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Metformin, Glucotoxicity And Islet Dysfunction

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by

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DEDICATION

This work is dedicated to my parents Paramjeet Kaur Baidwan and Kuldip Singh Baidwan, my brother Kultej Singh Baidwan and my fiancé Jasleen Antaal

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First and foremost, my thanks and appreciation to my academic advisor, Dr. Anjaneyulu Kowluru, who through his immense experience, enthusiasm and guidance always motivated and inspired me to work harder. I will forever be grateful to Dr. Kowluru for always being there to help me with any problem that I had during my stay in the lab and for always encouraging me to achieve my goals. I am glad that I got the opportunity to work under Dr. Kowluru's mentorship.

I would like to express my gratitude to my committee members; Dr. Randall Commissaris and Dr. Timothy Hadden for being on my committee and for providing encouraging suggestions and teachings throughout the duration of my project. I would also like to thank the Department of Pharmaceutical Sciences at Wayne State University.

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Last but not the least, I would like to thank my parents, my brother, my fiancé and my friends for always motivating me and supporting me. A special thanks to my parents and brother for always teaching me to be humble and thankful and that hard work always pays off.

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LIST OF ABBREVIATIONS

ATP- Adenosine triphosphate

CC3- Cleaved caspase-3

CD36- Cluster of differentiation 36

DMSO- Dimethyl sulfoxide

FBS- Fetal bovine serum

FTase- Farnesyl transferase

GEFs- Guanine nucleotide exchange factors

GGTase - Geranylgeranyl transferase/

GLUT2- Glucose transporter type 2

GPCR- G-protein coupled receptor

GSIS- Glucose stimulated insulin secretion

HEPES- 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HG- High glucose

JNK- cJun N-terminal kinases

LG- Low glucose

MF- Metformin

MTT- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

Nox2- phagocyte-like NADPH oxidase 2

p38MAPK- p38 mitogen activated protein kinase

PBS- Phosphate buffered saline

PBS-T- Phosphate buffered saline with tween

PI3K- phosphatidylinositol-4,5-biphosphate 3-kinase

PIP3- phosphatidylinositol (3,4,5)-triphosphate

PMSF- phenylmethylsulfonyl fluoride

Rac1- Ras-related C3 botulinum toxin substrate 1

RIPA- Radioimmunoprecipitation assay buffer

ROS- reactive oxygen species

RPMI- Roswell Park Memorial Institute medium

SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis

T2DM- Type 2 diabetes mellitus

Tiam1- T-lymphocyte invasive and metastasis protein 1

Vav2- Vav guanine nucleotide exchange factor 2

VDCC- Voltage dependent calcium channels

VLCD- very low calorie diet

VLED- very low energy diet

CHAPTER 1: INTRODUCTION

A cell is a basic structural and functional unit of all living organisms. The human body is composed of millions of cells; all these cells require an energy source for their normal functioning. Glucose, a monosaccharide is the primary source of energy for cells in the body. Glucose metabolism plays an important role in the human body and is regulated by an interplay between the pancreatic hormones glucagon and insulin to maintain glucose homeostasis **[1, 2]**. Glycogenolysis, gluconeogenesis and glycolysis also play a key role in glucose metabolism. It is well established that chronic exposure of pancreatic β-cells to hyperglycemic conditions leads to the onset of cell dysfunction and diabetes.

The pancreas, the key regulator of glucose metabolism, functions as an exocrine gland [secreting digestive enzymes] as well as an endocrine gland [secreting hormones such as insulin and glucagon]. The endocrine cells of the pancreas are grouped together to form the islets of Langerhans **[3]**. The different endocrine cells include α-cells, β-cells, δ-cells, γ-cells and ε-cells. The α-cells produce the hormone glucagon which increases the blood sugar levels and make about 15-20% of the total islet count whereas the β-cells produce the blood glucose level reducing hormone insulin and constitute about 65-80% of the total cells **[3]**. The δ-cells constitute 3-10% of the islets and produce somatostatin which inhibits the secretion of both glucagon and insulin **[3]**. The γ-cells (3-5%) produce pancreatic polypeptide (PP) **[4]** and ε-cells (<1%) produce ghrelin **[5]**.

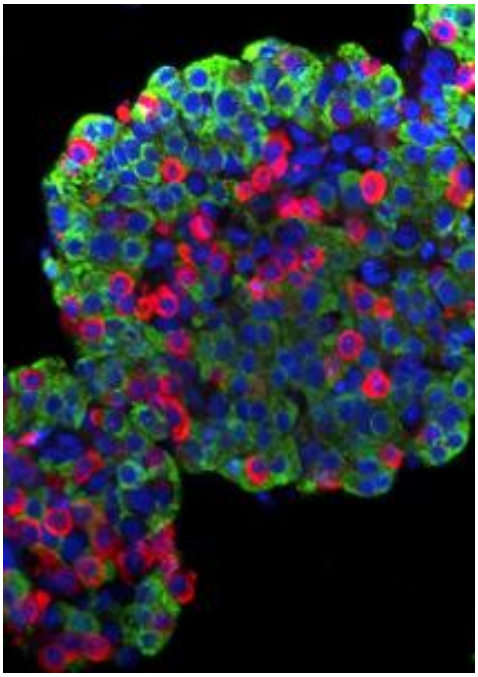


Figure 1-1: Image of a pancreatic islet: insulin (green) and glucagon (red). [diabetesresearch.org]

Glucotoxicity:

Prolonged exposure of the β-cells to high glucose [glucotoxic] conditions results in permanent damage to the β-cells, including defects in insulin gene transcription and expression **[6]**. Glucotoxic conditions promote generation of reactive oxygen species [ROS] mediated by Rac1-Nox2 signaling axis. This, in turn, leads to activation of stress kinases resulting in mitochondrial dysfunction and increased Caspase activity leading to β-cell death **[7]**. High glucose concentrations also cause an impaired **G**lucose **S**timulated **I**nsulin **S**ecretion (GSIS).

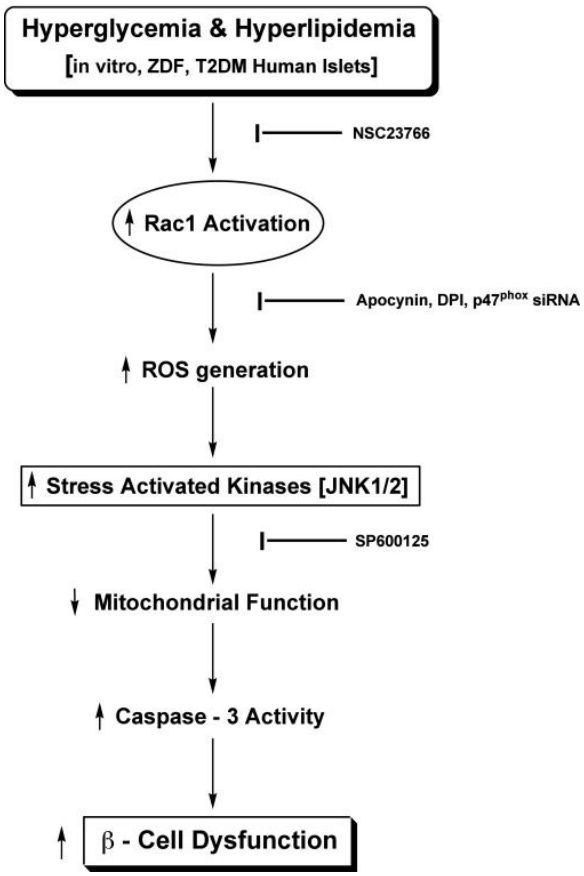


Figure 1-2: Glucotoxic conditions lead to β-cell death: Prolonged exposure to HG conditions activate Rac1-Nox2 mediated ROS generation pathway leading to stress kinase activation eventually culminating in β-cell death. [Image taken from Syed *et al.* ref **(7)**]

Glucose Stimulated Insulin Secretion (GSIS):

GSIS is initiated when glucose enters the pancreatic β-cell via GLUT2 transporters and then undergoes cationic and metabolic events that lead to increased intracellular ATP concentration causing the closure of K_{ATP} channel. The K_{ATP} channel closure causes membrane depolarization followed by opening of the Voltage dependent calcium channels (VDCC) causing an increased influx of Ca^{2+} ions into the β-cell and the Ca^{2+} influx mediates the mobilization of insulin granules to the plasma membrane for insulin exocytosis **[8, 9]**. High glucose concentrations/glucotoxic conditions induce impaired GSIS by partially inhibiting the K_{ATP} channel, thereby decreasing the K^+ efflux and Ca^{2+} influx **[6]**.

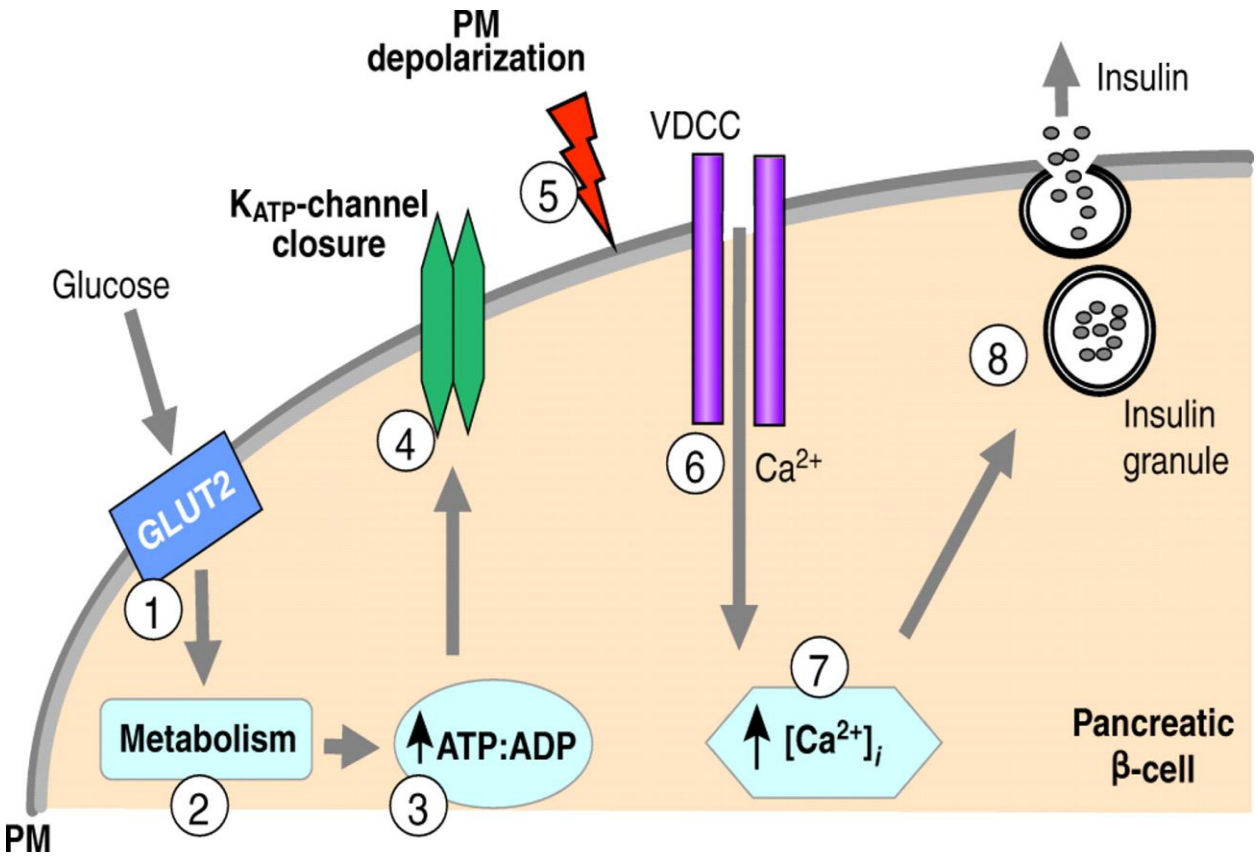


Figure 1-3: Glucose Stimulated Insulin Secretion (GSIS): a simplified model. [Wang *et al.* ref **(8)**].

Diabetes:

According to the International Diabetes Federation (IDF), diabetes is a chronic disease characterized by body's inability to produce insulin or use insulin effectively, thereby leading to increased glucose levels that impact the pancreatic β-cell negatively resulting in β-cell death and/or insulin resistance. There are 3 types of diabetes- Type 1, Type 2 and gestational diabetes. Diabetes can cause different complications leading to cardiovascular diseases, kidney failure, diabetic retinopathy, nerve diseases, limb amputations and pregnancy complications.

Type 1 Diabetes:

Type 1 diabetes, also referred to as insulin dependent diabetes mellitus (IDDM) is due to autoimmune reactions in which the pancreatic β-cells are destroyed by the immune system and insulin is no longer produced in the human body. Viral infections and family history of type 1 diabetes are the associated risk factors for type 1 diabetes. B lymphocytes have been viewed as key players in development of type 1 diabetes by producing autoantibodies and by presenting antigens to T lymphocytes **[10]**. Animal studies by Hu *et al.* and Guleria *et al.* have shown that anti B cell therapy restores normal glucose levels and reverses diabetes **[11, 12].** However, "In human disease, the function of B lymphocytes is less obvious and the desire to directly link murine pathogenesis of type 1 diabetes to that of humans (despite the profound differences in the immune systems between the two species) has led to various misconceptions and false expectations" **[13-16]**.

Type 2 Diabetes:

In type 2 diabetes, formerly known as non-insulin dependent diabetes mellitus (NIDDM), the pancreatic β-cells become resistant to insulin and are unable to utilize the insulin effectively in lowering blood glucose levels, thus, leading to hyperglycemia. T2DM alters the glucose homeostasis by inducing impaired nutrient storage and mobilization and triggers the pancreatic β-cells to enhance insulin secretion in response to insulin resistance **[17].** Obesity, unhealthy diet, high blood pressure are some of the known factors that can lead to type 2 diabetes. Other studies have shown that very low energy diet (VLED)/ very low calorie diet (VLCD) can prove beneficial to patients with type 2 diabetes **[18-20].**

Gestational Diabetes:

When the slightly elevated blood glucose levels in females during pregnancy cause an imbalance between elevated insulin secretion and pregnancy induced insulin resistance resulting in the onset of gestational diabetes **[21]**. Furthermore, in addition to insulin resistance, gestational diabetes is known to alter glucose metabolism and tolerance **[22].**

The number of people being diagnosed with diabetes is on the increase and according to an estimate by IDF, there will be 642 million individuals with diabetes in 2040 as opposed to 415 million in 2015. The number of deaths due to diabetes in 2015 were 5 million, which was higher than the deaths due to HIV/AIDS, tuberculosis and malaria combined **[23]**.

Diabetes around the world

International Diabetes Federation

The world at a glance

	2015	2040
Total world population	7.3 billion	9.0 billion
Adult population (20-79 years)	4.72 billion	6.16 billion
Child population (0-14 years)	1.92 billion	-
Diabetes (20-79 years)		
Global prevalence	8.8% (7.2-11.4%)	10.4% (8.5-13.5%)
Number of people with diabetes	415 million (340-536 million)	642 million (521-829 million)
Number of deaths due to diabetes	5.0 million	-
Health expenditure due to diabetes (20-79 years)		
Total health expenditure, R=2* 2015 USD	673 billion	802 billion
Hyperglycaemia in pregnancy (20-49 years)		
Proportion of live births affected	16.2%	-
Number of live births affected	20.9 million	-
Impaired glucose tolerance (20-79 years)		
Global prevalence	6.7% (4.5-12.1%)	7.8% (5.2-13.9%)
Number of people with impaired glucose tolerance	318 million (212.2-571.6 million)	481 million (317.1-855.7 million)
Type 1 diabetes (0-14 years)		
Number of children with type 1 diabetes	542,000	-
Number of newly diagnosed cases each year	86,000	-

Figure 1-4: Prevalence of Diabetes around the world [International Diabetes Federation Atlas 2015]

Metformin:

Metformin is an oral antidiabetic drug which is used as a first-line therapy for patients diagnosed with T2DM **[24-26]**. Metformin lowers elevated blood glucose levels by suppressing hepatic glucose production, increasing peripheral glucose uptake and ameliorating insulin sensitivity **[25, 27-29]**. It has been shown to improve vascular endothelial functions and reduce cardiovascular events in patients with type 2 diabetes **[30].** The oral bioavailability of Metformin is 50-60% with majority of the absorption being completed within ~ 6 hours in the small intestine and the drug gets minimally metabolized in the liver before being excreted by the kidneys **[31]**.

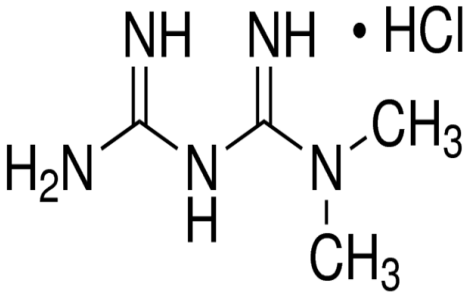


Figure 1-5: Structure of Metformin [https://pubchem.ncbi.nlm.nih.gov]

Hypothesis:

Glucotoxicity, as discussed earlier, induces the Rac1-Nox2 mediated stress kinase activation, thereby producing mitochondrial defects and eventually leading to β-cell death **[7].** Based on these observations, we asked if metformin, an oral antidiabetic drug for T2DM can potentially provide protection to the pancreatic β-cells from HG-induced mitochondrial dysfunction. Specifically, we hypothesized that metformin triggers its cyto-protective roles by inhibiting the HG-induced Rac1 mediated stress kinase activation signaling pathway. We validated this hypothesis in insulin-secreting INS-1 832/13 cells incubated cultured under

glucotoxic conditions in the absence or presence of clinically-relevant concentrations of metformin. Our findings indicate that metformin affords significant protection in pancreatic β-cell against HG-induced metabolic events leading to its dysfunction.

CHAPTER 2: MATERIALS AND METHODS

Chemicals and antibodies:

Rabbit polyclonal antibody for phospho-p38MAPK (Thr 180/Tyr 182), total-p38MAPK, Lamin B and mouse monoclonal antibody for CD36 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antisera directed against phospho-p53, total-p53, phospho-JNK1/2, total-JNK1/2 and cleaved caspase-3 antibodies were purchased from Cell Signaling (Danvers, MA). Antibodies against Bcl2 and actin were from Sigma–Aldrich (St. Louis, MO). Phospho-VAV2 and total-VAV2 antisera were purchased from Abcam (Cambridge, MA). NE-PER Nuclear and Cytoplasmic Extraction Kit was from Thermo Scientific (Waltham, MA). IRDye® 800CW anti-rabbit and anti-mouse secondary antibodies were obtained from LICOR (Lincoln, NE). Metformin hydrochloride, MTT, DMSO were purchased from Sigma-Aldrich (St. Louis, MO). Rac1 Antibody was from BD Transduction lab (San Jose, CA). Rac1 activation assay Biochem kit was purchased from Cytoskeleton, Inc (Denver, CO). EHT 1864 [Rac1 inhibitor], was purchased from R&D systems (Minneapolis, MN). All other reagents used in the studies were obtained from Sigma–Aldrich (St. Louis, MO).

Insulin-secreting INS-1 832/13 cells and culture conditions:

INS-1 832/13 cells were provided by Dr. Chris Newgard, Duke University Medical Center (Durham, NC). INS-1 832/13 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with antibacterial antifungal (100IU penicillin and 100IU/ml streptomycin), 1mM sodium pyruvate, 50 µM 2-mercaptoethanol and 10mM HEPES (pH 7.4) at 37°C and 5% CO_2 in a humidified incubator. INS-1 832/13 cells were sub cloned twice weekly following trypsinization and passages 53-61 were used for the studies. Following overnight incubation in 2.5 mM glucose and 2.5% serum RPMI media, the cells were treated with low

glucose (2.5 mM; LG) or high glucose (20mM; HG) in the absence or presence of MF (15 µM and 30 µM) and EHT 1864 (10 µM) for 24 hours.

Western Blotting:

After 24-hour incubation with glucose (2.5mM, LG and 20mM, HG) in the absence and presence of metformin (15 µM and 30 µM) and EHT 1864 (10 µM), cells were lysed using RIPA buffer containing protease inhibitor cocktail, 1mM NaF, 1mM PMSF and 1mM Na_3VO_4. Cell lysates (~45 µg for INS-1 832/13 cells) were then resolved by SDS-PAGE, and then transferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk in PBS-T buffer or 0.1% Casein in PBS-T and then incubated with appropriate primary antibody diluted with 5% non-fat dry milk in PBS-T buffer or 0.1% Casein in PBS-T, overnight at 4°C. The membranes were then washed 5 x 5 minutes with PBS-T, and then probed with the appropriate secondary antibody IRDye ® 800CW anti-rabbit or anti-mouse. The immune complexes were then detected using Odyssey ® Imaging Systems. The band intensities were quantifies using Carestream ® Molecular Imaging Software.

Rac1 activation assay:

Rac1 activation assay was performed using Rac1 pull-down activation assay kit (bead pull-down format; Cytoskeleton Inc.) using manufacturers' protocol. Briefly, INS-1 832/13 cells were grown to ~70% confluence in complete growth media (RPMI). Cells were then grown in low glucose (2.5mM) low serum (2.5%) starvation media overnight followed by culture in LG and HG media in the presence and absence of metformin (0-30 µM). After 24 hours, growth media was aspirated and cells were washed with ice cold PBS. After complete removal of PBS, ice cold lysis buffer containing 1X protease inhibitor cocktail were added to culture covering entire surface. Cell lysates were collected and snap frozen in liquid nitrogen and stored at -70 ^{0} C

until further processing. Pull-down assay was performed the same day using the snap frozen protein lysates.

Isolation of nuclear and non-nuclear fractions:

INS-1 832/13 cells were treated with low glucose or LG (2.5mM) and high glucose or HG (20mM) in the presence and absence of metformin (30µM) for 24hrs as described above. Adherent cells were harvested with trypsin-EDTA and the cell pellet was washed once with ice cold 1X PBS. The cytoplasmic and nuclear protein fractions were collected using NE-PER nuclear and cytoplasmic extraction Kit (Thermo Scientific) following manufacturer's protocol. Cytoplasmic protein extract and nuclear protein extract were further analyzed by western blotting. The purity of the nuclear fractions was assessed by probing for nuclear Lamin B.

Cell Viability Assay:

MTT assay was performed using INS-1 832/13 cells to quantify cell viability under LG (2.5mM) and HG (20mM) conditions in the presence and absence of MF (30 µM). INS-1 832/13 cells were starved in low glucose (2.5mM) low serum (2.5%) starvation medium for 12-18 hrs in a 96 well plate. Following starvation, the INS-1 832/13 cells were treated with LG or HG in the absence or presence of MF (30 µM) for 24 hrs. After the 24-hour incubation, the cells were incubated with MTT for a period of 2-4 hrs. At the end of the incubation, DMSO was added to the wells to solubilize the MTT crystals. The absorbance was measured at 540nm using GEN 5.0 software.

CHAPTER 3: GLUCOTOXICITY INDUCES Rac1 ACTIVATION AND NUCLEAR TRANSLOCATION AND CD36 EXPRESSION IN INS-1 832/13 CELLS

The studies conducted in this Chapter are based on the recent findings from our laboratory suggesting that glucotoxic conditions induce impaired GSIS, β-cell dysfunction and apoptosis by triggering a Rac1-Nox2 mediated stress kinase activation signaling pathway **[7, 9]**.

Three classes of G-proteins that have been identified in the pancreatic β-cells are: **1)** The heterotrimeric proteins involved in signal transduction via G-protein coupled receptors (GPCRs) to the intracellular effectors **[32, 33]**. **2)** small G-proteins, which are involved in membrane trafficking of secretory vesicles and cytoskeletal remodeling **[34]**. **3)** The third class of G-proteins includes the elongation factors and Tau proteins **[33, 34]**.

HG conditions promote nuclear accumulation of Rac-1 in INS-1 832/13 cells, normal rat islets and human islets

Previous observations from our laboratory have demonstrated sustained activation of Rac1 in INS-1 832/13 cells, normal rat islets and human islets exposed to HG conditions **[7, 35, 36].** We provided evidence to indicate a significant reduction in geranylgeranyltransferase [GGTase] activity, which regulates post-translational prenylation of small G-proteins, including Rac1. As an index for decreased prenylation, we observed significant accumulation of unprenylated proteins in pancreatic β-cells exposed to HG conditions. Therein, we speculated that unprenylated, but paradoxically active G-proteins might translocate into "inappropriate" compartments [e.g., nucleus] to induce metabolic defects in the effete β-cell **[37]**. Therefore, we undertook the current investigation to determine potential targeting of Rac1 into the nuclear compartment in pancreatic β-cells exposed to HG conditions. Data depicted in Figure 1 [Panels A and B] demonstrate a marked increase in the nuclear localization of Rac1 in INS-1 832/13

cells exposed to glucotoxic conditions. These findings were confirmed in normal rodent islets [Figure 1; Panels C and D] and human islets [Figure 1; Panel E]. Together, these observations validate our hypothesis that exposure of insulin-secreting cells to HG conditions leads to sustained activation of unprenylated Rac1 leading to its translocation to the nuclear compartment.

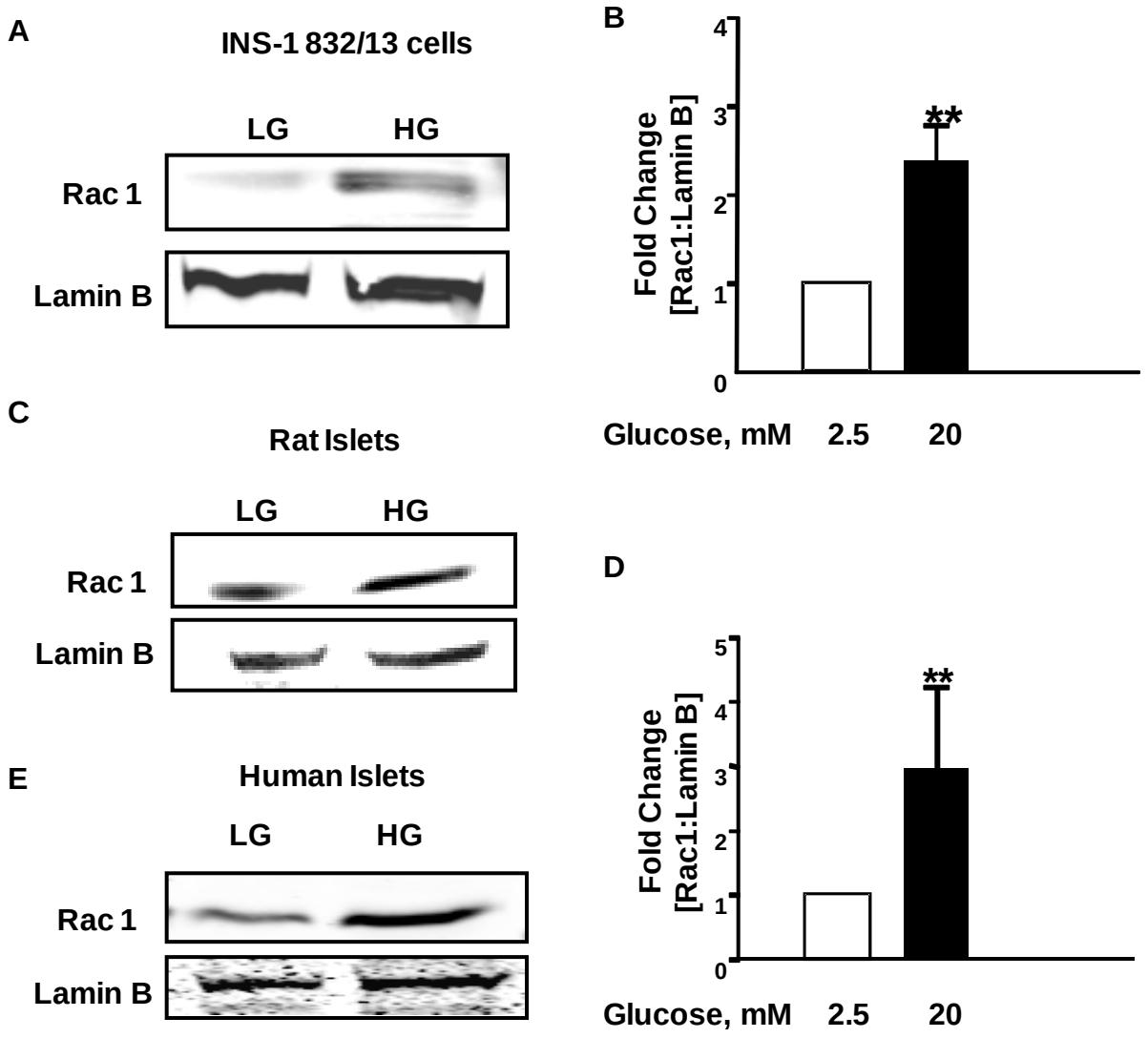


Figure 3-1: HG conditions promote nuclear accumulation of Rac-1 in INS-1 832/13 cells, normal rat islets and human islets: **Panel A:** Relative abundance of Rac1 in the nuclear fractions isolated from INS-1 832/13 cells exposed to LG or HG conditions was determined by Western blotting. **Panel B:** Pooled data from three independent experiments is shown. Accumulation of Rac1 was calculated as a ratio of Rac1 to Lamin B in the nuclear fraction [loading control as well as marker] and represented as fold change over basal. ** $p<0.005$ vs

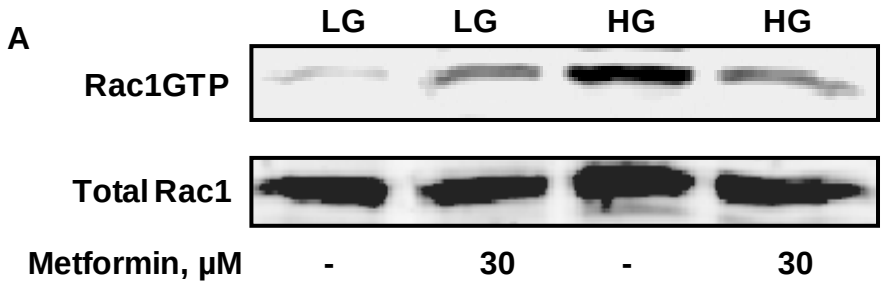
2.5mM glucose**. Panel C:** Relative abundance of Rac1 in the nuclear fractions isolated from normal rat islets exposed to LG or HG conditions was determined by Western blotting**. Panel D:** Pooled data from three independent experiments is represented herein. Accumulation of Rac1 was calculated as above. ** $p<0.005$ vs 2.5mM glucose. **Panel E:** Human pancreatic islets were incubated with LG or HG for 24 hours and relative abundance of Rac1 in the nuclear fraction was determined by Western blotting. Western blot of one batch of human islet lysates is provided here.

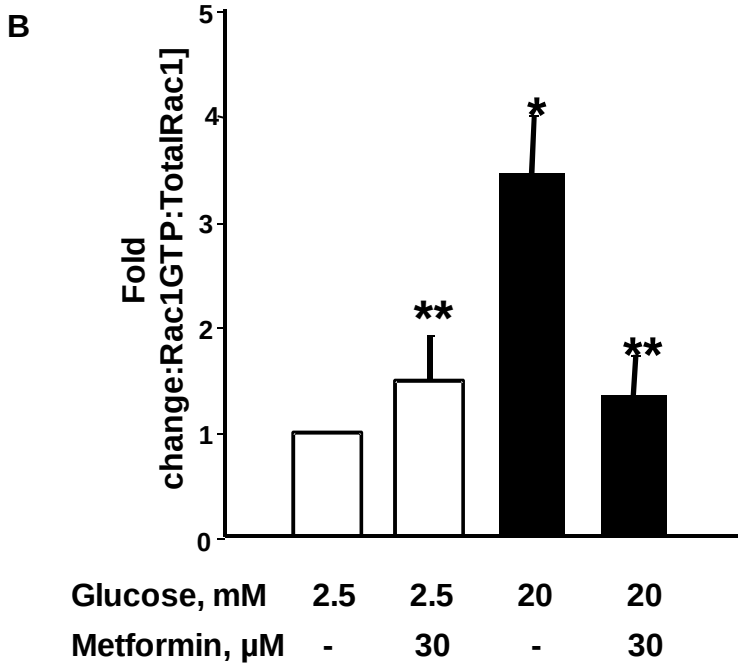
Metformin attenuates HG-Induced Rac1 activation and Rac1 nuclear translocation

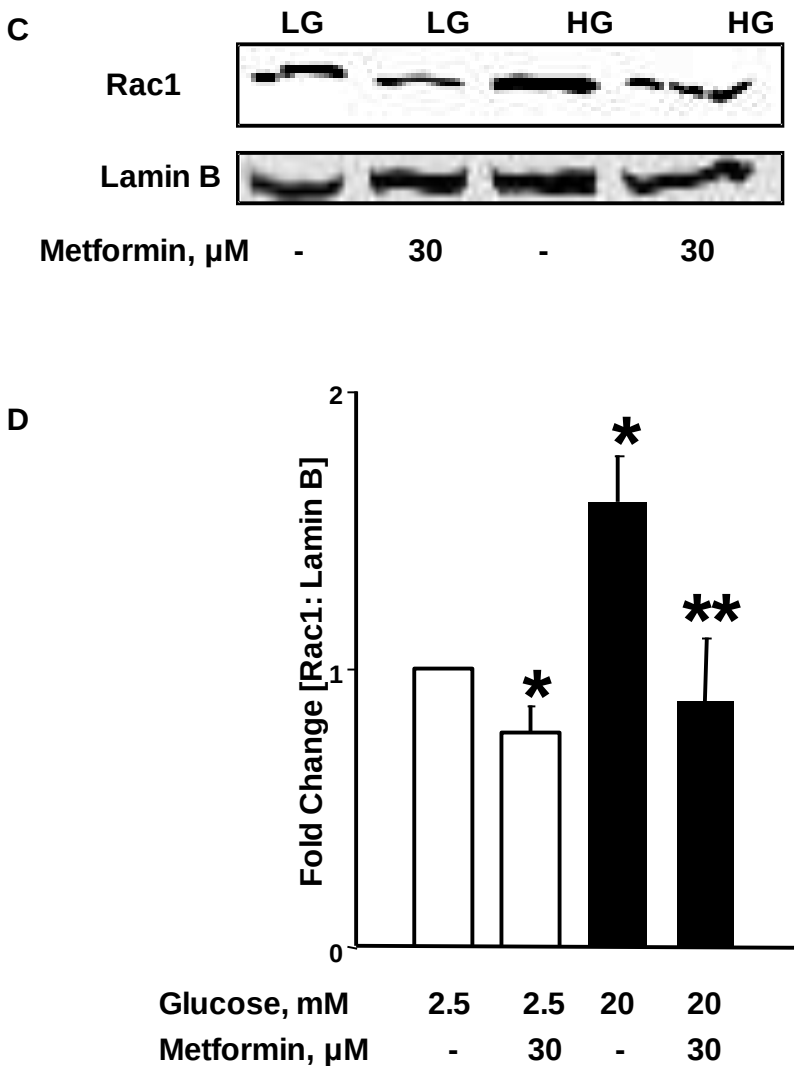
Small G-proteins play an important role in regulating glucose stimulated insulin secretion (GSIS). There are 3 major classes of small g-proteins. The first group includes Cdc42, Rac1, Rho and ADP-ribosylation factor-6 (ARF-6). The second group consists of Rap1, Rab3A and Rab27. The third group of small G-proteins consist of Rab2, Rhes and Rem2 **[34]**.

Our experimental model was focused on investigating whether clinically relevant concentrations of metformin, a biguanide antidiabetic compound, protects INS-1 832/13 cells from HG-induced sustained activation and translocation of Rac1, thereby halting the apoptotic signaling events leading to β-cell death. To address this, INS-1 832/13 cells were exposed to HG conditions in the presence and absence of metformin [30μM] and Rac1 activation was assessed using pull down assay. Data shown in figure 3-2 indicate significant increase in Rac1 activation and nuclear translocation in INS-1 832/13 cells exposed to HG conditions and co-provision of metformin significantly abated the HG-induced Rac1 activation and nuclear translocation. An interesting observation was that in addition to reducing HG-induced Rac1 activation, metformin induced slight Rac1 activation in cells exposed to basal [normal] glucose conditions [Figure 3-2, Panel A]. Pooled data from multiple experiments is depicted in Figure 3-2 [Panel B]. Metformin treatment also reduced HG-induced Rac1 nuclear translocation in INS-1 832/13 cells [Figure3-2, Panel C]. Quantified data from multiple experiments is represented in Figure 3-2 [Panel D].

Together, findings from these experiments demonstrate metformin's beneficial effect in inhibiting the HG-induced Rac1 activation and nuclear translocation in INS-1 832/13 cells.







<u>Figure 3-2: Metformin suppresses HG-induced Rac1 activation and Rac1 nuclear translocation in INS-1 832/13 cells:</u> **Panel A:** INS-1 832/13 cells were cultured in LG (2.5mM) or HG (20mM) for 24 hours in the absence and presence of Metformin (30 µM). Rac1 activation assay was performed using pull-down activation assay biochem kit [see Methods for additional details]. Cell lysates were separated and analyzed using western blotting. **Panel B:** Band intensities of Rac1 were quantified by densitometric analysis. Abundance of active Rac1 in pull-down samples was normalized by total Rac1. Pooled data from three experiments was represented in this panel. *$p < 0.05$ vs. 2.5mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone. **Panel C:** INS-1 832/13 cells (a) were incubated with LG (2.5mM) and HG (20mM) in the absence and presence of metformin (0-30 µM) for 24 h. Cell lysates were analyzed for Rac1 using western blotting. Purity of the nuclear fractions was verified by probing with Lamin B. **Panel D:** Band intensities for Rac1 were measured using densitometry and the ratios were calculated over Lamin B in the presence and absence of metformin (n=3 in INS-1 832/13 cells). *$p < 0.05$ vs. 2.5 mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone.

Guanine nucleotide exchange factors (GEFs)

There are various regulatory proteins/factors that enable the G-proteins to switch between the active (GTP-bound) and inactive (GDP-bound) conformations, and these factors are classified into 3 major categories. The first group consists of the guanine nucleotide exchange factors (GEFs), which facilitate GDP-GTP exchange [switch from inactive to active states]. The second group includes the GDP- dissociation inhibitors (GDIs) that retain putative G-proteins in their GDP-bound [inactive] conformation by complexing with GDP-bound G-proteins. The third group consists of GTPase-activating proteins (GAPs) which mediate inactivation of G-proteins by hydrolyzing the GTP-bound to G-proteins to their inactive GDP-bound forms **[33]**. As depicted in Figure 3-2, T-lymphocyte invasive and metastasis protein 1 (Tiam1) and Vav2 represent the two GEFs for Rac1 **[33]**. Our experimental design was focused on investigating whether co-provision of a clinically relevant concentration of metformin would inhibit Vav2 and thus, prevent conversion of inactive Rac1 to its active confirmation.

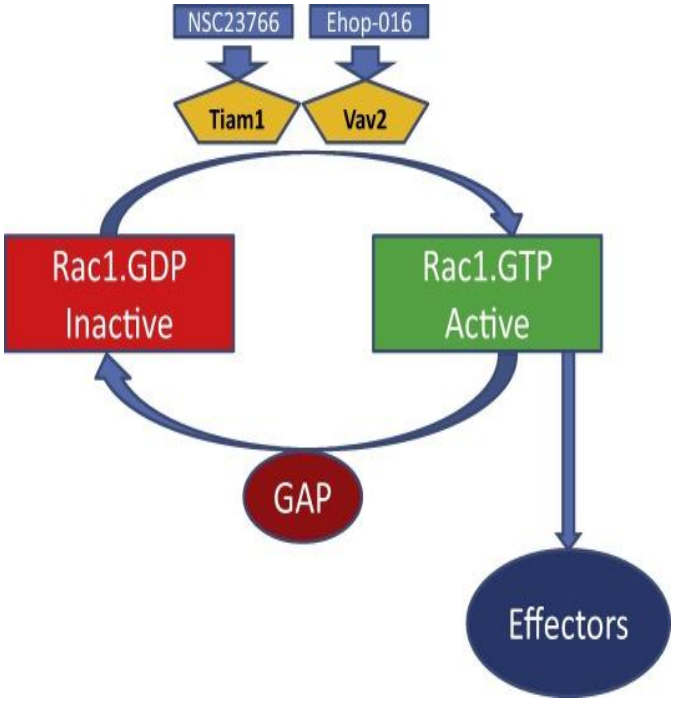


Figure 3-3: Guanine nucleotide exchange factors: [A schematic depicting the conversion of inactive Rac1 to its active GTP-bound confirmation by guanine nucleotide exchange factors (Tiam1 and Vav2) and conversion of active to inactive confirmation by GTPase-activation proteins. ref. (33)]

Guanine nucleotide exchange factor Vav2 (Vav2) and regulatory effects of metformin

Guanine nucleotide exchange factor Vav2 (Vav2) is one of the regulator proteins that induces the GDP/GTP exchange for Rac1 and belongs to the diffuse B-cell lymphoma (Dbl) family of proteins and is ubiquitously distributed **[38, 39]**. Vav2 is activated by tyrosine phosphorylation on epidermal growth factor receptor (EGFR) and interacts with phosphatidylinositol (3,4,5)-triphosphate (PIP3) generated by phosphatidylinositol-4,5-biphosphate 3-kinase (PI3K) **[40-42].**

According to the data published by Veluthakal *et al.***[39]**, Vav2 phosphorylation increases under glucotoxic conditions and it is the increased Vav2 phosphorylation that mediates the Rac1 activation and glucose stimulated insulin secretion. Based on these findings, we asked if metformin treatment of the INS-1 832/13 cells could result in Vav2 inhibition and thereby, inhibit the conversion of inactive Rac1 to active Rac1. The data accrued from the studies, showed that Vav2 phosphorylation increased in INS-1 832/13 cells exposed to HG-conditions. It is noteworthy, however, that metformin treatment did not restore the Vav2 phosphorylation to normal levels, but instead induced an increased Vav2 phosphorylation in cells exposed to LG conditions. Together, these findings suggest that metformin does not inhibit Vav2 phosphorylation even though it inhibited HG-induced Rac1 activation in INS-1 832/13 cells. These data indicate alternate mechanisms might exist for metformin-induced effects.

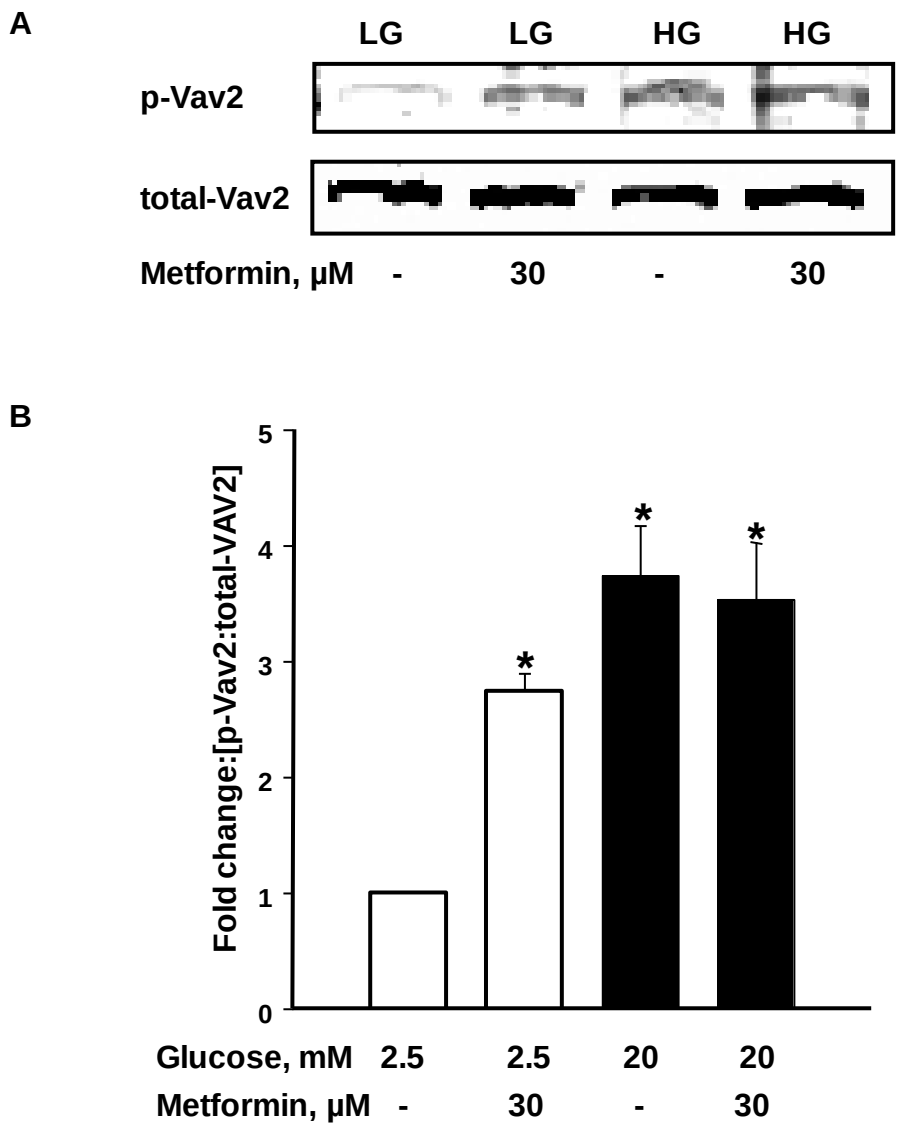


Figure 3-4: Metformin fails to inhibit HG-induced Vav2 phosphorylation in INS-1 832/13 cells: **Panel A:** INS-1 832/13 cells were treated with LG (2.5mM) and HG (20mM) in the absence or presence of Metformin (30 µM) for 24 h. Cell lysates were separated and analyzed using western blotting for phosphorylated and total Vav2. **Panel B:** Band intensities for phospho-Vav2 were measured using densitometry and the ratios were calculated over total-Vav2 in the presence of metformin (n=3 in INS-1 832/13 cells). *$p < 0.05$ vs. 2.5mM glucose alone.

Gluco or glucolipotoxic conditions induce CD36 expression

Cho *et al.* have demonstrated that CD36, a class B scavenger receptor, plays critical regulatory roles in numerous physiological and pathological functions by inducing distinct

cellular responses in multiple cell types such as cardiac muscle, skeletal muscle and adipocytes **[43].** **C**luster of **D**ifferentiation 36 or **C**luster **D**eterminant 36(CD36) is a fatty acid transporter, membrane protein that mediates glucotoxicity induced β-cell dysfunction by increasing the transport of fatty acids into the pancreatic beta cell and undergoes HG-induced increased expression in the intestinal epithelial cells **[26, 44-46].** According to the data published by Wallin and associates, overexpression of CD36 inhibits the glucose mediated fatty acid oxidation and also resulted in impaired glucose stimulated insulin secretion mediated by fatty acids **[47].**

Several recent studies have established novel roles for CD36 in the onset of HG-induced β-cell dysfunction and death. Based on these observations, we asked if HG-conditions increase expression of CD36 in INS-1 832/13 cells, and if so, if Rac1 activation represents an upstream signaling mechanism for HG-induced CD36 expression. We further questioned if metformin exerts any protective effects on HG-induced CD36 expression.

HG-induced CD36 expression in INS-1 832/13 cells: Protection by metformin

In the next of studies we asked if metformin prevents HG-induced CD36 expression in INS-1 832/13 cells. Data in Figure 3-5 demonstrate a significant increase in the expression of CD36 in INS-1 832/13 cells following exposure to HG conditions. In addition, co-provision of metformin markedly suppressed HG-induced expression of CD36. It is noteworthy, however, like Rac1 activation [Figure 3-1], metformin treatment slightly increased CD36 expression under basal glucose conditions [Figure 3-5; Panel A; lane 1 vs. 2]. Pooled data from multiple experiments are included in Figure 3-5 [Panel B]. Compatible with data described above [Figure 3-2], findings from this experiment suggest that HG-induced rac1 activation and nuclear translocation and downstream CD36 expression are sensitive to metformin.

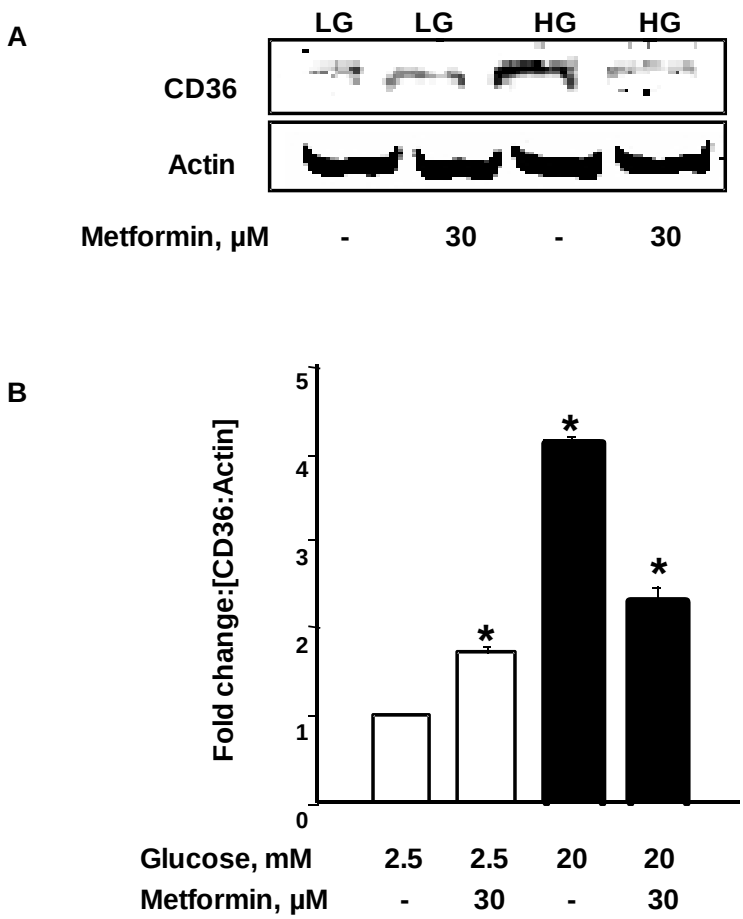


Figure 3-5: Metformin inhibits HG-induced CD36 expression: Panel A: INS-1 832/13 cells were treated with LG (2.5mM) and HG (20mM) with and without Metformin [30μM] for 24 hrs. Cell lysates for CD36 were separated using western blotting and actins were used as loading control. Data are representative of three experiments. **Panel B:** Quantification of the CD36 and actin bands was done using densitometric analysis and ratios were calculated over actins in the presence and absence of metformin. (n=3 in INS-1 832/13 cells). *$p < 0.05$ vs. 2.5mM glucose alone.

Reversal of glucotoxicity and lipotoxicity induced CD36 expression by EHT 1864

It has been established that glucotoxic and glucolipotoxic conditions mediate β-cell dysfunction by inducing CD36 overexpression **[26, 44-47].** EHT 1864 is a small molecule Rac1 inhibitor that prevents Rac1 activation by inhibiting the switch from inactive to active confirmation, thereby, keeping the G-protein in the inactive state **[48].** To address the question of whether Rac1 activation represents an upstream signaling mechanism for HG-induced CD36 expression, we quantified HG-induced expression of CD36 in INS-1 832/13 cells exposed to

EHT 1864. Data in Figure 3-4 indicate a significant increase in the CD36 expression in INS-1 832/13 cells exposed to glucotoxic and lipotoxic conditions. Moreover, HG-induced expression of CD36 was markedly reduced following inhibition of Rac1, thus suggesting that Rac1 activation may be upstream to CD36 expression in β-cells exposed to HG and lipotoxic conditions.

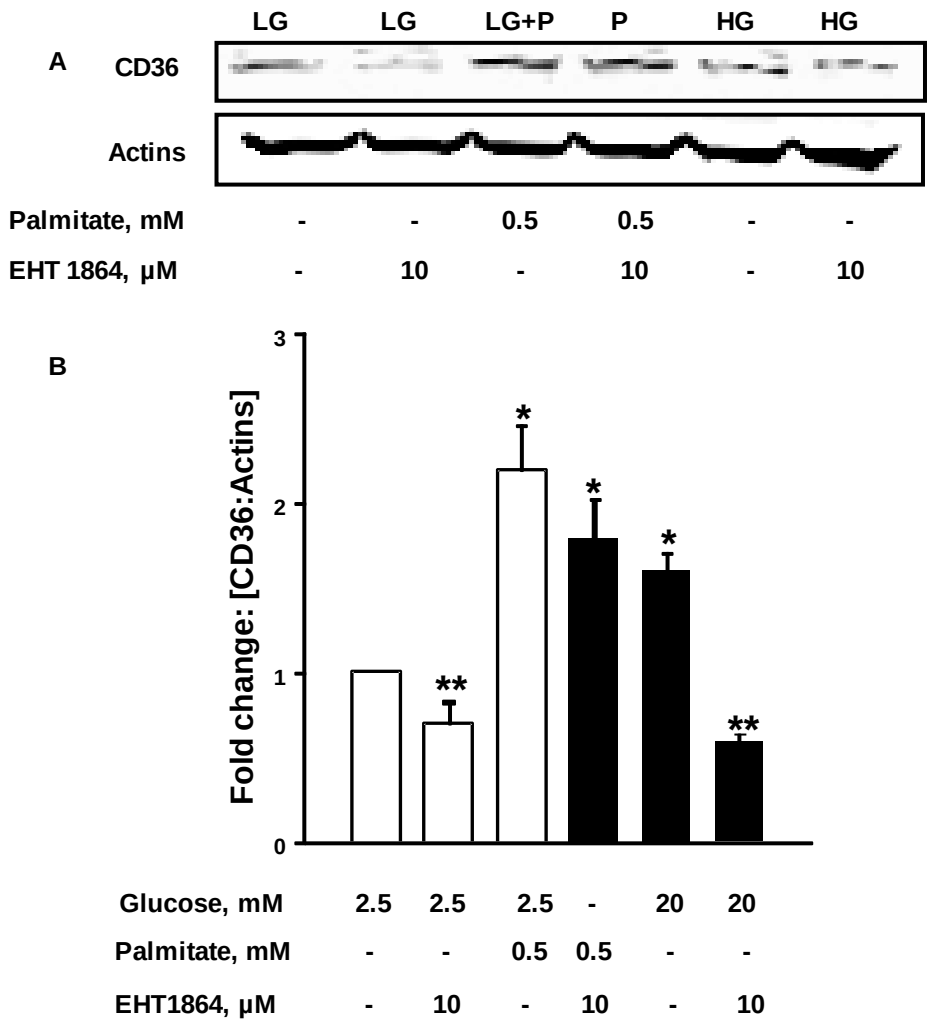


Figure 3-6: Glucolipotoxic conditions induce CD36 expression: regulation by Rac1: Panel A: INS-1 832/13 cells were treated with low glucose (2.5mM), high glucose (20mM) and palmitate (0.5mM) for 24 hrs in the presence and absence of EHT 1864 (10 µM). Cell lysates for CD36 were separated using western blotting and actins were used as loading control. Data are representative of three experiments. *$p < 0.05$ vs. 2.5 mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone.

Summary of findings:

- High glucose conditions induce increased Rac1 nuclear accumulation in INS-1 832/13 cells, primary rodent islets and human islets
- Glucotoxicity-induced Rac1 activation and nuclear translocation was attenuated by metformin
- HG-induced Vav2 phosphorylation was unaffected by metformin treatment
- Increased CD36 expression in response to HG conditions was reduced by metformin
- EHT 1864 inhibited CD36 expression
- CD36 may be downstream of Rac1

CHAPTER 4: GLUCOTOXIC CONDITIONS PROMOTE STRESS KINASE ACTIVATION AND PANCREATIC ISLET β-CELL DYSFUNCTION AND DEMISE

Based on the findings of Syed *et al.* and Sidarala *et al.*, we now have an understanding that glucotoxic conditions lead to β-cell dysfunction and apoptosis induced via stress kinase (p38MAPK and JNK1/2) and p53 activation **[7, 9, 49].** Inflammation and several other stress stimuli lead to the activation stress kinases (mainly serine/threonine kinases) and hence cause impaired insulin signaling **[50].** There are 3 types of Mitogen-activated protein kinases (MAPK) and these includes extracellular signal-regulated kinases (ERK), p38MAPK and the cJun N-terminal kinases (JNK). Differentiation signals and mitogens activate the ERKs whereas p38MAPK and JNK are activated by stress stimuli and are referred to as stress-activated kinases (SAPK) and these MAPK lead to the increased expression of certain inflammatory cytokines **[51, 52].** The tumor suppressor p53 plays a critical role in mediating apoptosis and tumor suppression via transcriptional regulation of downstream targets after the cells have been exposed to genotoxic stress **[53, 54].** Under normal conditions, the proteasome degradation pathway keeps a control on the levels of p53 **[54].** There are several enzymes such as kinases, phosphatases, acetyltransferases, deacetylases, ubiquitin ligases, deubiquitinases, methylases, and sumoylases that play a crucial role in stabilizing p53 **[54-56]**. Based on the findings mentioned above, we asked if HG-induced stress kinase (p38MAPK and JNK 1/2) and p53 activation could be inhibited by co-provision with clinically relevant concentrations of metformin in INS-1 832/13 cells.

p38MAPK and JNK 1/2 activation mediated by glucotoxic conditions is prevented by metformin

There are 4 different isoforms of p38MAPK and these include alpha, beta, gamma and delta **[52]**. Different tissues express these isoforms differently. The brain tissue expresses less p38α isoform whereas the δ isoform is abundant in tissues such as endocrine glands and neutrophils, p38γ is found in almost all the tissues with abundance in muscle tissue and p38β isoform is the main isoform **[51, 57-61].** "All p38 isoforms are activated, in response to appropriate stimuli, by dual phosphorylation in the activation loop sequence Thr-Gly-Tyr" **[51].**

It has been well established that glucotoxic conditions induce an increased p38MAPK activation which mediates pancreatic β-cell dysfunction and demise by mediating p53 expression and metabolic dysfunction **[49].** Based on the findings of Sidarala *et al.*, we designed an experiment to assess whether co provision with metformin (15 & 30 µM) attenuated p38MAPK phosphorylation in INS-1 832/13 cells exposed to HG conditions. Quantification of HG-induced activation of p38MAPK demonstrated a significant stimulation in INS-1 832/13 cells [Figure 4-1]. Metformin treatment significantly alleviated such effects. Interestingly, however, as in the case of Rac1 activation [Figure 3-1], Vav2 phosphorylation [Figure 3-3] and CD36 expression [Figure 3-5], metformin treatment increased p38MAPK activation under basal glucose conditions despite its protective effects against high glucose-induced Rac1 activation, CD36 expression and p38MAPK activation.

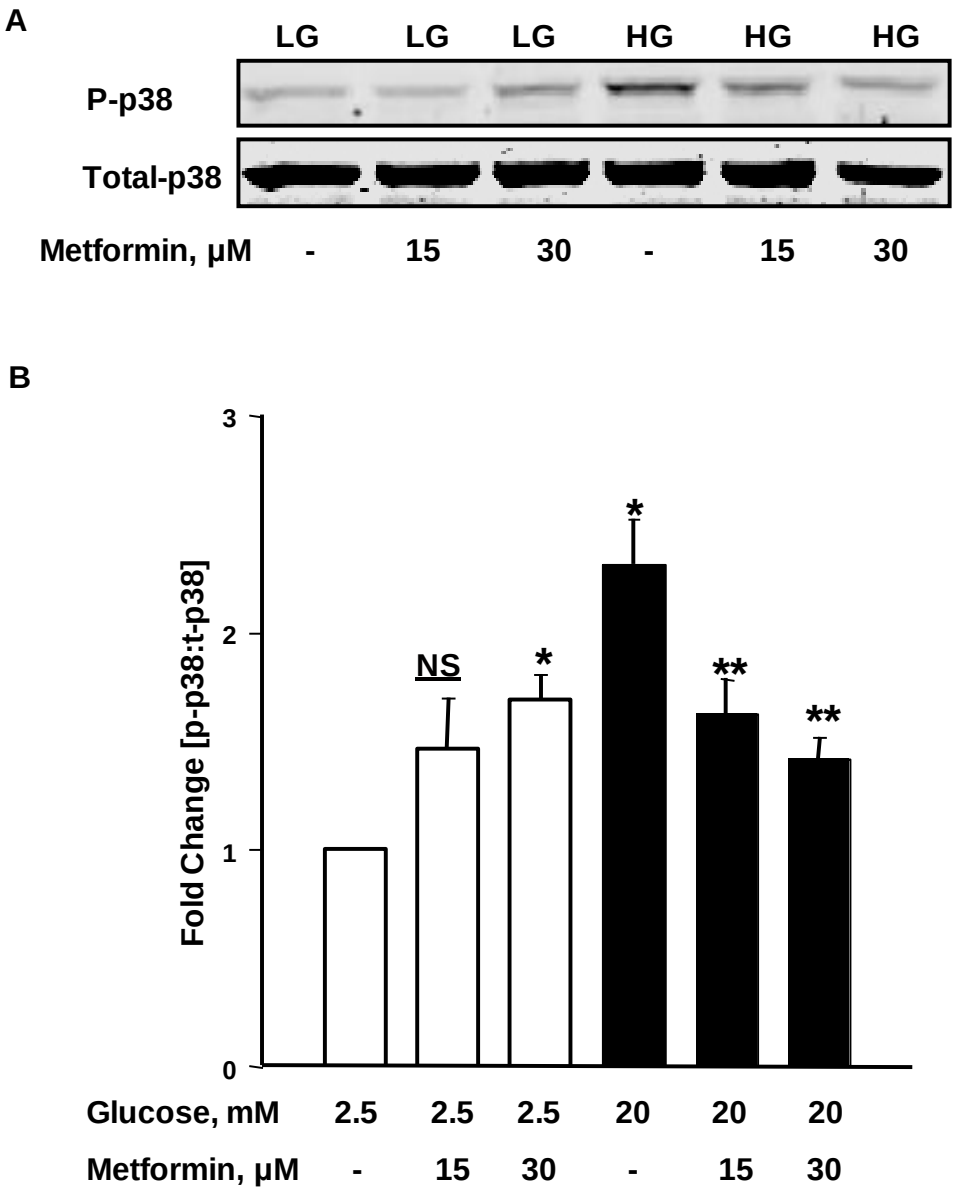


Figure 4-1: Metformin inhibits HG-induced p38MAPK activation: Panel A: INS-1 832/13 cells were incubated with LG [2.5mM] and HG [20mM] in the presence and absence of metformin [0-30 µM] for 24 h. Western blotting was used to separate and analyze the cell lysates for phopho-p38MAPK and total-p38MAPK. **Panel B:** Quantification of the phopho-p38 bands was done by densitometry and the ratios were calculated over total-p38 in the presence of metformin [n=5]. *$p < 0.05$ vs. 2.5mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone; NS: not significant.

HG-induces JNK1/2 activation: Regulation by metformin

cJun N-terminal kinase (JNK) is activated by stress stimuli and is also referred to as stress -activated kinase (SAPK). JNK1, JNK2 and JNK 3 are the three genes that encode for JNK **[51]**. JNK1 and JNK 2 are expressed ubiquitously whereas JNK 3 is expressed in brain, heart and testis **[62, 63]**. Apoptosis, cell proliferation and cell migration play an important role in mediating JNK to induce a specific response to a specific stimulus **[51]**. In case of dietary and genetically mediated obesity, tissues such as adipose tissue, muscle tissue and liver have highly activated JNK pathway **[64-67]**. In pancreatic β-cells, JNK pathway activation leads to the inhibition of fatty acid induced GSIS via phosphorylation and inhibition of IRS1 and IRS2 **[68]**. According to the data published by Syed *et al*. and Kaneto *et al.* increased oxidative stress induces JNK activation, thereby leading to pancreatic β-cell death **[7, 69, 70].**

Based on these findings, we questioned whether HG induces JNK 1/2 activation in INS-1 832/13 cells and if so, does metformin provide any protection to the cells by inhibiting the JNK 1/2 activation?. To address this question, we assessed the HG-induced JNK 1/2 activation in INS-1 832/13 cells and the data depicted in Figure 4-2 depicted a significant increase in HG-induced JNK 1/2 activation. It is noteworthy, however, that co-provision with clinically relevant concentrations of metformin only resulted in attenuating JNK 1 but not JNK 2 phosphorylation. This specific inhibition of JNK 1 by metformin could be attributed to the fact that it is JNK 1 which is the key player in the development of obesity and insulin resistance **[71]** and the substrate specificity due to splicing of one of the two alternate exons encoding for the kinase domain might influence the JNK interaction with the docking sites on the substrate **[72]**.

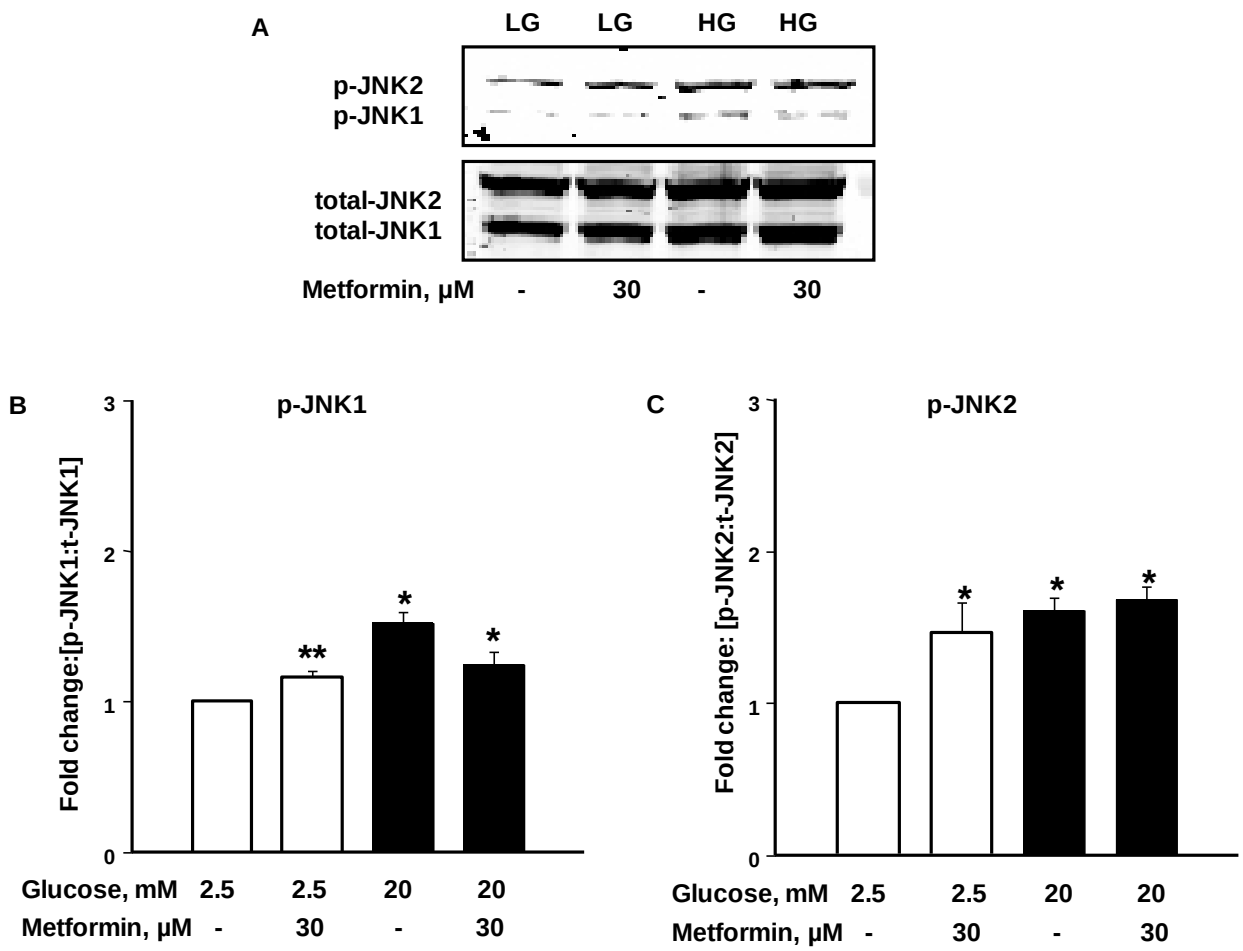


Figure 4-2: HG-induced JNK 1/2 phosphorylation: reversal by metformin: Panel A: INS-1 832/13 cells were treated with LG (2.5mM) and HG (20mM) in the absence and presence of metformin (30μM). Cell lysates for p-JNK 1/2 and total JNK 1/2 were analyzed and separated using western blotting. **Panel B:** Densitometric analysis of the bands for p-JNK 1 was done and the ratios were calculated over total JNK 1 in the presence and absence of metformin. **Panel C:** Bands for p-JNK 2 were analyzed by densitometry and the ratios were calculated over total-JNK 2 in the presence and absence of metformin. [n=3]. *$p < 0.05$ vs. 2.5mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone.

Metformin inhibits HG-mediated activation of p53 activation

In response to cell stress, p53 plays a pivotal role in activating/deactivating genes involved in cell cycle arrest, DNA repair, senescence or apoptosis via post-translational events such as ubiquitylation, phosphorylation, acetylation, sumoylation, methylation, and neddylation.**[73-76].** "p53 functions primarily as a transcription factor and is biologically active

as a homotetramer comprising 4 × 393 amino acid residues" **[77]**. As mentioned earlier, there are several enzymes such as kinases, phosphatases, acetyltransferases, deacetylases, ubiquitin ligases, deubiquitinases, methylases, and sumoylases that play a crucial role in stabilizing p53 **[54-56].** In pancreatic β-cells, streptozotocin and palmitic acid increase the p53 activity and this increased p53 activity leads to reduced β-cell proliferation, thereby, inducing glucose intolerance and hypoinsulinaemia **[78].** Data published by Sidarala *et al.* has shown HG induces p53 activation in INS-1 832/13 cells, rat islets, ZDF islets and human islets and treatment with several pharmacological inhibitors (EHT 1864, Simvastatin, GGTI-2147, SB203580) inhibited HG-induced p53 activation. Based on these findings, we questioned could metformin treatment impart protection to INS-1 832/13 cells from HG-induced p53 activation. Data from Figure 4-3 represents a significant stimulation of p53 activation induced by glucotoxic conditions and co-provision with clinically relevant concentrations (15 and 30 μM) attenuated HG-induced p53 activation in INS-1 832/13 cells. It is noteworthy that, unlike in the case of Rac1 activation, CD36 expression, p38MAPK and JNK1/2 activation, metformin did not exert any effects on p53 activation under basal glucose conditions.

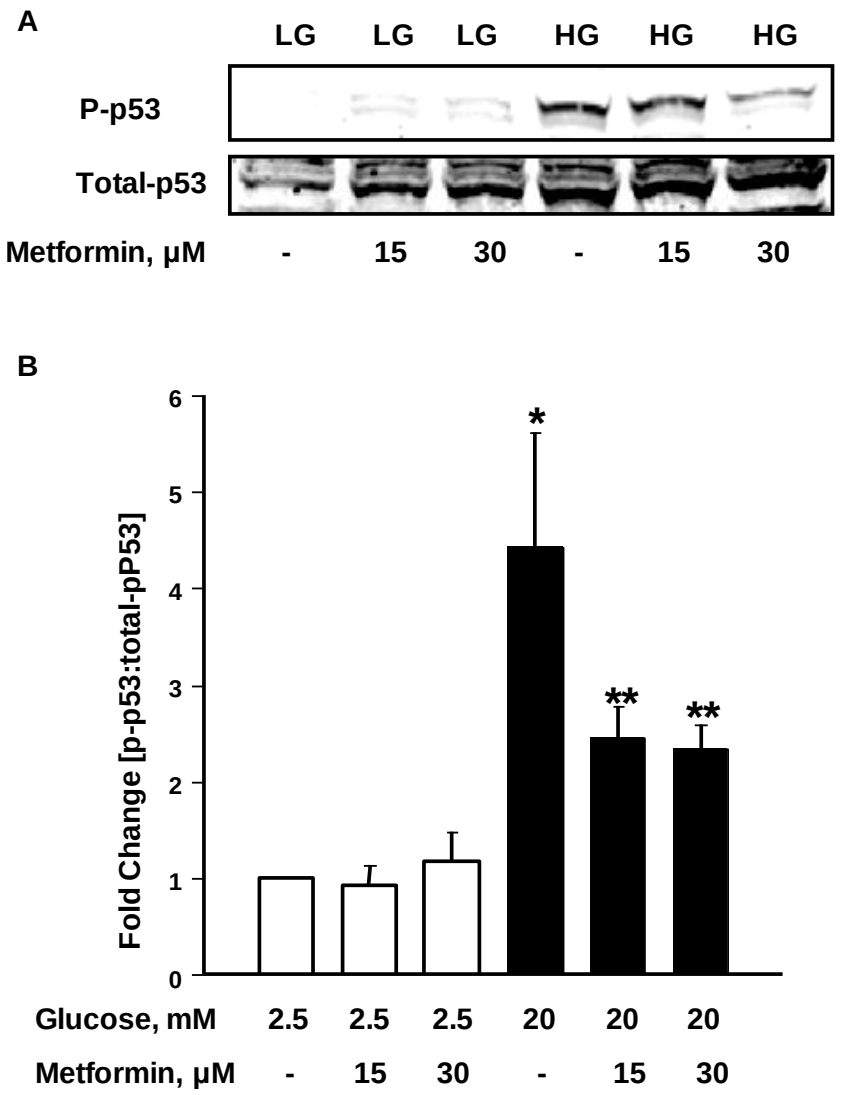


Figure 4-3: Metformin attenuates HG-induced p53 activation: Panel A: INS-1 832/13 cells were treated with LG [2.5mM] and HG [20mM] in the absence or presence of metformin [0-30 µM] for 24 h. Cell lysates were separated and analyzed using western blotting for phosphorylated and total p53. **Panel B:** Band intensities for phospho-p53 were measured using densitometry and the ratios were calculated over total-p53 in the presence of metformin. *$p < 0.05$ vs. 2.5mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone [n=3].

Summary of findings:

- HG-induced p38MAPK activation was reduced by metformin treatment
- Metformin inhibited HG-mediated phosphorylation of JNK 1 only
- p53 activation by glucotoxic conditions was prevented by metformin

CHAPTER 5: GLUCOTOXIC CONDITIONS INDUCE MITOCHONDRIAL DYSFUNCTION LEADING TO CASPASE ACTIVATION AND APOPTOSIS

β-cell failure because of chronic exposure to PA or palmitic acid and high glucose is induced by mitochondrial damage mediated by increased mitochondrial superoxide production resulting in increased expression of uncoupling proteins **[79, 80].** According to Fu *et al.* increased mitochondrial uncoupling and subsequent decreased glucose stimulated ROS production might lead to glucose or lipid induced β-cell death **[80].** In case of diabetes there is a change in the mitochondrial morphology as reported by Kabra *et al.* and Anello *et al.* **[81, 82].** According M. Anello et al. islets from diabetic patients show a decrease in glucose-induced mitochondrial membrane hyper polarization and decreased ATP levels, thereby resulting in an uneven ATP'ADP ratio **[81].** Mitochondrial dysfunction/damage results in release of cytochrome c, Bax (pro-apoptotic) and Bcl2 (pro survival).

Based on the findings, the next set of studies were focused on investigating whether exposure of INS-1 832/13 cells to glucose induce any changes in the levels of Bax and Bcl2 and how do these changes affect the Caspase activation. We further asked if the deleterious effects of HG-induced Bax, Bcl2 and Caspase activation could be reversed by co provision with metformin.

HG-mediated Bax and Bcl2 expression: regulation by metformin

Bax is a pro apoptotic factor whereas Bcl2 is a pro-survival factor. For a cell to avoid apoptosis, it is essential that the balance between Bax and Bcl2 activation remains unchanged **[83]**. Bax and Bcl2 are involved in the intrinsic or the mitochondrial pathway of apoptosis inducing alterations in the mitochondria leading to cytochrome c release and activation of

caspases **[84].** According to Schellenberg *et al.* Bax translocation from outer mitochondrial membrane to cytosol via retrotranslocation and in response to apoptosis, retrotranslocation is attenuated causing the mitochondrial Bax accumulation **[85].** Bcl2 plays an important role in regulating the decrease in mitochondrial membrane potential and inhibits the pro-apoptotic proteins, thereby maintaining a balance to avoid apoptosis **[85]**.

According to the data reported by Thurmond *et al.* INS-1 832/13 cells exposed to glucolipotoxic conditions showed a decrease in HG-induced Bcl2 phosphorylation, suggesting that glucolipotoxic conditions induce apoptosis **[86].** In the next set of studies, we asked if HG induced Bax activation and a Bcl2 deactivation. We also assessed whether metformin inhibited Bax activation or restored Bcl2 activation in INS-1 832/13 cells. Data depicted in Figure 5-1 [Panels A-C] shows an increase in HG-induced activation of 2 isoforms of Bax (Bax α and Bax β). It is noteworthy, however, that metformin treatment induced slight inhibition of only Bax α isoform and had no effect on Bax β isoform. Further data from Figure 5-1 [Panel D] shows a decrease in the activation of Bcl2 and co-provision with metformin was ineffective in restoring the Bcl2 levels in INS-1 832/13 cells.

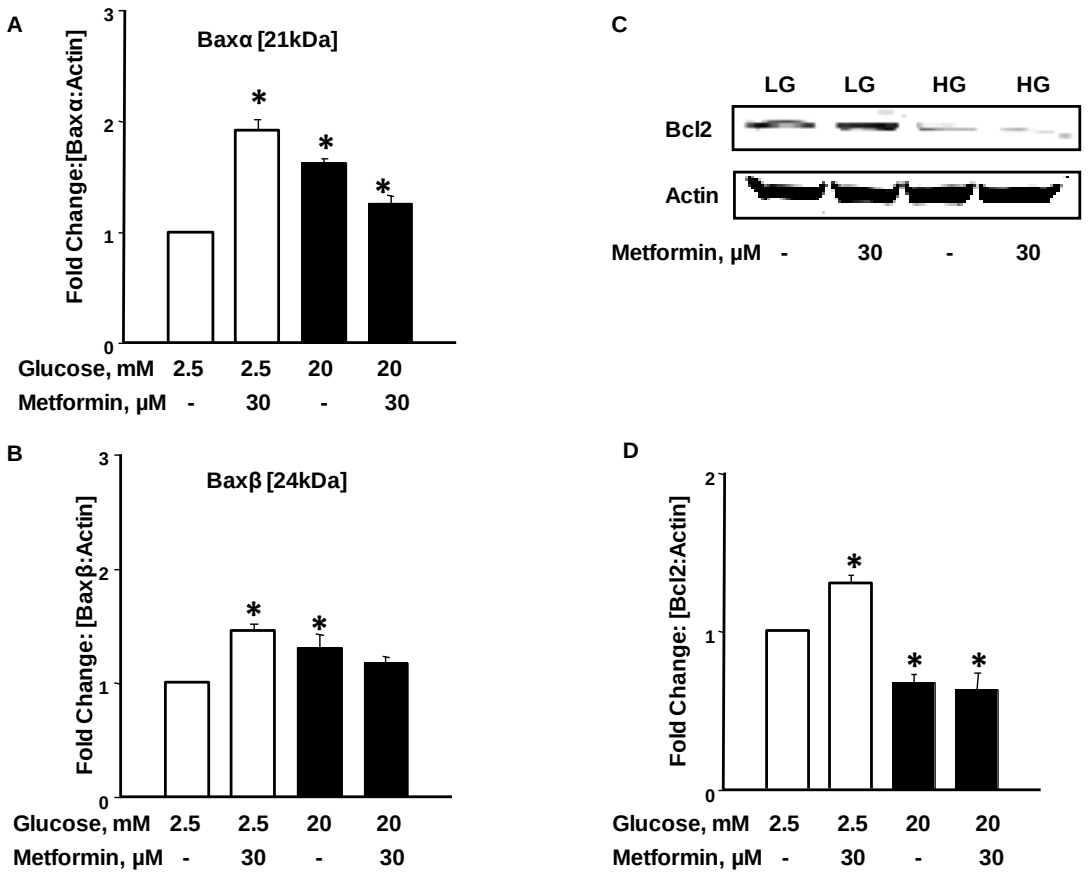


Figure 5-1: Metformin suppresses HG-induced Bax phosphorylation and ineffective in restoring Bcl2 levels: Panel A: INS-1 832/13 cells were treated with LG (2.5mM) and HG (20mM) in the absence and presence of metformin (0-30 μM) for 24 h. Cell lysates were analyzed for Bax using western blotting. Equal loading of proteins was confirmed using actin as a loading control. Band intensities of Baxα were quantified using densitometry and the ratios were calculated over actin in the presence of metformin. **Panel B:** Densitometric analysis was used to quantify the Baxβ isoform intensities and ratios were calculated over actin with and without metformin. *$p < 0.05$ vs. 2.5mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone. **Panel C:** INS-1 832/13 cells were treated with LG (2.5mM) and HG (20mM) in the absence and presence of metformin (0-30 μM) for 24 h. Cell lysates were analyzed for Bcl2 using western blotting. Actin was used as a loading control. **Panel D:** Band intensities of Bcl2 were quantified using densitometry and the ratios were calculated over actin in the presence and absence of metformin (n=3 in INS-1 832/13 cells). *$p < 0.05$ vs. 2.5mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone.

Metformin reverses HG-induced activation of Caspase-3

"Caspases are evolutionarily conserved cysteine-aspartyl specific proteases that play a key role in apoptosis" **[87]**. There are 14 caspases reported in mammals and some of them play a

key role in apoptosis while the other caspases participate in cytokine activation **[88, 89]**. The "c" in caspase represents the cysteine protease and the "aspase" refers to the caspase's activity to cleave after the aspartic acid residue **[90].** Caspases are inactive initially and it is only the cleavage of a specific aspartate cleavage site which makes a caspase functional in executing apoptosis **[90].** According to Fraser and Evan, some caspases activate the other caspases in a subsequent manner, for instance, caspase 8 or initiator protease activates caspase 1 or the amplifier protease which in turn induces the activation of machinery proteases or caspase 3/caspase 7 **[91].** According to the experiments performed by Liadis *et al.* caspase 3 plays an important role in β-cell apoptosis **[87].**

In the last series of experiments, we determined the degree of caspase-3 activation, a marker for mitochondrial dysregulation, in INS-1 832/13 cells exposed to HG conditions in the absence or presence of metformin. Our findings demonstrated a high degree of caspase-3 activation in cells exposed to HG conditions. This is evidenced by emergence of the cleaved [biologically- active] caspase-3 band under these conditions [Figure 5-2; Panel A]. We also observed a significant reduction in high glucose-induced caspase-3 activation in cells exposed to metformin. A modest increase in caspase-3 activation was also seen in cells under normal culture [basal] conditions. Pooled data from multiple experiments are provided in Figure 5-2 [Panel B].

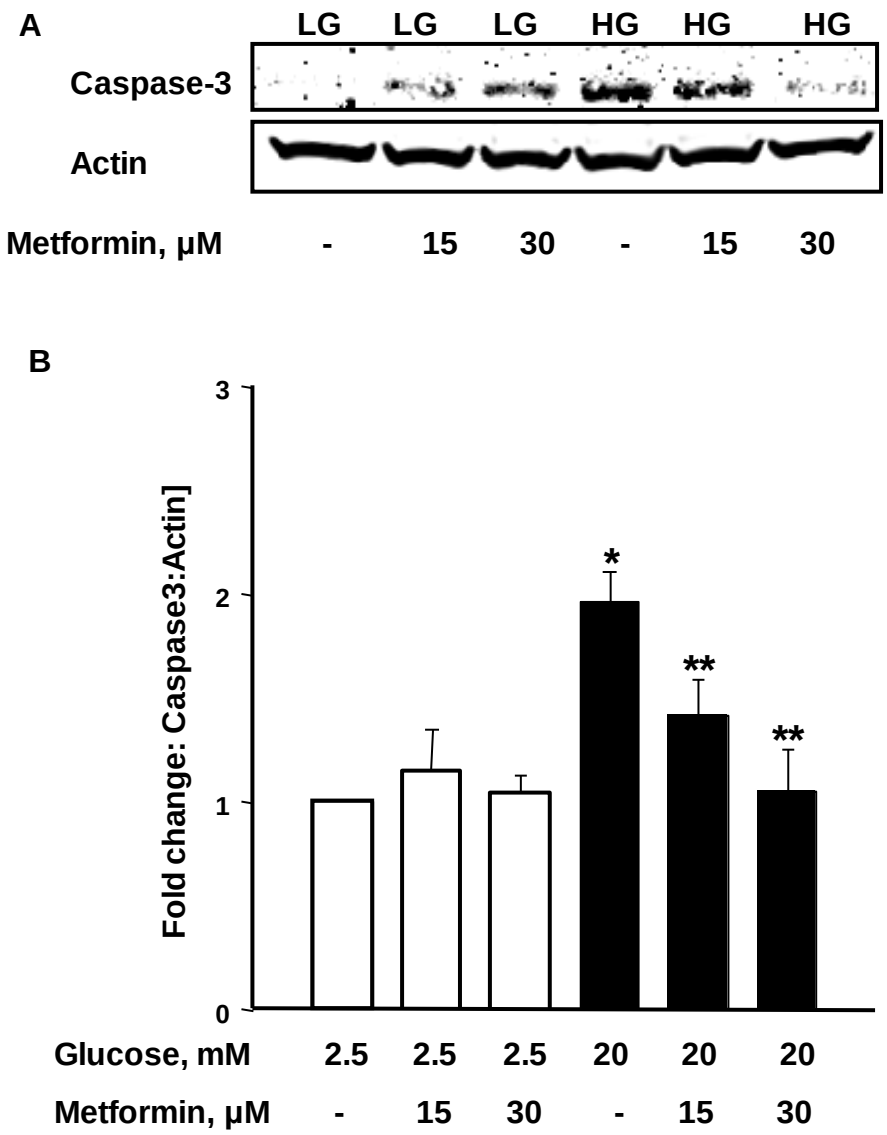


Figure 5-2: HG-mediated caspase-3 activation is reduced by metformin: Panel A: INS-1 832/13 cells were incubated with LG [2.5mM] and HG [20mM] in the absence and presence of metformin [0-30 µM] for 24 h. Cell lysates were analyzed for caspase-3 using Western blotting. **Panel B:** Densitometry was used to quantify the bands and the ratios were calculated over actin in the presence of metformin. *$p < 0.05$ vs. 2.5mM glucose alone, **$p < 0.05$ vs. 20mM glucose alone [n=3].

Metformin protects loss in cell viability induced by glucotoxic conditions in INS-1 832/13 cells

Compatible with above findings [Figure 5-2], we noticed significant protection by metformin of HG-induced loss in metabolic cell viability in these cells [Figure 5-3]. Our findings

demonstrated a significant reduction in metabolic cell viability in INS-1 832/13 cells incubated under HG conditions. Co-provision of metformin significantly protected these cells from metabolic alterations. Compatible with data described in the above sections, metformin treatment alone markedly suppressed cell viability under basal glucose concentrations. These data clearly imply dual regulatory roles of metformin.

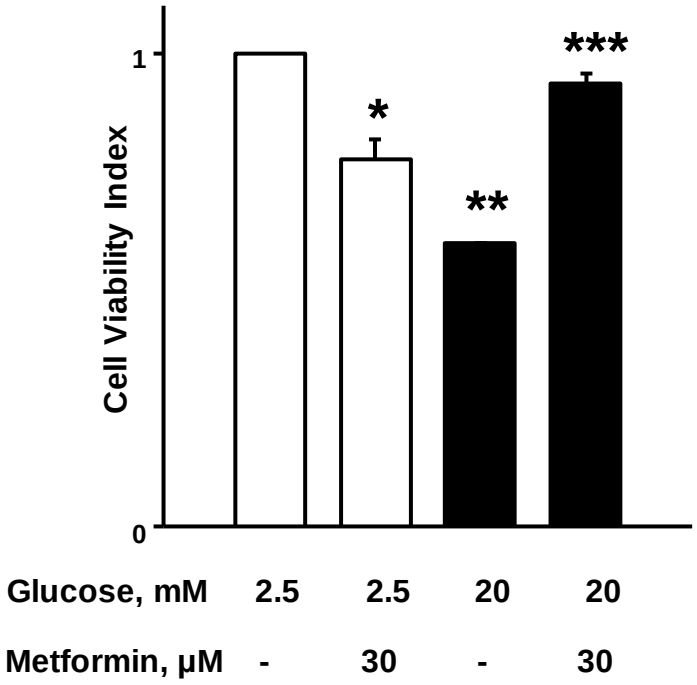


Figure 5-3: Cell viability assay: INS-1 832/13 cells were incubated with low [2.5 mM] or high [20mM] glucose for 24 hrs in the absence or presence of metformin [30 µM]. After 24 hrs of glucose treatment, the cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide [MTT] reagent for 4 hrs and absorbance was measured at 540 nm. Data are represented as mean ± SEM from 8-10 determinations in each condition. *p=0.033, ** p=0.0001; and *** p=0.78 [not significant] vs. basal conditions.

Based on the findings described in these studies, we conclude that HG conditions promote sustained activation and nuclear translocation of Rac1 and metabolic dysfunction [CD36 expression, stress kinase activation, Bax activation, decreased Bcl2 activation, caspase-3

activation and loss in metabolic cell viability] in pancreatic islet β-cells. We also provide evidence in support of significant protection of these metabolic defects by metformin. Together, these data provide evidence for novel targets for metformin, specifically at the level of pancreatic β-cell.

Summary of findings:

- Metformin attenuated expression of one Bax isoform and showed no effect on Bcl2 expression
- Caspase-3 activation was inhibited by metformin in a dose dependent manner
- Cell viability loss due to glucotoxic conditions was also abrogated by metformin

CHAPTER 6: DISCUSSION

It is well established that chronic exposure of pancreatic β-cells to HG conditions results in significant metabolic alterations and dysfunction, including loss in cell proliferation and GSIS leading to apoptotic demise of the β-cell **[92].** More recent findings from our laboratory have demonstrated novel regulatory roles for Rac1, a small G-protein, in the induction of islet β-cell dysfunction under the duress of glucotoxicity **[7, 34, 49, 93].** Specifically, we demonstrated that, under glucotoxic conditions, sustained activation of Rac1 results in accelerated Nox2 signaling leading to increased oxidative stress [ROS production], stress kinase [p38MAPK and p53] activation, mitochondrial [caspase 3 activation] and nuclear [Lamin degradation] dysfunction and cell death **[7, 34, 49, 93, 94].** During these investigations, we also identified two guanine nucleotide exchange factors [Tiam1 and Vav2] that mediate activation of Rac1 in eliciting damaging effects on β-cells **[33].** My studies described in this dissertation examined potential alterations, if any, in the subcellular distribution [mislocalization] of Rac1 in pancreatic β-cells exposed to glucotoxic conditions. Furthermore, I assessed the efficacy of metformin, an antidiabetic drug, against HG-mediated effects on β-cell function. Salient findings from my studies are exposure of INS-1 832/13 cells to HG-conditions result in nuclear association of Rac1. I also demonstrated that clinically-relevant concentrations of metformin prevent HG-induced; [i] Rac1 activation and nuclear translocation; [ii] CD36 expression; [iii] stress kinase, Bax and caspase-3 activation; and [iv] loss in cell viability. Implications of these findings in the context of regulatory roles of constitutively-active Rac1 in the pathology of islet dysfunction, and its prevention by metformin are discussed below.

Several recent studies from our laboratory have reported sustained activation of Rac1 in clonal INS-1 832/13 β-cells, normal rodent islets, and human islets under the duress of metabolic

stress, including glucotoxicity, lipotoxicity, exposure to proinflammatory cytokines, and biologically-active sphingolipids, such as ceramide **[7, 9, 33, 34, 49, 93, 95, 96].** These observations were also confirmed in islets derived from type 2 DM animal models and human donors with T2DM. Furthermore, pharmacological inhibition [NSC23766] of Tiam1, a GEF for Rac1, attenuated Rac1 activation in all the above experimental conditions, thus suggesting that Tiam1 represents one of the GEFs that mediate hyper-activation of Rac1. More importantly, inhibition of Tiam1-Rac1 signaling axis also prevented HG-induced, Nox2 activation and downstream stress kinase activation and mitochondrial dysfunction in pancreatic β-cells exposed to HG conditions **[33].** Together, these findings implicate Rac1 as a key mediator of islet β-cell dysfunction in metabolic stress and diabetes.

Several recent studies have investigated beneficial effects of metformin against islet β-cell function. It is noteworthy that these *in vitro* investigations utilized a wide range of metformin concentrations [10 μM-1 mM]. For example, Simon-Szabo and associates **[17]** have reported significant attenuation of palmitate-induced [lipoapoptosis] ER stress [elF2α phosphorylation and CHOP expression] and stress kinase [JNK1/2] activation by metformin [10-100 μM] in rat insulinoma cells. Using murine islets and human islets Lundquist *et al.* **[97]** have demonstrated a marked reduction by metformin [20 μM] in nitric oxide synthase-derived nitric oxide, insulin secretory dysfunction and loss in cell viability under conditions of long-term exposure to glibenclamide and HG. Using rodent islets, Hashemitabar and associates have demonstrated beneficial effects of metformin [15 μM] on insulin gene expression, insulin secretion and islet cell viability **[98]**. Natalichhio and coworkers have shown significant restoration of GLP-1 receptor impairment by metformin [0.5-1.0 mM] in murine islets following exposure to palmitate **[99].** Together, the above studies provide supporting evidence for

beneficial/protective effects of metformin against gluco-, or lipotoxicity and ER stress. Our current findings demonstrate marked protection of INS-1 832/13 cells, by metformin, against HG-induced metabolic defects at concentrations as low as 30 µM.

A growing body of evidence implicates CD36, a fatty acid transport protein, in cell apoptosis under glucolipotoxic conditions **[26, 45]**. Data from our current studies have provided evidence to suggest that Rac1 activation is upstream to CD36 expression since EHT1864, a known inhibitor of Rac1 **[48, 100],** attenuated HG-induced CD36 expression in INS-1 832/13 cells. Our findings are also compatible with recent observations of Elumalai and associates demonstrating regulatory roles for Rac1-Nox2 signaling axis promotes CD36 expression in INS-1 cells under the duress of glucotoxic conditions **[45]**. Using specific inhibitors of Tiam1-Rac1 [NSC23766] and Nox2 [VAS2870] these researchers were able to identify Tiam1-Rac1-Nox2 signaling steps as upstream modulators of CD36 expression under HG glucose exposure conditions. It should be noted that the findings of Elumalai *et al.* **[45]** further validate our original proposal that Tiam1-Rac1-Nox2 signaling pathway contributes to islet β-cell dysfunction under metabolic stress conditions **[33, 34, 93, 101].** Data from our current investigations involving a structurally distinct inhibitor of Rac1 [EHT1864] further support this working model. Our current observations also demonstrated a significant reduction in HG-induced CD36 expression by metformin at 30 µM concentration. Further, inhibition of Rac1-CD36 pathway appears to regulate the downstream stress kinase [p38MAPK and p53] activation and mitochondrial dysregulation [Bax and caspase-3 activation] in INS-1 832/13 cells. In further support of our findings are the recent observations of Moon and associates demonstrating significant protective effects of metformin [0.5 mM] against oxidative- and endoplasmic reticulum stress-induced CD36 expression in clonal β-cells and rodent islets **[26].**

It is noteworthy that metformin appears to exert dual regulatory roles in pancreatic β-cells. For example, in the current studies, we consistently noted that under basal glucose conditions, metformin increased Rac1-CD36-Stress kinase activation to a modest, but significant degree while affording protection against HG-induced effects on these signaling steps. Along these lines, using insulin-secreting MIN6 cells, Jiang and associates have provided evidence to suggest dual regulatory roles for metformin in pancreatic β-cell function. First, under normal growth conditions metformin significantly suppressed MIN6 cell proliferation and triggered apoptosis *via* a mechanism involving AMPK-activation and autophagy-related signaling steps **[102].** Interestingly, however, metformin significantly protected MIN6 cells against palmitate-induced mitochondrial dysfunction [caspase activation] and cell death. While these data appear to support our findings of significant protective effects of metformin on HG-induced effects in INS-1 832/13 cells, it should be noted that studies of Jiang and associates **[102]** used relatively high concentrations of metformin [2 mM] compared to much less concentration of metformin we used in our current studies [15-30 μM].

Based on the available evidence, we propose a working model [Figure 6-1] that HG-conditions stimulate Rac1 activation. It is also proposed that hyperactive Rac1 might regulate other apoptotic function including CD36 expression, other stress kinase [p38MAPK and JNK1/2] activation to initiate signaling events leading to mitochondrial dysregulation [Cleaved Caspase-3 and Bax activation] and nuclear collapse [Lamin-B degradation] terminating in loss in GSIS, inhibition of proliferation and cellular apoptosis **[7, 49, 94, 95].** We also propose that metformin affords protection against above mentioned glucotoxic effects at clinically relevant concentrations [15-30 μM]. Future studies will determine potential targets for metformin, specifically regulatory factors for Rac1 activation including GEFs, GTPase-activating proteins

and the Rho GDP dissociation inhibitor in the islet β-cell, the interplay of which is expected to retain Rac1 in its active, GTP-bound conformation to promote downstream signaling events that could contribute to metabolic dysregulation and onset of type 2DM **[34, 103].**

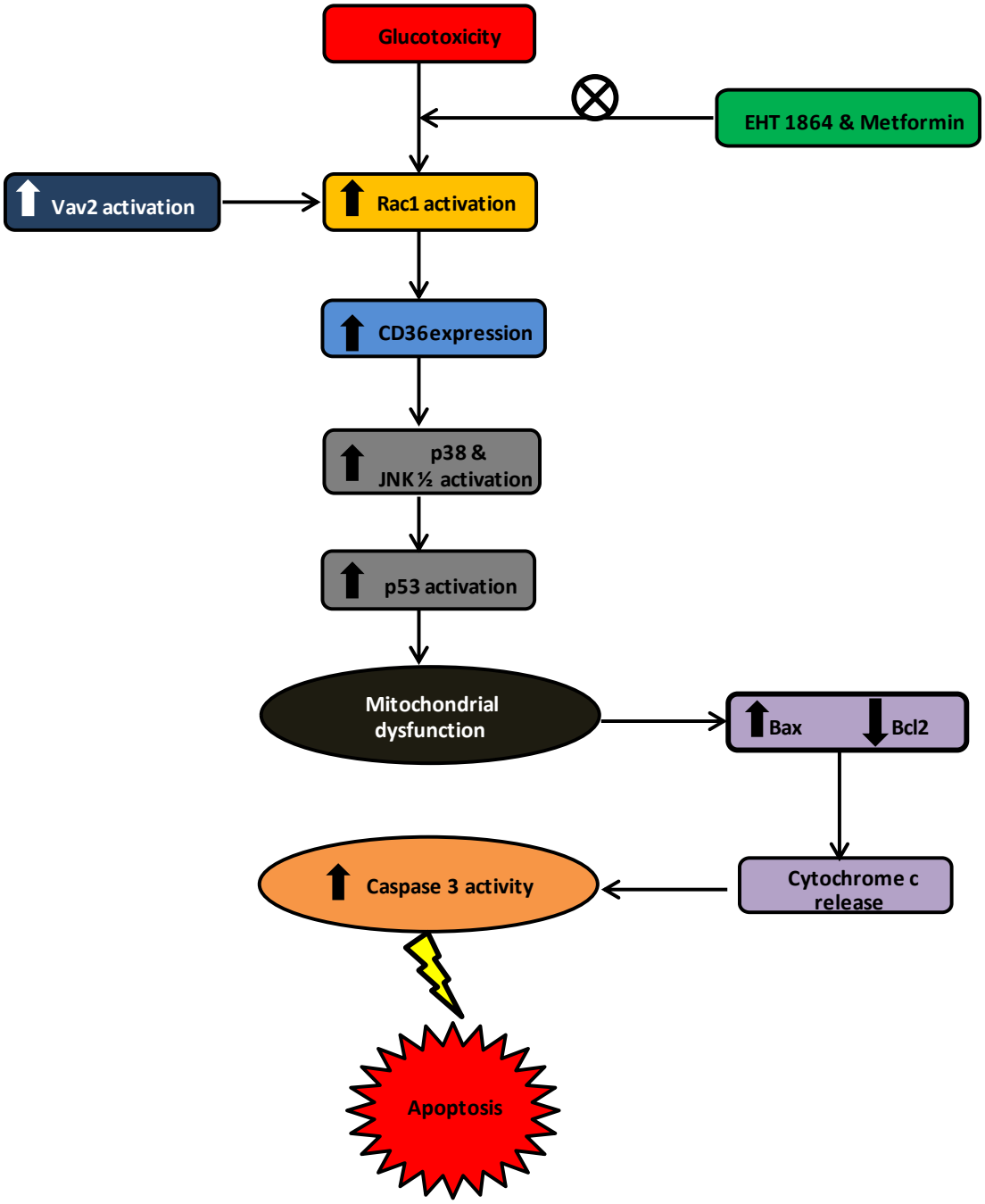


Figure 6-1: A proposed model for metabolic stress induced dysfunction of pancreatic islet β-cells: Reversal by metformin

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

Based on the findings accrued in my studies, I conclude that hyperactive Rac1 might regulate other apoptotic function including CD36 expression, other stress kinase [p38MAPK and JNK1/2] activation to initiate signaling events leading to mitochondrial dysregulation and nuclear collapse [Lamin-B degradation] terminating in loss in GSIS, inhibition of proliferation and cellular apoptosis. My findings further support the existing evidence in the literature **[7, 49, 94, 95].** My findings suggested that metformin affords protection against above mentioned glucotoxic effects at clinically relevant concentrations [15-30 μM]. Future studies will determine potential targets for metformin, specifically regulatory factors for Rac1 activation including GEFs, GAPs and GDI in the islet β-cell, the interplay of which is expected to retain Rac1 in its active, GTP-bound conformation to promote downstream signaling events that could contribute to metabolic dysregulation and onset of type 2DM **[34, 103].**

Data from my studies are summarized below:

1. Metformin reduced the HG-induced Rac1 activation and nuclear translocation
2. HG-induced CD36 expression is downstream to Rac1 activation
3. EHT 1864 and metformin attenuated HG-induced CD36 expression
4. Metformin abrogated HG-induced p38MAPK and JNK 1 activation
5. HG-induced p53 activation was suppressed by metformin
6. Metformin inhibited one isoform of Bax and had no effect on Bcl2
7. Caspase-3 activation was reduced in a dose dependent manner by metformin
8. Metformin protected INS-1 832/13 cells against HG-induced loss in metabolic cell viability.

Future Directions:

The results from my studies have enriched our current understanding of how HG-induced small G-proteins (Rac1) activation and their mislocalization plays an important role in activating fatty acid transporters, stress kinases, p53 and metabolic dysfunction, ultimately leading to β-cell dysfunction and demise. My studies have also provided novel insights into the mechanism of action of metformin in suppressing the sustained activation and mistargetting [nuclear localization] of Rac1, thereby, eliciting protective effects on high glucose-induced metabolic dysregulation of pancreatic β-cells. In my opinion, my work laid foundation to future work in further validating my observations and hypothesis in in vivo models of obesity , impaired insulin secretion and T2DM.

Following is the list of studies that need to be carried out to further assess the validity of my model:

- Recent studies have reported that HG conditions promote the degradation of the common α-subunit of FTase/GGTase, thereby causing Rac1 activation and nuclear translocation, resulting in the activation of Rac1 mediated downstream signaling mechanism contributing to β-cell death. It would we worthwhile to assess protective effects of metformin against HG-induced defects in G-protein prenylation in pancreatic β-cells.
- Furthermore, it is essential that we confirm our observations on potential cytoprotective effects of metformin on HG-induced metabolic defects in primary rodent and human islets.

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ABSTRACT

METFROMIN, GLUCOTOXICITY AND ISLET DYSFUNCTION

by

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MAY 2017

Advisor: Dr. Anjaneyulu Kowluru

Major: Pharmaceutical Sciences

Degree: Master of Science

Glucotoxicity is the leading cause for β-cell dysfunction [e.g., defective glucose-stimulated insulin secretion] in Type 2 Diabetes [T2DM]. Recent studies from our lab have shown sustained Rac1 activation leading to the activation of downstream signaling steps including stress kinase [p53, p38MAPK] activation and mitochondrial dysregulation [caspase-3 activation] in pancreatic islet beta-cells exposed to glucotoxic [HG] conditions [20 mM; 24 hrs]. Metformin [MF] is an oral anti-diabetic drug that is being widely prescribed to T2DM. MF works by suppressing hepatic glucose production and increasing glucose uptake by the target tissues. However, potential beneficial effects of MF on pancreatic beta-cell dysfunction under HG conditions have not been studied to date. Therefore, in the current studies, we asked if MF [0-30 μM; clinically relevant concentrations] affords protective effects against HG-induced metabolic dysfunction of the pancreatic beta [INS-1 832/13] cells. Since recent studies from our laboratory have demonstrated activation of Rac1, a small G-protein, as an upstream signaling event to stress kinase activation, we asked if protective effects of MF may, in part, be due to inhibition of HG-induced Rac1 activation in INS-1 832/13 cells. Data from these studies have suggested nearly 40% inhibition in HG-induced Rac1 activation [3.43±0.57 fold over basal; n=4;

p<0.05] by MF. Evidence is also presented to highlight novel roles for sustained activation of Rac1 in HG-induced expression of **C**luster of **D**ifferentiation 36 [CD36], a fatty acid transporter protein, which is implicated in cell apoptosis. Western blot analysis indicated a significant increase in the phosphorylation of p38MAPK [2.31±0.21 fold over basal; n=5; p<0.05], JNK1/2 and phosphorylation of p53 [4.42±1.20 fold over basal; n=3; p<0.05] in INS-1 832/13 cells. MF [15µM] markedly attenuated HG-induced p38MAPK [74.8%], JNK 1 and p53 [55.7%] activation under these experimental conditions. Our data from Bax phosphorylation [an indicator of cell dysregulation] studies demonstrated an increase in the phosphorylation of two Bax isoforms [Baxα by 1.63± 0.04 fold over basal; n=3; p<0.05; and Baxβ by 1.32±0.11 over basal; n=3; p<0.05]. MF [30µM] attenuated the phosphorylation of only Baxα isoform [by 77.3%]. Lastly, our data also suggested that co-provision of MF significantly reduced [72.4%] HG-induced caspase-3 activation. Together, these findings suggest significant protection by MF against HG-induced metabolic defects [activation of Rac1-stress kinase-caspase-3 signaling module] in the islet beta-cell. Potential implications of these findings in the context of novel and direct regulation of islet β-cell function by metformin are discussed.

AUTOBIOGRAPHICAL STATEMENT

EDUCATION

- MS in pharmaceutical sciences, Eugene Applebaum College of Pharmacy and Healthy Sciences, Wayne State University, Detroit, MI, USA
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ABSTRACTS

- Metformin protects pancreatic islet beta-cell dysfunction under glucotoxic conditions
- Glucotoxicity promotes aberrant activation and mislocalization of Ras-related C3 botulinum toxin substrate 1 [Rac1] and metabolic dysfunction in pancreatic islet β-cells: Reversal of such metabolic defects by metformin

PUBLICATION

- Glucotoxicity promotes aberrant activation and mislocalization of Ras-related C3 botulinum toxin substrate 1 [Rac1] and metabolic dysfunction in pancreatic islet β-cells: Reversal of such metabolic defects by metformin [Under review]