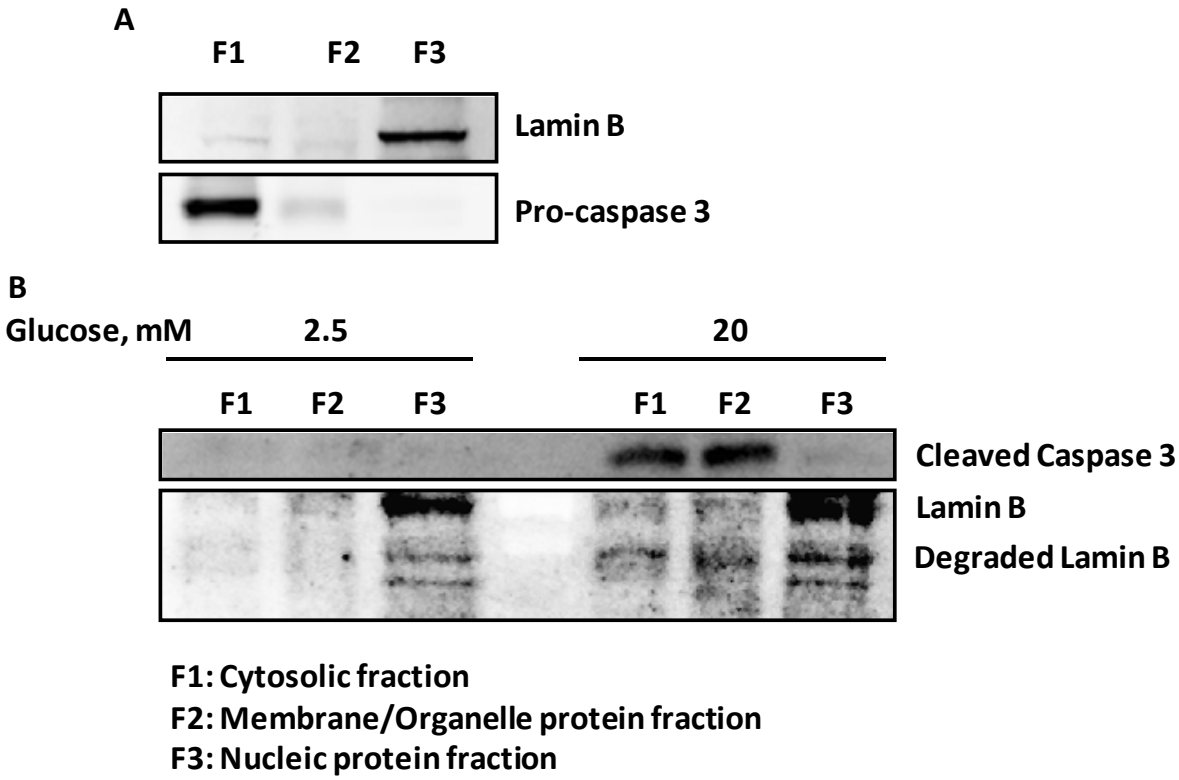


Figure 3-7: Exposure to high glucose results in altered subcellular distribution of caspase 3 and degraded product of lamin B in INS-1 832/13 cells. INS-1 832/13 cells were subjected to subcellular fractionation using ProteoExtract Subcellular Proteome Extraction Kit at basal level [Panel A] and after treatment with low [2.5 mM] and high [20 mM] glucose for 24 hrs [Panel B]. 20–30 µg of lysate proteins were loaded onto a SDS-Polyacrylamide gel and transferred to a nitrocellulose membrane. Lamin B, pro-caspase [Panel A] and cleaved caspase 3 and degraded lamin B [Panel B] were detected using antibodies against each protein. Data are representative of two experiments with identical results.

Increased activation of caspases and associated degradation of lamins are also demonstrable in diabetic rat islets and in islets from human donors with type 2 diabetes

As an extension to the above studies, we quantified caspase 3 and 6 activity and lamin A and B degradation in islets derived from the ZDF rat, a known animal model for T2D and in islets obtained from human donors with Type 2 diabetes. Islets from age-matched ZLC rats were used as controls in these studies. Data in **Figure 3-8** represent a Western blot study demonstrating a significant increase in the activation of caspase 3 and degradation of lamin B

[Panel A] and activation of caspase 6 [Panel D] and cleavage of lamin A [Panel E] in islets from the ZDF rat compared to the ZLC rat islets. Pooled data in islets from multiple ZLC and ZDF rats are given in Panels B, C, F and G. Compatible with our findings above, we also noticed a marked increase in lamin A and B degradation in islets from a human donor with T2DM along with activation of caspases depicted in **Figure 3-9** [Panel A and B]. T2D human islets studies were performed using single islet preparation.

	ZLC Mean ± SEM	**ZDF Mean ± SEM**
Body Weight [g]	275 ± 8	365 ± 7
Blood Glucose [mg/dl]	80 ± 12	276 ± 37

Table 3-1: Body weights and blood glucose levels of ZLC and ZDF rats measured at the time of sacrifice.

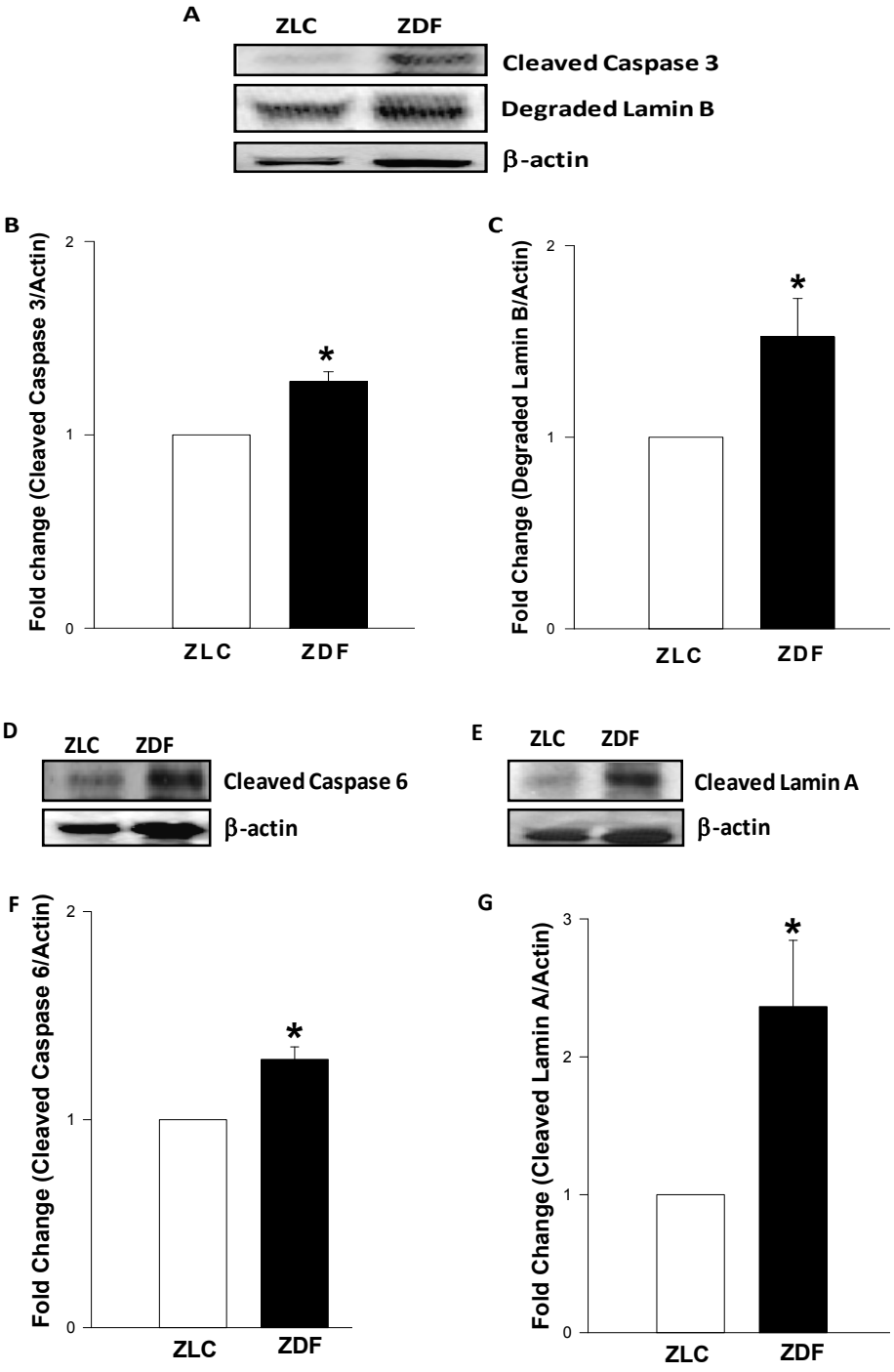
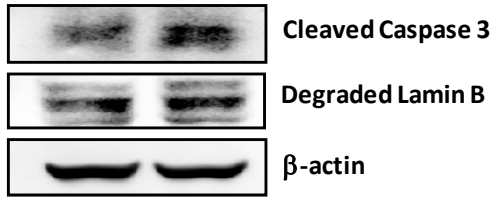


Figure 3-8: Activation of caspase and lamin degradation are observed in islets obtained from ZDF rats. Islets isolated from ZDF and ZLC rats by collagenase digestion method were lysed using RIPA buffer. Lysate proteins [40 µg] were resolved by SDS-PAGE and the abundance of cleaved caspase 3 and 6 and lamin A and B were determined by Western blotting. To assess equal protein loading, the membrane was probed for β-actin. Representative blots from three to four ZLC and ZDF islet preparations are shown in Panel A, D and E. Intensity of protein bands

was quantified by densitometry and data represent mean ± SEM from three to four islet preparations and are expressed as fold change in cleaved caspase 3 [Panel B], degraded lamin B [Panel C], cleaved caspase 6 [Panel F] and lamin A [Panel G]. *$P < 0.05$ *vs.* ZLC

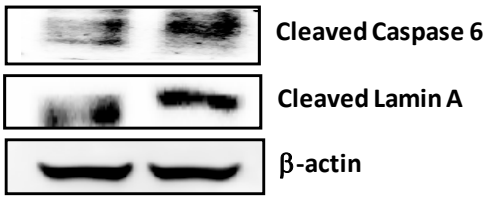
A



B

Fold Change(Cleaved Caspase 3/Actin)		Fold Change (Degraded Lamin B/Actin)	
Control	T2D	Control	T2D
1.0	1.41	1.0	1.71

C



D

Fold Change (Cleaved Caspase 6/Actin)		Fold Change (Cleaved Lamin A/Actin)	
Control	T2D	Control	T2D
1.0	1.43	1.0	1.36

Figure 3-9: Caspase 3 and 6 activation and lamin A and B degradation in diabetic human islets. Islets obtained from normal and diabetic individuals were lysed using RIPA buffer. Proteins from cell lysates were separated by gel electrophoresis and transferred to a nitrocellulose membrane. Cleaved caspase 3 and 6, and lamin A and B and β-actin antibodies were used to probe the same membrane after stripping. Human islet data from normal and diabetic individuals were accrued from a single batch of islet preparation.

Collectively, data shown in **Figures 3-1 to 3-9** can be summarized as follows:

- Glucotoxic conditions promote the activation of executioner caspases 3 and 6, which leads to the degradation of their downstream targets, the nuclear lamins A and B in INS-1 832/13 cells, normal rodent and human islets.

- Evidence of the expression of ZMPSTE24, a lamin A processing enzyme, was seen in INS-1 832/13 cells and human islets.

- ❖ Under conditions of high glucose exposure, a significant impairment in the insulin secretion capacity of the beta cells was observed.
- ❖ Significant alterations in the subcellular distribution of lamin B were observed under glucotoxic conditions.
- ❖ Islets obtained from the ZDF rat, a model for T2D and islets from T2D human donors also exhibit similar effects on activation of caspases and proteolytic cleavage of lamins.

CHAPTER 4

IDENTIFICATION OF DOWNSTREAM SIGNALING EVENTS INVOLVED IN GLUCOTOXICITY INDUCED ENDOPLASMIC RETICULUM [ER] STRESS LEADING TO DYSFUNCTION IN PANCREATIC BETA CELLS

Portions of this work have been published [copies of the published manuscripts are appended]

- **Khadija S**, Veluthakal R, Sidarala V, Kowluru A. Glucotoxic and diabetic conditions induce caspase 6-mediated degradation of nuclear lamin A in human islets, rodent islets and INS-1 832/13 cells. Apoptosis. 2014; 19[12]:1691-701.
- **Syeda K**, Mohammed AM, Arora DK, Kowluru A. Glucotoxic conditions induce endoplasmic reticulum stress to cause caspase 3 mediated lamin B degradation in pancreatic β-cells: protection by nifedipine. Biochem Pharmacol. 2013 1;86[9]:1338-46.

Thapsigargin, a known inducer of ER stress, markedly increases caspase 3 activation and lamin B degradation in INS 1-832/13 cells

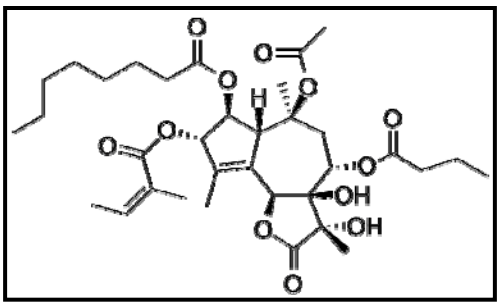
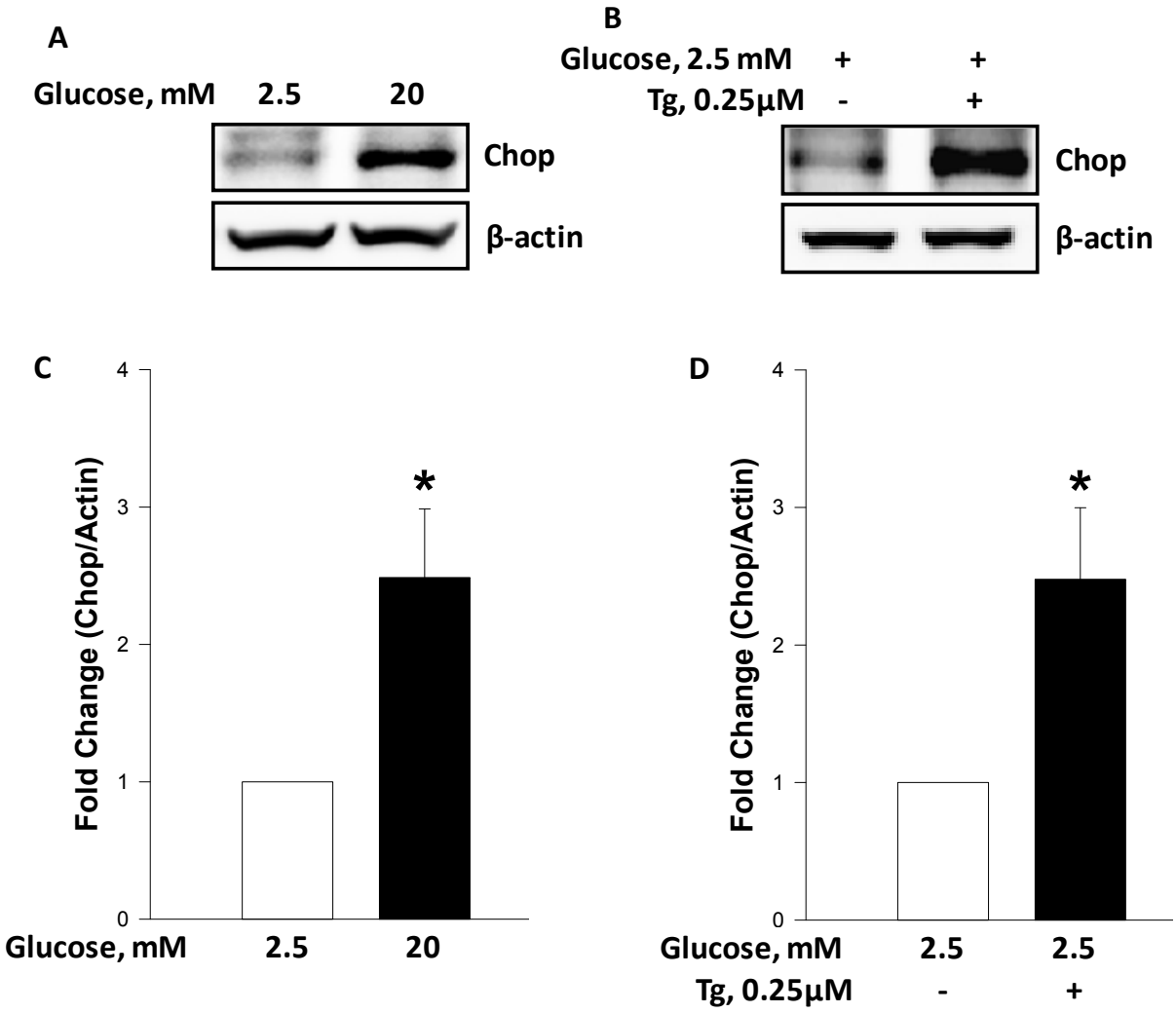


Figure 4-1: Chemical Structure of Thapsigargin

Several lines of experimental evidence implicate ER stress as one of intermediate steps involved in glucotoxicity of the islet beta cell **[93].** To determine if high glucose induces ER stress in our current experimental model, we quantified CHOP expression, an ER stress marker, in INS-1 832/13 cells exposed to low and high glucose conditions. Data depicted in **Figure 4-2**

[Panel A] demonstrate a significant increase in CHOP expression in cells exposed to glucotoxic conditions and a similar result was obtained with thapsigargin [Panel B]. To further assess if ER stress induces caspase 3 activation and lamin B degradation, we incubated INS-1 832/13 cells with thapsigargin and then quantitated caspase 3 activation and lamin B degradation in these cells. Data depicted in Figure 4-2, Panel E demonstrate significant increase in caspase 3 activation in cells treated with thapsigargin as early as 2 hrs of incubation [3.4- fold; Figure 4-2 Panels E and F]. Note that thapsigargin effects were maximal at 2 hrs since no further increase in caspase 3 activation was seen at 4 hrs [3.1-fold activation] and 6 hrs [3-fold activation; Panels E and F]. Under these conditions, we also noticed a significant increase in lamin B degradation induced by thapsigargin within 2 hrs [1.9-fold], which remain plateaued at 4 hrs [1.8- fold] and 6 hrs [1.7-fold] as shown in Panels E and G. Together, our findings suggest ER stress as one of the mechanisms underlying glucose-mediated effects on caspase 3 activation and lamin B degradation. In the next set of studies, we further tested this hypothesis using a pharmacological approach.


A
Glucose, mM 2.5 20
Chop
β-actin
B
Glucose, 2.5 mM + +
Tg, 0.25µM - +
Chop
β-actin
C
Fold Change (Chop/Actin)
0
1
2
3
4
*
Glucose, mM 2.5 20
D
Fold Change (Chop/Actin)
0
1
2
3
4
*
Glucose, mM 2.5 2.5
Tg, 0.25µM - +

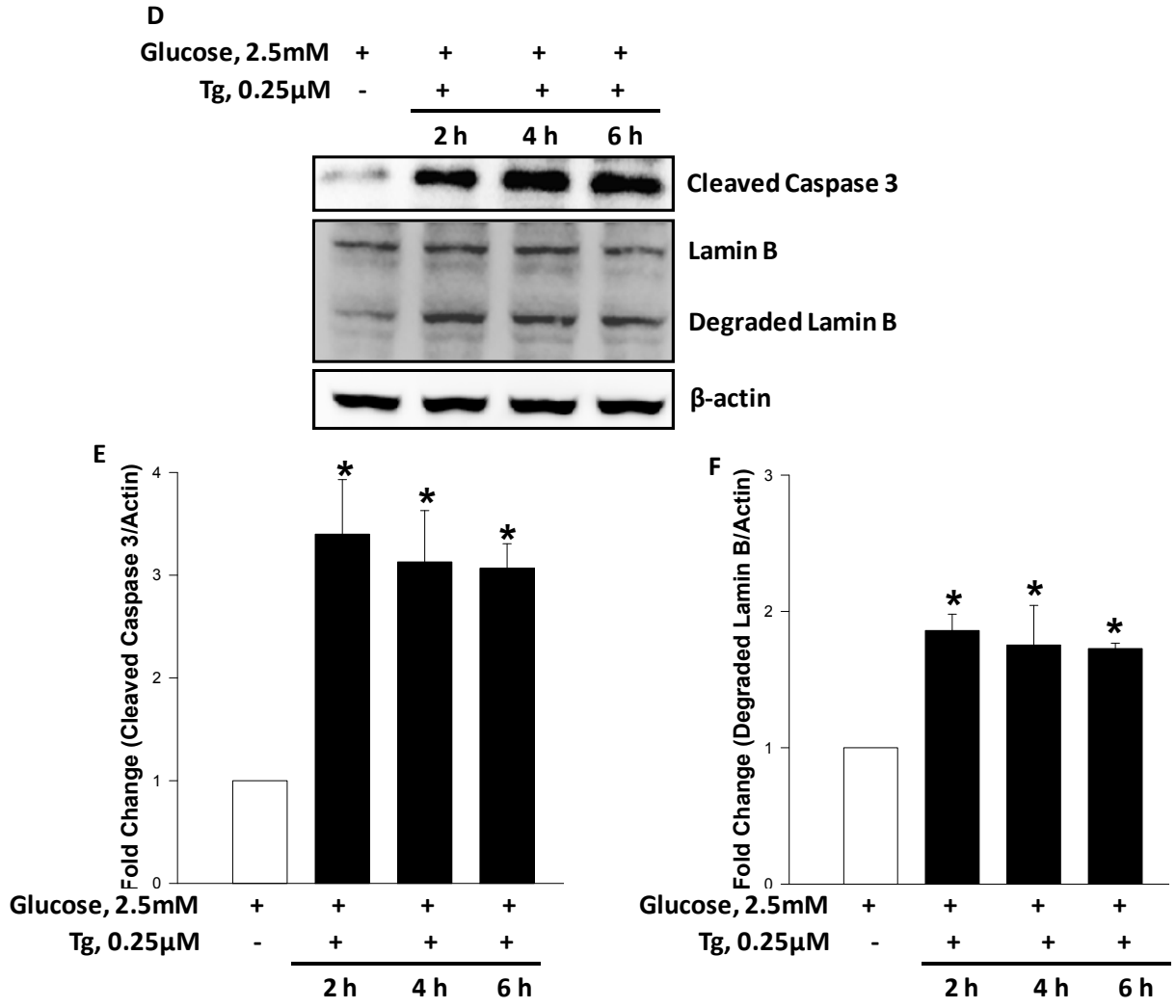


Figure 4-2: Thapsigargin, a known inducer of ER stress, also promotes caspase 3 activation and lamin B degradation in INS-1 832/13 cells. Cells were incubated with low glucose [2.5 mM] or high glucose [20 mM] for 24 hrs. Expression of CHOP was determined in cell lysates by Western blotting [Panel A]. The same membranes were reprobed using antibody against β-actin. Densitometry was used to measure CHOP expression. The statistical significance of the differences between the control and the experimental groups was determined by t-test. Panel C represents data from three independent experiments. *$P < 0.05$ *vs.* 2.5 mM glucose. Panel B: INS-1 832/13 cells were incubated with low glucose [2.5 mM] or thapsigargin [Tg; 0.25 µM for 6 hrs]. Expression of CHOP was determined by Western blotting. Uniform protein loading was assessed by re-probing the same membrane with antiserum against β-actin. Quantification of CHOP expression was carried out by densitometry. The statistical significance of the differences in Mean ± SEM values between the control and the experimental groups was evaluated by student's t-test. Data in panel D represent mean ± SEM from three independent experiments and expressed as fold change. *$P < 0.05$ *vs.* 2.5 mM glucose. Panel E: INS-1 832/13 cells were treated with low glucose [2.5 mM] or thapsigargin [Tg; 0.25 µM] for 2, 4 and 6 hrs as indicated in

the figure. Lysate proteins were loaded onto a SDS-Polyacrylamide gel and electro-transferred onto a nitrocellulose membrane. The membrane was probed with antisera against cleaved caspase 3 and degraded lamin B. To ascertain uniform loading of proteins, the membrane was stripped and reprobed with antibody against β-actin. Quantification of caspase 3 activation and lamin B degradation were carried out by densitometry. Data in Panel E and F represent mean ± SEM from three independent experiments and expressed as fold change. *$P < 0.05$ *vs.* 2.5 mM glucose.

Caspase 3 activation and Lamin B degradation observed in *Akita* beta cells: an ER stress mouse model

The *Akita* mouse model has a spontaneous mutation in the *Ins2* gene, which causes misfolding of the translated insulin in the endoplasmic reticulum. Therefore, $Ins2^{Akita}$ mice serve as a suitable model for ER stress in the beta cell, seen in diabetic conditions. They develop insulin dependent diabetes, including hyperglycemia, hypoinsulinemia, polydipsia, and polyuria. We wanted to examine if the effects on caspase 3 and lamin B seen under glucotoxic conditions could be mimicked in cells obtained from *Akita* mice in order to decipher the role of ER stress in our model. Results acquired from a Western blot study, after running the lysates obtained from wild type [WT] and *Akita* cells, are depicted in **Figure 4-3.** We observed a higher level of active caspase 3 and degradation of lamin B in cells from the *Akita* mouse compared to the wild type.

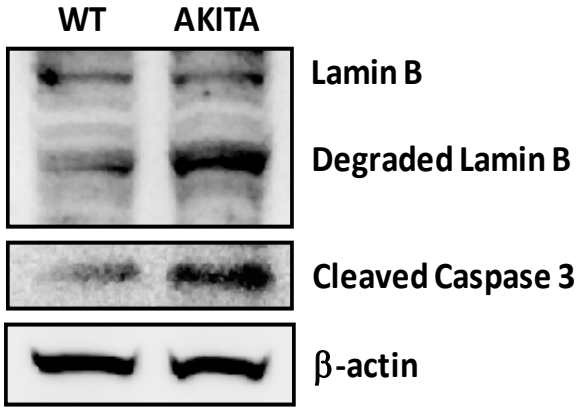


Figure 4-3: *Akita* mouse cells show noticeable caspase 3 activation and lamin B degradation. Lysate proteins from Akita and WT cells were resolved by gel electrophoresis, and then electro-transferred onto a nitrocellulose membrane. The abundance of cleaved caspase 3 and lamin B were determined by Western blotting. Equal protein loading was ensured by reprobing the membrane with antibody against β-actin.

4-Phenylbutyric acid [PBA], a known inhibitor of ER-stress, markedly attenuates glucose-induced CHOP expression, caspase 3 activation and lamin B degradation

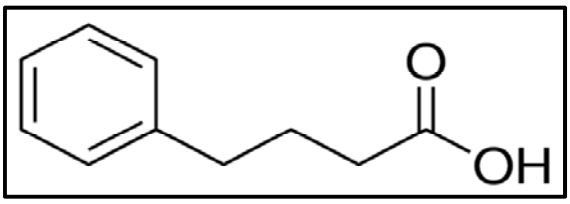


Figure 4-4: Chemical Structure of PBA

As a logical extension to the above studies, we attempted to establish a causal role for ER-stress in high glucose-induced caspase 3 activation and lamin B degradation. To accomplish this, we used PBA **[Figure 4-4],** a known inhibitor of ER-stress, on glucose-induced caspase 3 activation and lamin B degradation. We also quantified CHOP expression in these cells to affirm that PBA inhibits glucose-induced ER-stress under current experimental

conditions. Data depicted in **Figure 4-5** demonstrate a significant increase in CHOP expression in glucose-treated cells. We also observed complete inhibition of glucose-induced CHOP expression by PBA [Figure 4-5, Panel A]. These data thus validate the use of PBA as an inhibitor of ER-stress in INS-1 832/13 cells. More importantly, we also noticed a complete inhibition of glucose-induced caspase 3 activation and lamin B degradation by PBA in these cells [Figure 4-5; Panel B]. Taken together, these data provide the first evidence that glucotoxicity promotes caspase 3 activation and lamin B degradation in an ER-stress sensitive fashion.

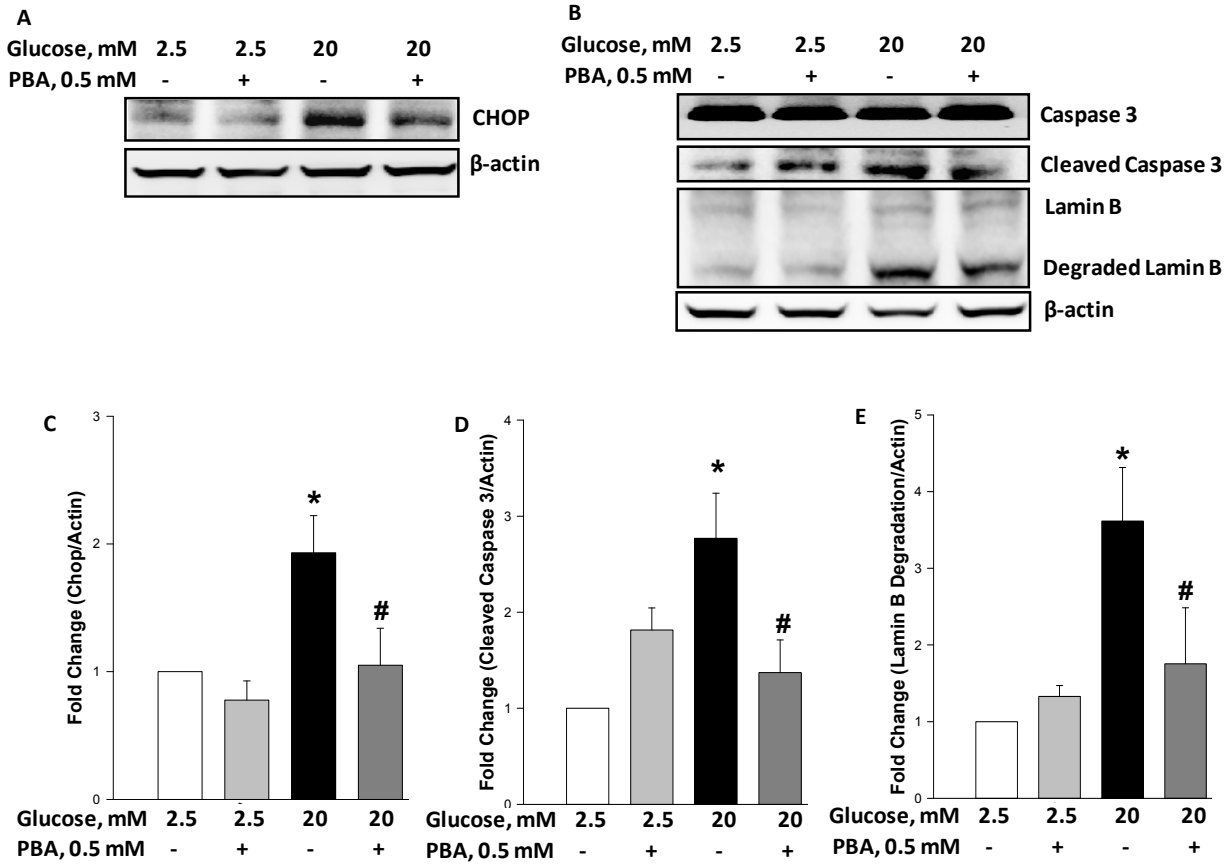


Figure 4-5: PBA, a known inhibitor of ER-stress, markedly attenuates glucose-induced CHOP expression, caspase 3 activation and lamin B degradation in INS-1 832/13 cells. Cells were

incubated in the presence of low or high [2.5 or 20 mM] glucose for 24 hrs in the presence and absence of PBA [0.5 mM]. Approximately 40 µg protein lysates were loaded onto SDS-Polyacrylamide gels and electro-transferred onto nitrocellulose membranes. The membranes were probed with antibodies against CHOP [Panel A], active caspase 3 and degraded lamin B [Panel B]. Uniform protein loading was assessed by reprobing the membranes antibody against β-actin. Intensity of protein bands was quantified by densitometry [Panels C-E]. Data represent mean ± SEM from three independent experiments and expressed as fold change in CHOP expression, caspase 3 activation and lamin B degradation. *P < 0.05 *vs.* 2.5 mM glucose without inhibitor, #P < 0.05 *vs.* 20 mM glucose without inhibitor.

Nifedipine, a calcium channel blocker, inhibits glucose-induced caspase 3 activation and lamin B degradation in INS-1 832/13 cells

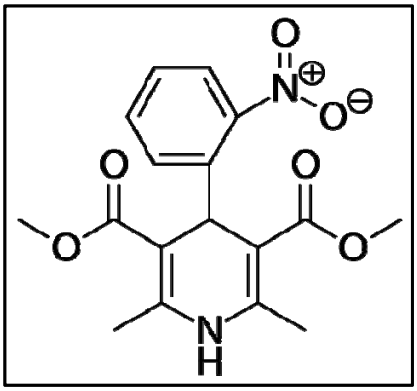


Figure 4-6: Chemical Structure of Nifedipine

We recently reported that calcium overload may represent one of the signaling mechanisms involved in caspase-3 activation under conditions of cellular apoptosis in isolated beta cells. For example, using etoposide, a known inducer of loss in metabolic *via*bility in beta cells, we reported a marked increase in caspase 3 activity in INS-1 832/13 cells and normal rat islets. More importantly, we have been able to prevent etoposide-induced metabolic dysfunction in these cells by nifedipine **[Figure 4-6],** a known blocker of L-type calcium channel activation and calcium entry **[93].** Furthermore, recent findings from the laboratory of Wang and associates **[94]** have also demonstrated significant protective effects of nifedipine against high glucose-induced ER stress and apoptosis. Therefore, as a logical extension to findings that we

reported above, we undertook a study to see if glucose-induced caspase 3 activation and lamin B degradation are prevented by nifedipine. Data presented in **Figure 4-7**, Panel A indicate a modest, but insignificant [1.2-fold] increase in caspase 3 activation in INS-1 832/13 cells incubated with nifedipine alone under basal conditions. No significant effects of nifedipine were demonstrable on lamin B degradation under these conditions. As shown above, high glucose-treatment markedly enhanced caspase 3 activation [2.5- fold] and lamin B degradation [1.9-fold; Figure 4-7, Panels B and C]. Interestingly, co-provision of nifedipine with glucose markedly reduced high glucose-induced effects on caspase 3 activation [2.5-fold *vs.* 1.2- fold in the absence and presence of nifedipine, respectively]. In a manner akin to these findings, nifedipine also attenuated lamin B degradation induced by glucose [1.9-fold in the absence of nifedipine and 1.2-fold in its presence; Figure 4-7].

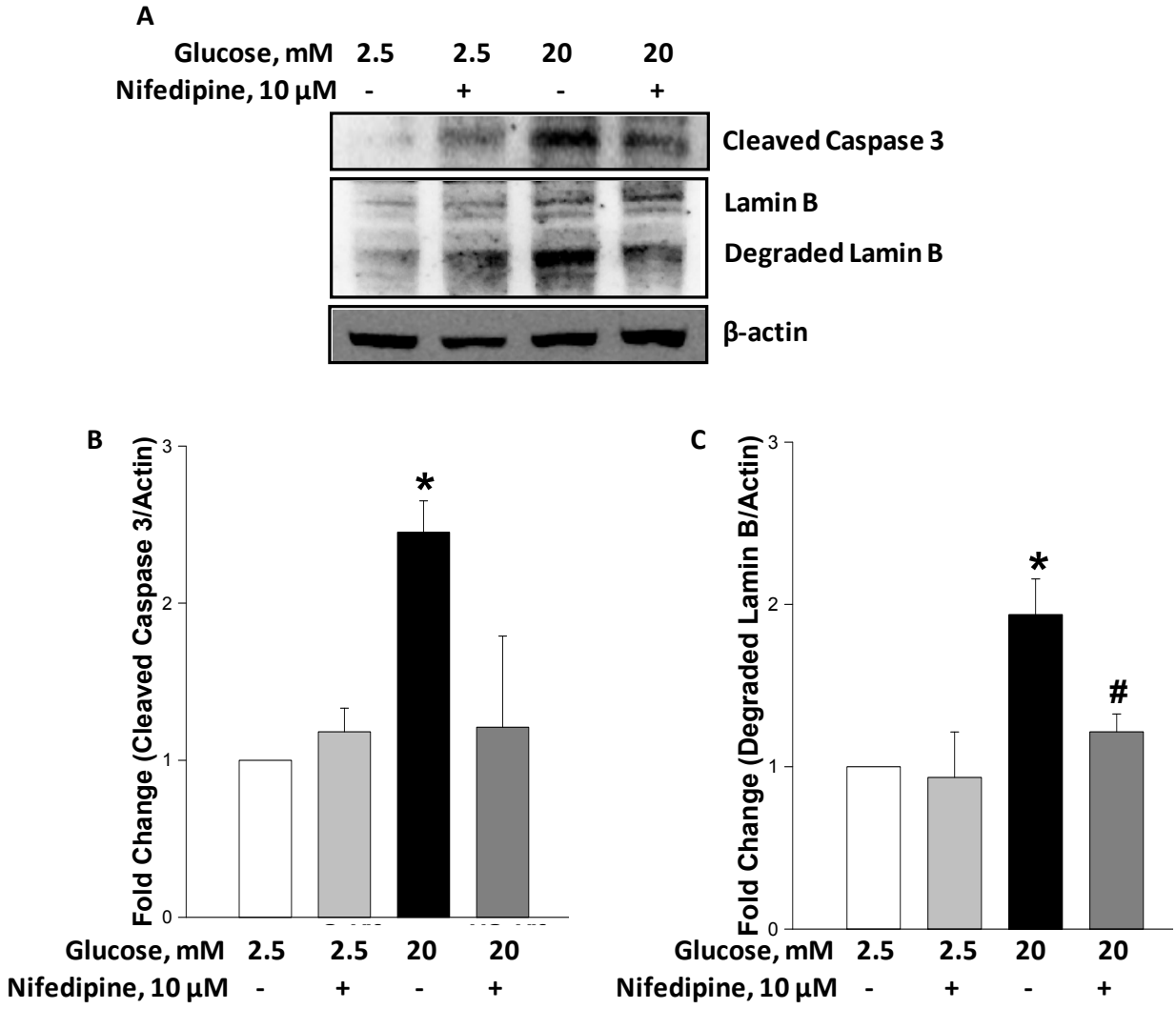


Figure 4-7: Nifedipine, a calcium channel blocker, inhibits glucose-induced caspase 3 activation and lamin B degradation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were incubated with low glucose or high glucose [2.5 or 20 mM] for 24 hrs in the absence [diluent] or presence of nifedipine [10 µM]. Proteins obtained after cell lysis were resolved by gel electrophoresis and electro-transferred onto a nitrocellulose membrane. The membrane was probed with antibodies against cleaved caspase 3 and lamin B. To ensure uniformity in protein loading, the same membranes were re-probed with β-actin antibody. Band densities of caspase 3 activation and lamin B degradation were measure. Data represent mean ± SEM from three independent experiments and expressed as fold change [Panel B and C]. **P* < 0.05 *vs.* 2.5 mM glucose without nifedipine and #*P* < 0.05 *vs.* 20 mM without nifedipine.

Z-DEVD-FMK, a known inhibitor of caspase 3, markedly attenuates high glucose-induced caspase 3 activation and lamin B degradation in INS-1 832/13 cells

In vitro studies on beta cell lines utilizing caspase inhibitors have demonstrated that the caspase 2 inhibitor is effective in protecting the HIT-T15 beta cell line against an experimental model of cell death **[95].** Furthermore, murine βTC-1 cell lines transfected with human Fas were shown to be protected against Fas-induced beta cell apoptosis by the caspase 3 inhibitor Z-Asp-Glu-Val-Asp-fluoromethyl ketone **[96]** and palmitate-induced beta cell apoptosis, could be prevented by executioner caspase 6 inhibitors **[97].** The preferential recognition of different caspases for specific amino acid sequences on the substrate proteins has 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 an attempt to investigate the dependency of lamin degradation on upstream caspase 3 activation, observed in glucotoxicity induced apoptotic signaling, we used a cell permeable peptide inhibitor, specific for caspase 3 [Z-DEVD-FMK]. We measured lamin B degradation and caspase 3 activation in cells treated with low and high glucose conditions in the absence and presence of Z-DEVD-FMK. Data in **Figure 4-8** Panel A show a marked inhibition of lamin B degradation and caspase 3 activation after treatment with the inhibitor. Panels B and C represent data collected from three to four experiments.

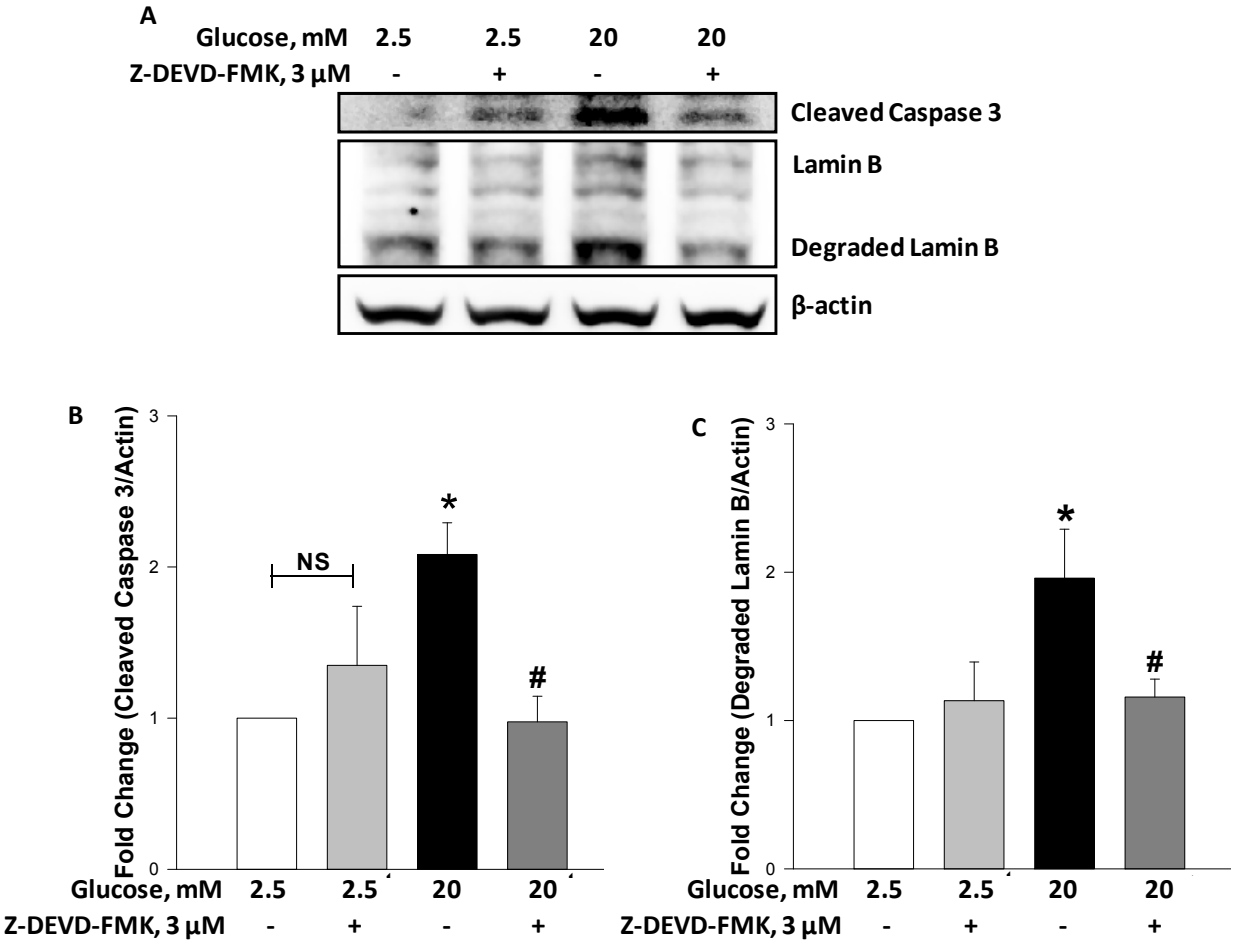
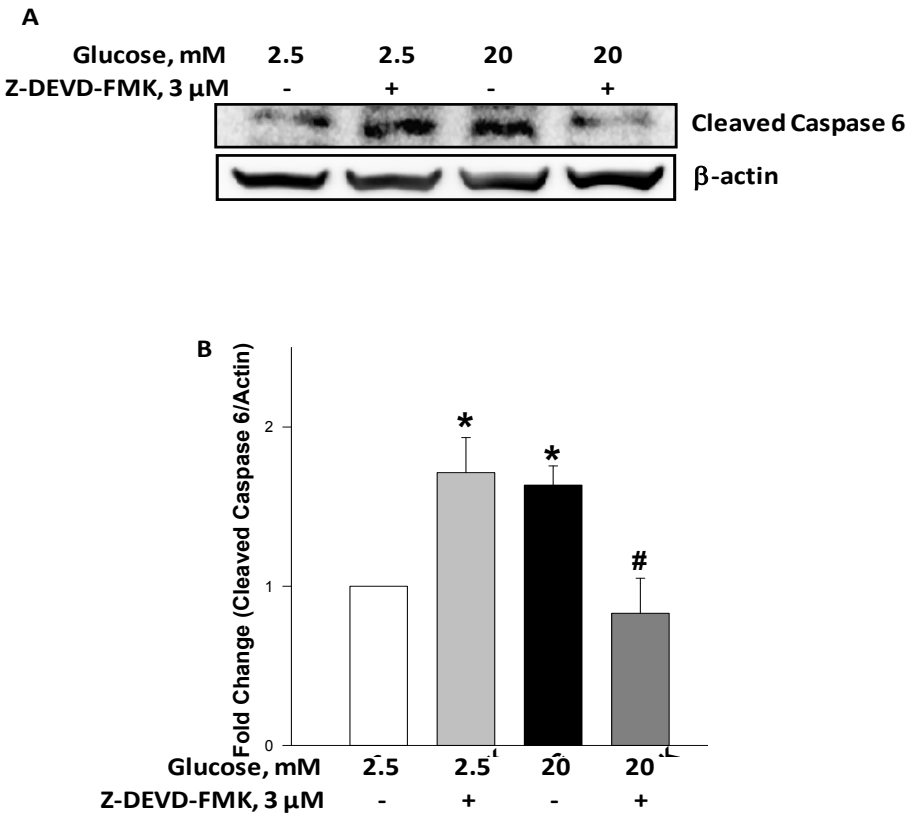


Figure 4-8: Z-DEVD-FMK, a known inhibitor of caspase 3, prevented high glucose induced caspase 3 activation and lamin B breakdown in INS-1 832/13 cells. INS-1 832/13 cells were preincubated with Z-DEVD-FMK [3µM] for 1 hr and further treated with low [2.5 mM; LG] or high [20 mM; HG] glucose in the presence and absence of Z-DEVD-FMK for 6 hrs. Caspase 3 activation and lamin B degradation [Panel A] were determined by Western blotting. To ensure equal protein loading, the membranes were probed with β-actin antiserum. Data represent mean ± SEM from three to four independent experiments and expressed as fold change in caspase 3 and lamin B degradation [Panel B and C]. *P < 0.05 *vs.* 2.5 mM glucose in the absence of the inhibitor; # P < 0.05 *vs.* 20 mM glucose without inhibitor. NS: Not significant *vs.* 2.5 mM glucose in the absence of the inhibitor.

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

Evidence in different cell types suggests that event of caspase 3 activation in the caspase cascade is upstream to activation of caspase 6 **[98],** and/or both these executioner caspases are cleaved and thereby activated under conditions induced by apoptotic stimuli **[99].** Therefore, in the next set of experiments, we examined if caspase 3 activation precedes caspase 6 activation in beta 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 4-9** [Panel A], demonstrate significant inhibition of glucose-induced caspase 6 activation following inhibition of caspase 3. Panel B includes data accumulated from multiple experiments. It should be noted that 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 4-9; 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 **[100]**, 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.


A
Glucose, mM 2.5 2.5 20 20
Z-DEVD-FMK, 3 µM - + - +
Cleaved Caspase 6
β-actin
B
Fold Change (Cleaved Caspase 6/Actin)
0
1
2
*
*
#
Glucose, mM 2.5 2.5 20 20
Z-DEVD-FMK, 3 µM - + - +

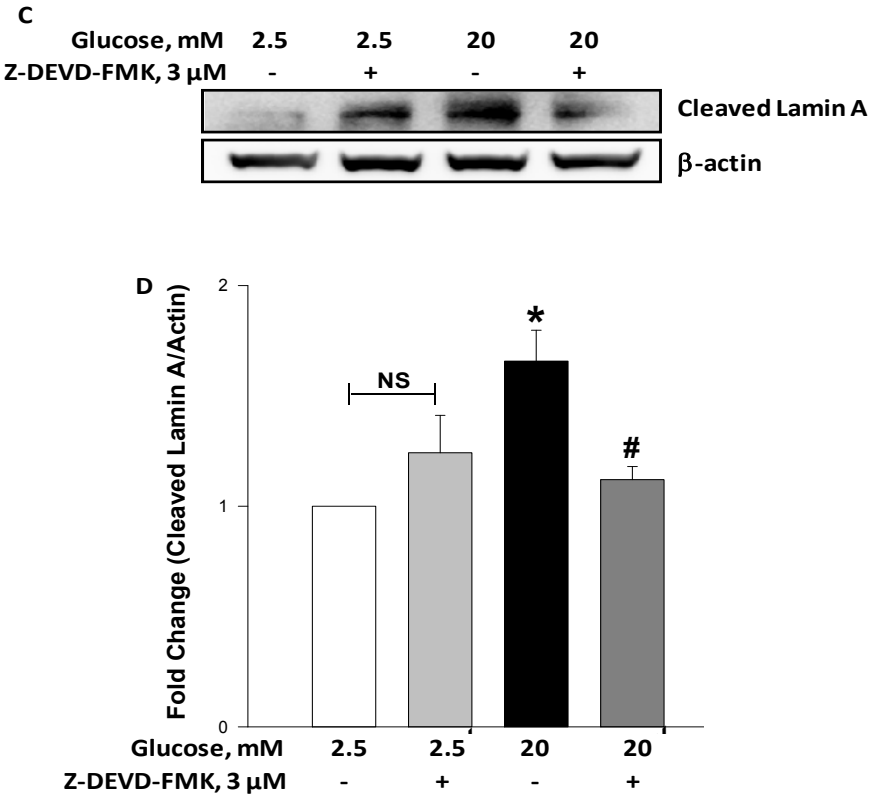
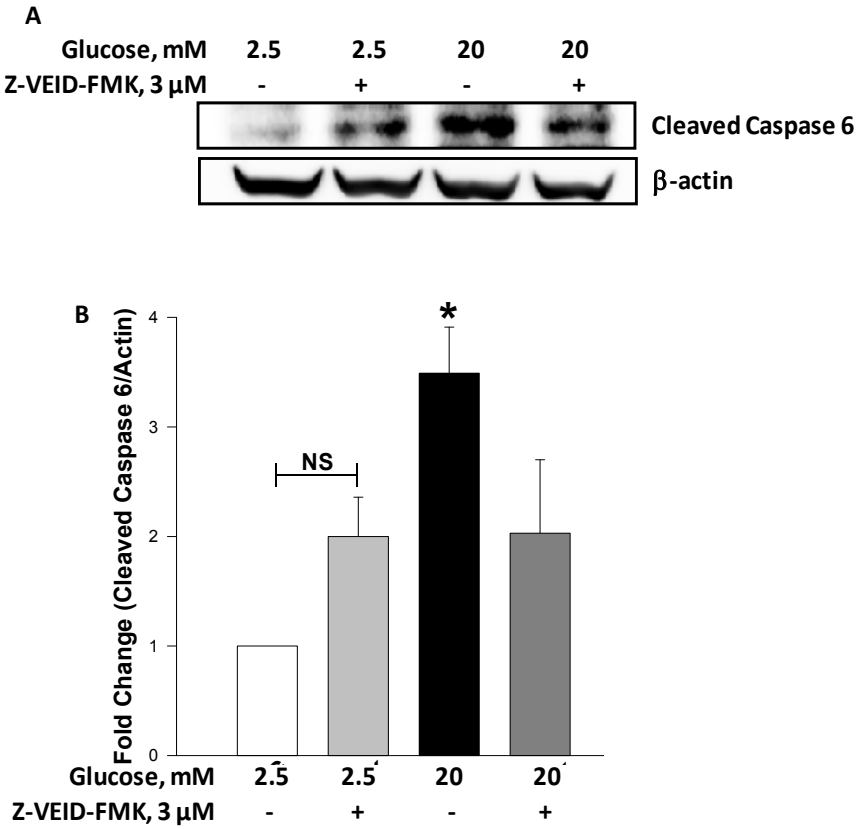
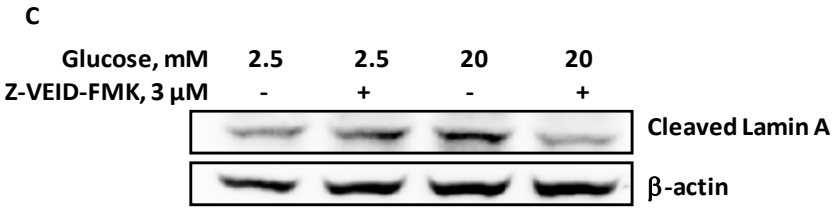

C
Glucose, mM 2.5 2.5 20 20
Z-DEVD-FMK, 3 µM - + - +
Cleaved Lamin A
β-actin
D
Fold Change (Cleaved Lamin A/Actin)
0
1
2
NS
*
#
Glucose, mM 2.5 2.5 20 20
Z-DEVD-FMK, 3 µM - + - +

Figure 4-9: 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 [3μM] for 1 hr and further treated with low [2.5 mM] or high [20 mM] glucose in the presence and absence of Z-DEVD-FMK for 6 hrs. 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. Data represent mean ± 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.* 2.5 mM glucose in absence of inhibitor; # P < 0.05 *vs.* 20 mM glucose without inhibitor. NS: Not significant *vs.* 2.5 mM glucose in absence of inhibitor.

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

We further 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 4-10** [Panel A] represents a Western blot demonstrating directional 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 4-10; Panel A]. However, data from multiple experiments indicated that this increase was not significant [Figure 4-10; Panel B]. Furthermore, Z-VEID-FMK inhibited high glucose-induced degradation of lamin A under the conditions it inhibited caspase 6 [Figure 4-10; Panel C]; these findings provide support to the notion that caspase 6 does play a contributory role, although not entirely, in the cleavage of lamin A in pancreatic beta cells under glucotoxic and diabetic conditions. Figure 4-10; Panel D displays data combined from multiple experiments. 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 a contributory role of caspase 6 in lamin A degradation under glucotoxic conditions.


A
Glucose, mM 2.5 2.5 20 20
Z-VEID-FMK, 3 µM - + - +
Cleaved Caspase 6
β-actin
B
Fold Change (Cleaved Caspase 6/Actin)
0
1
2
3
4
NS
*
Glucose, mM 2.5 2.5 20 20
Z-VEID-FMK, 3 µM - + - +


C
Glucose, mM 2.5 2.5 20 20
Z-VEID-FMK, 3 µM - + - +
Cleaved Lamin A
β-actin

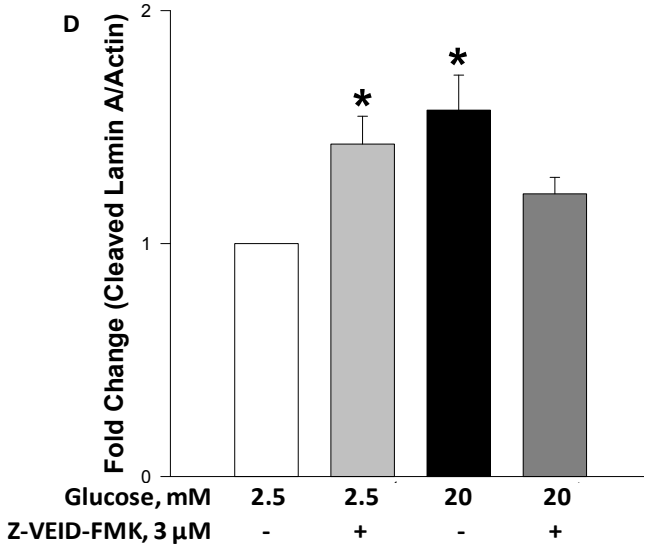

D
Fold Change (Cleaved Lamin A/Actin)
0
1
2
*
*
Glucose, mM 2.5 2.5 20 20
Z-VEID-FMK, 3 µM - + - +

Figure 4-10: 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 [3μM] for 1 hr and further treated with low [2.5 mM] or high [20 mM] glucose in the presence and absence of Z-VEID-FMK for 6 hrs. Caspase 6 activation and lamin A cleavage were determined by Western blotting. 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. Data represent mean ± 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.* 2.5 mM glucose without inhibitor. NS: Not significant *vs.* 2.5 mM glucose without inhibitor.

Studies using rottlerin, an inhibitor of PKC-δ, on the activation of caspase 3 and degradation of lamin B

Several studies have shown that in order for caspases to target the nuclear lamins for proteolytic degradation, phosphorylation of the lamin filaments by PKC-δ is required. In an attempt to gain deeper insight into this mechanism we tested the effects of rottlerin [5 and 10 μM], an inhibitor of PKC-δ on lamin B under the duress of glucotoxicity. However we could not see an inhibitory effect of this compound on the degradation of lamin B in our experiment in INS-1 832/13 cells [Figure 4-11]. Further we also tested siRNA against PKC-δ but could not attain sufficiently high transfection efficiency even at 200 nm concentration of the siRNA [data not shown]. Since PKC-δ is one of 11 isoforms of the PKC family and has a number of splice variants [PKC-δI, PKC-δII] which could be pro-apoptotic or anti-apoptotic **[101],** it would be interesting to study each splice variant individually and identify the role of each distinct splice variant in the processing of lamins.

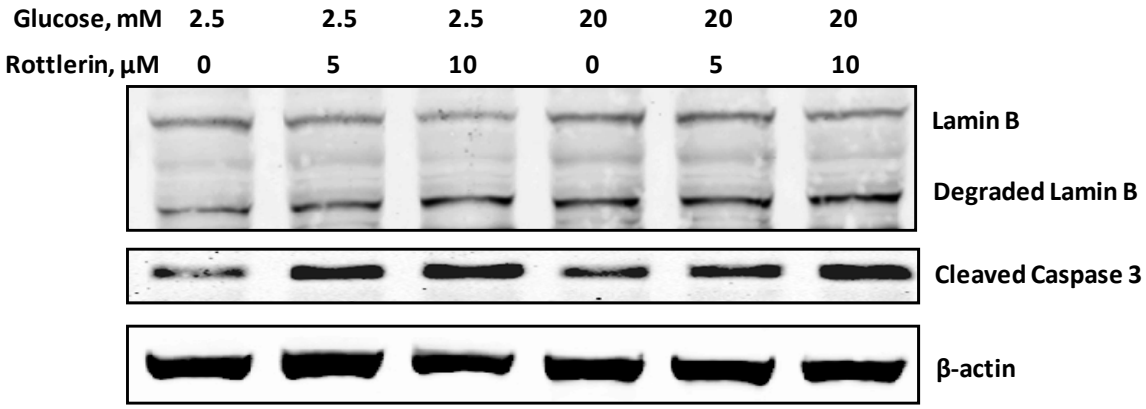


Figure 4-11: Rottlerin had no effect on high glucose induced activation of caspase 3 and lamin B degradation. INS-1 832/13 cells were preincubated with rottlerin [5 and 10 µM] for 1 hr and further treated with low [2.5 mM] or high [20 mM] glucose in the presence and absence of rottlerin for 6 hrs. Activation of executioner caspase 3 and lamin B degradation were examined by Western blotting. To ensure equal protein loading, the membranes were probed for β-actin. Representative blots from 3 individual experiments are shown in the figure above.

Results obtained from these studies can be summarized as follows:

- Effects of glucotoxicity on CHOP expression are comparable to those of thapsigargin, a potent inducer of ER stress in many cell types. Thapsigargin treatment also induced caspase 3 activation and lamin B degradation, as seen under high glucose treatment, thereby implicating an ER stress mediated pathway.
- PBA, an inhibitor of ER stress, markedly reduced glucose-induced CHOP expression, caspase 3 activation and lamin B degradation.
- Blocking of membrane associated L-type calcium ion channels, with nifedipine, markedly attenuated high glucose-induced effects on the beta cell.
- Z-DEVD-FMK, a specific inhibitor of caspase 3, inhibited high glucose-induced caspase 3, 6 activation and proteolysis of lamin A, B and Z-VEID-FMK, which specifically inhibits caspase 6 activation blocked high glucose-induced caspase 6 activation and lamin A degradation.

CHAPTER 5

CONSEQUENCES OF INHIBITION OF REQUISITE POST TRANSLATIONAL PRENYLATION OF LAMINS ON THE PANCREATIC BETA CELL

The nuclear lamins, in their original post transcription stage, are translated as native pre-lamins, which further need to undergo a series of PTMs and are then processed into their mature form for localization into the nuclear membrane, protein interactions and performing their functions within the nucleus. Apart from other modifications [Figure 1-5], lamins undergo farnesylation at the cysteine residue of the C-terminal CAAX motif **[21].** How then, does inhibition of prenylation of lamins affect nuclear envelope integrity? What would be the overall effects of inhibition of lamin farnesylation on the pancreatic beta cell? To address these questions, we made use of pharmacological inhibitors of protein prenylation. Firstly, we used simvastatin, which blocks the biosynthesis of mevalonic acid [MVA] and other key intermediates in the cholesterol biosynthesis pathway, namely: farnesyl pyrophosphate [FPP] and geranylgeranyl pyrophosphate [GGPP], that are involved in post translational prenylation of several proteins [Rho, Rab, Ras] **[Figure 5-1].** Secondly, we used a farnesyl transferase inhibitor, FTI-277, which inhibits the catalytic addition of 15 carbon farnesyl moiety to target proteins [e.g., Ras, nuclear lamins]. In addition, we also tested the effects of GGTI-2147, which inhibits the enzyme geranylgeranyl transferase [GGTase I]. We examined the effects of these inhibitors on the activation of caspase 3, lamin B degradation and overall cell morphology and viability in INS-1 832/13 cells and rat islets.

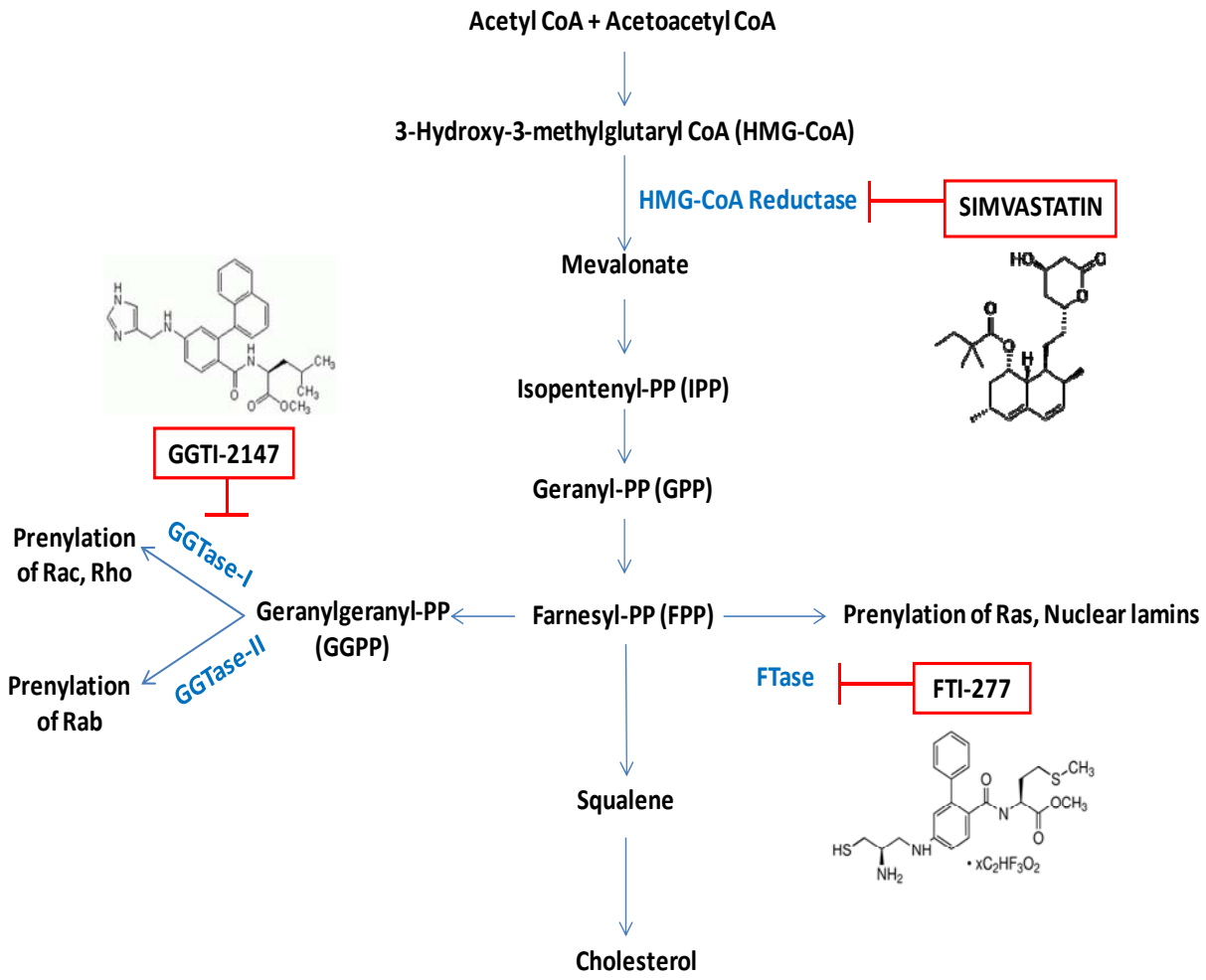


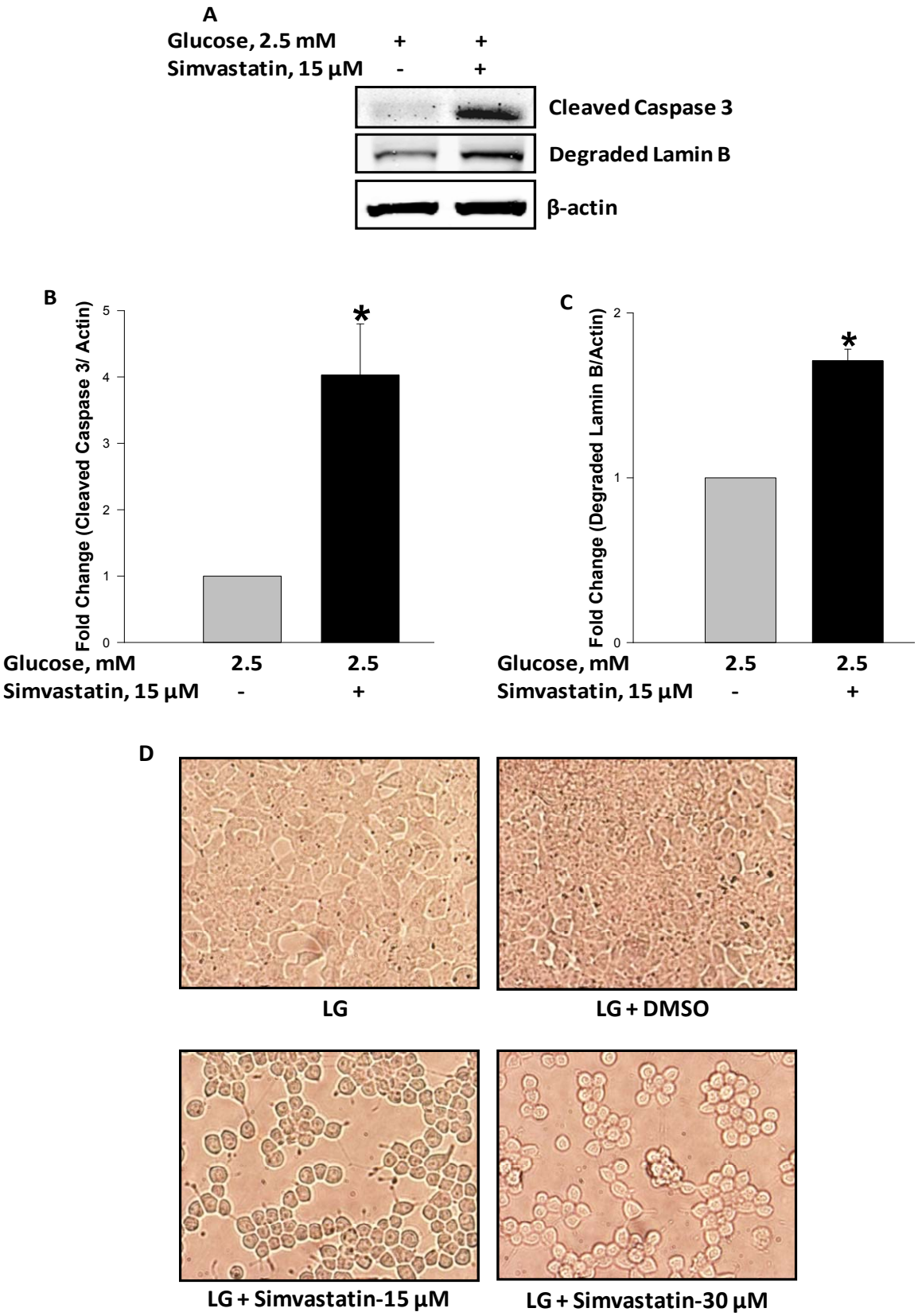
Figure 5-1: The cholesterol biosynthesis pathway. The formation of cholesterol begins with acetyl CoA and acetoacetyl CoA generating 3-hydroxy 3 methylglutaryl CoA [HMG-CoA], followed by a series of steps catalyzed by several enzymes along with generation of a number of intermediate metabolites. HMG-CoA reductase, the rate limiting enzyme of this pathway, catalyses the synthesis of mevalonate. Simvastatin inhibits the enzyme HMG-CoA reductase, thus blocking the subsequent steps which lead to cholesterol biosynthesis. It also prevents the formation of metabolites of this pathway, namely: FPP and GGPP that are involved in post translational prenylation of several proteins [Rho, Rab, Ras etc.]. FTI-277, inhibits the farnesyl transferase enzyme which catalyses the addition of 15 carbon farnesyl moiety to target proteins [eg: Ras, nuclear lamins] while GGTI-2147 inhibits GGTase I, therefore blocking geranylgeranylation of Rac and Rho.

A] Studies on effects of Statins:

INS-1 832/13 cells and rat islets treated with Simvastatin exhibit increased activation of caspase 3 and subsequent degradation of lamin B along with changes in cell morphology.

At the outset, we treated INS-1 832/13 cells, cultured in RPMI medium, with low glucose [2.5 mM] as control and low glucose with Simvastatin [15 μM] for 24 hrs and examined the effects of the treatment on caspase 3 and its downstream target lamin B. As shown in **Figure 5-2**, Panel A, we observed an increase in cleaved caspase 3, which is an indicator of caspase 3 activation, and a corresponding increase in the degraded product of lamin B. Pooled data from 3 independent Western blotting studies, shown in Panels B and C, demonstrate a significant increase in band densities of cleaved caspase 3 and degraded lamin B. Along with these observations, we also noticed changes in morphology in cells incubated for 24 hrs with Simvastatin. Microscopic images in Panel C demonstrate the rounding up of cells treated with 15 and 30 μM Simvastatin and no visible changes in morphology of cells incubated in media containing low glucose alone or with diluent [DMSO].

As a logical extension of these experiments we performed the above studies in primary cells from rat pancreatic islets. A representative blot from 2 such independent studies is depicted in Panel E, wherein islets obtained from normal Sprague Dawley, rats were exposed to low glucose in the absence and presence of Simvastatin [15 μM and 30 μM] for 24 hrs. We observed a similar increase in caspase 3 activation and lamin B degradation which support our observations in INS-1 832/13 cells.


A
Glucose, 2.5 mM + +
Simvastatin, 15 µM - +
Cleaved Caspase 3
Degraded Lamin B
β-actin
B
Fold Change (Cleaved Caspase 3/ Actin)
0
1
2
3
4
5
*
Glucose, mM 2.5 2.5
Simvastatin, 15 µM - +
C
Fold Change (Degraded Lamin B/Actin)
0
1
2
*
Glucose, mM 2.5 2.5
Simvastatin, 15 µM - +
D
LG
LG + DMSO
LG + Simvastatin-15 µM
LG + Simvastatin-30 µM

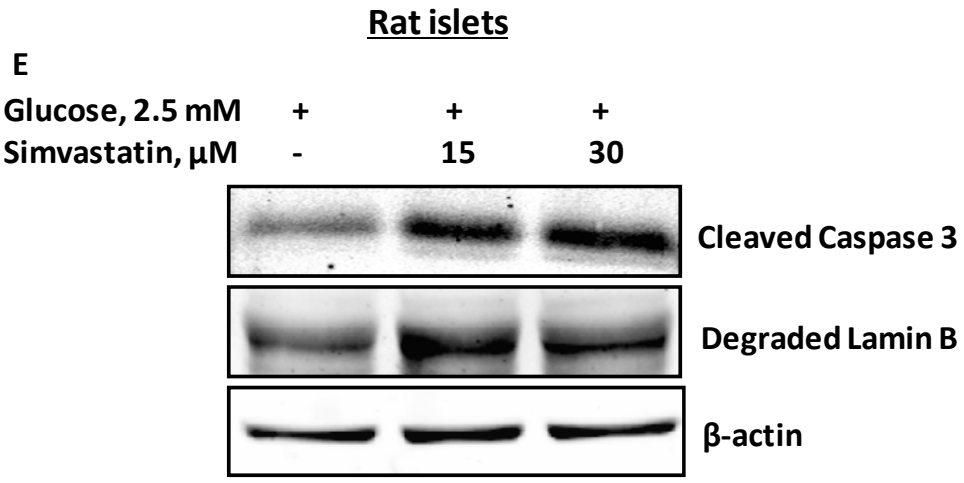


Figure 5-2: Exposure of INS-1 832/13 cells, rat pancreatic islets to simvastatin results in caspase 3 activation and degradation of lamin B along with changes in cell morphology.
(A) INS-1 832/13 cells were treated with RPMI media containing 2.5 mM glucose alone and with 15 µM Simvastatin for 24 hrs. Proteins obtained after cell lysis were resolved by SDS-PAGE and electro-transferred onto a nitrocellulose membrane. Antibodies against cleaved caspase 3 and lamin B were used to probe the membrane. Uniform protein loading was ensured by re-probing the blots with antibody against β-actin. Student's t-test was utilized to measure the statistical significance of the differences in values between the control and experimental conditions. Panel B and C represent mean ± SEM values and are expressed as fold change in caspase 3 activation **[Panel B]** and lamin B degradation **[Panel C]**. **P* < 0.05 *vs.* 2.5 mM glucose. **[D]** INS-1 832/13 cells, incubated overnight in RPMI medium containing 2.5 mM glucose and 2.5% FBS, were treated the following day with media containing low glucose [2.5 mM] alone and in the presence of diluent [DMSO] or simvastatin [15 µM and 30 µM] for 24 hrs. Changes in cell morphology were visualized by light microscopy. **[E]** Rat islets isolated by collagenase digestion method were incubated in media containing low glucose [2.5 mM] in the absence and presence of simvastatin [15 µM and 30 µM] for 24 hrs. Activation of executioner caspase 3 and lamin B degradation were evaluated by Western blotting. The membrane was re-blotted with anti β-actin to ensure equal protein loading.

Simvastatin promotes phosphorylation of p38 MAPK and reduces ERK1/2 phosphorylation in INS-1 832/13 cells

In the next set of experiments we examined the effects of Simvastatin treatment on two mitogen activated protein kinases [MAPKs] namely, p38 and ERK1/2, both of which are regulated by various extracellular stress stimuli and play a critical role in the cell stress signaling/survival pathways. INS-1 832/13 cells were exposed to low glucose [2.5 mM] in the

absence and presence of 15 μM simvastatin as described in the previous experiments. Under these conditions, we looked for the phosphorylation status of p38 using an antibody specific for phospho-p38 and observed a significant increase in the phosphorylated, active form of p38 MAPK in comparison to the total protein [**Figure 5-3** Panel A]. Besides, we also noted a marked decrease in ERK1/2 phosphorylation/activation using a monoclonal antibody, which recognizes the dual phosphorylation at Thr202 and Tyr204 of ERK1 and Thr185 and Tyr187 of ERK2, as shown in Figure 5-3 Panel C. Bar graphs in Panels B and D represents data pooled from 3 independent experiments and represent fold change in the intensities of the phosphorylated protein bands *vs.* the intensity of total protein levels. Under the same treatment conditions we assessed the metabolic cell viability and found a decrease [~12.4%] in viability with Simvastatin treatment [Panel E].

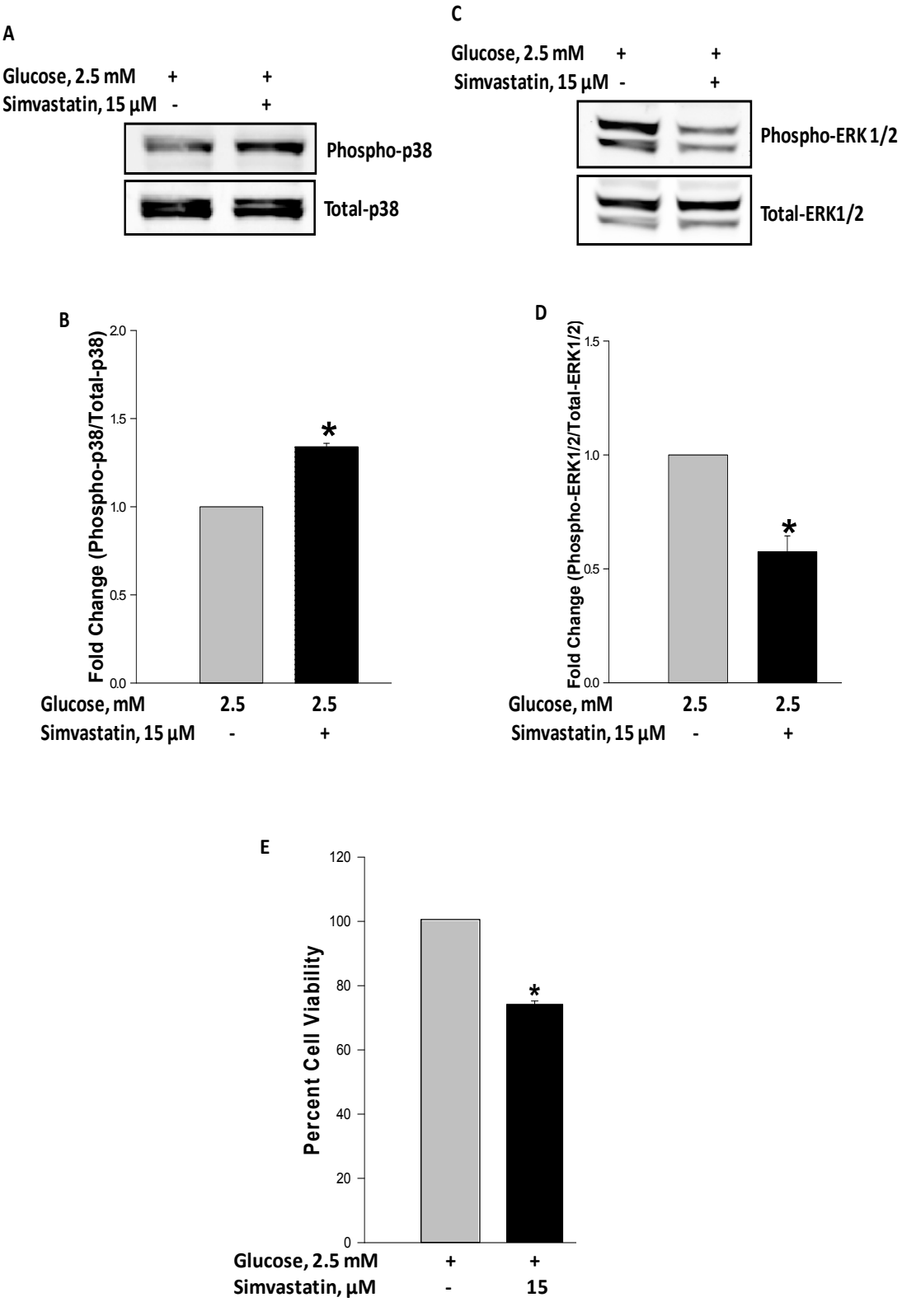

A
Glucose, 2.5 mM + +
Simvastatin, 15 µM - +
Phospho-p38
Total-p38
C
Glucose, 2.5 mM + +
Simvastatin, 15 µM - +
Phospho-ERK 1/2
Total-ERK1/2
B
Fold Change (Phospho-p38/Total-p38)
2.0
1.5
1.0
0.5
0.0
*
Glucose, mM 2.5 2.5
Simvastatin, 15 µM - +
D
Fold Change (Phospho-ERK1/2/Total-ERK1/2)
1.5
1.0
0.5
0.0
*
Glucose, mM 2.5 2.5
Simvastatin, 15 µM - +
E
Percent Cell Viability
120
100
80
60
40
20
0
*
Glucose, 2.5 mM + +
Simvastatin, µM - 15

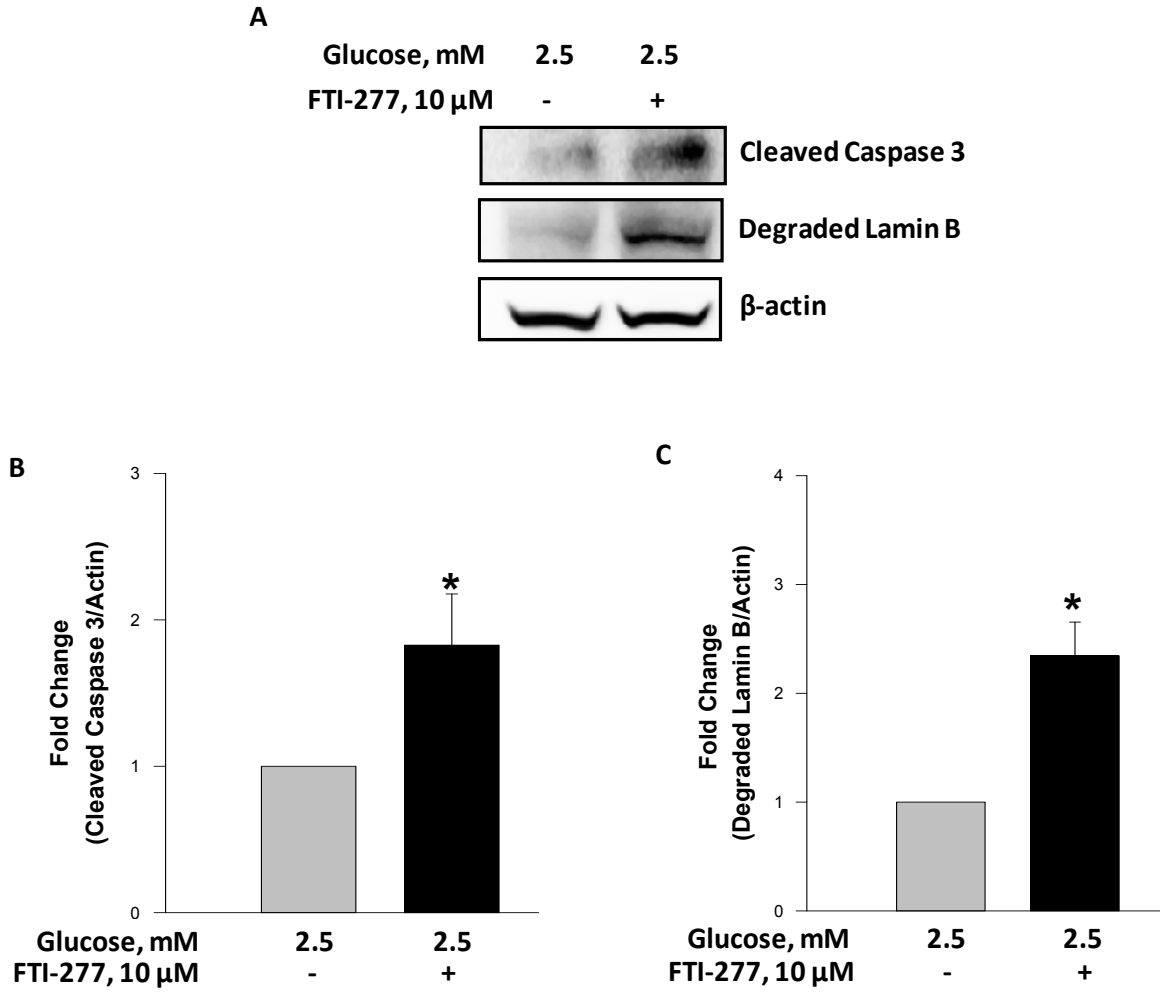
Figure 5-3: Simvastatin treatment causes a marked increase in phosphorylation of p38 MAP Kinase and significantly decreases phosphorylation of phospho-p44/42 ERK1/2 and a reduction in cell viability. Lysates from INS-1 832/13 cells incubated in RPMI media containing 2.5 mM glucose alone and with 15 μM Simvastatin for 24 hrs were resolved by SDS-PAGE and transferred to nitrocellulose membranes by wet transfer. The blots were probed for phosphorylated p38MAPK and total p38MAPK, phospho-p44/42 ERK1/2 and total p44/42 ERK1/2. Band densities were calculated by densitometry. Data provided in Panel B and D represent mean ± SEM and are expressed as fold change in p38 MAPK phosphorylation [Panel B] and p44/42 ERK1/2 phosphorylation [Panel D]. *$P < 0.05$ *vs.* 2.5 mM glucose. Panel E; INS-1 832/13 cells were incubated in low glucose [2.5 mM] in the absence and presence of Simvastatin [15 μM]. After 24 hrs cell viability was assessed by the MTT assay. Data are represented as mean ± SEM values and are expressed as percent cell viability. *$P < 0.05$ *vs.* 2.5 mM glucose.

B] Studies on effects of FTIs:

Inhibition of farnesylation with FTI-277 treatment of INS-1 832/13 cells has similar effects on lamins as Simvastatin

The above effects of simvastatin on lamins and caspases [Figure 5-2], could be due to the depletion of FPP and/or GGPP, thereby inhibiting requisite prenylation of key proteins. In order to identify the distinct role of geranylgeranylation and farnesylation, we conducted a few experiments using inhibitors of each post translational modification. FTI-277 is a specific inhibitor of the enzyme FTase, which catalyses the addition of the 15 carbon farnesyl moiety onto target proteins e.g., Ras, nuclear Lamins. Herein, we incubated INS-1 832/13 with low glucose [2.5 mM] alone and with FTI-277 [10μM] for 24 hrs. Results obtained from these studies demonstrated that reduction in the requisite farnesylation of lamin B by exposing cells to FTI-277 promotes caspase 3 activation and degradation of lamin B [**Fig 5-4** Panel A]. Panels B and C represent data obtained from three Western blotting experiments. Under the same treatment conditions with low glucose in the absence and presence of FTI-277, we performed phase partitioning assay using Triton X-114. Here we observed the appearance of the degraded fragment of lamin B in the hydrophobic fraction of the membrane component as well as in the cytosolic fraction [Panel D]. In addition we assessed the metabolic cell viability by measuring,

colorimetrically, the reduction of tetrazolium salt to formazan crystals and found a decrease [~12.4%] in viability under these treatment conditions [Panel F]. However these effects were not accompanied by any significant changes in cell morphology [Panel E] after 24 hrs of FTI-277 treatment.



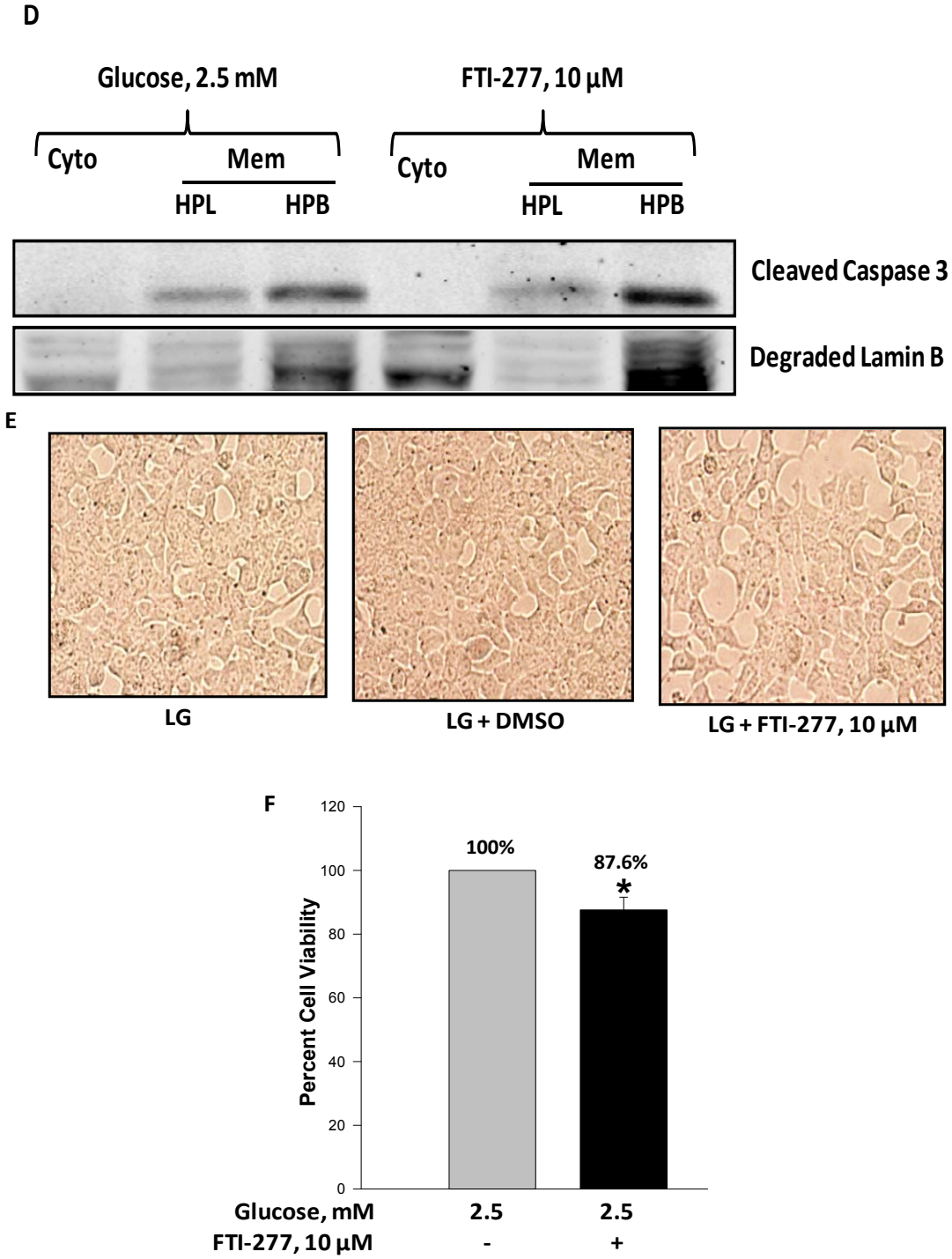


Figure 5-4: FTI-277 treatment results in caspase 3 mediated degradation of lamin B along with alterations in its subcellular distribution and reduction in cell viability. **[A]** INS-1 832/13 cells were incubated in media containing low glucose [2.5 mM] in the absence and presence of FTI-277 [10 µM] for 24 hrs. Caspase 3 activation and lamin B degradation for each condition was determined by Western blotting. Equal protein loading was ensured by re-probing the membrane with anti β-actin. Data represent mean ± SEM from three independent experiments and are expressed as fold change in caspase 3 activation **[Panel B]** and lamin B degradation

[Panel C]. *P < 0.05 *vs.* 2.5 mM glucose. **[D]** Lysates from INS-1 832/13 cells treated with low glucose [2.5 mM] in the absence and presence of FTI-277 [10 μM] were subjected to a single-step centrifugation to separate the supernatant cytosolic [Cyto] and the membrane-pellet [Mem] fractions followed by hydrophobic [HPB] and hydrophilic [HPL] phase partitioning with Triton X-114 detergent. Proteins obtained after cell lysis were resolved by SDS-PAGE and electro-transferred onto a nitrocellulose membrane. The membrane was probed for cleaved caspase 3 and lamin B. **[E]** INS-1 832/13 cells were treated with media containing low glucose [2.5 mM; LG] alone and in the presence of diluent [LG+DMSO], FTI-277 [10 μM] for 24 hrs. Changes in cell morphology were visualized by light microscopy. **[F]** INS-1 832/13 cells were incubated in low glucose [2.5 mM] in the absence and presence of FTI-277 [10 μM]. After 24, hrs cell viability was assessed by the MTT assay. Data are represented as mean ± SEM values expressed as percent cell viability. *P < 0.05 *vs.* 2.5 mM glucose.

C] Studies on effects of GGTIs:

GGTI-2147, a geranylgeranyl transferase inhibitor does not exhibit an increase in the activation of caspases and degradation of lamins

As an extension of the above studies we wanted to study the effect of blocking geranylgeranylation on the integrity of the nuclear lamins. To this end, we exposed INS-1 832/13 cells and primary rat pancreatic islets to GGTI-2147, a specific inhibitor of the enzyme GGTase I. A representative blot from 2 such independent studies is depicted in **Figure 5-5**, wherein islets obtained from normal Sprague Dawley rats were exposed to low glucose in the absence and presence of GGTI-2147 [10μM] for 24 hrs. Interestingly, blocking of geranylgeranylation had minimal effect on the integrity of lamin B and only a slight increase in the activation of caspase 3 which point out the possibility that farnesylation of lamins is essential for the stability and functioning of these nuclear proteins.

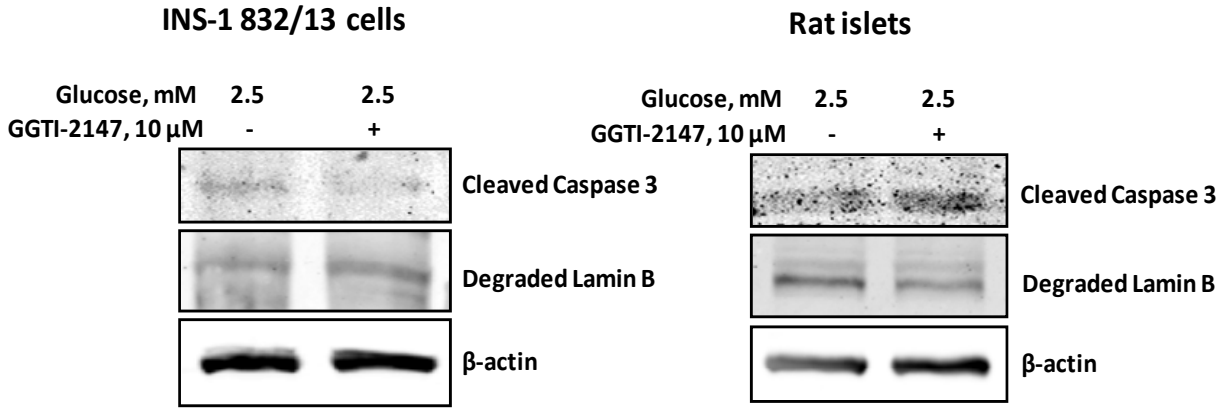


Figure 5-5: GGTI-2147 has minimal effects on the integrity of nuclear lamin B and caspase 3. INS-1 832/13 cells and rat islets [isolated by collagenase digestion method] were incubated in media containing low glucose [2.5 mM] in the absence and presence of GGTI-2147 [10 µM] for 24 hrs. Western blotting was used to evaluate caspase 3 activation and lamin B degradation. The membranes were re-blotted with anti β-actin to ensure equal protein loading.

Data obtained from the above studies can be summarized as follows:

- Inhibition of protein prenylation [farnesylation and geranylgeranylation] with simvastatin, induced significant alterations in mitochondrial and nuclear stability in pancreatic beta-cells, as evidenced by activation of executioner caspase-3 and degradation of nuclear lamin B, which were also seen in primary rodent islets.
- Simvastatin mediated blocking of prenylation also resulted in the activation of p38 MAPK, a stress kinase, which we have recently implicated in islet dysfunction under conditions of glucotoxicity and diabetes **[102].** In addition, we noticed a marked reduction in the phosphorylation of ERK1/2 in simvastatin treated INS-1 832/13 cells, an event necessary for G-protein activation, cytoskeletal organization and insulin secretion along with loss in cell viability.
- Results obtained after testing specific inhibitors of FTase [FTI-277] and GGTase-1 [GGTI-2147] suggest that simvastatin-mediated effects on degradation of lamin B are

more likely due to inhibition of farnesylation of lamins [a key step in the processing and intracellular localization of lamins] rather than blocking of geranylgeranylation.

CHAPTER 6

DISCUSSION

Hyperglycemia in T2DM is a consequence of progressive increase in insulin resistance in the peripheral tissues [adipose, muscle] along with decreasing pancreatic beta cell function. Several studies indicate that pancreatic islet beta cells, when subjected to glucotoxic and lipotoxic environment or when exposed to proinflammatory cytokines [e.g., IL-1β, TNFα and IFNγ] exhibit metabolic dysfunction as a result of oxidative and endoplasmic reticulum stress **[84, 103-105]**. A number of potential mechanisms underlying chronic hyperglycemia-induced metabolic dysfunction have been put forward, among which oxidative stress as well as endoplasmic reticulum stress induced mitochondrial defects and subsequent downstream effects are most common **[84, 106, 107]**. Despite this existing evidence, the precise effects that the elevated glucose levels have on the nuclear component of the cell are relatively lesser known. Therefore, one of the primary objectives of my doctoral work was to gain mechanistic insights into the role of ER-mitochondria axis in the activation of executioner caspases and their downstream targets; the nuclear lamins [lamin A and B], in insulin-secreting cells under the duress of glucotoxic conditions and diabetes.

The nuclear lamins are type V intermediate filament proteins, which assemble in a mesh-like pattern, forming the lamina layer within the inner-nuclear membrane. Studies have shown that the ER stress-induced effects may have an impact on the integrity of other subcellular organelles, such as the mitochondria, resulting in loss of mitochondrial membrane permeability transition [MPT] and leakage of cytochrome C into the cytosol **[108-111].** Under these conditions, executioner caspases [caspase 3 and 6] are activated which then cause the hydrolysis of proteins such as PARP, PKC-δ and nuclear lamins. Earlier reports from our own laboratory have exhibited a marked augmentation in the degraded product of lamin B as a result

of caspase 6 activation in cells subjected to proinflammatory cytokine, IL-1β, treatment. In addition there appeared to be an alteration in the distribution of lamin B within the nuclear compartment under these conditions **[112].**

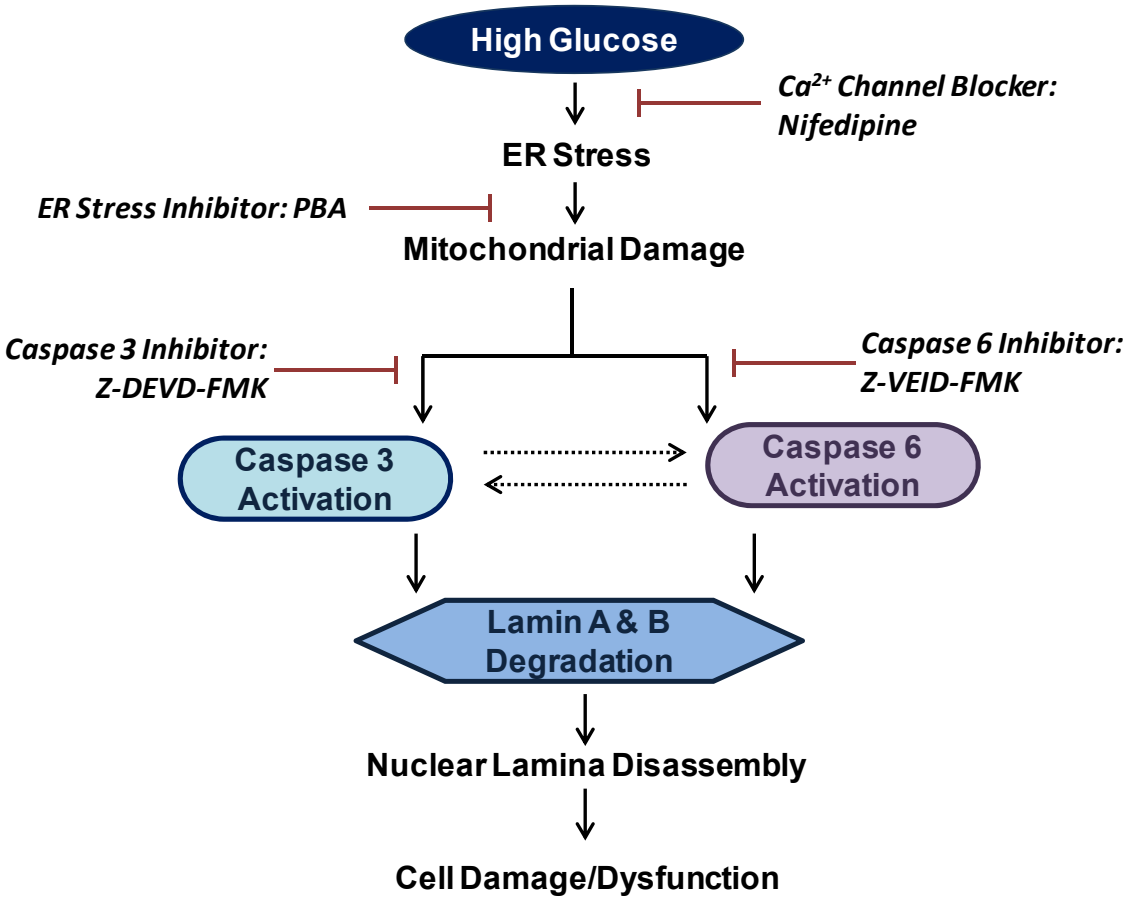


Figure 6-1: Working model for high glucose-mediated, ER stress-induced beta cell damage/dysfunction via caspase 3 mediated degradation of lamin B in pancreatic beta cells. Our current findings suggest that glucose-induced metabolic dysfunction in these cells may, in part, be due to increase in ER stress. Our model also highlights potential cytoprotective effects of calcium channel blockers [e.g., nifedipine], ER stress inhibitors [e.g., PBA] and caspase 3 and 6 inhibitors [e.g., Z-DEVD-FMK and Z-VEID-FMK respectively] against noxious effects of hyperglycemia as demonstrated in our study.

In this context, we explored the effects of glucotoxic conditions on the activation status of executioner caspases and the integrity of the nuclear lamina in cells exposed to chronic hyperglycemic conditions. Herein, we conducted experiments on insulin secreting INS-1 832/13 cells, normal rodent and human islets by incubating with high glucose concentrations [20 mM for INS-1 832/13 and rat islets and 30 mM for human islets] for 24 hrs. Findings from our experiments in INS-1 832/13 cells indicated that high glucose conditions lead to an increase in the cleaved form of both caspases 3 and caspase 6, indicating the activation of these executioner caspases, which resulted in a corresponding increase in the degraded product of lamin A as well as lamin B. Similarly, studies by Kivinen and associates have implicated the involvement of caspase 3 in the proteolysis of Nuclear Mitotic apparatus [NuMA] protein and the nuclear lamins in MCF-7:WS8 human breast cancer cell line **[91].** Another *in vitro* study demonstrates that PC12 cells exposed to palmitic acid [PA/BSA 2:1 molar ratio] showed a significant increase in caspase activity measured fluorimetrically which could be co-related to an increase in PARP and lamin B cleavage as seen by Western blotting **[92].** In the context of lamin A, Mintzer *et al.* have demonstrated that active caspase 6 has specificity for lamin A. The study shows that proteolytic cleavage of recombinant lamin A [GST-lamin A] increases with an increasing concentrations of human active caspase 6. They also state that a modest increase in GST-lamin A cleavage does occur at higher concentrations of caspase 3 **[113].** In order to validate our preliminary findings in INS-1 832/13 cells, we conducted experiments under similar incubation conditions in primary rodent islets and islets obtained from human donors. Interestingly, we observed an increase in the activation of caspases 3 and 6 accompanied by the proteolytic cleavage of the nuclear lamins in the islet cells in a manner akin to our observations in INS-1 832/13 cells. In the above experiments, our findings indicated no significant effect of high glucose treatment on the expression of ZMPSTE24, the metallo-

proteolytic enzyme involved in post translational processing of lamin A, in INS-1 832/13 cells and human islets. However, prenylation of these proteins, under conditions of glucolipotoxicity, needs to be studied in islet cells in a systematic manner.

Having examined the effects of glucotoxicity on caspases and lamins in INS-1 832/13 cells and islets, we next tested our hypothesis in islets derived from animal models of type 2 diabetes, namely the Zucker diabetic fatty rats. ZDF rats exhibit obesity along with diabetes and are widely used for research on type 2 diabetes [T2D]. These rats develop obesity and insulin resistance at a young age, and then with aging, progressively develop hyperglycemia. This hyperglycemia is associated with impaired pancreatic beta cell function and loss of pancreatic beta cell mass. Earlier reports from our own laboratory have shown an increase in the generation of NOX-2 induced reactive oxygen species [ROS] in the ZDF model **[103].** This study also demonstrated an increase phosphorylation of JNK 1/2, a type of mitogen activated protein kinases [MAPK] which respond to stress stimuli and a significant decrease in the phosphorylated form of ERK1/2, another MAPK which plays a role in the cell survival pathway. Finding an increase in the activated caspases and cleaved lamins in islets obtained from the ZDF rat model and type 2 diabetic humans, further affirmed our hypothesis.

Our next aim was to elucidate the effect of chronic high glucose exposure on beta cell function. Insulin release is one of the direct measures of beta cell function and any impairment in either phase of insulin secretion, chiefly first phase, may be the earliest evidence of an individual's progression towards type 2 diabetes. Numerous lines of evidence indicate that metabolic stress due to hyperglycemic conditions leads to a decline in glucose stimulated insulin secretion in the pancreatic beta cells. Hence, in the next set of studies, we assessed the insulin secretion capacity of INS-1 832/13 cells under high glucose exposure. The amount of insulin released into the culture medium under static incubation conditions was assessed by ELISA and

measured spectrophotometrically. Herein, we detected an increase in insulin secretion by the beta cells on short term glucose stimulus. However, cells that were chronically exposed to high glucose concentrations exhibited a significant impairment in insulin secretion, indicating that chronic hyperglycemia has a detrimental effect on beta cell function and contributes to the progression of type 2 diabetes.

Our findings also revealed considerable modifications in the subcellular distribution of lamin B in INS-1 832/13 cells under conditions of exposure to high glucose. What then might be the mechanisms underlying abnormal distribution of lamin B that we observed in beta cells exposed to high glucose conditions? To address this question, we made use of pharmacological inhibitors of the ER stress-mediated mitochondrial damage pathway in order to establish the causal role of ER stress in the release of cytochrome c into the cytoplasm, the subsequent activation of caspases and loss of integrity of the nuclear lamina. Thapsigargin, a known inducer of ER stress in many cell types inhibits the sarcoplasmic/ ER calcium ATPase [SERCA] pump, which regulates the influx of calcium ions from the cytoplasm of the cell into the ER lumen. Induction of ER stress by thapsigargin as evidenced by the upregulation of CHOP expression also induced activation of caspases in the islet beta cell, along with an increase in lamin B degradation. Extant studies and data obtained in our study suggest that increased oxidative and ER stress leading to mitochondrial dysfunction could be a result of hyperglycemic conditions. As a consequence, executioner caspases in the cytoplasm are activated by cytochrome C released from the dysregulated mitochondria. Moreover, the significant inhibition by PBA of CHOP expression, caspase 3 activation and lamin B degradation, that were augmented under high glucose exposure, further strengthens the notion of the involvement of an ER mediated pathway in the beta cell under glucotoxic conditions. Lastly, our observations also support reports by

Tang *et al.* establishing clear roles for the oxidative stress-ER stress signaling axis in glucose-induced beta cell dysfunction *in vivo* in rats **[114]**

Investigation of the association of intracellular calcium levels in this pathway, led to the suggestion that accumulation of cytosolic calcium plays a contributory role in regulation of glucose-induced effects on caspase 3 activation and lamin B degradation. Studies conducted by Wang *et al.* demonstrate the participation of intracellular calcium in ER stress pathway in the pancreatic beta cell. To this end they have uncovered the cytoprotective effects of nifedipine, a dihydropyridine calcium channel blocker *via* a decrease in the phosphorylation of eukaryotic initiation factor 2α [eIF2α], expression of CHOP and active caspase 3 **[94].** Another phenylalkylamine calcium channel blocker, verapamil, has been utilized by Xu and colleagues, wherein they demonstrated the inhibitory effect of verapamil on increased expression of thioredoxin-interacting protein [TXNIP], which is upregulated in diabetic conditions and reestablished insulin secretion *in vivo* in 8 week old, leptin-deficient, type 2 diabetic $BTBR^{ob/ob}$ mice. This background and our current findings further reinforce the idea that accumulation of calcium in the cytoplasm due to increased influx via the L-type calcium channels located on the cell membrane would eventually lead to mitochondrial damage due to calcium overload and further cause disruption of cellular function. Inhibitors of long lasting calcium channels [e.g., verapamil and nifedipine] seem to support the beta cells in defense against stress stimuli and enhance beta cell survival and function **[93, 94, and 115].** Moreover, a recent study by Zhou *et al*. has demonstrated the protective effects of nifedipine-mediated inhibition of calcium channels in pancreatic beta [MIN6] cells and mouse islets treated with palmitic acid. Further they have also suggested the involvement of ER stress in lipotoxicity, evidenced by an increase in phosphorylation of eukaryotic initiation factor 2α [eIF2α] and expression of CHOP which were reversed by nifedipine **[116].** Along these lines we put forward a working model that implicates

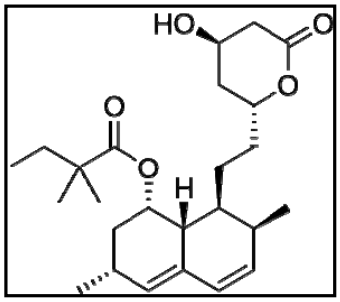
excessive influx and build up of cytosolic calcium as one of the causal factors leading to hyperglycemia-induced activation of caspase 3 and 6 and proteolytic degradation of lamins A and B.

The preferential detection of certain amino acid chain arrangements on the substrate proteins by individual caspases has been utilized in building up specific peptide inhibitors of these proteases. These peptide inhibitors of caspases readily permeate the cell membrane and bind to the catalytic sites on the caspases based on preferential sequence specificity and in doing so, impede the events downstream of caspase activation. *In vitro* studies by Huo *et al.* in the HIT-T15 beta cell line have shown that Z-VDVAD-FMK, a specific inhibitor of caspase 2 has a protective effect on the cells against detrimental effects of mycophenolic acid [MPA], thereby aiding their survival **[95].** Furthermore, another study which utilized human Fas cDNA for transfection into murine βTC-1 cell lines demonstrated the involvement of caspase 3 in Fas-mediated death signaling pathway. In addition, on using Z-Asp-Glu-Val-Asp fluoromethyl ketone [Z-DEVD-FMK] a specific inhibitor of caspase 3, protected against Fas-induced cell death **[96].** In a similar mouse model transfected with human Fas, a caspase 6 inhibitor was observed to exert protective effects against palmitic acid-induced effects on the cells **[97].** Our own data obtained from studies using Z-DEVD-FMK and Z-VEID-FMK specific inhibitors of caspase 3 and caspase 6 activation respectively, in INS-1 832/13 cells points out the involvement of both these executioner caspases in the disassembly of the nuclear lamina *via* proteolytic cleavage and the reversal of these effects by the peptide inhibitors **[117].** 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 beta cells under low glucose conditions. Additional studies to test the

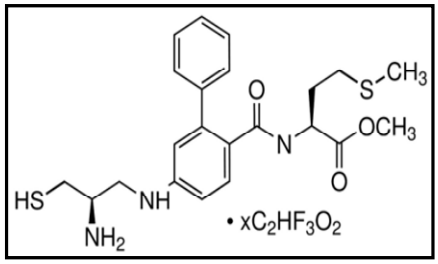
possibility that the addition of the chemical or peptide inhibitors to cells under basal conditions may trigger such a response would help in gaining further understanding of this observed phenomenon.

Further to elucidate the role of post translational prenylation of nuclear lamins towards cell stability and function in our studies we utilized pharmacological inhibitors of prenylation such as, statins [simvastatin], FTIs and GGTIs. Statins belong to the cholesterol lowering class of drugs and are one of the most widely used drugs in the treatment of cardiovascular diseases. The first ever statins isolated from the *Penicillium* species were compactin and mevastatin in the year 1976, followed by lovastatin in 1980. Subsequent studies revealed their property of inhibition of the HMG-CoA reductase enzyme and the associated blocking of cholesterol biosynthesis. However the most important benchmark in the discovery of statin molecules was in the year 1987; when Simvastatin, a semi-synthetic compound derived using the lovastatin parent structure was approved for use in humans. In the following years several purely synthetic statin moieties were derived, namely; fluvastatin, atorvastatin, cerivastatin, with rosuvastatin and pitavastatin being the most recently developed in 2003 and 2009 respectively **[118-121].** In 2001, cerivastatin was withdrawn from the market due to renal malfunction associated deaths that were reported in the post marketing surveys **[122].** The basic mechanism of action is by inhibition of HMG-coA reductase in the cholesterol biosynthesis pathway **[123].** The inhibition of the reductase enzyme blocks the production of mevalonic acid MVA which is a precursor to cholesterol. The primary effects of statins are inhibition of cholesterol synthesis and increasing LDL uptake **[124, 125].** However, based on recent evidence from a number of studies, trials and meta analysis, the U.S. Food and Drug Administration [FDA] has approved important safety label changes for statins that indicate the association of statins with an increase in hemoglobin A1C and fasting glucose levels. Despite

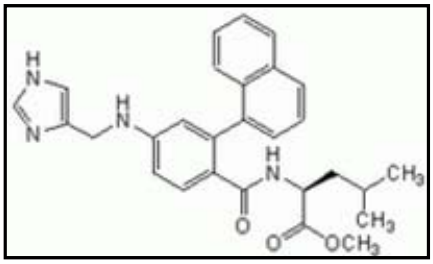
these reports, the underlying molecular mechanisms of these effects of statins need to be further explored in the context of pancreatic beta cells and type 2 Diabetes. In addition to blocking cholesterol synthesis, statins also prevent the formation of intermediate metabolites of this pathway, namely: farnesyl pyrophosphate [FPP] and geranylgeranyl pyrophosphate [GGPP] that are involved in post translational prenylation of several proteins [Rho, Rab, Ras, lamins], thereby preventing post translational prenylation of these proteins. In this context, a variety of pharmacological inhibitors that interfere with the activities of the enzymes farnesyl transferase [FTase] and geranylgeranyltransferase [GGTase] have been developed. Figure 6-2 depicts the structures of a few inhibitors of prenylation, namely; simvastatin, farnesyl transferase inhibitor [FTI-277] and geranylgeranyltransferase inhibitor [GGTI-2147].



SIMVASTATIN



FTI-277



GGTI-2147

Figure 6-2: Chemical structures of inhibitors of protein prenylation

A recent study carried out by Ishikawa and associates reported that different statin molecules have differential effects on the insulin release by the beta cell. In this study they

utilized MIN6 cells to study beta cell function by measuring glucose stimulated insulin secretion in the cells post treatment with various statin drugs and demonstrated that decreasing hydrophobicity of the molecules enhances their capacity to impair insulin secretion in the cells. Therefore, the more lipophilic statins such as lovastain and simvastatin demonstrated a more pronounced effect **[126].** Based on their studies with lovastatin in MIN6 beta cells, Tsuchiya *et al.* propose that statin-mediated inhibition of cholesterol biosynthesis pathway and thereby depletion of the intermediate metabolites such as mevalonate, squalene and geranylgeranyl pyrophosphate is a contributory factor towards the reduction in insulin content of the cell, as well as its capacity to efficiently secrete insulin **[127].** Early studies by Metz *et al.* demonstrated that inhibition of PTM of GTP binding proteins brought about a decrease in the insulin secretion in intact rat islets. In this study, they also showed that lovastatin treatment of the islets caused a hindrance in their association with the membrane and markedly diminished glucose stimulated insulin secretion, which could be precluded by supplementing the culture media with mevalonic acid **[128].** 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 and can have detrimental effects on the cell. 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 **[129].** Likewise, more recent studies by Chang *et al.* have demonstrated abnormalities in lamin A processing following inhibition of its farnesylation using FTIs and also GGTIs. They reported significant accumulation of unprocessed lamin A intracellularly **[130].**

In order to study the regulatory role of farnesylation in lamins, several studies utilizing farnesyltransferase inhibitors have been conducted. In one such study Adam *et al.* reported an

increase in unprocessed lamin B2 and lamin A and a decrease in mature lamin B1 in human fibroblasts treated with FTIs **[131].** Hutchinson-Gilford Progeria Syndrome [HGPS] is a rare premature aging disorder caused by point mutation G608G [GGC>GGT] within exon 11 of *LMNA* gene encoding A-type nuclear lamins. This mutation results in the expression of a truncated protein, progerin, deficient in 50 amino acids in the carboxyl-terminus of prelamin A, which still retains a farnesylated cysteine at its carboxyl terminus, a modification involved in HGPS pathogenesis. Previous studies in cellular and animal models of HGPS have demonstrated that FTI treatment improves abnormal nuclear morphology and phenotype **[132].** Mallampalli *et al.* also demonstrated that FTI [rac-R115777] significantly improved abnormal nuclear morphology in HeLa cells **[133]** and also FTI-276 improved nuclear shape abnormalities in keratinocytes of transgenic mice expressing progerin in epidermis or a combination therapy of pravastatin and zoledronate by immunofluorescence microscopy **[134].** In HGPS mouse model dose-dependent administration of the FTI tipifarnib [R115777, Zarnestra] significantly prevented loss of vascular smooth muscle cells in the large arteries and also delayed progression of cardiovascular disease **[135].** In another study along these lines, R115777 a potent, orally active FTI, was recently investigated in a phase I dose-ranging study in patients with acute leukemias. R115777 dose dependently inhibited farnesylation of lamin A and HDJ-2 **[136]** suggesting further evaluation of this compound in clinical studies. In a recent clinical trial study in children prone to HGPS, 25 patients were subjected to a 2 year treatment with lonafarnib [FTI]. All subjects improved in one or more of the following: gaining additional weight, better hearing, improved bone structure and/or, most importantly increased flexibility of blood vessels. This study presented preliminary evidence that lonafarnib may improve the detrimental phenotypical changes associated with this disorder **[137].**

Interestingly, significant effects on mitochondrial and nuclear stability indicated by increased activation of caspase 3 and degradation of lamin B were observed in our studies with simvastatin, which demonstrate the consequences of inhibition of post-translational prenylation in INS-1 832/13 cells as well as rat islets. In addition, we observed clear alterations in cell morphology [rounding], and a marked decrease in cell survival and GSIS. Moreover, a significant increase in the activation of p38 MAP Kinase, which has been implicated in hyperglycemia associated beta cell dysfunction, was observed along with a marked decrease in the phosphorylation of ERK 1/2 which plays a crucial role in cell survival and function. The effects of this lipophilic statin on caspase 3 and lamin B were mimicked by a specific inhibitor of farnesyl transferase, FTI-277. However GGTI-2147, which specifically inhibits geranylgeranyltransferase-I, could not produce the same effects as seen in INS-1 cells and rat islets. These observations point out the possibility that the caspase 3 activation and lamin B degradation effects are farnesylation dependent. However, whether this is a result of depletion of the substrate farnesyl pyrophosphate [FPP] or if it is due the inhibition of the enzyme FTase or a combination of the two needs to be further investigated. In this context, recent reports from our laboratory in insulin secreting cells have shown that the degradation of the regulatory subunit of FTase was caspase 3 mediated **[93].** Also, evidence in other cell types indicated a caspase 3 dependent mechanism involved in the degradation of the common α subunit of FTase and GGTase **[138].**

In an attempt to identify the patho-mechanism of inhibition of prenylation using statins or farnesylation inhibitors Pelaia *et al.* have demonstrated in 2 lung cancer cell lines, namely CALU-1 [squamous cell line] and GLC-82 [adenocarcinoma cell line] that inhibition of prenylation with these agents caused a decrease in the phosphorylation of ERK 1/2 and showed detrimental effects on cell morphology, thereby implicating the Ras/Raf/MEK/ERK pathway

[139]. This observation was confirmed in glioma cell lines by Afshordel and associates wherein they inhibited prenylation with lovastatin and perillyl alcohol **[140].** Bonifacio and colleagues have identified the AKT/mTOR pathway as a target for statin mediated detrimental effects in myotubes. 24 hr incubations of C2C12 myotubes with 10 – 50 μM concentrations of simvastatin and atorvastatin caused a decrease in AKT phosphorylation thereby inhibiting its activation. In addition they observed a parallel increase in the activation of caspases and the downstream cleavage of PARP. Comparable effects observed in mice skeletal muscles indicate that probing further into statin-mediated effects on the AKT/mTOR pathway would provide important leads in the identification of prospective therapeutic targets **[141].** Collectively, these observations point out the significance of post translational modifications, specifically farnesylation in maintaining the function and integrity of the nuclear lamins.

CHAPTER 7

CONCLUSION AND FUTURE DIRECTIONS

The overall goal of my Ph.D. project is to understand the cellular mechanisms involved in the pathology of islet beta cell dysfunction under conditions of chronic exposure to high glucose [glucotoxicity]. Salient features of my research work are:

- Exposure of insulin-secreting pure beta cells [INS-1 832/13 cells], normal rodent islets and human islets to glucotoxic conditions results in the activation of executioner caspases 3 and 6, and subsequent degradation of their downstream target proteins, such as nuclear lamins A and B. I also observed a significant inhibition of glucose-stimulated insulin secretion under these conditions.

- Glucotoxic conditions promoted significant alterations in the subcellular distribution of nuclear lamins [e.g., increased abundance in cytosol] suggesting the collapse of nuclear compartment under these conditions.

- Specific inhibitors of caspase 3 and caspase 6 attenuated high glucose-induced caspase activation and lamin degradation.

- Similar increases in the activation of executioner caspases and degradation of nuclear lamins A and B were seen in islets derived from the ZDF rat, a model for T2DM and islets from T2DM human donors.

- In a manner akin to thapsigargin, a known inducer of ER stress, glucotoxic conditions also significantly increased ER stress as evidenced by increased expression of CHOP, a marker for ER stress. Moreover, exposure of beta cells to thapsigargin resulted in the

activation of executioner caspases and lamin degradation, thus suggesting that caspase activation and lamin degradation seen under the duress of high glucose may, in part, be due to increased ER stress. This is further confirmed by my observations that PBA, a known inhibitor of ER stress, markedly attenuated CHOP expression, caspase activation and lamin degradation in beta cells exposed to glucotoxic conditions.

- Nifedipine, a known inhibitor of membrane-associated L-type calcium channels, also inhibited high glucose-induced caspase activation and lamin degradation; these findings suggest that increased levels of intracellular calcium in beta cells exposed to glucotoxic conditions could contribute to the mitochondrial [caspase activation] and nuclear [lamin degradation] defects.

- Inhibition of protein [lamin] prenylation with simvastatin, a known inhibitor of farnesylation and geranylgeranylation, promoted mitochondrial and nuclear defects as evidenced by caspase activation and lamin degradation in INS-1 832/13 cells and normal rodent islets. More site-specific inhibitors of protein farnesylation [FTI-277] also caused caspase activation and lamin degradation suggesting potential involvement of a farnesylated protein in the induction of mitochondrial defects. It is also likely that inhibition of farnesylation of lamins by FTI-277 and simvastatin make lamins more vulnerable for degradation and nuclear collapse. My findings also suggested that inhibition of protein prenylation leads to increase in stress kinase [p38 kinase] and inhibition of ERK1/2, known for its cell survival roles. Collectively, these alterations in cell signaling pathways could promote intracellular stress and demise.

Based on the above findings, I conclude that chronic exposure of beta cells to hyperglycemic conditions leads to increased intracellular calcium levels [calcium overload] and

ER stress leading to loss in mitochondrial dysregulation, including loss in mitochondrial permeability pore transition, leakage of cytochrome C into cytosol and activation of executioner caspases. These events, in turn, lead to degradation of key cellular proteins, such as nuclear lamins, resulting in the collapse of nuclear compartment and accumulation of lamin degradation products in cytosolic compartment. Collectively, alterations in these cellular events lead to a loss in functional beta cell mass and onset of diabetes. I believe that my studies not only provided mechanistic insights into mitochondrial and nuclear dysfunction in beta cells under the duress of glucotoxic conditions, but they also provided fresh insights into potential beneficial [cytoprotective] effects of calcium channel blockers in the treatment of T2DM in humans.

Future directions:

While my research work provided novel insights into islet dysfunction in diabetes, I believe that there are several knowledge gaps that need to be filled. Some of those are listed below.

First, we need to quantify the intracellular calcium levels in the beta cell following exposure to glucotoxic conditions. Data from calcium channel blockers are encouraging, but it is important to quantify the levels of calcium in cells treated without or with glucose and calcium channel blockers and correlate those with cellular indices of apoptosis. Further, we need to verify these *in vitro* findings in animal models of T2DM to see if administration of calcium channel blockers prevents/halts islet dysfunction and diabetes in these model systems.

Second, we need to investigate metabolic alterations in beta cells exposed to saturated fatty acids, such as palmitate [lipotoxic conditions] and, ideally, glucolipotoxic conditions. These

studies would provide additional insights into combined effects of glucose and lipids in inducing mitochondrial and nuclear defects.

Third, additional studies are needed to further asses the roles of post-translational farnesylation of lamins in maintaining the integrity of the nuclear envelop. This can be addressed first by *in vitro* approaches involving use of inhibitors of protein prenylation and siRNA-mediated knockdown of prenyltransferases. My studies have identified the lamin processing enzyme in the islet, and therefore, it is likely that lamins might undergo additional PTMs [carboxylmethylation] that might be requisite for maintaining the integrity of nuclear envelop. Very little is known with regard to alterations in these modification signaling events in the diabetic islet.

Lastly, *LMNA* mutations have been shown in human subjects with severe metabolic syndrome. More needs to be understood with regard to potential alterations in post-translational prenylation and phosphorylation of lamins as cause for pathology of metabolic disorders including obesity and T2DM.

APPENDIX A

Biochemical Pharmacology 86 (2013) 1338–1346

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Glucotoxic conditions induce endoplasmic reticulum stress to cause caspase 3 mediated lamin B degradation in pancreatic β-cells: Protection by nifedipine

Khadija Syeda, Abiy M. Mohammed, Daleep K. Arora, Anjaneyulu Kowluru *

Beta-Cell Biochemistry Laboratory, John D. Dingell VA Medical Center and Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI 48201, United States

ARTICLE INFO

Article history:
Received 20 June 2013
Accepted 15 August 2013
Available online 27 August 2013

Keywords:
Caspase 3
Glucotoxicity
Nuclear lamin B
Pancreatic islet β-cell
Nifedipine

ABSTRACT

Nuclear lamins form the lamina on the interior of the nuclear envelope, and are involved in the regulation of various cellular processes, including DNA replication and chromatin organization. Despite this evidence, little is known about potential alterations in nuclear metabolism, specifically lamin structure and integrity in isolated β-cells subjected to stress conditions, including chronic exposure to hyperglycemia (i.e., glucotoxicity). Herein, we investigated effects of glucotoxic conditions on the catalytic activation of caspase 3 and the associated degradation of one of its substrate proteins, namely lamin-B. We report that incubation of insulin-secreting INS-1 832/13 cells, normal rat islets or human islets under glucotoxic conditions (20 mM; 12–48 h) results in the degradation of native lamin B leading to accumulation of the degraded products in non-relevant cellular compartments, including cytosol. Moreover, the effects of high glucose on caspase 3 activation and lamin B degradation were mimicked by thapsigargin, a known inducer of endoplasmic reticulum stress (ER stress). Nifedipine, a known blocker of calcium channel activation, inhibited high glucose-induced caspase 3 activation and lamin B degradation in these cells. 4-Phenyl butyric acid, a known inhibitor of ER stress, markedly attenuated glucose-induced CHOP expression (ER stress marker), caspase 3 activation and lamin B degradation. We conclude that glucotoxic conditions promote caspase 3 activation and lamin B degradation, which may, in part, be due to increased ER stress under these conditions. We also provide further evidence to support beneficial effects of calcium channel blockers against metabolic dysfunction of the islet β-cell induced by hyperglycemic conditions.

Published by Elsevier Inc.

1. Introduction

Apoptosis is the process of programmed cell death that is required for the maintenance of tissue homeostasis. It is characterized by specific changes in the morphology of the cell. The cell undergoes shrinkage, blebbing (bulges in the plasma membrane), nuclear fragmentation and chromatin condensation [1]. Basically, it is a natural process that balances cell growth and removal of damaged or injured cells. It is well established that β-cell failure is a characteristic feature of Type 1 and 2 diabetes. In both types of the disease, apoptosis is the most likely form of cell death. Therefore, it is necessary to study the intracellular events that the pancreatic β-cell undergoes during apoptosis, and understanding of such events will aid in searching for a possible drug target for the management and/or prevention of the disease [2,3].

The caspase (cysteine dependent aspartate-directed proteases) family is one of the most important factors in the apoptotic pathway. Many proteins in the cell including a large number of structural proteins are cleaved by activated caspases which culminates in apoptosis of the cell. The caspase 8, 9 and 10 are initiator caspases whereas caspase 3, 6 and 7 are effector caspases. The initiator caspases cleave and activate the effector caspases leading to cell death [1]. These caspases exist in the form of inactive zymogens (also referred to as pro-caspases), which are activated by the process of cleavage by other upstream proteases or by auto- or trans-activation [4]. In the recent years a variety of substrates of caspases have been recognized. Caspase-dependent degradation of nuclear lamins has been identified as a precursor to nuclear collapse in programmed cell death [5]. The nuclear lamina lines the

Abbreviations: CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; FTase, farnesyl transferase; FTI, farnesyl transferase inhibitor; Nif, nifedipine; PBA, 4-phenylbutyric acid; Tg, thapsigargin.

* Corresponding author at: B-4237 Research Service, John D. Dingell VA Medical Center, 4646 John R, Detroit, MI 48201, United States. Tel.: +1 313 576 4478; fax: +1 313 576 1112.
E-mail address: akowluru@med.wayne.edu (A. Kowluru).

0006-2952/$ – see front matter. Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.bcp.2013.08.023

interior of the nuclear envelope and is composed of three proteins; lamins A, B and C. The A type lamins, which include lamins A and C, are products of alternative splicing of the same gene LMNA whereas lamin B is encoded by the LMNB1 gene [6]. These lamins are type V intermediate filament proteins and are lined up on the inner face of the inner nuclear membrane. The nuclear lamina is a fundamental part of major nuclear activities, namely mitosis, chromatin organization and in DNA replication. Besides, lamins also play key functional roles in providing structural support thereby contributing to the nuclear architecture [7].

Degradation of lamins leads to the breakdown of nuclear lamina which is a preliminary stage of apoptosis [8] as this is followed by DNA degradation and chromatin condensation [9]. Previous results from our laboratory indicated that IL-1β treatment causes an increase in lamin B degradation mediated by caspases [10]. In addition, a recent study highlighted the involvement of caspase 3 in the breakdown of the nuclear matrix by cleaving nuclear lamin B. It has been stated that this cleavage probably occurs by activation of caspase 3 directly or by other downstream proteases [11]. Recent evidence in PC12 and rat cortical cells also implicates cell apoptosis via caspase 3 activation and cleavage of lamin-B under lipotoxic conditions induced by saturated fatty acids, such as palmitic acid [12].

It is well established that chronic exposure of isolated β-cells to hyperglycemic conditions leads to metabolic dysfunction and demise [13,14]. Several intracellular signaling events have been identified as causal to HG-induced metabolic dysregulation of the islet. These include endoplasmic reticulum (ER) and oxidative stress. It has been suggested that both oxidative and ER stress lead to mitochondrial dysfunction, cytochrome-C release, and caspase activation resulting in the demise of the β-cell [15]. Despite this growing body of evidence, very 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. The overall objective of this study therefore is to investigate the role of caspase 3 on the degradation of nuclear lamins, specifically lamin-B in insulin-secreting INS-1 832/13 cells, rat islets and human islets under glucotoxic conditions. Our findings provide the first evidence to implicate significant caspase 3 mediated degradation of lamin B in these cells under glucotoxic environment. We also provide evidence to suggest novel roles for ER stress in glucose-induced caspase 3 activation and lamin B degradation, and associated alterations in the subcellular distribution of lamin B under glucotoxic conditions.

2. Materials and methods

2.1. Materials

Antisera directed against lamin-B, procaspase-3, and cleaved caspase-3 (Asp 175), CHOP were obtained from Cell Signaling (Danvers MA and Santa Cruz Biotechnology, Santa Cruz, CA). Anti-β Actin was from Sigma–Aldrich (St. Louis, MO). Anti-mouse IgG and anti-rabbit IgG conjugated to horseradish peroxidase were from GE Healthcare UK. Glucose, nifedipine, thapsigargin, 4-phenylbutyric acid were obtained from Sigma–Aldrich (St. Louis, MO). Enhanced chemiluminescence (ECL) kit was from Amersham Biosciences (Piscataway, NJ). All other reagents were from Sigma–Aldrich (St. Louis, MO) unless stated otherwise.

2.2. Insulin-secreting INS-1 832/13 cells, rat islets, human islets and culture conditions

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 μM 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. Islets from normal 6-week-old male Sprague–Dawley rats (Harlan Laboratories, Oxford, MI) were isolated by the collagenase digestion method [16]. Human islets and islet culture medium were obtained from Prodo Laboratories, Irvine, CA. All protocols, including isolation of pancreatic islets from rats, were reviewed and approved by the Wayne State University and John D. Dingell VA Medical Center Institutional Animal Care and Use Committee.

INS-1 832/13 cells and normal rat islets were incubated in the presence of low (2.5 mM) and high glucose (20 mM or 30 mM in case of human islets) for 12–48 h as indicated in the text. Low and high glucose solutions were prepared by supplementing glucose to glucose-free medium where appropriate. At the end of the incubation period the cells were harvested and lysed in RIPA buffer containing 1 mg/ml protease inhibitor cocktail, 1 mM NaF, 1 mM PMSF and 1 mM Na_3VO_4.

2.3. Isolation of subcellular fractions

Extraction of cytosolic, membrane/organelle and nucleic protein fraction was carried out as per the manufacturer's instructions using the ProteoExtract® Subcellular Proteome Extraction Kit. Briefly, INS-1 832/13 cells were incubated with low (2.5 mM) and high (20 mM) glucose for 24 h. The cells were scraped and suspended in wash buffer and pelleted by centrifugation for 10 min at 300 × *g* at 4 °C. The pellet obtained was then resuspended in the extraction buffer-I and protease inhibitor cocktail, provided in the kit. After incubation for 10 min at 4 °C the cells were centrifuged for 10 min at 1000 × *g*, the supernatant obtained was the cytosolic fraction. The pellet was then resuspended in Extraction buffer-II and protease inhibitor cocktail and incubated for 30 min and then centrifuged for 10 min at 6000 × *g*. The supernatant thus obtained was the membrane/organelle fraction. Finally the pellet was resuspended in extraction buffer-III, protease inhibitor cocktail and benzonase, incubated for 10 min at 4 °C and centrifuged at 6800 × *g* for 10 min. The supernatant obtained was the nucleic protein fraction and the remaining pellet, resuspended in buffer IV and protease inhibitor cocktail was the cytoskeletal matrix protein fraction.

2.4. Western blotting

Cellular lysate proteins (30–50 μg/lane) were separated by SDS-PAGE on 10% (w/v) polyacrylamide mini gels and electro transferred to a nitrocellulose membrane. The membranes were blocked with 5% milk in 10 mM Tris–HCl, pH 7.6, 1.5 M NaCl and 0.1% Tween 20 followed by incubation with primary antibodies (lamin B—1:250, cleaved caspase 3—1:400) in TBS-T containing 5% BSA at room temperature for 1 h and washed 5 times 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 h. 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 used to probe for β-actin to ensure equal loading and transfer of proteins.

2.5. 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.

3. Results

3.1. Exposure of INS-1 832/13 cells, normal rat islets and human islets to glucotoxic conditions induce caspase 3 activation and degradation of lamin B

At the outset, INS-1 832/13 cells were incubated with either low (2.5 mM) or high (20 mM) glucose for 12, 24 and 48 h, and caspase 3 activation, as evidenced by the emergence of caspase-3 degradation fragment, was monitored by Western blotting, and the data are then quantitated by densitometry. Data depicted in Fig. 1 demonstrate a marked increase in caspase 3 activation as early as 12 h (1.8-fold; Panel A), which continued to increase as a function of time (2.2- and 2.6-fold increase at 24 and 48 h, respectively; Panels B and C). Furthermore, we noticed a marked increase in the degradation of lamin B under these conditions (Fig. 1). For example the fold increase in lamin B degradation represented 1.6-fold at 12 h (Panel A), 1.8-fold at 24 h (Panel B) and 2.3-fold at 48 h (Panel C). Pooled data from multiple experiments are provided in Panel D. Together, data in Fig. 1 suggested activation of caspase 3 and degradation of lamin B under glucotoxic conditions. It should be noted that the observed effects of glucose on caspase 3 activation and lamin B degradation are not due to osmotic effects of glucose since incubation of these cells with mannitol (20 mM), used as an osmotic control, did not elicit any clear effects on caspase-3 activation and lamin-B degradation under these conditions ($n = 2$ experiments; additional data not shown).

The above studies in INS-1 832/13 cells were repeated in normal rat islets to further validate the observed effects of glucotoxicity (20 mM glucose for 24 h) on caspase 3 activation and lamin B degradation are attributable to the primary islets as well. Data depicted in Fig. 2 (Panels A and B) indicate a 2.6-fold increase in caspase 3 activation followed by a corresponding increase in lamin B degradation under these conditions (Fig. 2; Panels A and B). Likewise, we noticed a 1.9-fold increase in caspase-3 activation and 2-fold increase in lamin-B degradation in human islet preparations incubated with glucose (30 mM; 24 h; Fig. 2; Panel C). These data in primary islets (rat and human) further support our observations in INS-1 832/13 cells (Fig. 1).

3.2. Glucotoxic conditions promote alterations in the subcellular distribution of cleaved caspase 3 and lamin B in INS-1 832/13 cells

In these studies, we determined potential alterations, if any, in the subcellular localization of active caspase 3 fragment and lamin B degradation products in INS-1 832/13 cells following exposure to glucotoxic conditions. To determine this, INS-1 832/13 cells were incubated with low (2.5) or high glucose (20 mM). Individual subcellular fractions, namely the cytosolic fraction (fraction F1), membrane/organelle protein fraction (fraction F2) and the pure nucleic protein fraction (fraction F3) were isolated using a ProteoExtract subcellular proteome extraction kit (see Section 2). At the outset, we determined subcellular distribution of native caspase (pro-caspase) and lamin B in INS-1 832/13 lysates. Data depicted in Fig. 3 (Panel A), as expected, suggest that native lamin B and pro-caspase are localized predominantly in the nuclear (F3) and cytosolic (F1) fractions, respectively. However, glucotoxic conditions caused significant alterations in the subcellular distribution of biologically active (cleaved) caspase 3 and degraded lamin B (Fig. 3; Panel B). For example, we noticed significant accumulation of degraded lamin B in cytosolic (F1) and membrane/organelle protein (F2) fractions under high glucose-treatment conditions. Interestingly, we also noticed significant accumulation of cleaved caspase 3 in these fractions. Together, these observations demonstrate abnormal distribution of lamin B under glucotoxic conditions further suggestive of disassembly of nuclear structure under these conditions.

3.3. Thapsigargin, a known inducer of ER stress, markedly increases caspase 3 activation and lamin B degradation in INS 1-832/13 cells

Several lines of experimental evidence implicate ER stress as one of intermediate steps involved in glucotoxicity of the islet

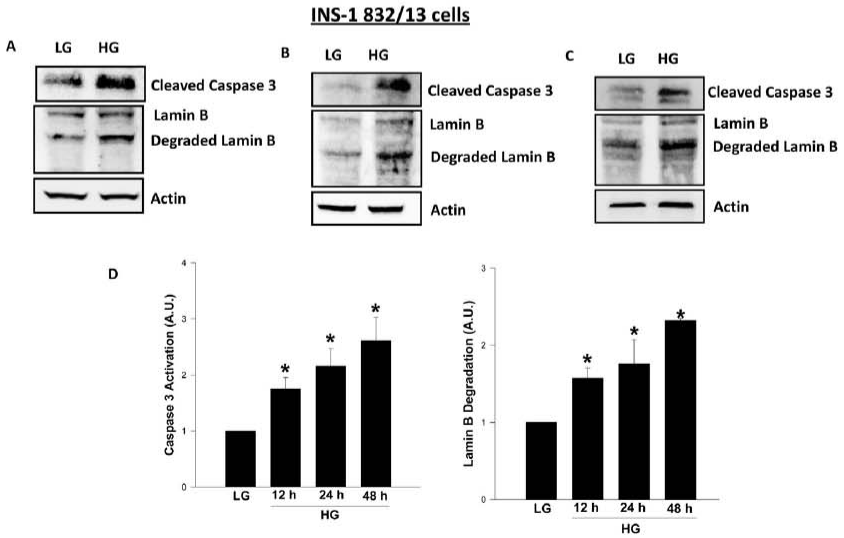


Fig. 1. Exposure of INS-1 832/13 cells to glucotoxic conditions results in caspase 3 activation and lamin B degradation. INS-1 832/13 cells were incubated in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 12 h (Panel A), 24 h (Panel B), and 48 h (Panel C), and protein lysates (50 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved (active) caspase 3 and degraded lamin B, and immune complexes were identified using ECL detection kit. To check equal protein loading, the membranes were stripped and reprobed for actin. Intensity of protein bands was quantitated by densitometry. The statistical significance of the differences between the control and experimental conditions was determined by *t*-test. Data in Panel D represent mean ± SEM from three to four independent experiments and expressed as fold change in caspase 3 activation and lamin B degradation. $^{*}p < 0.05$ vs. LG

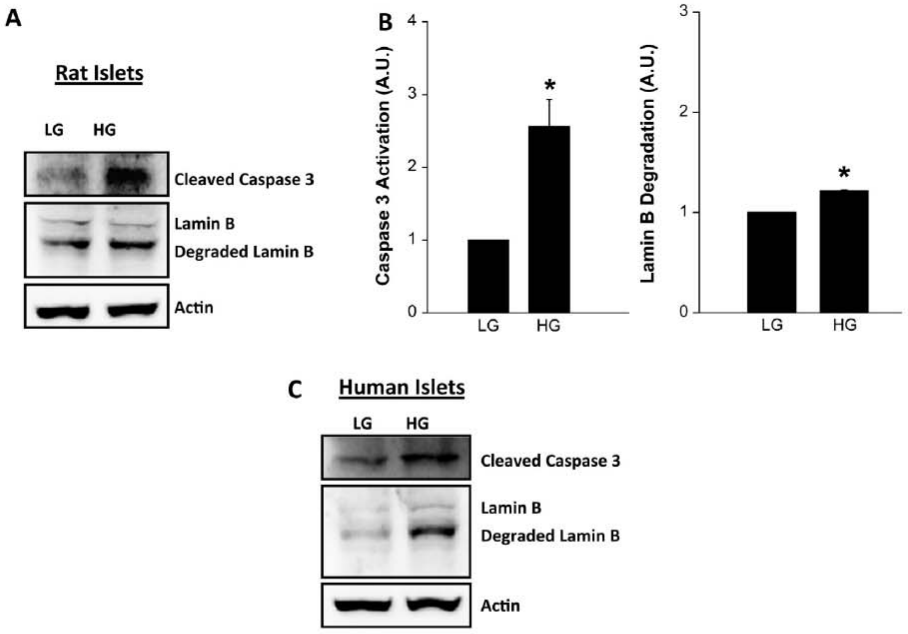


Fig. 2. Treatment of normal rat islets or human islets with high glucose results in caspase 3 activation and lamin B degradation. Rat islets (Panels A and B) or human islets (Panel C) were incubated in the presence of low (2.5 mM; LG) and high glucose (HG; 20 mM for rat islets, 30 mM for human islets) for 24 h as described in the text. Twenty-five micrograms lysate proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved caspase 3 and lamin B and immune complexes were identified using ECL detection kit. To check equal protein loading, the membrane for each protein was stripped and reprobed for actin. Quantitation of caspase 3 activation and lamin B degradation were carried out by densitometry. The statistical significance of the differences between the control and experimental conditions was determined by *t*-test. Data provided in Panel B represent mean ± SEM from three to four independent experiments and expressed as fold change for rat islets. Please note that human islet data (Panel C) were accrued from a single preparation. The ratios of cleaved caspase-3 to actin in LG and HG treated human islets were 0.068 vs. 0.120, respectively, and the ratios of degraded lamin-B to actin in LG and HG treated human islets were 0.142 vs. 0.283, respectively. $^{*}p < 0.05$ vs. LG.

β-cell [17–21]. To determine if high glucose induces ER stress in our current experimental model, we quantitated CHOP expression, an ER stress marker, in INS-1 832/13 cells exposed to low and high glucose conditions. Data depicted in Fig. 4 (Panel A) demonstrate a significant increase in CHOP expression in cells exposed to glucotoxic conditions and a similar result was obtained with thapsigargin (Panel B). To further assess if ER stress induces

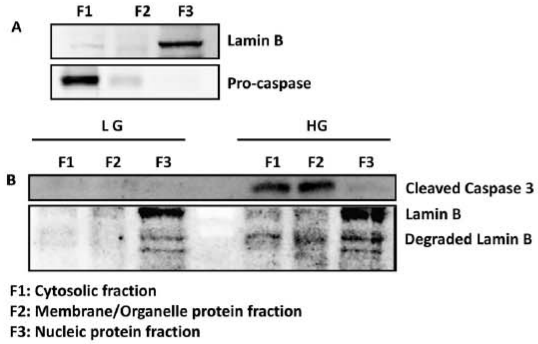


Fig. 3. Exposure to high glucose results in altered subcellular distribution of caspase 3 and degraded product of lamin B in INS-1 832/13 cells. INS-1 832/13 cells were subjected to subcellular fractionation using ProteoExtract® Subcellular Proteome Extraction Kit at basal level (Panel A) and after treatment with low (2.5 mm; LG) and high (20 mM; HG) glucose for 24 h (Panel B). The lysate proteins (~20–30 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for native lamin B and pro-caspase (Panel A) and cleaved caspase 3 and degraded lamin B (Panel B), and immune complexes were identified using ECL detection kit. Data are representative of two experiments with identical results.

caspase 3 activation and lamin B degradation, we incubated INS-1 832/13 cells with thapsigargin and then quantitated caspase 3 activation and lamin B degradation in these cells. Data depicted in Fig. 4 demonstrate significant increase in caspase 3 activation in cells treated with thapsigargin as early as 2 h of incubation (3.4-fold; Fig. 4; Panels C and D). Note that thapsigargin effects were maximal at 2 h since no further increase in caspase 3 activation was seen at 4 h (3.1-fold activation) and 6 h (3-fold activation; Fig. 4; Panels C and D). Under these conditions, we also noticed a significant increase on lamin B degradation induced by thapsigargin within 2 h (1.9-fold), which remain plateaued at 4 h (1.8-fold) and 6 h (1.7-fold). Together, our findings suggest ER stress as one of the mechanisms underlying glucose-mediated effects on caspase-3 activation and lamin-B degradation. This hypothesis was tested further using a pharmacological approach (see below).

3.4. 4-Phenylbutyric acid (PBA), a known inhibitor of ER-stress, markedly attenuates glucose-induced CHOP expression, caspase 3 activation and lamin B degradation

As a logical extension to the above studies, we attempted to establish a causal role for ER-stress in high glucose-induced caspase 3 activation and lamin B degradation. To accomplish this, we used PBA, a known inhibitor of ER-stress, on glucose-induced caspase 3 activation and lamin B degradation. We also quantified CHOP expression in these cells to affirm that PBA inhibits glucose-induced ER-stress under current experimental conditions. Data depicted in Fig. 5 demonstrate a significant increase in CHOP expression in glucose-treated cells. We also observed complete inhibition of glucose-induced CHOP expression by PBA (Fig. 5). These data thus validate the use of PBA as an inhibitor of ER-stress

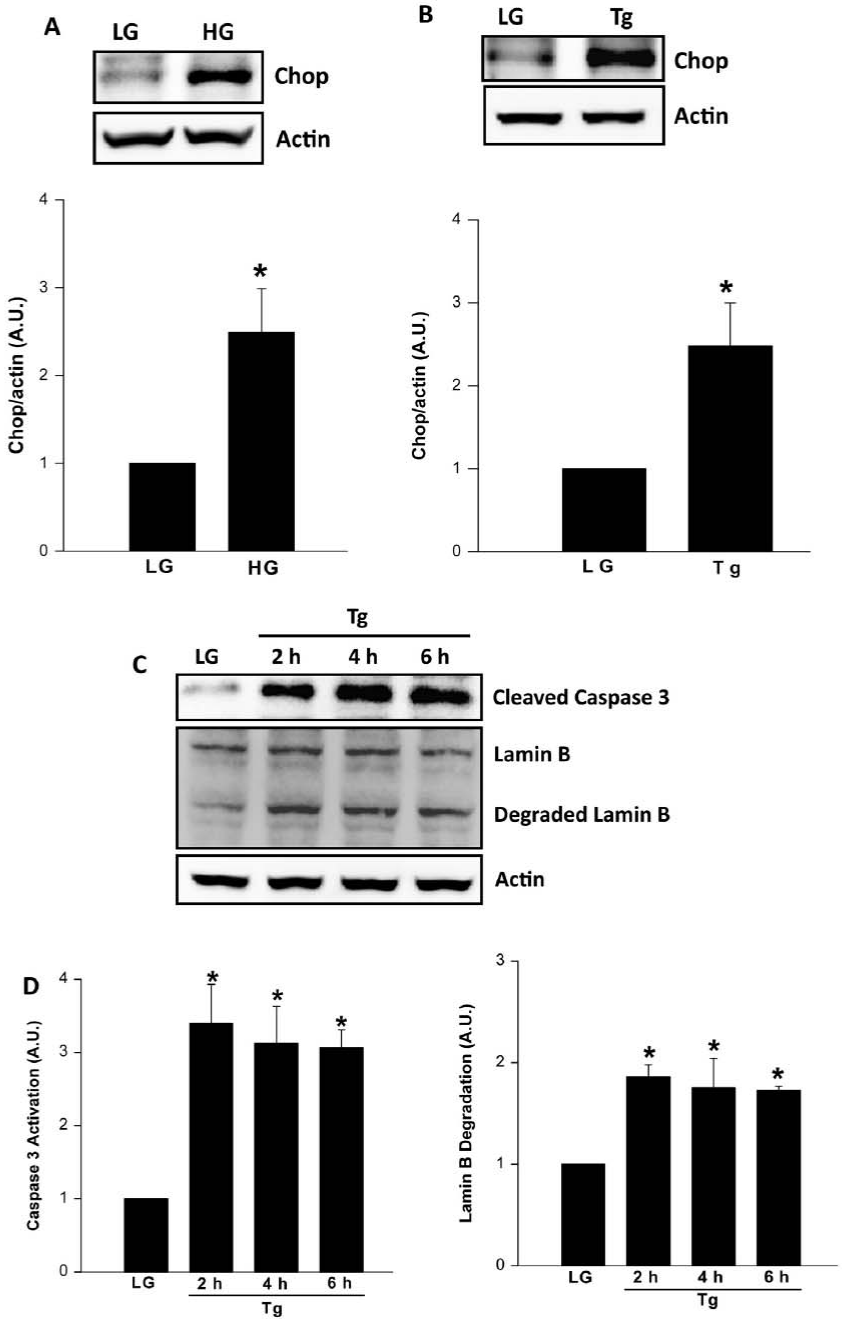


Fig. 4. Thapsigargin, a known inducer of ER stress, also promotes caspase 3 activation and lamin B degradation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells are incubated with low glucose (LG; 2.5 mM) or high glucose (HG; 20 mM) for 24 h. Expression of CHOP was determined in cell lysates by Western blotting. To check equal protein loading, the membrane for each proteins was stripped and reprobed for actin. Quantification of CHOP expression was carried out by densitometry. The statistical significance of the differences between the control and the experimental groups was determined by *t*-test. Data represent mean ± SEM from three independent experiments and expressed as fold change. $^{*}p < 0.05$ vs. LG. Panel B: INS-1 832/13 cells are incubated with low glucose (LG; 5 mM) or thapsigargin (Tg; 0.25 μM for 6 h). Expression of CHOP was determined by Western blotting. To check equal protein loading, the membrane for each proteins was stripped and reprobed for actin. Quantification of CHOP expression was carried out by densitometry. The statistical significance of the differences between the control and the experimental groups was determined by *t*-test. Data represent mean ± SEM from three independent experiments and expressed as fold change. $^{*}p < 0.05$ vs. LG. Panel C: INS-1 832/13 cells were incubated in the presence of low glucose (2.5 mM) or thapsigargin (Tg; 0.25 μM) for 2, 4 and 6 h as indicated in the figure. Forty micrograms lysate proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved caspase 3 and degraded lamin B, and immune complexes were identified using ECL detection kit. To check equal protein loading, the membrane for each proteins was stripped and reprobed for actin. Quantitation of caspase 3 activation and lamin B degradation were carried out by densitometry. The statistical significance of the differences between the control and experimental conditions was determined by ANOVA followed by Student–Newman–Keuls post hoc test. Data in Panel D represent mean ± SEM from three independent experiments and expressed as fold change. $^{*}p < 0.05$ vs. LG.

in INS-1 832/13 cells. More importantly, we also noticed a complete inhibition of glucose-induced caspase 3 activation and lamin B degradation by PBA in these cells (Fig. 5; Panels A and B). Taken together, these data provide the first evidence that glucotoxicity promotes caspase 3 activation and lamin B degradation in an ER-stress sensitive fashion.

3.5. Nifedipine, a calcium channel blocker, inhibits glucose-induced caspase 3 activation and lamin B degradation in INS-1 832/13 cells and normal rat islets

We recently reported that calcium overload may represent one of the signaling mechanisms involved in caspase-3 activation under conditions of cellular apoptosis in isolated β-cells [21]. For example, using etoposide, a known inducer of loss in metabolic viability in β-cells, we reported a marked increase in caspase 3 activity in INS-1 832/13 cells and normal rat islets. More importantly, we have been able to markedly prevent etoposide-induced metabolic dysfunction in these cells by nifedipine, a known blocker of calcium channel activation and calcium entry [22]. Furthermore, recent findings from the laboratory of Wang and associates [23] have also demonstrated significant protective effects of nifedipine against high glucose-induced ER stress and apoptosis. Therefore, as a logical extension to findings that we reported above, we undertook a study to see if glucose-induced caspase 3 activation and lamin B degradation are rescued by nifedipine. Data presented in Fig. 6 indicate a modest, but insignificant (1.2-fold) increase in caspase 3 activation in INS-1 832/13 cells incubated with nifedipine alone under basal conditions. No significant effects of nifedipine were demonstrable on lamin B degradation under these conditions. As shown above, high glucose-treatment markedly enhanced caspase 3 activation (2.5-fold) and lamin B degradation (1.9-fold; Fig. 6). Interestingly, coprovision of nifedipine with glucose markedly reduced high glucose-induced effects on caspase 3 activation (2.5-fold vs. 1.2-fold in the absence and presence of nifedipine, respectively). In a manner akin to these findings, nifedipine also attenuated lamin B degradation induced by glucose (1.9-fold in the absence of nifedipine and 1.2-fold in its presence; Fig. 6).

We next assessed protective effects of nifedipine against high glucose-induced caspase 3 activation and lamin B degradation in normal rodent islets. Further, we examined whether protection of these cellular events by nifedipine is mediated via inhibition of glucose-induced ER stress. To accomplish this, normal rat islets were incubated in the presence of low or high glucose in the absence or presence of nifedipine, and expression of CHOP, caspase 3 activation and lamin B degradation were quantified in cell lysates as described above. Data presented in Table 1 demonstrate a significant increase in the expression of CHOP in rat islets exposed to high glucose, which was inhibited by nifedipine. These data suggest that glucose-mediated effects involve increase in ER stress, which is sensitive to inhibition of calcium channels. In a manner akin to these effects, nifedipine significantly prevented glucose-induced activation of caspase 3 in rat islets. The extent of lamin B degradation reached to basal values in cells incubated simultaneously with high glucose and nifedipine (Table 1). It is important to note that nifedipine, by itself, increased caspase 3 activation and lamin B degradation under low glucose conditions; such effects might have masked its effects on glucose-induced caspase 3 activation and lamin B degradation (Table 1).

Taken together, our findings provide the first direct evidence to suggest that glucotoxic conditions induce ER stress to promote caspase 3 activation and lamin B degradation in INS-1 832/13 cells and normal rodent islets. Our data also suggest significant alterations in subcellular distribution of lamin B and its degradation products in these cells under the duress of glucotoxicity.

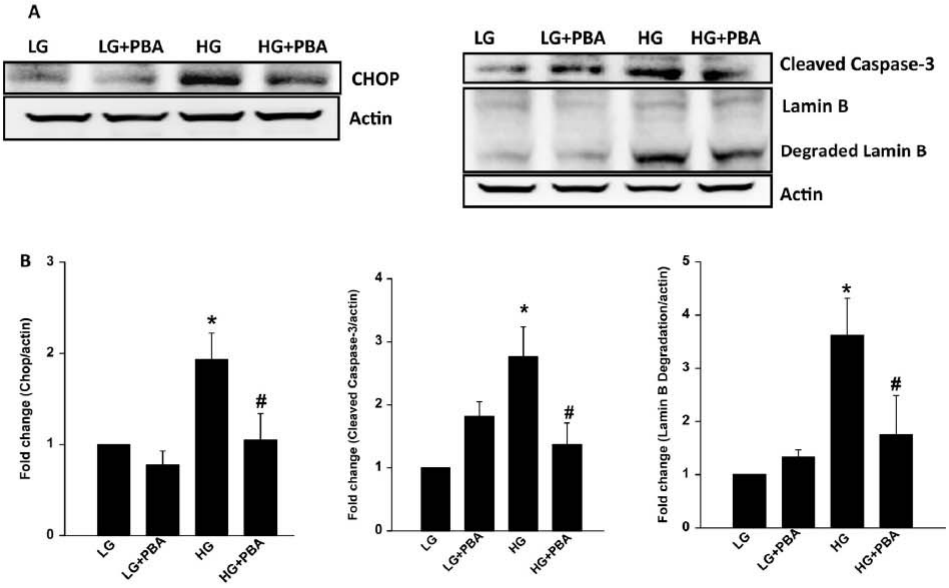


Fig. 5. 4-Phenylbutyric acid (PBA), a known inhibitor of ER-stress, markedly attenuates glucose-induced CHOP expression, caspase 3 activation and lamin B degradation in INS-1 832/13 cells. Panel A: INS-1 832/13 Cells were incubated in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 24 h in the presence and absence of PBA (0.5 mM). Lysate proteins (40 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for CHOP, active caspase 3 and degraded lamin B and immune complexes were identified using ECL detection kit. To check equal protein loading, the membranes were stripped and reprobed for actin. Intensity of protein bands was quantitated by densitometry (Panel B). The statistical significance of the differences between the experimental conditions was determined by ANOVA followed by Student–Newman–Keuls post hoc test. Data represent mean ± SEM from three independent experiments and expressed as fold change in CHOP expression, caspase 3 activation and lamin B degradation. $^{*}p < 0.05$ vs. LG, $^{\#}p < 0.05$ vs. HG.

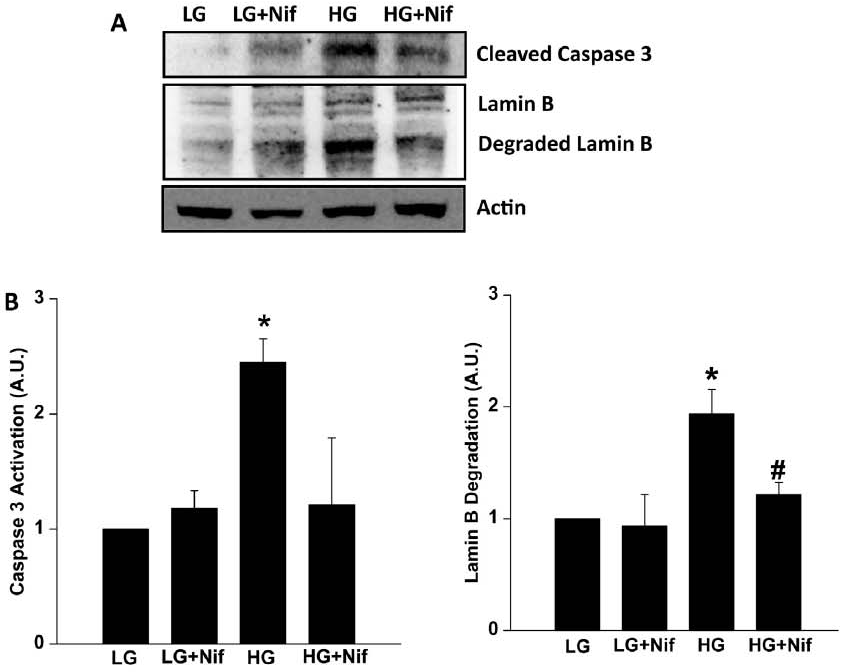


Fig. 6. Nifedipine, a calcium channel blocker, inhibits glucose-induced caspase 3 activation and lamin B degradation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were incubated with low glucose (LG; 2.5 mM) or high glucose (HG; 20 mM) for 24 h in the absence (diluent) or presence of nifedipine (Nif; 10 μM). Lysate proteins (40 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for degraded lamin B and cleaved caspase 3, and immune complexes were identified using ECL detection kit. To check equal protein loading, the membrane for each proteins was stripped and reprobed for actin. Quantitation of caspase 3 activation and lamin B degradation were carried out by densitometry. The statistical significance of the differences between control and the experimental conditions was determined by ANOVA followed by Student–Newman–Keuls post hoc test. Data represent mean ± SEM from three independent experiments and expressed as fold change (Panel B). $^{*}p < 0.05$ vs. LG and $^{\#}p < 0.05$ vs. HG.

4. Discussion

Several lines of evidence in clonal β-cells, normal rodent islets and human islets implicate that chronic exposure of these cells to elevated glucose induces metabolic dysfunction that leads to loss in their viability culminating in apoptosis [14,24–26]. Several mechanisms have been postulated for metabolic dysregulation of the islet including generation of oxidative stress and ER stress culminating in mitochondrial abnormalities [14,27,28]. However, very little is known to date with regard to alterations in the nuclear compartment induced by chronic hyperglycemic conditions. Our findings suggested that: (i) high glucose induces caspase 3 activation, which in turn, degrades nuclear lamin B; (ii) high glucose also induces significant alterations in the subcellular distribution of lamin B; (iii) thapsigargin, a known inducer of ER stress in many cell types, including the islet β-cell, also induces caspase 3 activation and lamin B degradation; (iv) nifedipine, a known inhibitor of plasma membrane-associated calcium channels, markedly attenuates glucose-induced caspase-3 activation and lamin-B degradation; and (v) PBA, a known inhibitor of ER-stress, markedly reduced glucose-induced CHOP expression, caspase 3 activation and lamin B degradation. Based on these findings we propose that nifedipine-sensitive influx and intracellular accumulation of calcium as one of the signaling events leading to glucose-induced metabolic dysfunction of the islet β-cell, leading to the activation of caspase-3 and lamin-B degradation (see below).

Lamins are intermediate filament proteins that constitute the main components of the lamina underlying the inner-nuclear membrane and serve to organize chromatin. Our current findings suggested significant alterations in the subcellular distribution of lamin B in INS-1 832/13 cells under conditions of exposure to high glucose. What then might be the mechanisms underlying abnormal distribution of lamin B that we observed in beta-cells

Table 1
Effect of nifedipine on glucose-induced CHOP expression, caspase 3 activation and lamin B degradation in normal rat islets.

Treatment conditions	CHOP expression	Caspase 3 activation	Lamin-B degradation
Low glucose	1.00	1.00	1.00
Low glucose + nifedipine	1.11 ± 0.16	1.42 ± 0.21	1.33 ± 0.39
High glucose	1.62 ± 0.11*	1.67 ± 0.12*	1.28 ± 0.17
High glucose + nifedipine	1.21 ± 0.10**	0.63 ± 0.31**	0.93 ± 0.19

Rat islets were incubated in the presence of low (2.5 mM) or high (20 mM) glucose for 24 h in the presence and absence of nifedipine (10 μM) as indicated. Lysate proteins (40 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for CHOP, cleaved caspase 3 and degraded lamin B, and immune complexes were identified using ECL detection kit. To check equal protein loading, the membranes were stripped and reprobed for actin. Intensity of protein bands was quantified by densitometry. The statistical significance of the differences between the experimental conditions was determined by *t*-test. Data represent mean ± SEM from three to four independent experiments and expressed as fold change over low glucose exposure condition. $^{*}p < 0.05$ vs. low glucose and $^{**}p < 0.05$ vs. high glucose.

exposed to high glucose conditions? It is well established that in a manner akin to small molecular weight G-proteins and the γ-subunits of trimeric G-proteins, nuclear lamins undergo post-translational farnesylation, catalyzed by the farnesyl transferase (FTase). Along these lines, we have recently reported caspase 3-mediated degradation of the α-subunit of FTase in insulin-secreting cells [22]. It has been shown in other cells that caspase-mediated degradation of FTase results in functional inactivation of the enzyme, which presumably could lead to impaired farnesylation of substrate proteins culminating in mistargeting of these proteins [29]. It is likely that similar alterations in the farnesylation of lamin B might be taking place in the beta-cell under high glucose-treatment conditions. Additional studies are needed to test such a possibility. Along these lines, recent studies by Chang and associates have demonstrated that inhibition of farnesylation using FTIs led to a marked reduction in the biogenesis of mature lamin A culminating in the accumulation of prelamin A intracellularly. Further, their data suggested a critical requirement for a geranylgeranylation signaling step in the conversion of farnesylated lamin A to its mature form mediated by a zinc metalloprotease [30]. Together, these findings implicate novel roles for post-translational prenylation steps in the functional regulation of lamins. It remains to be determined if glucotoxic conditions impair normal processing of lamins due to degradation of prenylating enzymes under glucotoxic conditions, and if so, whether unprenylated lamins are more vulnerable to degradation by caspase 3.

It is also noteworthy that our current observations also suggested regulatory roles for accumulation of cytosolic calcium in glucose-induced effects on caspase 3 activation and lamin B degradation. As described above, our findings are in agreement with observations by Wang et al. [23] who reported cytoprotective effects, by nifedipine, against endoplasmic reticulum stress and apoptosis induced by glucotoxic conditions in pancreatic β-cells. Therefore, it is likely that such conditions could also promote β-cell dysfunction via calcium-mediated mitochondrial dysregulation, caspase 3 activation and lamin B degradation pathways leading to loss in cell viability. This possibility needs to be verified experimentally. Indeed, recent observations by Xu and associates [31] have provided compelling evidence to suggest a marked reduction by verapamil, a calcium channel blocker, of increased expression of pro-apoptotic thioredoxin-interacting protein expression and restoration of normal β-cell survival and function in the BTBR ob/ob mice. Our current findings provide further evidence in support of the formulation that influx of extracellular calcium and subsequent overload of mitochondrial calcium leads to dysregulation of cellular function. Thus, calcium channel blockers such as verapamil [22,23,31] and nifedipine (current study) appear to exert cytoprotective effects in β-cells against noxious stimuli and improve β-cell survival and function.

Based on the published evidence and data accrued in the current study we propose that exposure of isolated β-cells to hyperglycemic conditions leads to increased oxidative and ER stress leading to mitochondrial dysfunction (Fig. 7). Consequentially, cytosolic caspase 3 is activated by cytochrome C released from the dysregulated mitochondria. As demonstrated in the current study, caspase 3 activation leads to degradation of lamin B culminating in nuclear damage and altered distribution of degraded lamin B into various subcellular compartments, and nuclear lamina disassembly and associated loss in cell viability. We also propose that glucose-induced metabolic dysfunction in these cells may, in part, be due to increase in the ER stress as evidenced by significant inhibition of glucose-induced CHOP expression, caspase 3 activation and lamin B degradation by PBA, a known inhibitor of ER stress. Our findings also suggest cytoprotective effects of calcium channel blockers (e.g., nifedipine) against noxious effects of glucose as demonstrated in this study and data from other laboratories [22,23,31]. Lastly, our studies provide further support to the recent observations by Tang and associates establishing clear roles for the oxidative stress-ER stress signaling axis in glucose-induced β-cell dysfunction in vivo in rats [34]

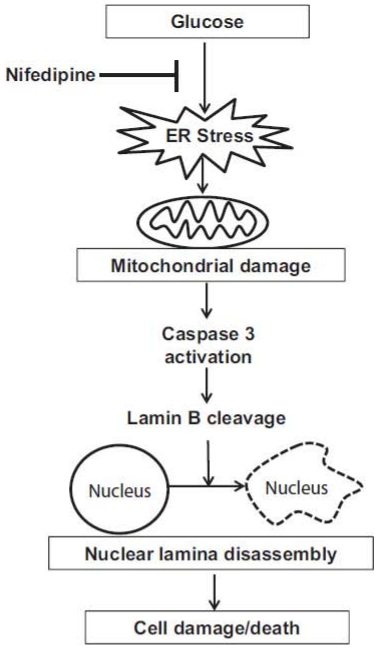


Fig. 7. Proposed model for high glucose-mediated, ER-stress-induced β-cell damage/death via caspase 3 mediated degradation of lamin B in pancreatic β-cells. We propose that exposure of insulin-secreting cells to high glucose leads to increased oxidative and ER stress leading to mitochondrial dysfunction [14,32,33]. As a consequence, cytosolic caspase 3 is activated by the released cytochrome C from the dysregulated mitochondria. As shown in this study, caspase 3 activation leads to degradation of lamin B culminating in nuclear damage and altered distribution of degraded lamin B into various subcellular compartments leading to nuclear lamina disassembly. Our current findings suggest that glucose-induced metabolic dysfunction in these cells may, in part, be due to increase in the ER stress. Our model also highlights potential cytoprotective effects of calcium channel blockers (e.g., nifedipine) against noxious effects of hyperglycemia as demonstrated in this study, and by other recent studies [22,23,31].

Acknowledgments

This research was supported in part by a Merit Review Award (to AK; 1BX000469) from the Department of VA, and the National Institutes of Health (RO1 DK74921). AK is also the recipient of a Senior Research Career Scientist Award from the Department of VA. KS is the recipient of Rumble Fellowship from Wayne State University. AMM is the recipient of Rumble Fellowship from Wayne State University and George Fuller Endowed Fellowship from the Eugene Applebaum College of Pharmacy and Health Sciences. We thank Prof. Chris Newgard for kindly providing INS-1 832/13 cells.

References

[1] Bantel H, Schulze-Osthoff K. Mechanisms of cell death in acute liver failure. Front Physiol 2012;3:79.
[2] Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Eizirik DL. Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes: many differences, few similarities. Diabetes 2005;54:S97–107.

[3] Chandra J, Zhivotovsky B, Zaitsev S, Juntti-Berggren L, Berggren PO, Orrenius S. Role of apoptosis in pancreatic beta-cell death in diabetes. Diabetes 2001;50: S44–7.
[4] Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770–6.
[5] Lazebnik YA, Takahashi A, Moir RD, Goldman RD, Poirier GG, Kaufmann SH, et al. Studies of the lamin proteinase reveal multiple parallel biochemical pathways during apoptotic execution. Proc Natl Acad Sci USA 1995;92:9042–6.
[6] Burke B, Stewart CL. The nuclear lamins: flexibility in function. Nat Rev Mol Cell Biol 2013;14:13–24.
[7] Gruenbaum Y, Wilson KL, Harel A, Goldberg M, Cohen M. Review: nuclear lamins—structural proteins with fundamental functions. J Struct Biol 2000;129: 313–23.
[8] Rao L, Perez D, White E. Lamin proteolysis facilitates nuclear events during apoptosis. J Cell Biol 1996;135:1441–55.
[9] Neamati N, Fernandez A, Wright S, Kiefer J, McConkey DJ. Degradation of lamin B, precedes oligonucleosomal DNA fragmentation in apoptotic thymocytes and isolated thymocyte nuclei. J Immunol 1995;154:3788–95.
[10] Veluthakal R, Amin R, Kowluru A. Interleukin-1 induces posttranslational carboxymethylation and alterations in subnuclear distribution of lamin B in insulin-secreting RINm5F cells. Am J Physiol Cell Physiol 2004;287:C1152–62.
[11] Kivinen K, Kallajoki M, Taimen P. Caspase-3 is required in the apoptotic disintegration of the nuclear matrix. Exp Cell Res 2005;311:62–73.
[12] Almaguel FG, Liu JW, Pacheco FJ, Casiano CA, De Leon M. Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid. J Neurosci Res 2009;87:1207–18.
[13] Campos C. Chronic hyperglycemia and glucose toxicity: pathology and clinical sequelae. Postgrad Med 2012;124:90–7.
[14] Poitout V, Robertson RP. Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev 2008;29:351–66.
[15] Jonas JC, Bensellam M, Duprez J, Elouil H, Guiot Y, Pascal SM. Glucose regulation of islet stress responses and beta-cell failure in type 2 diabetes. Diabetes Obes Metab 2009;11:65–81.
[16] Jayaram B, Syed I, Kyathanahalli CN, Rhodes CJ, Kowluru A. Arf nucleotide binding site opener [ARNO] promotes sequential activation of Arf6, Cdc42 and Rac1 and insulin secretion in INS 832/13 β-cells and rat islets. Biochem Pharmacol 2011;81:1016–27.
[17] Kwon MJ, Chung HS, Yoon CS, Lee EJ, Kim TK, Lee SH, et al. Low glibenclamide concentrations affect endoplasmic reticulum stress in INS-1 cells under glucotoxic or glucolipotoxic conditions. Korean J Intern Med 2013;28:339–46.
[18] Bachar E, Ariav Y, Ketzinel-Gilad M, Cerasi E, Kaiser N, Leibowitz G. Glucose amplifies fatty acid-induced endoplasmic reticulum stress in pancreatic beta-cells via activation of mTORC1. PLoS One 2009;4:e4954.
[19] Qian B, Wang H, Men X, Zhang W, Cai H, Xu S, et al. TRIB3 is implicated in glucotoxicity- and endoplasmic reticulum-stress-induced beta-cell apoptosis. J Endocrinol 2008;199:407–16.
[20] Wang H, Kouri G, Wollheim CB. ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. J Cell Sci 2005;118:3905–15.
[21] Marchetti P, Bugliani M, Lupi R, Marselli L, Masini M, Boggi U, et al. The endoplasmic reticulum in pancreatic beta cells of type 2 diabetes patients. Diabetologia 2007;50:2486–94.
[22] Arora DK, Mohammed AM, Kowluru A. Nifedipine prevents etoposide-induced caspase-3 activation, prenyl transferase degradation and loss in cell viability in pancreatic β-cells. Apoptosis 2013;18:1–8.
[23] Wang Y, Gao L, Li Y, Chen H, Sun Z. Nifedipine protects INS-1 β-cell from high glucose-induced ER stress and apoptosis. Int J Mol Sci 2011;12:7569–80.
[24] Kim WH, Lee JW, Suh YH, Hong SH, Choi JS, Lim JH, et al. Exposure to chronic high glucose induces beta-cell apoptosis through decreased interaction of glucokinase with mitochondria: downregulation of glucokinase in pancreatic beta-cells. Diabetes 2005;54:2602–11.
[25] Federici M, Hribal M, Perego L, Ranalli M, Caradonna Z, Perego C, et al. High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. Diabetes 2001;50:1290–301.
[26] Piro S, Anello M, Di Pietro C, Lizzio MN, Patanè G, Rabuazzo AM, et al. Chronic exposure to free fatty acids or high glucose induces apoptosis in rat pancreatic islets: possible role of oxidative stress. Metabolism 2002;51:1340–7.
[27] Leem J, Koh EH. Interaction between mitochondria and the endoplasmic reticulum: implications for the pathogenesis of type 2 diabetes mellitus. Exp Diabetes Res 2012;2012:242984.
[28] Supale S, Li N, Brun T, Maechler P. Mitochondrial dysfunction in pancreatic β cells. Trends Endocrinol Metab 2012;23:477–87.
[29] Kim KW, Chung HH, Chung CW, Kim IK, Miura M, Wang S, et al. Inactivation of farnesyltransferase and geranylgeranyltransferase I by caspase-3: cleavage of the common alpha subunit during apoptosis. Oncogene 2001;20: 358–66.
[30] Chang SY, Hudon-Miller SE, Yang SH, Jung HJ, Lee JM, Farber E, et al. Inhibitors of protein geranylgeranyltransferase-I lead to prelamin A accumulation in cells by inhibiting ZMPSTE24. J Lipid Res 2012;53:1176–82.
[31] Xu G, Chen J, Jing G, Shalev A. Preventing β-cell loss and diabetes with calcium channel blockers. Diabetes 2012;6:848–56.
[32] Syed I, Kyathanahalli CN, Jayaram B, Govind S, Rhodes CJ, Kowluru RA, et al. Increased phogocyte-like NADPH oxidase and ROS generation in type 2 diabetic ZDF rat and human islets: role of Rac1-JNK1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. Diabetes 2011;60: 2843–52.
[33] Kowluru A. Small G proteins in islet beta-cell function. Endocr Rev 2010;31: 52–78.
[34] Tang C, Koulajian K, Schuiki I, Zhang L, Desai T, Ivovic A, et al. Glucose-induced beta cell dysfunction in in vivo in rats: link between oxidative stress and endoplasmic reticulum stress. Diabetologia 2012;55:1366–79.

APPENDIX B

Apoptosis
DOI 10.1007/s10495-014-1038-4

ORIGINAL PAPER

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

Syeda Khadija · Rajakrishnan Veluthakal · Vaibhav Sidarala · Anjaneyulu Kowluru

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 h) and diabetic conditions. Under glucotoxic conditions, glucose-stimulated insulin secretion 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

Abbreviations

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 metallopeptidase (STE24 homolog)
Z-VEID-FMK	Caspase 6 inhibitor (Z-Val-Glu-Ile-Asp-fluoromethylketone)

S. Khadija · R. Veluthakal · V. Sidarala · A. Kowluru (✉)
B-4237 Research Service, β-Cell Biochemistry Laboratory, John D. Dingell VA Medical Center, 4646 John R, Detroit, MI 48201, USA
e-mail: akowluru@med.wayne.edu

S. Khadija · R. Veluthakal · V. Sidarala · A. Kowluru
Department of Pharmaceutical Sciences, Eugene Applebaum College of Pharmacy and Health Sciences, Wayne State University, Detroit, MI 48201, USA

Introduction

The nucleus is a complex subcellular organelle and represents the center of cellular events during the cell cycle

Published online: 08 October 2014

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 μM 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 h as indicated in the text. For the studies involving inhibitors, cells were pre-incubated for 1 h, in the presence and absence of caspase inhibitors, Z-DEVD-FMK (Caspase 3 inhibitor; 3 μM) and Z-VEID-FMK (Caspase 6 inhibitor; 3 μM) 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 h. 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 h) 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 h 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 h, the cells were incubated with KRB buffer for 1 h, 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 h 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 electro transferred 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 h and washed 5× for 5 min each with TBS-T. The membrane was then incubated with corresponding secondary antibodies conjugated to horseradish peroxidase (1:1,000) in 5 % non-fat dry milk in TBS-T at room temperature for 1 h. 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 h; referred to as glucotoxic conditions throughout) on GSIS in INS-1 832/13 cells. Data in Fig. 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 h incubation with high glucose [21]. Additional studies have suggested a 13 and 19 % reduction in metabolic cell viability in these

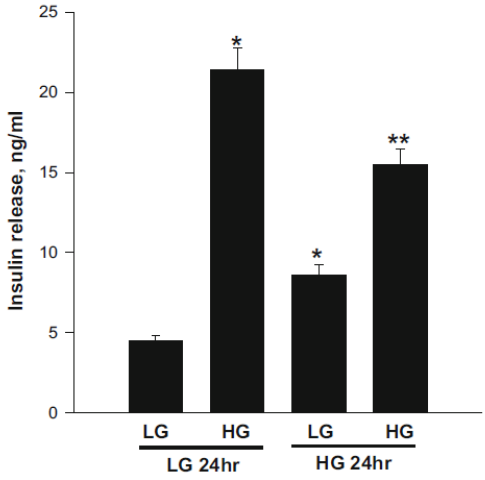


Fig. 1 Glucotoxic conditions attenuate GSIS in INS-1 832/13 β-cells. INS-1 832/13 cells were cultured in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 24 h following which they were stimulated with low (2.5 mM) or high (20 mM) glucose for 45 min. Insulin released into the medium was quantified by ELISA (see "Materials and methods" section for additional details). The data are expressed as insulin release (ng/ml) and are mean ± SEM from three independent experiments. *$P < 0.05$ vs. LG under 24 h low glucose treatment; **$P < 0.05$ vs. HG under 24 h low glucose treatment

cells following exposure to glucotoxic conditions at 24 and 48 h, respectively (n = 2 independent studies; additional data not shown). Together, these data indicate significant impairment in GSIS even at 24 h 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 Fig. 2a 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 (Fig. 3a–c), human islets (Fig. 4a) and in islets from a human donor with T2D (Fig. 4b) confirmed our observations in INS-1 832/13 cells. Together, these findings (Figs. 2, 3, 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 Fig. 5a 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 Figs. 2, 3, 4 and 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 Fig. 6a 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 (Fig. 6a). However, data from multiple experiments indicated that this increase was not significant (Fig. 6b). Furthermore, Z-VEID-FMK inhibited high glucose-induced degradation of lamin A under the conditions it inhibited caspase 6 (Fig. 6c); these

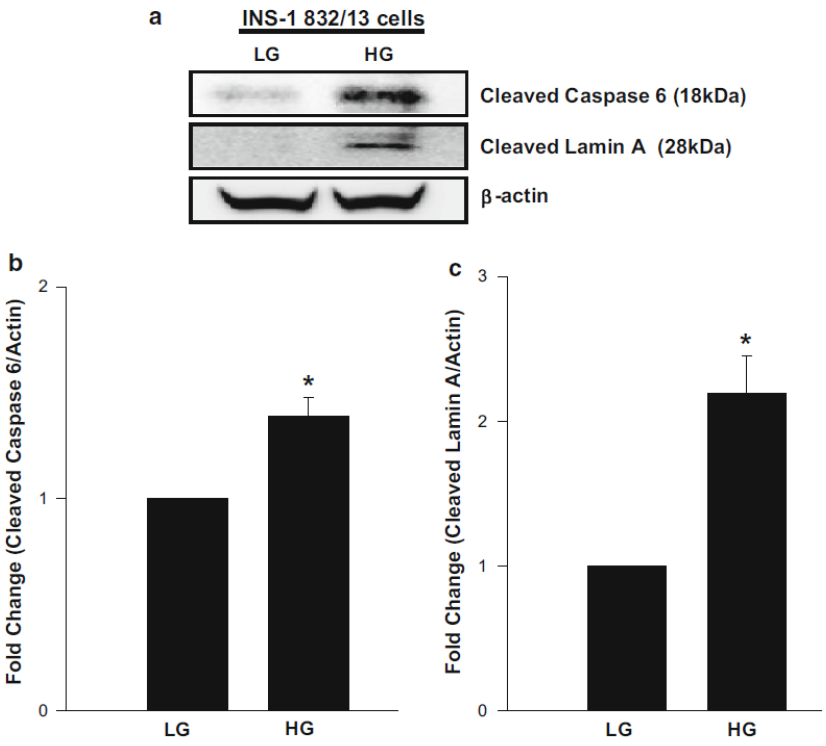


Fig. 2 High glucose treatment induces caspase 6 activation and lamin A cleavage in INS-1 832/13 cells. INS-1 832/13 cells were incubated in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 24 h. Caspase 6 activation and lamin A cleavage were determined by Western blotting. Protein lysates (40 μg) 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 (**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 ± SEM from three independent experiments and expressed as fold change in caspase 6 (**b**) and lamin A cleavage (**c**). *$P < 0.05$ vs. LG

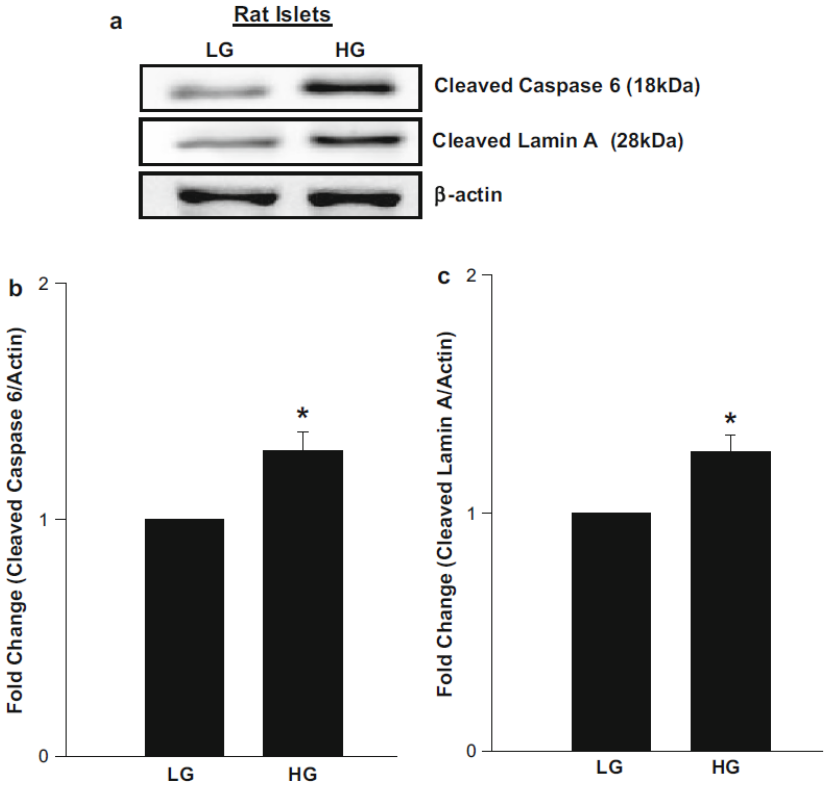


Fig. 3 High glucose treatment results in caspase 6 activation and lamin A cleavage in normal rat islets. Rat islets were incubated in the presence of low (2.5 mM; LG) and high glucose (20 mM; HG) for 24 h. Approximately 40 μg protein lysate was resolved by SDS-PAGE and the degree of abundance of cleaved caspase 6 and lamin A were determined by Western blot analysis. Immune complexes were identified using ECL detection kit (**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 ± SEM from three independent experiments and are expressed as fold change in caspase 6 (**b**) and lamin A (**c**) cleavage. *$P < 0.05$ vs. LG

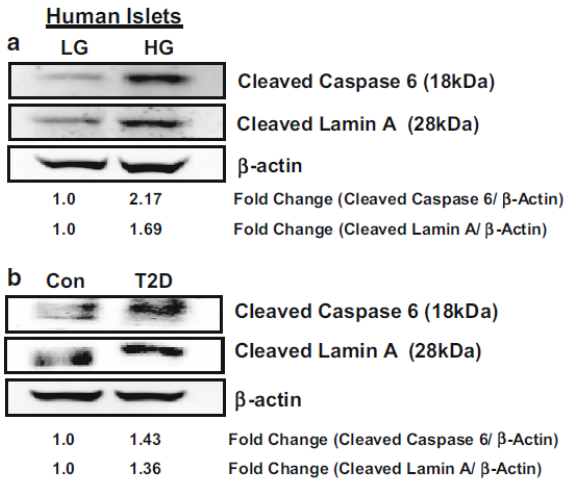


Fig. 4 Glucotoxic conditions promote caspase 6 activation and lamin A cleavage in normal human islets treated with high glucose and in diabetic human islets. Normal human islets were incubated in the presence of low (5.8 mM; LG) and high glucose (30 mM; HG) for 24 h as described in the text (**a**). Islets obtained from normal and diabetic individuals were lysed using RIPA buffer (**b**). Lysate proteins (25 µg) 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

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 Fig. 6d. 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 Fig. 7a, 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 (Fig. 7c), 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 Fig. 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" section).

Discussion

One of the objectives of the current study was to investigate regulatory roles of caspase 6 on nuclear lamin A

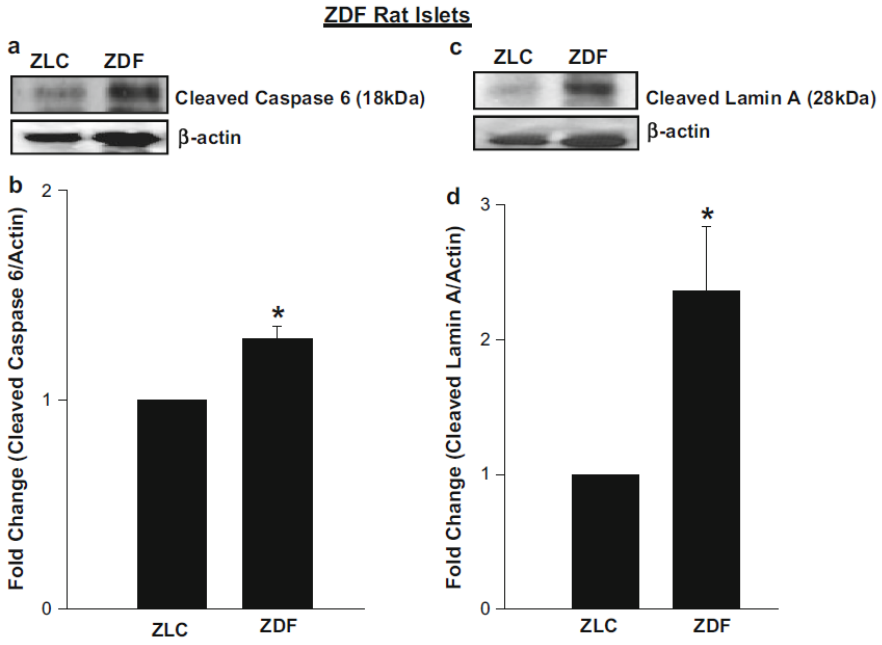


Fig. 5 Activation of caspase 6 and lamin A degradation are observed in ZDF rat islets. Islets isolated from ZDF and ZLC rats by collagenase digestion method were lysed using RIPA buffer. Lysate proteins (40 μg) were resolved by SDS-PAGE and the abundance of cleaved caspase 6 and lamin A was determined 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 **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 (**b**) and lamin A cleavage (**d**). $*P < 0.05$ vs. ZLC

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-1β, TNFα and IFNγ) 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-1β 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-1β-treated cells [26].

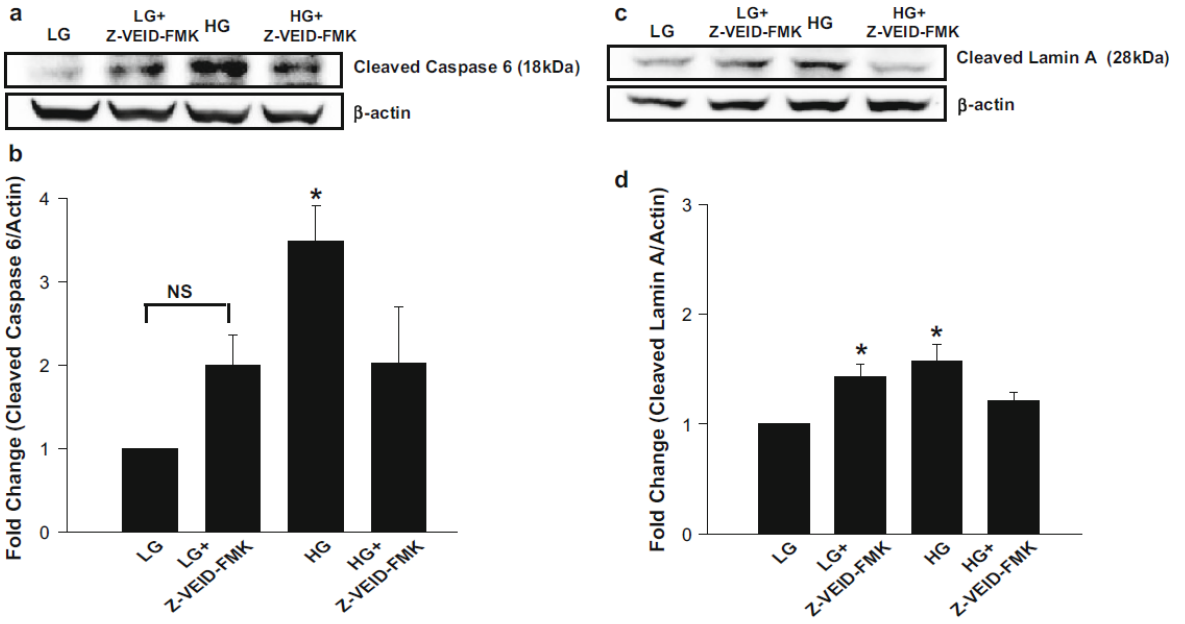


Fig. 6 High glucose-induced caspase 6 activation and breakdown of lamin A cleavage were attenuated by Z-VEID-FMK, a specific inhibitor of caspase 6, in INS-1 832/13 cells. INS-1 832/13 cells were preincubated with Z-VEID-FMK (3 μM) for 1 h and further treated with low (2.5 mM; LG) or high (20 mM; HG) glucose in the presence and absence of Z-VEID-FMK for 6 h. Caspase 6 activation and lamin A cleavage were determined by Western blotting. Protein lysates (40 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved caspase 6 (**a**) and cleaved lamin A (**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 ± SEM from three independent experiments and expressed as fold change in caspase 6 (**b**) and lamin A cleavage (**d**). $*P < 0.05$ vs. LG. *NS* not significant vs. LG

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-1β, followed by NFκB 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

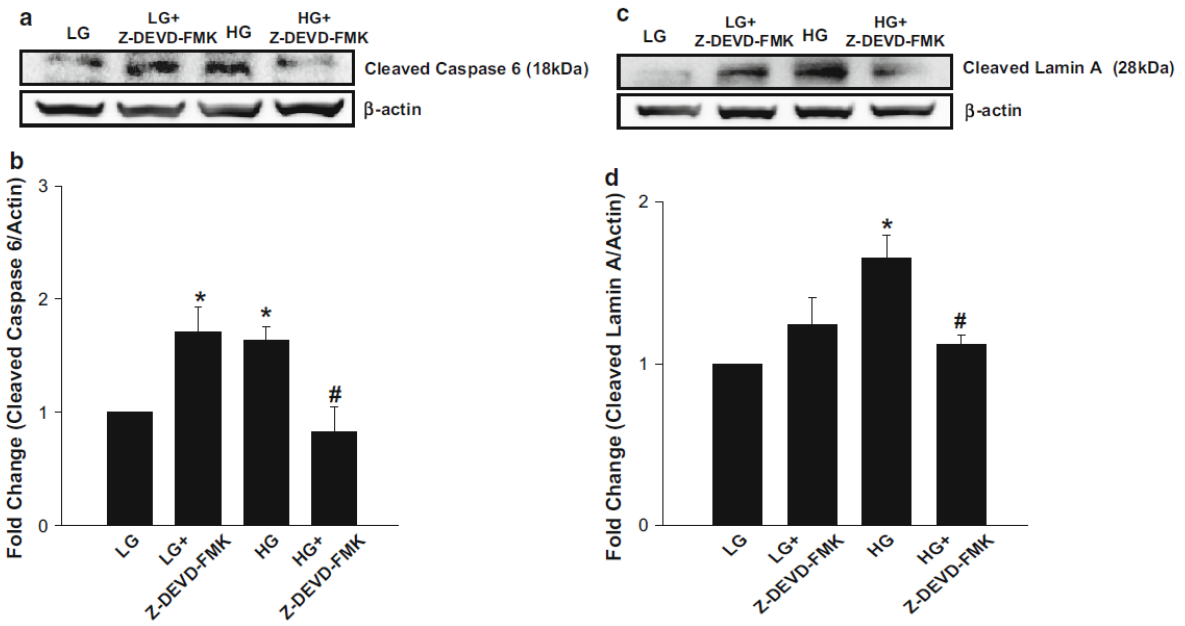


Fig. 7 Z-DEVD-FMK, a known inhibitor of Caspase 3, prevented high glucose induced caspase 6 activation and lamin A breakdown in INS-1 832/13 cells. INS-1 832/13 cells were preincubated with Z-DEVD-FMK (3 μM) for 1 h and further treated with low (2.5 mM; LG) or high (20 mM; HG) glucose in the presence and absence of Z-DEVD-FMK for 6 h. Caspase 6 activation (**a**) and lamin A cleavage (**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 ± SEM from three independent experiments and expressed as fold change in caspase 6 (**b**) and lamin A cleavage (**d**). $^{*}P < 0.05$ vs. LG; $^{\#}P < 0.05$ vs. HG

against an experimental model of cell death [42]. Furthermore, murine βTC-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 knock-down 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 et al. 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.

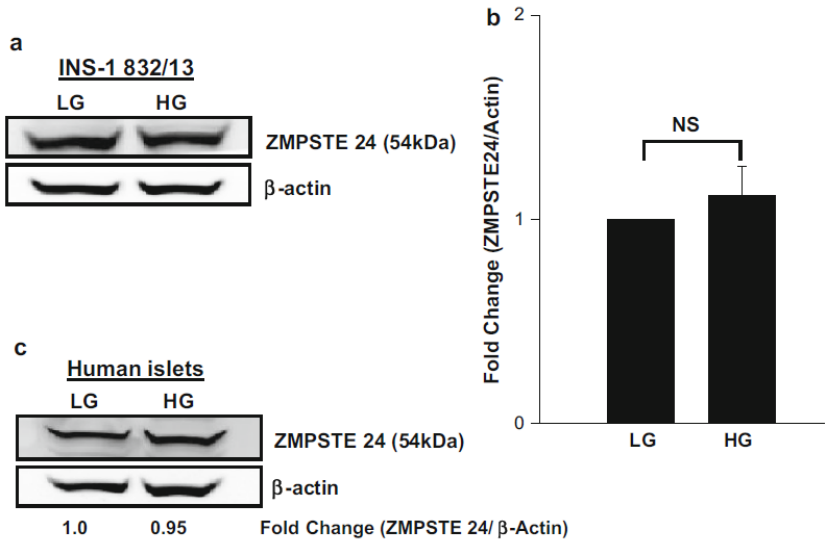


Fig. 8 Immunological evidence to indicate expression of lamin A processing protease (ZMPSTE 24) in INS-1 832/13 cells and normal human islets: Lack of effects of high glucose exposure on the expression of ZMPSTE 24. INS-1 832/13 cells were incubated in the presence of low (2.5 mM; LG) or high (20 m M; HG) glucose for 24 h (as in Fig. 2). Protein lysates (40 μg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. A representative blot from three studies is shown in **a**. Intensity of protein bands was quantified by densitometry and data represent mean ± SEM from three independent experiments and are expressed as fold change in ZMPSTE 24 (**b**). *NS* not significant vs. LG. Normal human islets were incubated in the presence of low (5.8 mM; LG) and high glucose (30 mM; HG) for 24 h (as in Fig. 4a). 25 μg 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 **c**

Acknowledgments This research was supported in part by a Merit Review award (to AK; 1BX000469) from the Department of Veterans Affairs, the National Institutes of Health (DK94201 and EY022230), the Juvenile Diabetes Research Foundation (5-2012-257), and Research Stimulation Funds from the Office of Vice President for Research-Wayne State University. AK is also the recipient of a Senior Research Career Scientist Award from the Department of VA (13S-RCS-006). KS is the recipient of Rumble Fellowship from Wayne State University. We thank Prof. Chris Newgard for providing INS-1 832/13 cells.

Conflict of interest The authors declare no conflict of interests.

References

1. Gruenbaum Y, Wilson KL, Harel A, Goldberg M, Cohen M (2000) Review: nuclear lamins–structural proteins with fundamental functions. J Struct Biol 129:313–323
2. Moir RD, Spann TP (2001) The structure and function of nuclear lamins: implications for disease. Cell Mol Life Sci 58:1748–1757
3. Moir RD, Spann TP, Goldman RD (1995) The dynamic properties and possible functions of nuclear lamins. Int Rev Cytol 162:141–182
4. Moir RD, Spann TP, Herrmann H, Goldman RD (2000) Disruption of nuclear lamin organization blocks the elongation phase of DNA replication. J Cell Biol 149:1179–1192
5. Shahzidi S, Brech A, Sioud M, Li X, Suo Z, Nesland JM, Peng Q (2013) Lamin A/C cleavage by caspase-6 activation is crucial for apoptotic induction by photodynamic therapy with hexaminolevulinate in human B-cell lymphoma cells. Cancer Lett 339:25–32
6. Attur M, Ben-Artzi A, Yang Q, Al-Mussawir HE, Worman HJ, Palmer G, Abramson SB (2012) Perturbation of nuclear lamin A causes cell death in chondrocytes. Arthritis Rheum 64:1940–1949
7. Chang KH, Multani PS, Sun KH, Vincent F, de Pablo Y, Ghosh S, Gupta R, Lee HP, Lee HG, Smith MA, Shah K (2011) Nuclear envelope dispersion triggered by deregulated Cdk5 precedes neuronal death. Mol Biol Cell 22:1452–1462
8. Yeo HS, Shehzad A, Lee YS (2012) Prostaglandin E2 blocks menadione-induced apoptosis through the Ras/Raf/Erk signaling pathway in promonocytic leukemia cell lines. Mol Cells 33:371–378
9. Martínez-Poveda B, Rodríguez-Nieto S, García-Caballero M, Medina MÁ, Quesada AR (2012) The antiangiogenic compound aeroplysinin-1 induces apoptosis in endothelial cells by activating the mitochondrial pathway. Mar Drugs 10:2033–2046
10. Burke B (2001) Lamins and apoptosis: a two-way street? J Cell Biol 153:F5–F7
11. Syeda K, Mohammed AM, Arora DK, Kowluru A (2013) Glucotoxic conditions induce endoplasmic reticulum stress to cause caspase 3 mediated lamin B degradation in pancreatic β-cells: protection by nifedipine. Biochem Pharmacol 86:1338–1346
12. Lenin R, Maria MS, Agrawal M, Balasubramanyam J, Mohan V, Balasubramanyam M (2012) Amelioration of glucolipotoxicity-induced endoplasmic reticulum stress by a "chemical chaperone" in human THP-1 monocytes. Exp Diabetes Res. doi:10.1155/2012/356487
13. Jing G, Wang JJ, Zhang SX (2012) ER stress and apoptosis: a new mechanism for retinal cell death. Exp Diabetes Res. doi:10.1155/2012/589589
14. Roy S, Trudeau K, Roy S, Tien T, Barrette KF (2013) Mitochondrial dysfunction and endoplasmic reticulum stress in

diabetic retinopathy: mechanistic insights into high glucose-induced retinal cell death. Curr Clin Pharmacol 8:278–284
15. Newsholme P, Haber EP, Hirabara SM, Rebelato EL, Procopio J, Morgan D, Oliveira-Emilio HC, Carpinelli AR, Curi R (2007) Diabetes associated cell stress and dysfunction: role of mitochondrial and non-mitochondrial ROS production and activity. J Physiol 583:9–24
16. Lim S, Rashid MA, Jang M, Kim Y, Won H, Lee J, Woo JT, Kim YS, Murphy MP, Ali L, Ha J, Kim SS (2011) Mitochondria-targeted antioxidants protect pancreatic β-cells against oxidative stress and improve insulin secretion in glucotoxicity and glucolipotoxicity. Cell Physiol Biochem 28:873–886
17. Veluthakal R, Palanivel R, Zhao Y, McDonald P, Gruber S, Kowluru A (2005) Ceramide induces mitochondrial abnormalities in insulin-secreting INS-1 cells: potential mechanisms underlying ceramide-mediated metabolic dysfunction of the beta cell. Apoptosis 10:841–850
18. Inoue S, Browne G, Melino G, Cohen GM (2009) Ordering of caspases in cells undergoing apoptosis by the intrinsic pathway. Cell Death Differ 16:1053–1061
19. Seki K, Yoshikawa H, Shiiki K, Hamada Y, Akamatsu N, Tasaka K (2000) Cisplatin (CDDP) specifically induces apoptosis via sequential activation of caspase-8, -3 and -6 in osteosarcoma. Cancer Chemother Pharmacol 45:199–206
20. Jayaram B, Kowluru A (2012) Phagocytic NADPH oxidase links ARNO-Arf6 signaling pathway in glucose-stimulated insulin secretion from the pancreatic β-cell. Cell Physiol Biochem 30:1351–1362
21. Arora DK, Machhadieh B, Matti A, Wadzinski BE, Ramanadham S, Kowluru A (2014) High glucose exposure promotes activation of protein phosphatase 2A in rodent islets and INS-1 832/13 β-cells by increasing the posttranslational carboxylmethylation of its catalytic subunit. Endocrinology 155:380–391
22. Slee EA, Adrain C, Martin SJ (2001) Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem 276:7320–7326
23. Simon DJ, Weimer RM, McLaughlin T, Kallop D, Stanger K, Yang J, O'Leary DD, Hannoush RN, Tessier-Lavigne M (2012) A caspase cascade regulating developmental axon degeneration. J Neurosci 32:17540–17553
24. Okinaga T, Kasai H, Tsujisawa T, Nishihara T (2007) Role of caspases in cleavage of lamin A/C and PARP during apoptosis in macrophages infected with a periodontopathic bacterium. J Med Microbiol 56:1399–1404
25. Zhao H, Zhao W, Lok K, Wang Z, Yin M (2014) A synergic role of caspase-6 and caspase-3 in Tau truncation at D421 induced by H_2O_2. Cell Mol Neurobiol 34:369–378
26. Veluthakal R, Amin R, Kowluru A (2004) Interleukin-1 induces posttranslational carboxymethylation and alterations in subnuclear distribution of lamin B in insulin-secreting RINm5F cells. Am J Physiol Cell Physiol 287:1152–1162
27. Veluthakal R, Wadzinski BE, Kowluru A (2006) Localization of a nuclear serine/threonine protein phosphatase in insulin-secreting INS-1 cells: potential regulation by IL-1beta. Apoptosis 11:1401–1411
28. Reddy S, Comai L (2012) Lamin A, farnesylation and aging. Exp Cell Res 318:1–7
29. Burke B, Stewart CL (2013) The nuclear lamins: flexibility in function. Nat Rev Mol Cell Biol 14:13–24
30. Dechat T, Adam SA, Taimen P, Shimi T, Goldman RD (2010) Nuclear lamins. Cold Spring Harb Perspect Biol. doi:10.1101/cshperspect.a000547
31. Kowluru A (2000) Evidence for the carboxyl methylation of nuclear lamin-B in the pancreatic beta cell. Biochem Biophys Res Commun 268:249–254
32. Poitout V, Robertson RP (2008) Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev 29:351–366
33. Syed I, Kyathanahalli CN, Jayaram B, Govind S, Rhodes CJ, Kowluru RA, Kowluru A (2011) Increased phagocyte-like NADPH oxidase and ROS generation in type 2 diabetic ZDF rat and human islets: role of Rac1-JNK1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. Diabetes 60:2843–2852
34. Mohammed AM, Syeda K, Hadden T, Kowluru A (2013) Upregulation of phagocyte-like NADPH oxidase by cytokines in pancreatic beta-cells: attenuation of oxidative and nitrosative stress by 2-bromopalmitate. Biochem Pharmacol 85:109–114
35. Eizirik DL, Miani M, Cardozo AK (2013) Signalling danger: endoplasmic reticulum stress and the unfolded protein response in pancreatic islet inflammation. Diabetologia 56:234–241
36. Wang H, Kouri G, Wollheim CB (2005) ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. J Cell Sci 118:3905–3915
37. Jonas JC, Bensellam M, Duprez J, Elouil H, Guiot Y, Pascal SM (2009) Glucose regulation of islet stress responses and beta-cell failure in type 2 diabetes. Diabetes Obes Metab 11:65–81
38. Lei X, Zhang S, Bohrer A, Barbour SE, Ramanadham S (2012) Role of calcium-independent phospholipase A(2)β in human pancreatic islet β-cell apoptosis. Am J Physiol Endocrinol Metab 303:1386–1395
39. Maedler K, Sergeev P, Ris F, Oberholzer J, Joller-Jemelka HI, Spinas GA, Kaiser N, Halban PA, Donath MY (2002) Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. J Clin Invest 110:851–860
40. Donath MY, Ehses JA, Maedler K, Schumann DM, Ellingsgaard H, Eppler E, Reinecke M (2005) Mechanisms of beta-cell death in type 2 diabetes. Diabetes 54:S108–S113
41. Boni-Schnetzler M, Thorne J, Parnaud G, Marselli L, Ehses JA, Kerr-Conte J, Pattou F, Halban PA, Weir GC, Donath MY (2008) Increased interleukin (IL)-1beta messenger ribonucleic acid expression in beta-cells of individuals with Type 2 diabetes and regulation of IL-1beta in human islets by glucose and autostimulation. J Clin Endocrinol Metab 93:4065–4074
42. Huo J, Luo RH, Metz SA, Li G (2002) Activation of caspase-2 mediates the apoptosis induced by GTP-depletion in insulin-secreting (HIT-T15) cells. Endocrinology 143:1695–1704
43. Yamada K, Ichikawa F, Ishiyama-Shigemoto S, Yuan X, Nonaka K (1999) Essential role of caspase-3 in apoptosis of mouse beta-cells transfected with human Fas. Diabetes 48:478–483
44. Hirota N, Otabe S, Nakayama H, Yuan X, Yamada K (2006) Sequential activation of caspases and synergistic beta-cell cytotoxicity by palmitate and anti-Fas antibodies. Life Sci 79:1312–1316
45. Di Matola T, D'Ascoli F, Luongo C, Bifulco M, Rossi G, Fenzi G, Vitale M (2001) Lovastatin-induced apoptosis in thyroid cells: involvement of cytochrome c and lamin B. Eur J Endocrinol 145:645–650
46. Chang SY, Hudon-Miller SE, Yang SH, Jung HJ, Lee JM, Farber E, Subramanian T, Andres DA, Spielmann HP, Hrycyna CA, Young SG, Fong LG (2012) Inhibitors of protein geranylgeranyltransferase-I lead to prelamin A accumulation in cells by inhibiting ZMPSTE24. J Lipid Res 53:1176–1182

REFERENCES

1. Lin Y, Sun Z. Current views on type 2 diabetes. J Endocrinol. 2010;204(1):1-11.
2. Kulkarni RN. The islet beta-cell. The Interrnational Journal of Biochemistry and Cell Biology. 2004;36(3):365-71.
3. Brissova M, Fowler MJ, Nicholson WE, Chu A, Hirshberg B, Harlan DM, Powers AC. Assessment of human pancreatic islet architecture and composition by laser scanning confocal microscopy. Journal of Histochemistry and Cytochemistry. 2005; 53(9):1087-97.
4. Zhuo Fu, Elizabeth R. Gilbert, and Dongmin Liu. Regulation of Insulin Synthesis and Secretion and Pancreatic Beta-Cell Dysfunction in Diabetes. Curr Diabetes Rev. 2013; 9(1): 25–53.
5. Layden, B. T., Durai, V. and Lowe, Jr., W. L. G-Protein-Coupled Receptors, Pancreatic Islets, and Diabetes. *Nature Education.* 2010; 3(9):13.
6. Seino S, Shibasaki T, Minami K. Dynamics of insulin secretion and the clinical implications for obesity and diabetes. *The Journal of Clinical Investigation*. 2011;121(6):2118-2125.
7. Ferrannini E, Mari A. β-Cell function in type 2 diabetes. Metabolism. 2014;63(10):1217-27.
8. Cersosimo E, Solis-Herrera C, Trautmann ME, Malloy J, Triplitt CL. Assessment of pancreatic β-cell function: review of methods and clinical applications. Curr Diabetes Rev. 2014;10(1):2-42.
9. Wang YW, Sun GD, Sun J, Liu SJ, Wang J, Xu XH, Miao LN. Spontaneous type 2 diabetic rodent models. J Diabetes Res. 2013;2013:401723.

10. Srinivasan K, Ramarao P. Animal models in type 2 diabetes research: an overview. Indian J Med Res. 2007;125(3):451-72.

11. King AJ. The use of animal models in diabetes research. Br J Pharmacol. 2012;166(3):877-94.

12. Burke B, Stewart CL. Life at the edge: the nuclear envelope and human disease. Nat. Rev. Mol. Cell. Biol 2002; 3: 575–85

13. Polychronidou M1, Grobhans J. Determining nuclear shape: the role of farnesylated nuclear membrane proteins. Nucleus 2011; 2(1): 17-23.

14. Harr JC, Luperchio TR, Wong X, Cohen E, Wheelan SJ, Reddy KL. Directed targeting of chromatin to the nuclear lamina is mediated by chromatin state and A-type lamins. J Cell Biol 2015; 208(1): 33-52.

15. Butin-Israeli V, Adam SA, Jain N, Otte GL, Neems D, Wiesmüller L, *et al.*. Role of lamin b1 in chromatin instability. Mol Cell Biol 2015; 35(5): 884-98.

16. Butin-Israeli V, Adam SA, Goldman RD. Regulation of nucleotide excision repair by nuclear lamin b1. PLoS One 2013; 8(7): e69169.

17. Ho CY, Lammerding J. Lamins at a glance. J Cell Sci 2012; 125(Pt 9): 2087-93.

18. Furukawa K, Hotta Y. cDNA cloning of a germ cell specific lamin B3 from mouse spermatocytes and analysis of its function by ectopic expression in somatic cells. EMBO J 1993; 12(1): 97-106.

19. Stuurman N, Heins S, Aebi U. Nuclear lamins: their structure, assembly, and interactions. J Struct Biol 1998; 122(1-2): 42-66.

20. Dechat T, Adam SA, Taimen P, Shimi T, Goldman RD. Nuclear lamins. Cold Spring Harb Perspect Biol 2010; 2(11): a000547.

21. Burke B, Stewart CL. The nuclear lamins: flexibility in function. Nat Rev Mol Cell Biol 2013; 14(1): 13-24.

22. Aebi U, Cohn J, Buhle L, Gerace L. The nuclear lamina is a meshwork of intermediate-type filaments. Nature 1986; 323(6088): 560-4.

23. Goldman AE, Maul G, Steinert PM, Yang HY, Goldman RD. Keratin-like proteins that coisolate with intermediate filaments of BHK-21 cells are nuclear lamins. Proc Natl Acad Sci U S A 1986; 83(11): 3839-43.

24. Gerace L, Burke B. Functional organization of the nuclear envelope. Annu Rev Cell Biol 1988; 4: 335-74.

25. Lammerding J, Fong LG, Ji JY, Reue K, Stewart CL, Young SG, Lee RT. Lamins A and C but not lamin B1 regulate nuclear mechanics. J Biol Chem. 2006;281(35):25768-80.

26. Panorchan P, Schafer BW, Wirtz D, Tseng Y. Nuclear envelope breakdown requires overcoming the mechanical integrity of the nuclear lamina. J Biol Chem 2004; 279(42): 43462-7.

27. Taniura H, Glass C, Gerace L. A chromatin binding site in the tail domain of nuclear lamins that interacts with core histones. J Cell Biol 1995; 131(1): 33-44.

28. Sullivan T, Escalante-Alcalde D, Bhatt H, Anver M, Bhat N, Nagashima K*, et al.*. Loss of A-type lamin expression compromises nuclear envelope integrity leading to muscular dystrophy. J Cell Biol 1999; 147(5): 913-20.

29. Nikolova V, Leimena C, McMahon AC, Tan JC, Chandar S, Jogia D*, et al.*. Defects in nuclear structure and function promote dilated cardiomyopathy in lamin A/C-deficient mice. Clin Invest 2004; 113(3): 357-69.

30. Goldman RD, Shumaker DK, Erdos MR, Eriksson M, Goldman AE, Gordon LB*, et al.*. Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. Proc Natl Acad Sci U S A 2004; 101(24): 8963-8.

31. Moir RD, Montag-Lowy M, Goldman RD. Dynamic properties of nuclear lamins: lamin B is associated with sites of DNA replication.J Cell Biol 1994; 125(6): 1201-12.

32. Forbes DJ, Kirschner MW, Newport JW. Spontaneous formation of nucleus-like structures around bacteriophage DNA microinjected into Xenopus eggs. Cell 1983; 34(1): 13-23.

33. Meier J, Campbell KH, Ford CC, Stick R, Hutchison CJ. The role of lamin LIII in nuclear assembly and DNA replication, in cell-free extracts of Xenopus eggs. J Cell Sci 1991; 98 (Pt 3): 271-9.

34. Ellis DJ, Jenkins H, Whitfield WG, Hutchison CJ. GST-lamin fusion proteins act as dominant negative mutants in Xenopus egg extract and reveal the function of the lamina in DNA replication. J Cell Sci 1997; 110 (Pt 20): 2507-18.

35. Spann TP, Goldman AE, Wang C, Huang S, Goldman RD. Alteration of nuclear lamin organization inhibits RNA polymerase II-dependent transcription. J Cell Biol 2002; 156(4):603-8.

36. Kowluru A. Protein prenylation in glucose-induced insulin secretion from the pancreatic islet beta cell: a perspective. J Cell Mol Med 2008; 12(1): 164-73.

37. Amaya M, Baranova A, van Hoek ML. Protein prenylation: a new mode of host-pathogen interaction. Biochem Biophys Res Commun 2011; 416(1-2): 1-6.

38. Manolaridis I, Kulkarni K, Dodd RB, Ogasawara S, Zhang Z, Bineva G*, et al.*. Mechanism of farnesylated CAAX protein processing by
the intramembrane protease Rce1. Nature 2013; 504(7479): 301-5.

39. Barrowman J, Hamblet C, Kane MS, Michaelis S. Requirements for efficient proteolytic cleavage of prelamin A by ZMPSTE24. PLoS One. 2012;7(2):e32120.

40. Reddy S, Comai L. Lamin A, farnesylation and aging. Exp Cell Res 2012; 318(1): 1-7.

41. Jung HJ, Nobumori C, Goulbourne CN, Tu Y, Lee JM, Tatar A*, et al.*. Farnesylation of lamin B1 is important for retention of nuclear chromatin during neuronal migration. Proc Natl Acad Sci U S A 2013; 110(21): E1923-32.

42. Corrigan DP, Kuszczak D, Rusinol AE, Thewke DP, Hrycyna CA, Michaelis S*, et al.*. Prelamin A endoproteolytic processing in vitro by recombinant Zmpste24. Biochem J 2005; 387(Pt 1): 129-38.

43. Boyartchuk V.L, Ashby MN, Rine J. Modulation of Ras and a-factor function by carboxyl-terminal proteolysis. Science 1997; 275(5307): 1796-800.

44. Young SG, Fong LG, Michaelis S. Prelamin A, Zmpste24, misshapen cell nuclei, and progeria--new evidence suggesting that protein farnesylation could be important for disease pathogenesis. J Lipid Res 2005; 46(12): 2531-58.

45. Kowluru A. Evidence for the carboxyl methylation of nuclear lamin-B in the pancreatic beta cell. Biochem Biophys Res Commun 2000; 268(2): 249-54.

46. Maske CP, Hollinshead MS, Higbee NC, Bergo MO, Young SG, Vaux DJ. A carboxyl-terminal interaction of lamin B1 is dependent on
the CAAX endoprotease Rce1 andcarboxymethylation. J Cell Biol 2003; 162(7): 1223-32.

47. Jayaram B, Syed I, Singh A, Subasinghe W, Kyathanahalli CN, Kowluru A. Isoprenylcysteine carboxyl methyltransferase facilitates glucose-induced Rac1 activation, ROS generation and insulin secretion in INS 832/13 β-cells. Islets. 2011;3(2):48-57.

48. Moir RD, Yoon M, Khuon S, Goldman RD. Nuclear lamins A and B1: different pathways of assembly during nuclear envelope formation in living cells. J Cell Biol. 2000;151(6):1155-68.

49. Dechat T, Pfleghaar K, Sengupta K, Shimi T, Shumaker DK, Solimando L, Goldman RD. Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. Genes Dev. 2008;22(7):832-53.

50. Davidson PM, Lammerding J. Broken nuclei--lamins, nuclear mechanics, and disease. Trends Cell Biol. 2014;24(4):247-56.

51. THE CELL, Third Edition, Fig. 8.4 ASM Press and Sinauer Associates, 2003

52. Gerace L, Huber MD. Nuclear lamina at the crossroads of the cytoplasm and nucleus. J Struct Biol. 2012;177(1):24-31.

53. Broers JL, Ramaekers FC. Dynamics of nuclear lamina assembly and disassembly. Symp Soc Exp Biol. 2004;(56):177-92.

54. Margalit A, Vlcek S, Gruenbaum Y, Foisner R. Breaking and making of the nuclear envelope. J Cell Biochem. 2005;95(3):454-65.

55. Moir RD, Goldman RD. Lamin dynamics. Curr Opin Cell Biol. 1993;5(3):408-11.

56. Cohen S, Marr AK, Garcin P, Panté N. Nuclear envelope disruption involving host caspases plays a role in the parvovirus replication cycle. J Virol. 2011;85(10):4863-74.

57. Reyland ME. Protein kinase Cdelta and apoptosis. Biochem Soc Trans. 2007;35(Pt 5):1001-4.

58. Zhao M, Xia L, Chen GQ. Protein kinase cδ in apoptosis: a brief overview. Arch Immunol Ther Exp (Warsz). 2012;60(5):361-72.

59. Cross T, Griffiths G, Deacon E, Sallis R, Gough M, Watters D, Lord JM. PKC-delta is an apoptotic lamin kinase. Oncogene. 2000;19(19):2331-7.

60. Eitel K, Staiger H, Rieger J, Mischak H, Brandhorst H, Brendel MD, Bretzel RG, Häring HU, Kellerer M. Protein kinase C delta activation and translocation to the nucleus are required for fatty acid-induced apoptosis of insulin-secreting cells. Diabetes. 2003;52(4):991-7.

61. Goldman RD, Goldman AE, Shumaker DK. Nuclear lamins: building blocks of nuclear structure and function. Novartis Found Symp. 2005;264:3-16.

62. Rao L, Perez D, White E. Lamin proteolysis facilitates nuclear events during apoptosis. J Cell Biol. 1996 Dec;135(6 Pt 1):1441-55.

63. Worman HJ, Ostlund C, Wang Y. Diseases of the nuclear envelope. Cold Spring Harb Perspect Biol. 2010 Feb;2(2):a000760.

64. Emery-Dreifuss muscular dystrophy. Available at: http://ghr.nlm.nih.gov/condition/emery-dreifuss-muscular-dystrophy (Accessed on February 11, 2015).

65. Bonne G, Di Barletta MR, Varnous S, Bécane HM, Hammouda EH, Merlini L, *et al.*. Mutations in the gene encoding lamin A/C cause autosomal dominant Emery-Dreifuss muscular dystrophy. Nat Genet 1999; 21(3): 285-8.

66. Puckelwartz M, McNally EM. Emery-Dreifuss muscular dystrophy. Handb Clin Neurol 2011; 101: 155-66.

67. Khanna P, Opitz JM, Gilbert-Barness E. Restrictive dermopathy: report and review. Fetal Pediatr Pathol 2008; 27(2): 105-18.

68. Morais P, Magina S, Ribeiro Mdo C, Rodrigues M, Lopes JM, Thanh Hle T, *et al.*. Restrictive dermopathy--a lethal congenital laminopathy. Case report and review of the literature. Eur J Pediatr 2009; 68(8): 1007-12.

69. Thill M, Nguyen TD, Wehnert M, Fischer D, Hausser I, Braun S, et al. Restrictive dermopathy: a rare laminopathy. Arch Gynecol Obstet 2008; 278(3): 201-8.

70. Hutchinson-Gilford Progeria Syndrome. Available at: http://ghr.nlm.nih.gov/condition/hutchinson-gilford-progeria-syndrome (Accessed on February 27, 2015).

71. Gonzalez JM, Pla D, Perez-Sala D, Andres V. A-type lamins and Hutchinson-Gilford progeria syndrome: pathogenesis and therapy. Front Biosci (Schol Ed) 2011; 3: 1133-46.

72. Hegele RA. Familial partial lipodystrophy: a monogenic form of the insulin resistance syndrome. Mol Genet Metab 2000; 71(4): 539-44.

73. LIPODYSTROPHY, FAMILIAL PARTIAL, TYPE 2; FPLD2. Available at: http://www.omim.org/entry/151660 (Accessed on February 27, 2015).

74. Hegele RA. Insulin resistance in human partial lipodystrophy. Curr Atheroscler Rep. 2000; 2(5): 397-404.

75. Lin ST, Ptácek LJ, Fu YH. Adult-onset autosomal dominant leukodystrophy: linking nuclear envelope to myelin. J Neurosci. 2011;31(4):1163-6.

76. Bombelli F, Stojkovic T, Dubourg O, Echaniz-Laguna A, Tardieu S, Larcher K, Amati-Bonneau P, Latour P, Vignal O, Cazeneuve C, Brice A,Leguern E. Charcot-Marie-Tooth disease type 2A: from typical to rare phenotypic and genotypic features. JAMA Neurol. 2014;71(8):1036-42.

77. Pareyson D, Marchesi C. Diagnosis, natural history, and management of Charcot-Marie-Tooth disease. Lancet Neurol. 2009;8(7):654-67.

78. Colella R, Hollensead SC. Understanding and recognizing the Pelger-Huët anomaly. Am J Clin Pathol. 2012;137(3):358-66.

79. Rankin J, Ellard S. The laminopathies: a clinical review. Clin Genet. 2006;70(4):261-74.

80. Neamati N, Fernandez A, Wright S, Kiefer J, McConkey DJ. Degradation of Lamin B, Precedes Oligonucleosomal DNA Fragmentation in Apoptotic Thymocytes and Isolated Thymocyte Nuclei. J Immunol 1995;154:3788-95.

81. Hegele RA. Familial partial lipodystrophy: a monogenic form of the insulin resistance syndrome. Mol Genet Metab. 2000;71(4):539-44.

82. Bantel H, Schulze-Osthoff K. Mechanisms of cell death in acute liver failure. Front Physiol 2012;3:79.

83. Hengartner MO. The biochemistry of apoptosis. Nature 2000;407:770–6.

84. Bensellam M, Laybutt DR, Jonas JC. The molecular mechanisms of pancreatic β-cell glucotoxicity: recent findings and future research directions. Mol Cell Endocrinol. 2012;364(1-2):1-27.

85. Poitout V, Robertson RP. Glucolipotoxicity: fuel excess and beta-cell dysfunction. Endocr Rev 2008;29:351–66.

86. Malhotra JD, Kaufman RJ. ER stress and its functional link to mitochondria: role in cell survival and death. Cold Spring Harb Perspect Biol. 2011;3(9):a004424.

87. Simmen T, Aslan JE, Blagoveshchenskaya AD, Thomas L, Wan L, Xiang Y, Feliciangeli SF, Hung CH, Crump CM, Thomas G. PACS-2 controls endoplasmic reticulum-mitochondria communication and Bid-mediated apoptosis. EMBO J. 2005 Feb 23;24(4):717-29.

88. Bordier C. Phase separation of integral membrane proteins in Triton X-114 solution. J Biol Chem. 1981;256(4):1604-7.

89. Gruenbaum Y, Wilson KL, Harel A, Goldberg M, Cohen M. Review: nuclear lamins—structural proteins with fundamental functions. J Struct Biol 2000;129: 313–23.

90. Veluthakal R, Amin R, Kowluru A. Interleukin-1 induces posttranslational carboxymethylation and alterations in subnuclear distribution of lamin B in insulin-secreting RINm5F cells. Am J Physiol Cell Physiol 2004;287:C1152–62.

91. Kivinen K, Kallajoki M, Taimen P. Caspase-3 is required in the apoptotic disintegration of the nuclear matrix. Exp Cell Res 2005;311:62–73.

92. Almaguel FG, Liu JW, Pacheco FJ, Casiano CA, De Leon M. Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid. J Neurosci Res 2009;87:1207–18.

93. Arora DK, Mohammed AM, Kowluru A. Nifedipine prevents etoposide-induced caspase-3 activation, prenyltransferase degradation and loss in cell viability in pancreatic b-cells. Apoptosis 2013;18:1–8.

94. Wang Y, Gao L, Li Y, Chen H, Sun Z. Nifedipine protects INS-1 b-cell from high glucose-induced ER stress and apoptosis. Int J Mol Sci 2011;12:7569–80.

95. Huo J, Luo RH, Metz SA, Li G. Activation of caspase-2 mediates the apoptosis induced by GTP-depletion in insulinsecreting (HIT-T15) cells. Endocrinology 2002;143:1695–1704.

96. Yamada K, Ichikawa F, Ishiyama-Shigemoto S, Yuan X, Nonaka K. Essential role of caspase-3 in apoptosis of mouse betacells transfected with human Fas. Diabetes 1999; 48:478–483.

97. Hirota N, Otabe S, Nakayama H, Yuan X, Yamada K. Sequential activation of caspases and synergistic beta-cell cytotoxicity by palmitate and anti-Fas antibodies. Life Sci 2006;79:1312–1316.

98. Slee EA, Adrain C, Martin SJ. Executioner caspase-3, -6, and -7 perform distinct, non-redundant roles during the demolition phase of apoptosis. J Biol Chem2001; 276:7320 7326.

99. Zhao H, Zhao W, Lok K, Wang Z, Yin M. A synergic role of caspase-6 and caspase-3 in Tau truncation at D421 induced by H2O2. Cell Mol Neurobiol 2014;34:369–378

100. Syeda K, Mohammed AM, Arora DK, Kowluru A. Glucotoxic conditions induce endoplasmic reticulum stress to cause caspase 3 mediated lamin B degradation in pancreatic b-cells: protection by nifedipine. Biochem Pharmacol. 2013; 86:1338–1346.

101. Patel NA, Song SS, Cooper DR.
PKCdelta alternatively spliced isoforms modulate cellular apoptosis in retinoic acid-induced differentiation ofhuman NT2 cells and mouse embryonic stem cells. Gene Expr. 2006;13(2):73-84.

102. Sidarala V, Veluthakal R, Syeda K, Vlaar C, Newsholme P, Kowluru A. Phagocyte-like NADPH oxidase (Nox2) promotes activation of p38MAPK in pancreatic β-cells under glucotoxic conditions: Evidence for a requisite role of Ras-related C3 botulinum toxin substrate 1 (Rac1). Biochem Pharmacol. 2015;95(4):301-10.

103. Syed I, Kyathanahalli CN, Jayaram B, Govind S, Rhodes CJ, Kowluru RA, Kowluru A. Increased phagocyte-like NADPH oxidase and ROS generation in type 2 diabetic ZDF rat and human islets: role of Rac1-JNK1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. Diabetes. 2011; 60:2843–2852.

104. Mohammed AM, Syeda K, Hadden T, Kowluru A. Upregulation of phagocyte-like NADPH oxidase by cytokines in pancreatic beta-cells: attenuation of oxidative and nitrosative stress by 2-bromopalmitate. Biochem Pharmacol. 2013 85:109–114.

105. Eizirik DL, Miani M, Cardozo AK. Signalling danger: endoplasmic reticulum stress and the unfolded protein response in pancreatic islet inflammation. Diabetologia. 2013; 56:234–241.

106. Wang H, Kouri G, Wollheim CB. ER stress and SREBP-1 activation are implicated in beta-cell glucolipotoxicity. J Cell Sci. 2005; 118:3905–3915.

107. Leem J, Koh EH. Interaction between mitochondria and the endoplasmic reticulum: implications for the pathogenesis of type 2 diabetes mellitus. Exp Diabetes Res 2012;2012:242984.

108. Supale S, Li N, Brun T, Maechler P. Mitochondrial dysfunction in pancreatic b cells. Trends Endocrinol Metab 2012;23:477–87.

109. Veluthakal R, Palanivel R, Zhao Y, McDonald P, Gruber S, Kowluru A. Ceramide induces mitochondrial abnormalities in insulin-secreting INS-1 cells: potential mechanisms underlying ceramide-mediated metabolic dysfunction of the beta cell. Apoptosis. 2005; 10:841–850.

110. Jonas JC, Bensellam M, Duprez J, Elouil H, Guiot Y, Pascal S. Glucose regulation of islet stress responses and beta-cell failure in type 2 diabetes. Diabetes Obes Metab. 2009;11:65–81.

111. Lei X, Zhang S, Bohrer A, Barbour SE, Ramanadham S. Role of calcium-independent phospholipase A(2)b in human pancreatic islet b-cell apoptosis. Am J Physiol Endocrinol Metab. 2012;303:1386–1395.

112. Veluthakal R, Amin R, Kowluru A. Interleukin-1 induces posttranslational carboxymethylation and alterations in subnuclear distribution of lamin B in insulin secreting RINm5F cells. Am J Physiol Cell Physiol. 2004 287:1152–1162.

113. Mintzer R, Ramaswamy S, Shah K, Hannoush RN, Pozniak CD, Cohen F, Zhao X, Plise E, Lewcock JW, Heise CE. A whole cell assay to measure caspase-6 activity by detecting cleavage of lamin A/C. PLoS One. 2012;7(1):e30376.

114. Tang C, Koulajian K, Schuiki I, Zhang L, Desai T, Ivovic A, *et al.* Glucose-induced beta cell dysfunction in in vivo in rats: link between oxidative stress and endoplasmic reticulum stress. Diabetologia 2012;55:1366–79.

115. Xu G, Chen J, Jing G, Shalev A. Preventing b-cell loss and diabetes with calcium channel blockers. Diabetes 2012;6:848–56.

116. Zhou Y, Sun P, Wang T, Chen K, Zhu W, Wang H.
Inhibition of Calcium Influx Reduces Dysfunction and Apoptosis in Lipotoxic Pancreatic β -Cells via Regulation ofEndoplasmic Reticulum Stress. PLoS
One. 2015;10(7):e0132411.

117. Khadija S, Veluthakal R, Sidarala V, Kowluru A. Glucotoxic and diabetic conditions induce caspase 6-mediated degradation of nuclear lamin A in human islets, rodent islets and INS-1 832/13 cells. Apoptosis. 2014;19(12):1691-701.

118. Endo A, Kuroda M, Tsujita Y. ML-236A, ML-236B, and ML-236C, new inhibitors of cholesterogenesis produced by Penicillium citrinium. J Antibiot (Tokyo). 1976;29(12):1346-8.

119. Brown AG, Smale TC, King TJ, Hasenkamp R, Thompson RH. Crystal and molecular structure of compactin, a new antifungal metabolite from Penicillium brevicompactum. J Chem Soc Perkin 1. 1976;(11):1165-70.

120. Alberts AW, Chen J, Kuron G, Hunt V, Huff J, Hoffman C, Rothrock J, Lopez M, Joshua H, Harris E, Patchett A, Monaghan R, Currie S, Stapley E, Albers-Schonberg G, Hensens O, Hirshfield J, Hoogsteen K, Liesch J, Springer J. Mevinolin:

a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and acholesterol-lowering agent. Proc Natl Acad Sci U S A. 1980;77(7):3957-61.

121. Hajar R. Statins: past and present. Heart Views. 2011;12(3):121-7.

122. Furberg CD, Pitt B. Withdrawal of cerivastatin from the world market. Curr Control Trials Cardiovasc Med. 2001;2(5):205-207.

123. Corsini A, Maggi FM, Catapano AL. Pharmacology of competitive inhibitors of HMG-CoA reductase. Pharmacol Res. 1995;31(1):9-27.

124. Chao YS, Kroon PA, Yamin TT, Thompson GM, Alberts AW. Regulation of hepatic receptor-
dependent degradation of LDL by mevinolin in rabbits with hypercholesterolemia induced by a wheat starch-casein diet. Biochim Biophys Acta. 1983;29;754(2):134-41.

125. Bilheimer DW, Grundy SM, Brown MS, Goldstein JL. Mevinolin and colestipol stimulate receptor-mediated clearance of low density lipoprotein from plasma in familial hypercholesterolemia heterozygotes. Proc Natl Acad Sci U S A. 1983;80(13):4124-8.

126. Ishikawa M, Okajima F, Inoue N, Motomura K, Kato T, Takahashi A, Oikawa S, Yamada N, Shimano H. Distinct effects of pravastatin, atorvastatin, and simvastatin on insulin secretion from a beta-cell line, MIN6 cells. J Atheroscler Thromb. 2006;13(6):329-35.

127. Tsuchiya M, Hosaka M, Moriguchi T, Zhang S, Suda M, Yokota-Hashimoto H, Shinozuka K, Takeuchi T. Cholesterol biosynthesis pathway intermediates and inhibitors regulate glucose-stimulated insulin secretion and secretory granule formation in pancreatic beta-cells. Endocrinology. 2010;151(10):4705-16.

128. Metz SA, Rabaglia ME, Stock JB, Kowluru A. Modulation of insulin secretion from normal rat islets by inhibitors of the post-translational modifications of GTP-binding proteins. Biochem J. 1993;295 (Pt 1):31-40.

129. Di Matola T, D'Ascoli F, Luongo C, Bifulco M, Rossi G, FenziG, Vitale M. Lovastatin-induced apoptosis in thyroid cells: involvement of cytochrome c and lamin B. Eur J Endocrinol 2001;145:645–650.

130. Chang SY, Hudon-Miller SE, Yang SH, Jung HJ, Lee JM, Farber E, Subramanianb T, Andres DA, Spielmann HP, Hrycyna CA, Young SG, Fong LG. Inhibitors of protein geranylgeranyltransferase- I lead to prelamin A accumulation in cells by inhibiting ZMPSTE24. J Lipid Res 2012;53:1176–1182.

131. Adam SA, Butin-Israeli V, Cleland MM, Shimi T, Goldman RD. Disruption of lamin B1 and lamin B2 processing and localization by farnesyltransferase inhibitors. Nucleus. 2013;4(2):142-50.

132. Capell BC, Erdos MR, Madigan JP, Fiordalisi JJ, Varga R, Conneely KN, Gordon LB, Der CJ, Cox AD, Collins FS. Inhibiting farnesylation of progerin prevents the characteristic nuclear blebbing of Hutchinson-Gilford progeria syndrome. Proc Natl Acad Sci U S A. 2005;102(36):12879-84.

133. Mallampalli MP, Huyer G, Bendale P, Gelb MH, Michaelis S. Inhibiting farnesylation reverses the nuclear morphology defect in a HeLa cell model for Hutchinson-Gilford progeria syndrome. Proc Natl Acad Sci U S A. 2005;102(40):14416-21.

134. Wang Y, Ostlund C, Worman HJ. Blocking protein farnesylation improves nuclear shape abnormalities in keratinocytes of mice expressing the prelamin A variant in Hutchinson-Gilford progeria syndrome. Nucleus. 2010;1(5):432-9.

135. Capell BC, Olive M, Erdos MR, Cao K, Faddah DA, Tavarez UL, Conneely KN, Qu X, San H, Ganesh SK, Chen X, Avallone H, Kolodgie FD, Virmani R, Nabel EG, Collins FS. A farnesyltransferase inhibitor prevents both the onset and late progression of

cardiovascular disease in a progeria mouse model. Proc Natl Acad Sci U S A. 2008;105(41):15902-7.

136. Karp JE. Farnesyl protein transferase inhibitors as targeted therapies for hematologic malignancies. Semin Hematol. 2001;38(3 Suppl 7):16-23.

137. Gordon LB, Kleinman ME, Miller DT, Neuberg DS, Giobbie-Hurder A, Gerhard-Herman M, Smoot LB, Gordon CM, Cleveland R, Snyder BD, Fligor B, Bishop WR, Statkevich P, Regen A, Sonis A, Riley S, Ploski C, Correia A, Quinn N, Ullrich NJ, Nazarian A, Liang MG, Huh SY, Schwartzman A, Kieran MW. Clinical trial of a farnesyltransferase inhibitor in children with Hutchinson-Gilford progeria syndrome. Proc Natl Acad Sci U S A. 2012;109(41):16666-71.

138. Kim KW, Chung HH, Chung CW, Kim IK, Miura M, Wang S, *et al*. Inactivation of farnesyltransferase and geranylgeranyltransferase I by caspase-3: cleavage of the common alpha subunit during apoptosis. Oncogene 2001;20: 358–66.

139. Pelaia G, Gallelli L, Renda T, Fratto D, Falcone D, Caraglia M, Busceti MT, Terracciano R, Vatrella A, Maselli R, Savino R. Effects of statins and farnesyl transferase inhibitors on ERK phosphorylation, apoptosis and cell viability in non-small lung cancer cells. Cell Prolif. 2012;45(6):557-65.

140. Afshordel S, Kern B, Clasohm J, König H, Priester M, Weissenberger J, Kögel D, Eckert GP. Lovastatin and perillyl alcohol inhibit glioma cell invasion, migration, and proliferation--impact of Ras-/Rho-prenylation. Pharmacol Res. 2015;91:69-77.

141. Bonifacio A, Sanvee GM, Bouitbir J, Krähenbühl S. The AKT/mTOR signaling pathway plays a key role in statin-induced myotoxicity. Biochim Biophys Acta. 2015;1850(8):1841-9.

ABSTRACT

ISLET DYSFUNCTION IN DIABETES

by

KHADIJA G. SYEDA

August 2015

Advisor: Dr. Anjan Kowluru

Major: Pharmaceutical Sciences

Degree: Doctor of Philosophy

Type 2 Diabetes [T2DM] is a chronic condition resulting from gradual failure of pancreatic beta cells to synthesize and secrete sufficient insulin to meet the metabolic demands and the inability of tissues [muscle, adipose and liver] to efficiently utilize the secreted insulin leading to an overall increase in blood glucose levels [hyperglycemia]. As indicated by recent estimates from the International Diabetes Federation, the prevalence of the disease in the year 2014 has risen to a record 387 million worldwide. The main objective of my project was to study the mechanisms involved in pancreatic beta cell dysfunction in diabetes, specifically in elucidating the role of endoplasmic reticulum [ER] - mitochondria axis, executioner caspases and their target substrates; specifically nuclear lamins.

Results obtained from our studies in pure beta cells [INS-1 832/13], primary rodent and human islets strongly suggest that glucotoxicity induced pancreatic beta cell damage involves the degradation of nuclear lamins A and B, *via* ER stress-mediated activation of executioner caspases 3 and 6. We confirmed this by employing pharmacological approaches [inhibitors of -ER stress, -caspase activation and calcium channel activation] to gain mechanistic insights into

beta cell dysfunction under the duress of chronic hyperglycemia. Further, we were able to corroborate these findings in the ZDF rat, an animal model for T2DM and in islets obtained from human donors with T2DM. Also, our findings revealed significant attenuation of glucose-stimulated insulin secretion [GSIS] in beta cells exposed to glucotoxic conditions suggesting cellular dysfunction under these conditions. Post-translational prenylation of lamins is important for their localization into the nuclear membrane, and subsequent interaction with other proteins. Our results indicate that inhibition of prenylation by simvastatin and a site-specific inhibitor of protein farnesylation [FTI-277], promoted mitochondrial and nuclear defects as evidenced by caspase activation and lamin degradation in INS-1 832/13 cells and normal rodent islets. Our findings also suggest that inhibition of protein prenylation leads to increase in stress kinase [p38 kinase] and inhibition of ERK1/2, known for its cell survival roles. Collectively, these alterations in cell signaling pathways could promote intracellular stress and demise.

We hope that data accrued in these studies will provide fresh insights into the identification of the intracellular mechanisms involved in beta cell malfunction in nutrient overload and metabolic stress. These studies will also aid in the identification of potential drug targets for the management and/or prevention of diabetes.

AUTOBIOGRAPHICAL STATEMENT

EDUCATION:

- **Masters in Pharmacy,** (2011), University of Pune, Pune, India
- **Bachelors in Pharmacy,** (2009), University of Pune, Pune, India

AWARDS:

- George C. Fuller Endowed Scholarship, Department of Pharmaceutical Sciences, Wayne State University, 2015.
- Thomas C. Rumble - Graduate Fellowship, Department of Pharmaceutical Sciences, Wayne State University, 2011, 2013.

PUBLICATIONS:

1. Phagocyte-like NADPH oxidase (Nox2) promotes activation of p38MAPK in pancreatic β-cells under glucotoxic conditions: Evidence for a requisite role of Ras-related C3 botulinum toxin substrate 1 (Rac1). Sidarala V, Veluthakal R, **Syeda K**, Vlaar C, Newsholme P, Kowluru A. Biochem Pharmacol. 2015.;95(4):301-10.
2. EHT 1864, a small molecule inhibitor of Ras-related C3 botulinum toxin substrate1 (Rac1), attenuates glucose-stimulated insulin secretion in pancreatic β-cells. Sidarala V, Veluthakal R, **Syeda K**, Kowluru A. Cellular Signalling. 2015; 1159-1167.
3. Glucotoxic and diabetic conditions induce caspase 6-mediated degradation of nuclear lamin A in human islets, rodent islets and INS-1 832/13 cells. **Khadija S**, Veluthakal R, Sidarala V, Kowluru A. Apoptosis. 2014;19(12):1691-701.
4. Glucotoxic conditions induce endoplasmic reticulum stress to cause caspase 3 mediated lamin B degradation in pancreatic β-cells: protection by nifedipine. **Syeda K**, Mohammed AM, Arora DK, Kowluru A. Glucotoxic conditions induce endoplasmic reticulum stress to cause caspase 3 mediated lamin B degradation in pancreatic β-cells: protection by nifedipine. Biochem Pharmacol. 2013;86[9]:1338-46.
5. Upregulation of phagocyte-like NADPH oxidase by cytokines in pancreatic beta-cells: attenuation of oxidative and nitrosative stress by 2-bromopalmitate. Mohammed AM, **Syeda K**, Hadden T, Kowluru A. Biochem Pharmacol. 2013;85(1):109-14.
6. VAV2, a guanine nucleotide exchange factor for Rac1, regulates glucose-stimulated insulin secretion in pancreatic beta cells. Veluthakal R, Tunduguru R, Arora DK, Sidarala V**, Syeda K,** Vlaar CP, Thurmond DC, Kowluru A. Diabetologia 2015 [In Press]