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Mechanisms Of Cytokine-Induced Metabolic Dysfunction Of The Pancreatic Beta-Cell

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**MECHANISMS OF CYTOKINE-INDUCED METABOLIC DYSFUNCTION OF THE
PANCREATIC BETA-CELL**

by

ABIY MUSSA MOHAMMED

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

2013

Major: PHARMACEUTICAL SCIENCES

Approved by:

Advisor

Date

DEDICATION

This work is dedicated to all my family members who supported me throughout my educational career.

ACKNOWLEDGEMENTS

First and foremost, I would like to extend my heartfelt gratitude to Dr. Anjaneyulu Kowluru for his continuous support and guidance throughout the program, without his support and acceptance I would not have completed the program. I would like to extend my appreciation for previous and current members of Dr. Kowluru`s lab especially Dr. Arora, Dr. Veluthakal and Khadija for their support and morale while I was doing my project and write-up. I would like also to extend my appreciation to Dr. David Thomas for his guidance and support while I was in his laboratory.

Concerning my project, I would like to extend my sincere gratitude to my committee members, Dr. Commissaris, Dr. Chen, Dr. Yi and Dr. Hadden for their advice, comments and suggestions through the course of the project.

I would like also to extend my special thanks and appreciation to all my families especially, my wife Bruktawit for their support and encouragement throughout my educational life.

Last but not least, I would like to be thankful for the Department of Pharmaceutical Sciences, Wayne State University for giving me the chance to join the program; without the support of the department and Dr. Corcoran, all these would have been impossible.

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LIST OF ABBREVIATIONS

APC: Antigen presenting cell

BB rats: Bio-breeding rats

2-BP: 2-Bromopalmitate

CAT: Catalase

CER: Cerulenin

CHAP: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate

CHOP: C/EBP homologous protein

CMT: Carboxyl methyltransferase

DCHFDA: 2` 7-Dichlorofluorescein diacetate

ER: Endoplasmic reticulum

ETC: Electron transport chain

FPP: Farnesyl pyrophosphate

FTase: Farnesyl transferase

FTI: Farnesyl transferase inhibitor

GAPs: GTPase activating proteins

GDIs: GDP dissociation inhibitors

GEFs: Guanine nucleotide exchange factors

GGTase: Geranylgeranyltransferase

GGTI: Geranylgeranyltransferase inhibitor

GPCRs: G-protein coupled receptors

GSH: Reduced glutathione

GSSG: Oxidized glutathione

GSIS: Glucose-stimulated insulin secretion

GSHPER: Glutathione peroxidase
GSHRED: Glutathione reductase
IDDM: Insulin-dependent diabetes mellitus
IKK: IκB kinase
iNOS: Inducible nitric oxide synthase
IR: insulin receptor
IRS: insulin receptor substrate
JNK: c-Jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
NFκB: Nuclear factor-kappa B
NO: Nitric oxide
NOD mice: Non obese diabetic mice
NOX: NADPH oxidase
PI3K: Phosphatidylinositol 3-kinase
PKB/AKT: Protein kinase B
PMT: Palmitoyltransferase
ROS: Reactive oxygen species
SAM: S-adenosyl methionine
SOD: Superoxide dismutase
Tiam1: T-lymphoma invasion and metastasis 1
Type I DM: Type I diabetes mellitus
Type II DM: Type II diabetes mellitus
UPR: Unfolded protein response

Chapter 1: Introduction

Diabetes mellitus

Diabetes is a disease characterized by elevated blood glucose level either due to deficit in insulin production and/or the inability of the body to utilize it. Diabetes mellitus can be broadly classified as Type I DM [Type I Diabetes Mellitus] and Type II DM [Type II Diabetes Mellitus]. Type I DM is manifested by relative lack of insulin secretion due to loss of pancreatic β -cells by autoimmune aggression. Type II DM is a metabolic disorder characterized by an increase in blood glucose levels either due to a decrease in insulin secretion from pancreatic β -cells, or tissue resistance to the secreted insulin and accounts for 90% of the disease. Hypersecretion of insulin by the β -cells due to tissue resistance to insulin worsens the situation leading to hyperglycemia and hyperinsulinemia [1]. According to the WHO, 346 million people are living with diabetes mellitus globally and 80% of deaths associated with the disorder occur in middle- and low- income countries [Table 1-1 and 1-2].

Country	Millions
China	90.0
India	61.3
United States of America	23.7
Russian Federation	12.6
Brazil	12.0
Japan	10.1
Mexico	10.3
Bangladesh	8.4
Egypt	7.3
Indonesia	7.0
Pakistan	6.4
Germany	5.0
Philippines	4.2
Italy	4.0
Thailand	4.0

Table 1-1: Top fifteen countries of the world with diabetes mellitus in 2012 [2, 3].

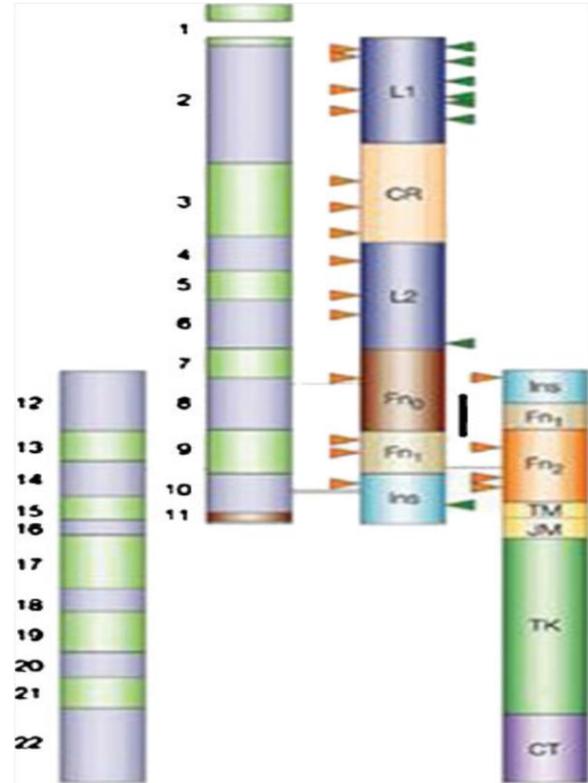
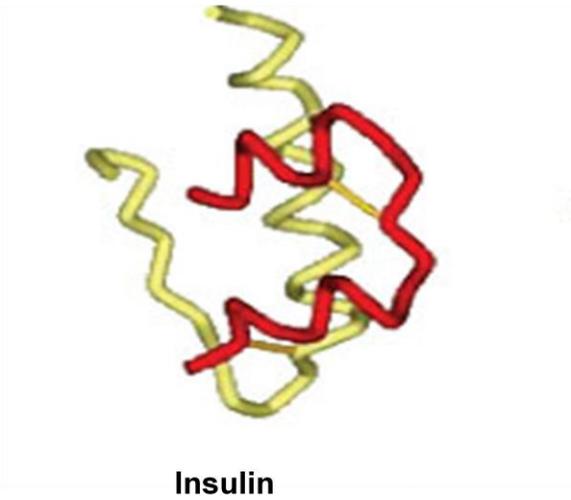
Country	Millions
China	129.7
India	101.2
United States of America	29.6
Brazil	19.6
Bangladesh	16.8
Mexico	16.4
Russian Federation	14.1
Egypt	12.4
Indonesia	11.8
Pakistan	11.4

Table 1-2: Projection for top ten countries of the world with diabetes mellitus by 2030 [2, 3].

Insulin and its receptor

The discovery of insulin in 1920s by Drs Frederick Banting, Charles Best and John Macleod revolutionized the treatment of diabetes in young patients where starvation therapy was the only option for those in need. Insulin is a peptide hormone secreted by the β -cells of the pancreas that facilitates the uptake of glucose, amino acids and fatty acids by the body cells and inhibits the hydrolysis of their precursors namely glycogen, proteins and fats, respectively thereby maintaining the plasma blood glucose concentration between 4-7mM [4-6].

The physiologically active form of insulin in human has two chains. The A chain and B chain having 21 and 30 amino acids respectively. With the exception of few organs in the body such as brain, the majority of body cells depend on insulin for glucose uptake. Insulin binds to its receptor which is tetrameric in nature with two extracellular α - and intracellular β -subunits localized on the cell surface of target organs such as liver, kidney and muscle [Figure 1-1]. Upon binding of insulin to extracellular subunits, the β -subunits which have a tyrosine kinase activity autophosphorylate and transduce the signals to intracellular targets. The biological action of insulin such as expression of GLUT4, enzyme activation [increase in the synthesis of glycolytic and fatty acid synthetic enzymes; eg., acetyl-CoA carboxylase] and enzyme deactivation [decrease in gluconeogenic enzymes; eg., glucose-6-phosphatase] are mainly due to the concerted actions of IR/IRS, PI3K and AKT/PKB pathways [5, 7 8].



Insulin receptor

De Meyts, Bioessays. 2004;26(12):1351-1362

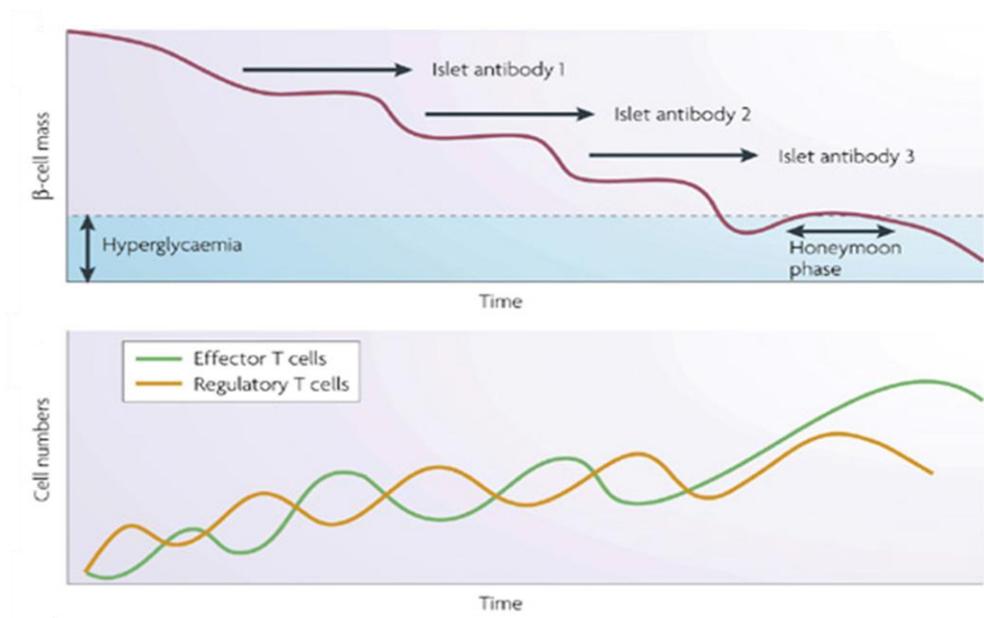
Figure 1-1: Insulin and its receptor.

Structure of the A chain [red; 21 amino acids] and B chain [yellow; 30 amino acids] of human insulin linked by two disulfide bridges [A7–B7 and A20–B19] and insulin receptor which is a tetramer consisting of two extracellular α - and two intracellular β -subunits [5].

Pathogenesis of Type I DM

Based on the therapeutic approach and epidemiological occurrence, it is also known as insulin-dependent diabetes mellitus or juvenile onset diabetes mellitus. It is characterized by an absolute insulin deficiency due to a chronic recurring immune-mediated attack on functional β -cells by the auto-reactive effector T-cells which dominate over the regulatory T-cell through time that leads to major loss in β -cell mass [Figure 1-2]. The presence of auto-antibodies against insulin, glutamic acid decarboxylase 65, islet cell antigen 2, islet

specific glucose-6-phosphatase catalytic subunit related protein, glial fibrillary acidic protein puts susceptible individual at risk for acquiring the disease [9, 10].



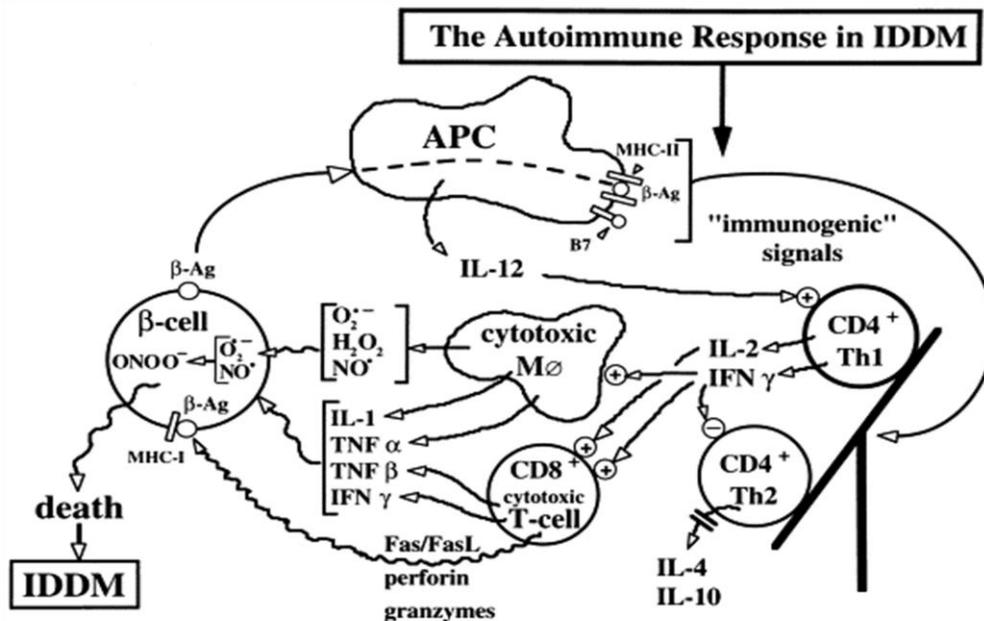
von Herrath M et al. Nat Rev Immunol. 2007;7(12):988-994

Figure 1-2: Beta-cell mass and autoimmune reaction.

The loss of β -cell mass over time due to autoimmune reaction leading to a deficit in insulin secretion and an increase in blood glucose level, and the opposing effect of regulatory T-cells over the auto-reactive effector T-cell at the earlier time point followed by an increase in number of auto-reactive effector T-cells [9].

Although the exact cause of the disease is under scientific scrutiny, it is widely accepted that environmental [e.g., viral infection, presence of nitrosamine in food] and genetic [e.g., susceptibility loci in human leukocyte antigen region] predisposition factors play a role in autoimmune mediated destruction of the pancreatic β -cells. Studies from animal models of type I diabetes and human type I diabetic patients revealed that antigen presenting cells [APC] for instance macrophages and dendritic cells, lymphocytes [CD4+ and CD8+ T-cells] and proinflammatory cytokines released by those immune cells exert significant roles in the pathogenesis of the disease. The auto-antigens such as islet cell antigen 2 and/or

insulin are processed by antigen presenting cells and serve as immunologic signals that disturb the balance of the regulatory and effector T-cell function [Figure 1-3]. The hyperactivation of the effector T-cell further activates cytotoxic T-cell [CD8+] and macrophages that lead to the destruction of pancreatic β -cells either through generation of reactive oxygen and/or nitrogen species, through Fas/FasL, granzymes, perforin and release of proinflammatory cytokines. Among known cytokines, IL-1 β , TNF α , and INF γ have been shown to play a major role in mediating the disease progression [11-14].



Rabinovitch A and Suarez-Pinzon WL, *Biochem Pharmacol.* 1998;55(8):1139-1149

Figure 1-3: Autoimmune destruction of pancreatic β -cell.

Self antigen processed by antigen presenting cell disturbs the balance of effector and regulatory T-cell functions leading to autoimmune destruction of pancreatic β -cell. The hyperactivation of effector T-cell function mediates pancreatic β -cell destruction through proinflammatory cytokines and reactive oxygen species [14].

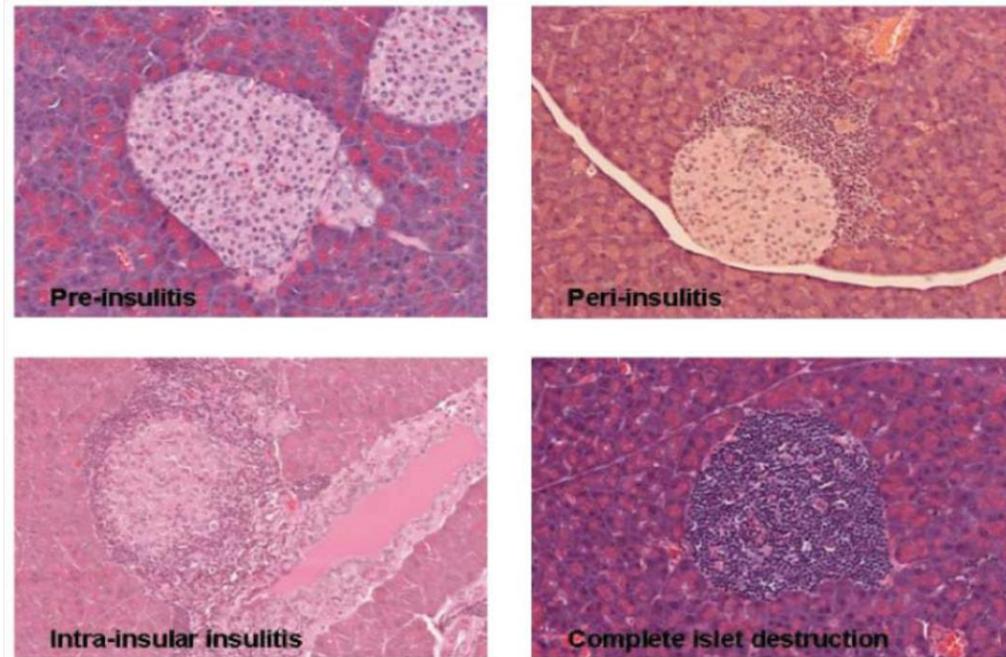
The two principal pathways of apoptosis in pancreatic β -cell due to cytokine insult are the intrinsic pathway and the extrinsic pathway. In intrinsic pathway, cytokines disturb the balance of anti-apoptotic and pro-apoptotic activities of the Bcl-2 family of the mitochondrial

proteins which leads to the release of cytochrome complex to the cytoplasm and activation of the initiator [e.g., caspase-9] and effector caspases [e.g., caspase-3]. The extrinsic pathway is initiated by binding of cytokines to death receptors [e.g., CD95] located on the cell surface followed by activation of adaptor proteins, kinases and transcription factors which culminate in the activation of the initiator and effector caspases that cleave different cellular substrates such as prenylating enzymes [eg., FTase and GGTase] and nuclear envelop proteins [eg., lamin B] [13, 15, 16-18].

Animal models

Non obese diabetic [NOD] mice and bio-breeding [BB] rats are the two commonly used genetic animal models for type I diabetes. Both NOD mice and BB rats share similar pathogenetic mechanisms and diabetic symptoms. The incidence rate of the disease is much higher in the female NOD mice than their male counterparts, whereas both sexes of BB rats equally manifest the disease symptoms. The major histocompatibility complex products namely RT1^{u/u} in BB rats and I-A^{g7} in the NOD mice make them genetically susceptible to the disease [19-21].

NOD mice were established as animal model for type I diabetes in the 1970`s in Shionogi Aburahi Laboratories in Japan by Makino and colleagues from out bred JC1-ICR mice that are prone to cataracts. Unlike other breeds of mice, there is an increased frequency and localization of antigen presenting cells in the pancreas of NOD mice since their birth. This is followed by pre-insulinitis, peri-insulinitis and intra-insular insulinitis [Figure 1-4] caused by auto-reactive T-cell, dendritic cell, CD4+T cell, CD8+T cell, macrophage and B-cells leading to a complete destruction of the islets, and the mice show diabetic symptoms starting from 12 weeks of age and die from the complications around 30 weeks of age, if not treated [22-24].



Bluestone JA et al. Nature. 2010; 464(7293):1293-1300

Figure 1-4: Pathogenesis of type I diabetes in the NOD mice.

Localization of antigen presenting cells such as dendritic cell to the pancreas starts around 3 weeks of age and the different stages of the inflammatory conditions [pre-insulinitis, peri-insulinitis, and intra-insular insulinitis] ensue up to 12 weeks of age. Complete islet destruction and clinical manifestation start from 12 weeks onwards [22, 24].

GTP-binding proteins in islet function

There are three different types of G-proteins in pancreatic β -cell: trimeric G-proteins consisting of α -[39-53 kDa], β -[~37 kDa] and γ -[7-10 kDa] subunits, small molecular weight [17-30 kDa] monomeric G-proteins consisting of Ras, Rho, Rab, Sar1/Arf, Ran families and the elongation factors and *Tau* proteins. The trimeric G-proteins are involved in receptor mediated cellular activation primarily due to hormones and neurotransmitters; the monomeric G-proteins function in vesicular transport and cytoskeletal organization, and the elongation factor and *Tau* proteins have a role in protein biosynthesis [25-27].

Post-translational modification of G-proteins

The functions of small monomeric G-proteins are regulated by post-translational modifications such as prenylation, palmitoylation and methylation. Prenylation is the addition of either 15 carbon [farnesyl group] or 20 carbon [geranyl group] derivatives of mevalonic acid into the carboxyl terminal of the cysteine residues by a thioether linkage. Acetyl CoA, which is an important intermediary metabolite in energy production in the body and the starting material for cholesterol synthesis, is the precursor for farnesyl and geranylgeranyl pyrophosphate **[Figure 1-5] [25]**.

Prenylation is catalyzed by a group of enzymes called prenyltransferases which include farnesyltransferase [FTase], geranylgeranyltransferase [GGTase] I and II **[28, 29]**. Based on the specific structural motif of the substrate proteins for prenyltransferase and the subfamily of the protein they prenylate, the FTase and GGTase-I are referred to as CAAX prenyltransferase and GGTase-II is called non-CAAX prenyltransferase or Rab GGTase, where C stands for cysteine, A is aliphatic amino acid and X is the terminal amino acid. Addition of farnesyl group, the 15 carbon derivatives of the mevalonic acid, is catalyzed by FTase, and examples of farnesylated proteins include H-Ras, Lamin A or B and Gy subunits. Incorporation of geranyl group, the 20 carbon derivatives of mevalonic acid, is catalyzed by GGTase and some of the geranylated proteins include Rac1, Rho and Cdc42 **[25, 30]**.



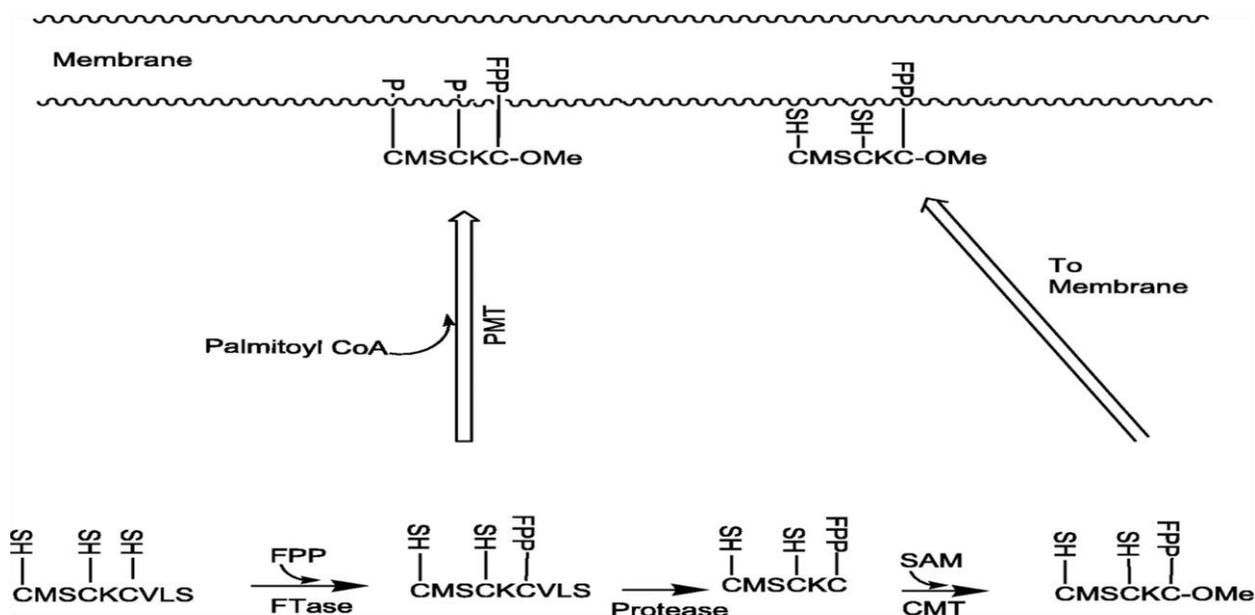
Kowluru A, Endocr Rev. 2010; 31(1):52-78

Figure 1-5: Biosynthesis of farnesyl and geranyl pyrophosphates.

HMG-CoA synthetase and reductase catalyzes the synthesis of mevalonate from acetyl-CoA and acetoacetyl-CoA. Mevalonate then serves as a precursor for farnesyl pyrophosphate [farnesyl-pp], geranylgeranyl pyrophosphate [geranylgeranyl-pp] and cholesterol. The farnesyl pyrophosphate and geranylgeranyl pyrophosphate are incorporated into substrate proteins catalyzed by FTase and GGTase respectively. Lovastatin inhibits mevalonate synthesis; FTI and GGTI inhibit FTase and GGTase, respectively [25].

Prenylation is the first step of the post-translational modifications and it is followed by protease-dependent removal of the three amino acids of the CAAX motif after the prenylated cysteine. In the presence of S-adenosyl methionine [SAM] as a methyl donor, the exposed carboxyl group is methylated by carboxymethyltransferase. Moreover, certain proteins require an additional palmitoylation [addition of palmitate] step upstream of the prenylated

cysteine. These modifications will help the proteins to associate with the membrane and/or activate other proteins in the signal transduction pathways [Figure 1-6] [27].



Kowluru A, Am J Physiol Endocrinol Metab. 2003 ;285(4):E669-E684

Figure 1-6: Steps implicated in post-translational modification of small G-proteins.

Farnesylation/Geranylgeranylation is the first modification followed by protease dependent removal of three amino acids after the prenylated cysteine. These proteins then undergo carboxymethylation reaction in the presence of S-adenosyl methionine and/or palmitoylation at cysteine residue upstream to the prenylated cysteine. These post-translational modifications are requisite for the functions of G-proteins [27].

Inhibition of G-protein activation

Studies using Clostridial toxins, molecular biological techniques [siRNA and dominant negative mutants] and pharmacological inhibitors have shown that G-proteins play important roles in pancreatic β -cell function including insulin secretion [31-36]. G-proteins are also involved in the metabolic dysfunction of β -cells. Studies from our laboratory and others have indicated that small G-proteins, namely H-Ras and Rac1, are involved in nitrosative and oxidative stress-induced pancreatic β -cell death. Both H-Ras and Rac1 are monomeric G-

proteins with molecular weight of ~21 kDa that shuttle back and forth between cytosol and membrane thus acting as molecular switches in signal transduction pathways between the receptor and effector systems. Both H-Ras and Rac1 proteins undergo post-translational prenylation, which is catalyzed by FTase and GGTase, respectively [25, 37-39].

I. Clostridial toxins: Clostridial toxins irreversibly glucosylate and inactivate small G-proteins. Our laboratory provided the first evidence that small G-proteins [e.g., H-Ras] are involved in cytokine-induced metabolic dysfunction of the islet β -cell. These studies were further confirmed using pharmacological inhibitors [40].

II. Inactive mutants and siRNA: Overexpression of an inactive mutant of the regulatory α -subunit of protein prenyltransferase markedly attenuated glucose but not KCl induced insulin secretion [35]. siRNA mediated knockdown of α - and β -subunits of GGTase II, Rab escort protein 1 and isoprenylcysteine carboxyl methyltransferase markedly attenuates glucose stimulated insulin secretion [41].

III. Pharmacological inhibitors: Generic [eg., lovastatin] and site specific inhibitors of protein prenylation [eg., 3-vinyl farnesol, 3-vinyl geraniol, GGTI-2147] as well as the inhibitor of carboxymethylation acetyl farnesylcysteine [AFC] indicated that G-proteins are involved in nutrient induced insulin secretion [30, 32, 34]. The significance of palmitoylation reaction in pancreatic β -cell was also confirmed by using two structurally different inhibitors of palmitoylation reaction, namely, cerulenin and 2-bromopalmitate. These inhibitors protected the insulin-secreting β -cells against noxious effects of cytokines [IL-1 β] [42].

Regulators of G-protein function

There are three major types of proteins that regulate the activity of small G-proteins. These include GEFs [Guanine nucleotide Exchange Factors], GDIs [GDP Dissociation Inhibitors] and GAPs [GTPase Activating Proteins]. During the inactive state, GDI causes G-

proteins to be in the GDP-bound form. Following appropriate stimulation, GDI dissociates from the corresponding G-protein and GEF facilitates the exchange of GTP for GDP, causing the G-protein to be in the active form to regulate its effector proteins. The intrinsic GTPase activity of G-protein along with GAP causes GTP hydrolysis converting the G-protein into inactive state [25].

NADPH oxidase in islet function

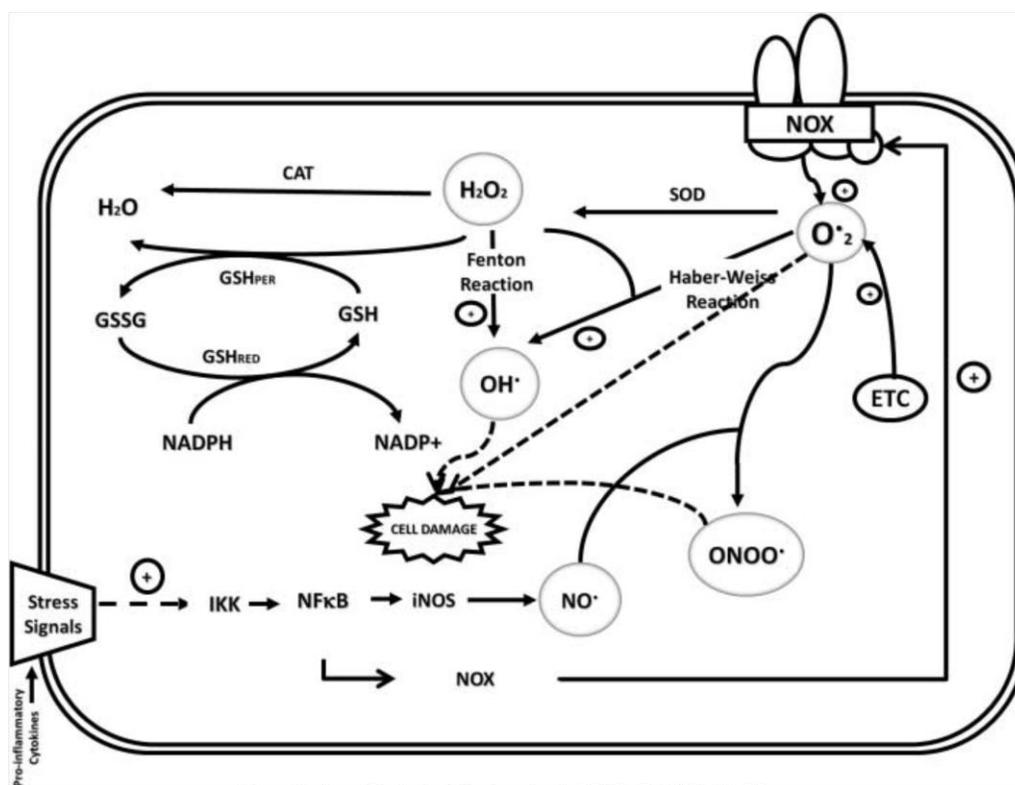
NADPH oxidase [NOX] represents a class of enzymes that has a membrane and cytosolic components, and it is involved in the generation of intracellular reactive oxygen species [ROS] in phagocytic and non-phagocytic cells. From the different isoforms of NOX that exist in the mammalian cells NOX1, NOX2 and NOX4 are found in pancreatic β -cells [43, 44]. NOXA1 [NOX Activator Protein 1] and NOXO1 [NOX Organizer Protein 1] constitute the cytosolic components for NOX1. gp91phox, p22^{phox} and Rap1 makes the membrane bound catalytic subunits and p67^{phox}, p47^{phox}, p40^{phox} and Rac1 form the cytosolic subunits for NOX2. Rac1 is the only cytosolic component for NOX4 [44, 45].

NOX catalyzes one electron reduction of molecular oxygen using NADPH as a cofactor and this process is associated with the generation of superoxide [$O_2^{\cdot-}$] [Figure 1-7]. In addition to the normal physiological stimuli, a number of chemical, physical, and inflammatory stimuli activate the NOX system. The activation of a prototypical NOX with proinflammatory cytokines such as IL-1 β , TNF α , and INF γ causes Rac activation which in turn activates, phosphorylates and translocates other cytosolic components to the membrane-associated components to complete the holoenzyme assembly and function [25, 45, 46].

Rac1, part of the cytosolic components of the NOX2 enzyme system, undergoes geranylgeranylation reactions. It is also regulated by GEF such as T-lymphoma invasion and

Oxidative and Nitrosative stress

Pancreatic β -cells, like other cells in the body, use oxygen to fulfill their energy demand; therefore, they are constantly exposed to oxidants that result from metabolism. In contrast to majority of the cells in the body, pancreatic β -cells have low antioxidant machinery; this makes the islet β -cells more vulnerable to damage by oxidative and nitrosative stress due to reactive oxygen and nitrogen species, respectively. The reactive oxygen and nitrogen species include superoxide radical [$O_2^{\cdot-}$], hydrogen peroxide [H_2O_2], hydroxyl radical [OH^{\cdot}], nitric oxide [NO^{\cdot}] and peroxynitrite [$OONO^{\cdot}$] [Figure 1-8] [49-52].



Newsholme P et al. J Endocrinol. 2012; 214(1):11-20

Figure 1-8: A model for oxidative and nitrosative stress pathways and the corresponding antioxidants system.

Stimuli such as proinflammatory cytokines activate NOX and iNOS gene expression and these activations lead to the generation of superoxide and nitric oxide, respectively. In the presence of iron, superoxide and hydrogen peroxide will be converted to hydroxyl radical through Fenton and Haber-Weiss reactions causing cell damage. Superoxide also reacts with nitric oxide leading to the generation of a highly reactive species called peroxynitrite. The presence of antioxidants such as superoxide dismutase, catalase, glutathione peroxidase and reductase counteract the oxidant effect and prevent the cell damage [52].

Although physiological levels of reactive oxygen species are needed for islet function and insulin secretion, excessive ROS-mediated oxidative stress has been shown to be detrimental to the cells [47, 51]. The two well known cellular sites for the generation of the reactive oxygen species are the mitochondria respiratory system and phagocyte-like NADPH oxidase system. Nutrient-induced insulin secretion from pancreatic β -cell depends on glucose metabolite, ATP production and ROS generation. The NOX in the pancreatic β -cell is the major contributory factor for pathological roles of ROS in β -cell [53, 54]. Proinflammatory cytokines such as IL-1 β , TNF α and INF γ also induce iNOS gene expression through NF κ B [Figure 1-8] leading to generation of reactive nitrogen species such as NO, culminating in β -cell death [52]. Several extant studies demonstrating the role of oxidative and nitrosative stress in cytokine-induced pancreatic β -cell dysfunctions are summarized in Table 1-3.

Model	Cytokines	Findings	Ref.
Rat islets, RINm5F cells	IL-1 β /IL-1 β , TNF α , INF γ	ROS dependent	17
Human and mouse islet, INS1, β TC3 cells	IL-1 β / TNF α , INF γ	ROS dependent	55
MIN6 cells, mouse islets	TNF α , INF γ	ROS dependent	56
Rat islet, BRIN BD 11	IL-1 β / TNF α , INF γ	ROS dependent	57
HIT-T15 cells	IL-1 β	NO dependent	40
HIT-T15, INS-1	IL-1 β	NO dependent	42
RINm5F cells, rat islets	IL-1 β , INF γ	NO dependent	58
RINm5F cells	IL-1 β / TNF α , INF γ	NO dependent	59
Rat (LEW.1W and BB/OK) islets	IL-1 β , INF γ	NO dependent	60
Pancreatic islet, RINm5F cells	IL-1 β , INF γ	NO dependent	61
RINm5F, rat islets	IL-1 β , INF γ	NO dependent	62
Mouse islet, RINm5F cells,	IL-1 β / TNF α , INF γ	NO dependent	63
RINm5F, rat islets	IL-1 β , INF γ	NO dependent	64
RINm5F	IL-1 β / TNF α , INF γ	NO dependent	65
INS1E cells	IL-1 β / TNF α , INF γ	NO dependent	66
Rat islets	IL-1 β , TNF α	NO dependent	67
Mouse islet	IL-1 β / TNF α , INF γ	NO independent	68
Bank vole	IL-1 β / TNF α , INF γ	NO independent	69
iNOS knock out mouse Islets	IL-1 β , INF γ	NO independent	70
INS 832/13 cells	IL-1 β / TNF α , INF γ	ROS and NO dependent	47
RINm5, rat islets	IL-1 β /IL1 β , TNF α , INF γ	ROS and NO dependent	71
BRIN-BD11 cells, mouse islets	IL-1 β / TNF α , INF γ	ROS and NO dependent	72
RINm5F cells	IL-1 β / TNF α , INF γ	ROS and NO dependent	73
Human islets	IL-1 β / TNF α , INF γ	Peroxynitrite dependent	74

Table 1-3: ROS generation and NO release in cytokines-induced pancreatic β -cell dysfunction.

Endoplasmic Reticulum [ER] stress

Endoplasmic reticulum is one of the major cellular organelles involved in the synthesis and folding of cellular proteins and maintaining physiological concentration of calcium in the cell. In the pancreatic β -cell, both physiological and pathological factors cause ER stress. Mutant proteins, hypoxia, viral infection, environmental toxins, proinflammatory cytokines are some of the pathological factors causing ER stress. The three principal ER membrane bound proteins that detect the ER stress are: inositol requiring 1 [IRE1], double-stranded-RNA-dependent protein kinase-like ER kinase [PERK] and activating transcription factor 6 [ATF6] [75-78].

The ER stress initiates a signaling network called unfolded protein response [UPR] and, depending on the nature of the stimuli and extent of damage, the UPR has either a homeostatic outcome that helps to resolve the stress or an apoptotic consequence. Studies have shown that proinflammatory cytokines deplete endoplasmic reticulum calcium by interfering with sarcoendoplasmic reticulum pump $\text{Ca}^{2+}\text{ATPase2b}$ [SERCA2b] causing pancreatic β -cell apoptosis through ER stress [77, 79].

Stress kinases

c-Jun N-terminal Kinase [JNK] and p38 are part of the Mitogen-Activated Protein [MAP] kinase family that are activated by protein kinase cascade following various stress inducing agents that affect cell survival [80-82]. There are three isoforms of JNK; JNK1 and JNK2 are expressed ubiquitously in different tissues of the body and JNK3 is expressed only in brain and heart tissues. There are four different isoforms of p38; p38- α , p38- β , p38- γ and p38- δ [82, 83]. Depending on the nature of the stimuli when these protein kinases get activated, they cause ER stress, activate mitochondrial apoptotic protein and transcription factors in the nucleus [83, 84]. Studies in pancreatic β -cells have shown that p38 MAP kinase and JNK are activated following exposure to proinflammatory cytokines and stress induced during islet isolation process [85-88].

Proposed working model

The overall objective of my doctoral dissertation is to determine putative cellular mechanisms underlying proinflammatory cytokine-induced metabolic dysfunction and demise of the islet β -cell. To accomplish this goal, I propose to:

- test the hypothesis that cytokines induce ROS generation and oxidative stress via activation of phagocyte-like NADPH-oxidase [NOX2];
- test the hypothesis that protein palmitoylation is a key regulatory step involved in cytokine-induced nitrosative and oxidative stress;
- determine the identity of palmitoylated G-proteins, which are involved in cytokine-induced metabolic dysfunction of the islet β -cell; and
- test the hypothesis that pharmacological inhibition of palmitoylation of specific G-proteins [e.g., H-Ras and Rac1] attenuates cytokine-induced activation of oxidative, nitrosative and other stress signaling pathways [e.g., JNK1/2] thereby halting metabolic dysregulation of the islet β -cell.

Based on the results obtained from my studies, I conclude that protein palmitoyl transferase is a novel therapeutic target for the prevention of cytokine-induced metabolic dysfunction of the islet β -cell.

Chapter 2: Materials and Methods

2.1. Materials

Chemicals: IL-1 β , TNF α , INF γ and Z-DEVD-FMK, a caspase inhibitor, were from R & D Systems [Minneapolis, MN]. 2',7-Dichlorofluorescein diacetate, 2-bromopalmitate, Griess reagent, N,N'-dimethyl-9,9'-biacridiniumdinitrate [Lucigenin], 4'-hydroxy-3'-methoxyacetophenone [apocynin] and 50 mM phosphate buffer solution were obtained from Sigma-Aldrich [St. Louis, MO]. NADPH and NSC23766 were from Calbiochem [Billerica, MA] and EHop-016 was kindly provided by Dr Vlaar, Department of Pharmaceutical Sciences, School of Pharmacy, University of Puerto Rico.

Antibodies: Antisera directed against phospho-p47^{phox} [NCF1] and p67^{phox} were from Abcam [Cambridge, MA]. Total-p47^{phox}, phospho-p38, total-p38 and FTase/GGTase- α subunit were from Santa Cruz Biotechnology [Santa Cruz, CA]. Rac1 and gp91^{phox} were from BD Biosciences [San Jose, CA]. Antisera directed against CHOP, phospho-JNK, total-JNK and cleaved caspase-3 [active form] were from Cell Signaling [Danvers, MA]. Anti-mouse and anti-rabbit IgG-horseradish peroxidase conjugates were from Amersham Biosciences [Piscataway, NJ].

Assay kits: Enhanced chemiluminescence kits were from Amersham Biosciences [Piscataway, NJ]. Re-blot plus strong antibody stripping solution was from Millipore [Billerica, MA]. Rac1 activation assay kit was from Cytoskeleton [Denver, CO]. All other reagents used in these studies were from Sigma Aldrich [St. Louis, MO] unless stated otherwise.

Animals: NOD/ShiLtJ and BALB/cJ mice were from Jackson Laboratory [Bar Harbor, ME]. Picolab Rodent Diet was from Lab Diet [Brentwood, MO]. The laboratory animal bedding, Bed-o'Cobs 1/8", was from Anderson [Maumee, OH].

2.2. Insulin-secreting cell line and mouse islets

INS-1 832/13 cells were kindly provided by Dr. Chris Newgard, Duke University Medical Center, Durham. 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, 1mM sodium pyruvate, 50 μ M 2-mercaptoethanol and 10 mM HEPES [pH 7.4]. The cultured cells were subcloned twice weekly following trypsinization and passages 53-61 were used for the studies. Islets from 7-8 weeks NOD/ShiLtJ and control BALB/cJ mice were isolated by collagenase digestion method [89]. All protocols, including isolation of pancreatic islets from mice, were reviewed and approved by Institutional Animal Care and Use Committee of the Wayne State University.

INS-1 832/13 cells or mouse islets were incubated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] or individual cytokines [25 ng/ml] for 0-24 hrs as indicated in the text. In select studies, INS-1 832/13 cells were incubated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] in the presence and absence of different pharmacological inhibitors for 0-24 hrs as indicated in the text. At the end of the incubation period the cells were harvested and lysed in RIPA buffer [50 mM Tris-HCl, pH7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1mM EDTA, 1 mM NaF, 1 mM PMSF, 1 mM Na₃VO₄ and 1 μ g/ml protease inhibitor cocktail].

2.3. Isolation of membrane fraction

INS-1 832/13 cells were treated with cytomix for the designated time points as indicated in the text. At the end of the incubation period, cells were washed with ice-cold PBS, harvested and pelleted by centrifugation at 2,000 rpm for 5 min at 4°C. After re-suspending in the homogenizing buffer [50 mM Tris-HCl [pH 7.4], 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PIC, 1 mM PMSF and 1 mM Na₃VO₄] and sonication, unbroken

cells and nuclei were separated by centrifugation at 1,300 rpm for 10 min. The cleared supernatant was further centrifuged at 40,000 rpm for 30 min [Optima™ MAX Ultracentrifuge]. The cytosol fraction was separated and the membrane fraction was dissolved in 2% CHAPS [10 mM Tris-HCl, pH 7.6, 1.5 M NaCl, 1 mM PIC, 1 mM PMSF and 1 mM Na₃VO₄].

2.4. ROS generation assay

INS-1 832/13 cells were plated in a 6 well plate and treated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] or individual cytokines [25 ng/ml] for 24 hrs as indicated in the text. Subsequently, the media was removed and the cells were incubated further with 2',7'-dichlorofluorescein diacetate [DCHF-DA] at 37°C for 30 min. The cells were washed twice with ice-cold PBS and harvested, followed by loading equal amount of protein [50 μ g] and reading the fluorescence using luminescence spectrophotometer [Ex:485nm and Em:535nm] [PerkinElmer, Waltham, MA].

2.5. NOX2 assay

INS-1 832/13 cells were plated in a 6 well plate and co-incubated with IL-1 β [25 ng/ml] and apocynin [100 μ M] for 30 min. The NOX2 activity was measured using the method described by Hwang and associates [90]. Control, cytokine [IL-1 β] and glucose-treated cells were homogenized using 50 mM phosphate buffer solution [pH 7.0] containing 1 mM EDTA and 1 mM PMSF. The homogenates were centrifuged at 3,000 g for 10 min and the clarified lysates [250 μ g protein/ml] were then incubated with N, N-dimethyl-9,9'-biacridinium dinitrate [lucigenin] for 2 min followed by the addition of NADPH [100 μ M]. The chemiluminescence signal resulting from reaction of superoxide anion and lucigenin was recorded every 1 min for

15 min using BioTek Synergy HT, Gen5 [Winooski, VT] and the activity was expressed as chemiluminescence units per mg lysate protein per minute.

2.6. NO release assay

INS-1 832/13 cells were plated in a 6 well plate and treated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] for 24 hrs as indicated in the text. At the end of the incubation period, the media was collected and centrifuged at 1,000 g for 5 min. Equal amount of media and Griess reagent were mixed and the absorbance [540nm] was measured using a microplate reader [Molecular Devices, Sunnyvale, CA].

2.7. Rac1 activation assay

Pull-down assay: INS-1 832/13 cells were pretreated with NSC23766 [20 μ M] followed by treatment with cytomix for 15 min in the absence and presence of the inhibitor [20 μ M]. Cell lysates [250–300 μ g] were clarified by centrifugation. Then PAK-PBD [p21-activated kinase-p21-binding domain] beads [20 μ l] were added to the supernatant, rotated for 1 h at 4°C, and pelleted. The resultant pellet was washed and reconstituted in Laemmli buffer. Proteins were resolved by SDS-PAGE and immunoblotted for Rac1 [47].

GLISA: Activated Rac1 was quantitated using the GLISA activation assay kit according to the manufacturer's instructions. Briefly, lysates were clarified by centrifugation at 14,000 rpm for 2 min. Equal amounts of protein were incubated in the Rac1-GTP affinity plate for 30 min at 4°C. The wells were washed twice with washing buffer and incubated with anti-Rac1 primary antibody and secondary antibody, followed by additional incubation with horseradish peroxidase-detection reagent. Horseradish peroxidase-stop buffer was added to stop the reaction, and the absorbance was measured at 490 nm using a microplate reader.

2.8. Protein phosphorylation assay

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ , 10 ng/ml each] in the presence and absence of pharmacological inhibitors for 0-24 hrs time point and the cells were lysed with RIPA buffer containing 1 μ g/ml protease inhibitor cocktail, 1 mM NaF, 1 mM PMSF, and 1 mM Na₃VO₄. The samples were incubated on ice for 10 minutes followed by clarification and determination of the total protein content by Pierce 600 nm Protein Assay. Equal amount of proteins were separated by SDS-PAGE on 10% [w/v] polyacrylamide mini gels and electro-transferred to a nitrocellulose membrane. The membranes were immunoprobed with corresponding primary phospho-antibodies and developed as indicated below. The same blots were used to probe for total-proteins for the respective phospho-proteins to ensure equal loading and transfer of the proteins.

2.9. Western blotting

Proteins [30-40 μ g/lane] were separated by SDS-PAGE on 10% [w/v] polyacrylamide mini gels and electro transferred to nitrocellulose membrane. The membranes were blocked with 5% BSA or 5% non fat dry milk in 10 mM Tris-HCl, pH 7.6, 1.5 M NaCl and 0.1% Tween 20 followed by incubation with corresponding primary antibody and secondary polyclonal rabbit or mouse antibody conjugated to horseradish peroxidase. The protein signal was enhanced by chemiluminescence system and developed using Kodak Pro Image 400 R [New Haven, CT]. The same blots were used to probe for β -actin to ensure equal loading and transfer of the proteins.

2.10. Pharmacological inhibitors

INS-1 832/13 cells were incubated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] or [IL-1 β ; 25 ng/ml] in the presence and absence of 2-bromopalmitate [100 μ M],

apocynin [100 μ M], EHop-016 [5 μ M], NSC23766 [20 μ M] for 0-24 hrs and their effect on cytomix/IL-1 β induced ROS generation, NO release, Rac1 and NOX2 activation, CHOP expression, JNK1/2 and p38 MAP kinase activation were analyzed as indicated in the text.

2.11. Statistical analysis of experimental data

Results are expressed as mean \pm SEM. Statistical significant difference between groups was evaluated by ANOVA followed by SNK Post-Hoc test where appropriate. $P < 0.05$ was considered to be statistically significant.

Chapter 3: Cytokines activate phagocyte-like NADPH oxidase in pancreatic β -cell.

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

Mohammed AM, Syeda K, Hadden T, Kowluru A. Upregulation of phagocyte-like NADPH oxidase by cytokines in pancreatic β -cells: attenuation of oxidative and nitrosative stress by 2-bromopalmitate. *Biochemical Pharmacology* 2013; 85(1):109 -114.

Mohammed AM, Kowluru A. Activation of apocynin-sensitive NADPH oxidase [NOX2] activity in INS-1 832/13 cells under glucotoxic conditions. *Islets* 2013; 5(3).

Type 1 diabetes is characterized by an absolute insulin deficiency arising from progressive autoimmune destruction of insulin-secreting pancreatic β -cells [1, 24, 91]. During the progression of this disease, several proinflammatory cytokines, including IL-1 β , TNF α and IFN γ are secreted into the islets by infiltrated and activated T cells and macrophages, which, in turn, elicit damaging effects on pancreatic β -cells. However, putative mechanisms and the underlying signaling mechanisms involved in cytokine-induced loss of β -cells remain only partially understood [55, 92]. Among numerous mechanisms proposed, IL-1 β has been demonstrated to mediate its effects via the induction of inducible nitric oxide synthase (iNOS), which, in turn, promotes cytotoxic NO release culminating in β -cell demise. In addition, TNF α and IFN γ elicit regulatory effects on β -cell function via regulation of individual metabolic signaling steps leading to cell death [16, 40, 71]. Along these lines, previous studies have suggested that IFN γ sensitizes human islets to the effects of IL-1 β [93]. In addition to NO, several recent studies including our own have suggested that phagocyte-like NADPH oxidase [NOX2] contributes to loss of β -cell function, metabolic dysregulation and

the loss of β -cell mass under the duress of pathological conditions such as glucolipotoxicity and exposure to cytokines. Specifically, we have demonstrated that chronic exposure of isolated β -cells to cytomix leads to NOX2 holoenzyme activation, ROS generation, mitochondrial dysfunction and cell death [44, 47, 53, 94]. Moreover, using selective inhibitors of Rac1, a small G-protein, which is a member of NOX2 holoenzyme, we have been able to demonstrate that inhibition of Rac1 activation leads to prevention of ROS generation and mitochondrial dysregulation in isolated β -cells [47, 51, 95]. Interestingly however, we failed to see any clear effects of Rac1 inhibitors on cytomix-induced NO generation suggesting involvement of a Rac1-independent mechanism for cytomix-induced NO release in these cells.

A recent Editorial Focus by Jastroch [96] appropriately pointed out a need for the development of pharmacological probes/reagents that can block both ROS and NO generation in pancreatic β -cells exposed to cytomix as simultaneous release of both ROS and NO could lead to the formation of peroxynitrite, which may be more damaging to the β -cell compared to ROS or NO alone. Therefore, as a logical extension, we undertook an investigation to assess the roles of protein palmitoylation in the cascade of events leading to cytomix-induced ROS and NO generation in INS-1 832/13 cells.

IL-1 β promotes ROS generation

Our laboratory recently reported a significant increase in NOX2-mediated generation of ROS in INS-1 832/13 cells. In the current set of experiments we examined effects of individual cytokines on ROS generation in order to determine which of the three cytokines exert stimulatory effects on ROS generation in INS-1 832/13 cells. Data in **Figure 3-1** indicated that of all the three cytokines tested, namely IL-1 β , TNF α or IFN γ [25 ng/ml each; 24 hr] only IL-1 β significantly augmented [\sim 2.5 fold] ROS generation; these values were

comparable to those demonstrated in the presence of all the three cytokines combined [referred to as cytomix throughout]. A modest, but insignificant, effect of TNF α [bar 1 vs. 3] or IFN γ [bar 1 vs.4], was demonstrated under these conditions. Therefore, we determined the effects of cytokines on NOX2 activation in most of the studies described from here on since the cytomix represents an appropriate model to determine effects of cytokines on islet β -cell dysfunction.

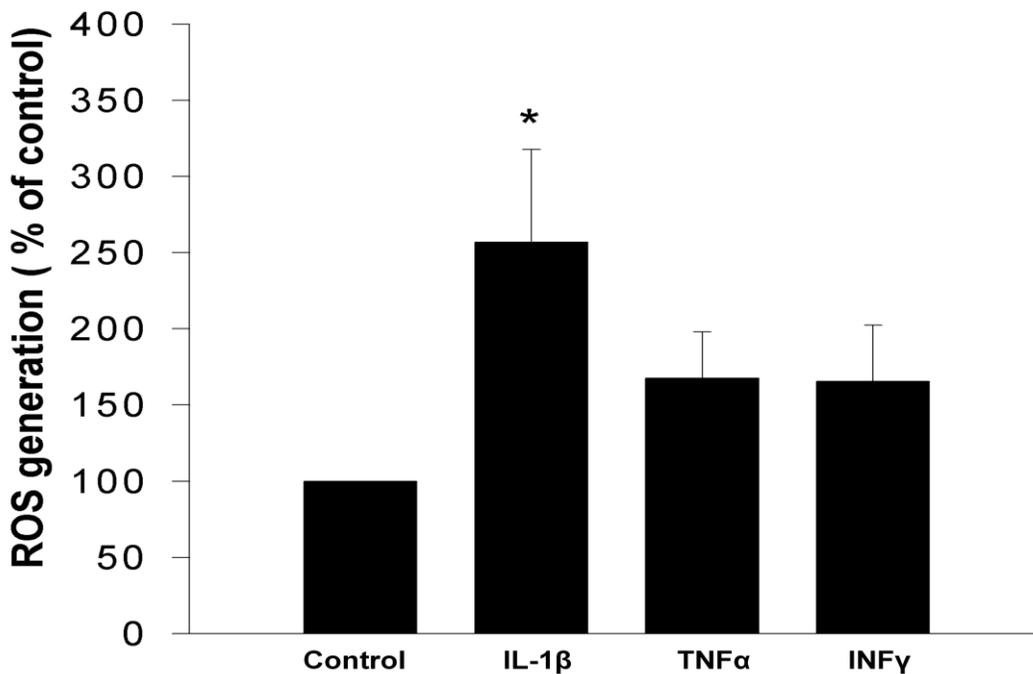


Figure 3-1: IL-1 β , but not TNF α and INF γ , activates ROS generation in INS-1 832/13 cells.

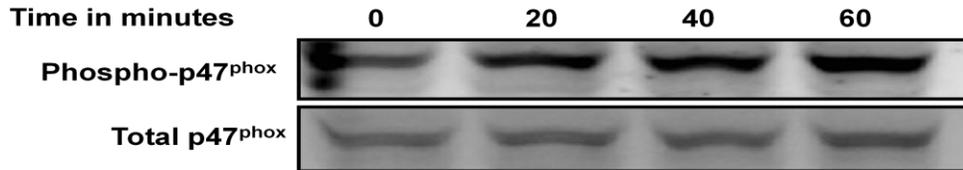
INS-1 832/13 cells were treated with individual cytokines [IL-1 β , TNF α and INF γ ; 25 ng/ml each] for 24 hrs and the amount of ROS generation was measured with 2',7-dichlorofluorescein diacetate assay. Data represent mean \pm SEM from three independent experiments and expressed as % of control. * $P < 0.05$ versus control.

Cytomix induces p47^{phox} phosphorylation

Recent studies from our laboratory and others have shown that cytokines induce the expression of p47^{phox}, and siRNA-mediated depletion of endogenous pools of p47^{phox}

markedly attenuated cytokine-induced NOX2-mediated ROS generation in insulin-secreting cells [47]. Furthermore, evidence in other cell types indicates that p47^{phox} is phosphorylated subsequent to agonist activation [97, 98] and the phosphorylation step is necessary for its translocation to the membrane fraction for association with other members of the NOX2 core proteins to complete holoenzyme assembly leading to the activation of NOX2. This has not been examined before in islet β -cells exposed to cytokines. Data in **Figure 3-2 [Panel A]** suggested a time-dependent activation of p47^{phox} [0-60 min] following exposure to cytokines. We observed nearly a 2-fold increase in the phosphorylation of p47^{phox} by cytokines within an hour of incubation [**Panel B**]. These data suggest that cytokines induce phosphorylation of p47^{phox}.

A)



B)

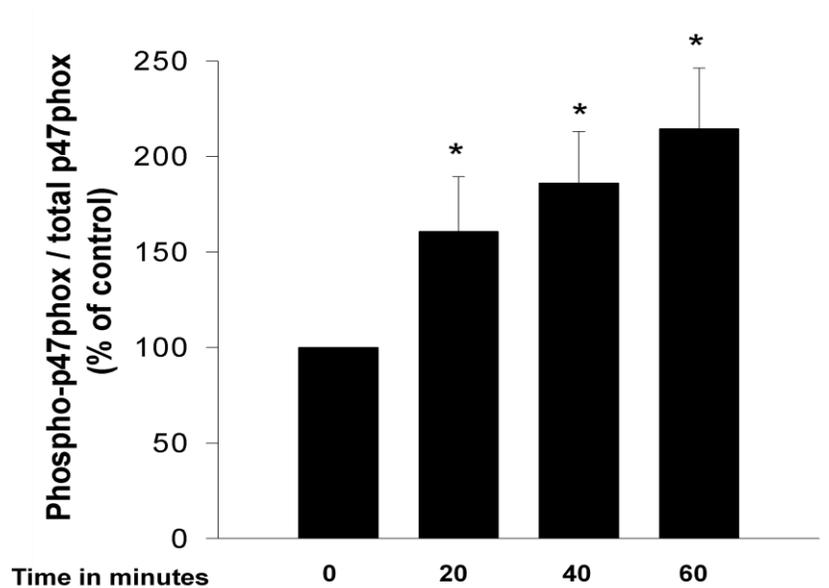


Figure 3-2: Cytomix induces phosphorylation of p47^{phox} in INS-1 832/13 cells.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for different time intervals as indicated in the figure. Degree of phosphorylation of p47^{phox} was determined by Western blot **[Panel A]** followed by densitometry **[Panel B]**. Data are normalized to total p47^{phox} content in corresponding lanes. Data represent mean \pm SEM from three independent experiments and expressed as percent control of the ratios between phosphorylated- and total-p47^{phox}. * $P < 0.05$ versus control.

Cytomix induces gp91^{phox} expression

We next investigated alterations, if any, in the expression of gp91^{phox} in cells following exposure to cytomix. To address this, INS-1 832/13 cells were incubated in the presence of cytomix for different time intervals [0-60 min] as above **[Figure 3-2]**. Relative abundance of

gp91^{phox} was determined in the total membrane fraction isolated by a single-step centrifugation method by Western blotting for gp91^{phox} **[Figure 3-3; Panel A]** and densitometry **[Figure 3-3; Panel B]**. These data indicated a time-dependent increase in the expression of gp91^{phox} in INS-1 832/13 cells following exposure to cytomix. A significant increase in the expression was seen as early as 20 min [\sim 2 fold], which appear to plateau with time. Together, these data are indicative of positive modulatory effects of cytomix on NOX2 in INS-1 832/12 cells. Such effects are comprised of increase in the activation of Rac1 **[47]**, phosphorylation of p47^{phox} and expression of gp91^{phox}; such conditions are essential for activation of NOX2 enzyme.

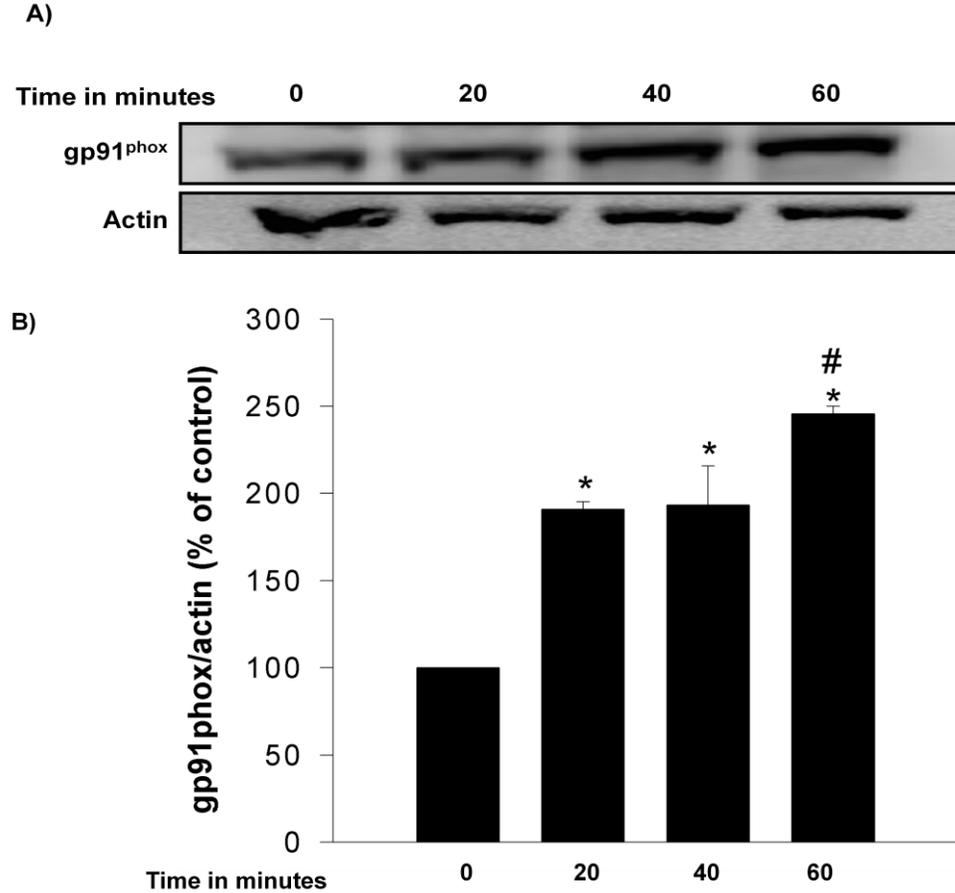


Figure 3-3: Cytomix increases the expression of gp91^{phox} in INS-1 832/13 cells.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for different time points as indicated in the figure. Degree of gp91^{phox} expression was measured by Western blot [**Panel A**] followed by densitometry [**Panel B**]. Data are normalized to β -actin content in individual lanes. Data represent mean \pm SEM from three independent experiments and expressed as percent control of the ratios between gp91^{phox} and β -actin. * $P < 0.05$ versus control and # $P < 0.05$ versus 20 min and 40 min.

IL-1 β increases apocynin-sensitive NOX2 activity

It is well documented that in addition to the phagocyte-like NADPH oxidase, other cellular organelles such as mitochondria, peroxisomes and endoplasmic reticulum produce reactive oxygen species [99-102]. Physiological amounts of ROS is required for normal functioning of the β -cells such as glucose stimulated insulin secretion. The presence and chronic activation of NADPH oxidase by high glucose, palmitate and proinflammatory

cytokines could lead to excessive amount of ROS in the β -cell. Hence, follow up studies were carried out in INS-1 832/13 cells to specifically measure the NOX2 activity under cytokine [IL-1 β] condition. To exclude other oxidoreductase enzyme activities in the assay, a relatively selective pharmacological inhibitor for NOX2 [apocynin] was used. The results showed that apocynin has no significant effect on basal NOX2 activation [bar 1 vs. bar 2]. However, exposure to IL-1 β [25 ng/ml] activates the enzyme [\sim 1.7 fold] as early as 30 min [bar 1 vs. Bar 3] and it was significantly blocked by apocynin [100 μ M] [bar 3 vs. bar 4] [Figure 3-4].

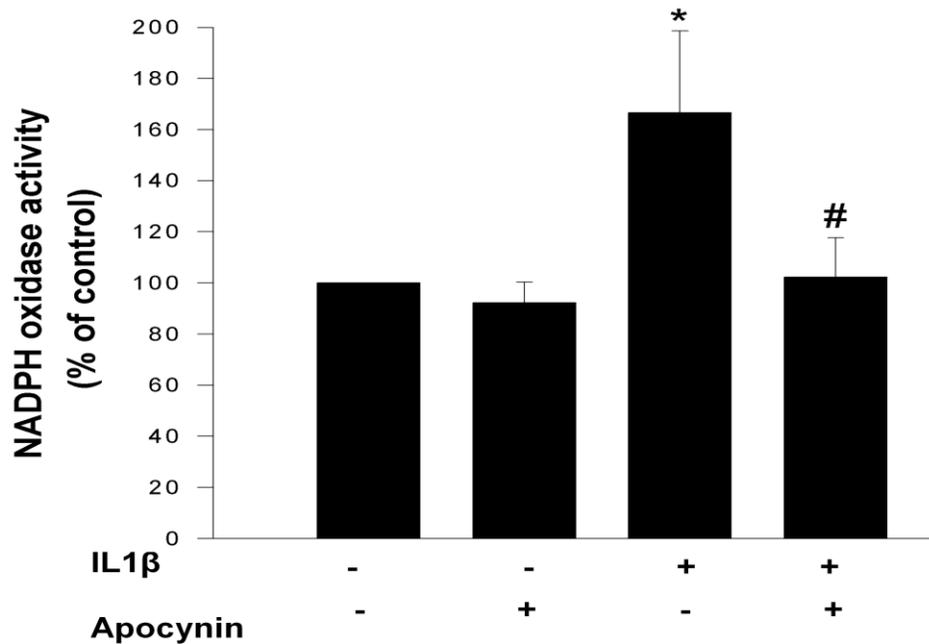


Figure 3-4: IL-1 β increases apocynin-sensitive NOX2 activity in INS-1 832/13 cells.

INS-1 832/13 cells were incubated with IL-1 β [25 ng/ml] in presence and absence of apocynin [100 μ M] for 30 min and NOX2 enzyme activity was measured for 15 min. The enzyme activity was expressed as chemiluminescence units per mg lysate protein per minute. Data represent mean \pm SEM from six independent experiments and expressed as percent of control. * P < 0.05 versus control; # P < 0.05 versus IL-1 β .

2-Bromopalmitate inhibits cytomix-induced ROS generation

Recent studies from our laboratory have demonstrated that activation of Rac1 is necessary for cytokine-induced NOX2 activation and ROS generation. In this context we reported that prior incubation of INS-1 832/13 cells with GGTI-2147, an inhibitor of geranylgeranylation of Rac1 or NSC23766, a known inhibitor of Rac1 mediated by Tiam1, a guanine nucleotide exchange factor for Rac1 markedly attenuated cytomix-induced ROS generation [**Figure 3-5**]. Therefore, in the present study we determined the effects of 2-bromopalmitate, a known inhibitor of protein palmitoylation on cytomix-induced ROS generation. These studies are based on the recent reports of palmitoylation of Rac1 at a cysteine residue upstream to the C-terminal cysteine. To address this, cytomix-induced ROS generation was quantitated in INS-1 832/13 cells incubated in the presence of diluent alone or 2-BP [100 μ M; 24 hr]. Data depicted in **Figure 3-6** indicated no significant effects of 2-BP on basal ROS generation [bar 1 vs. bar 2]. However, cytomix-induced ROS generation was completely abolished by 2-BP [bar 3 vs. bar 4]. These findings suggest that a palmitoylation-dependent signaling step is necessary for cytomix-induced ROS generation.

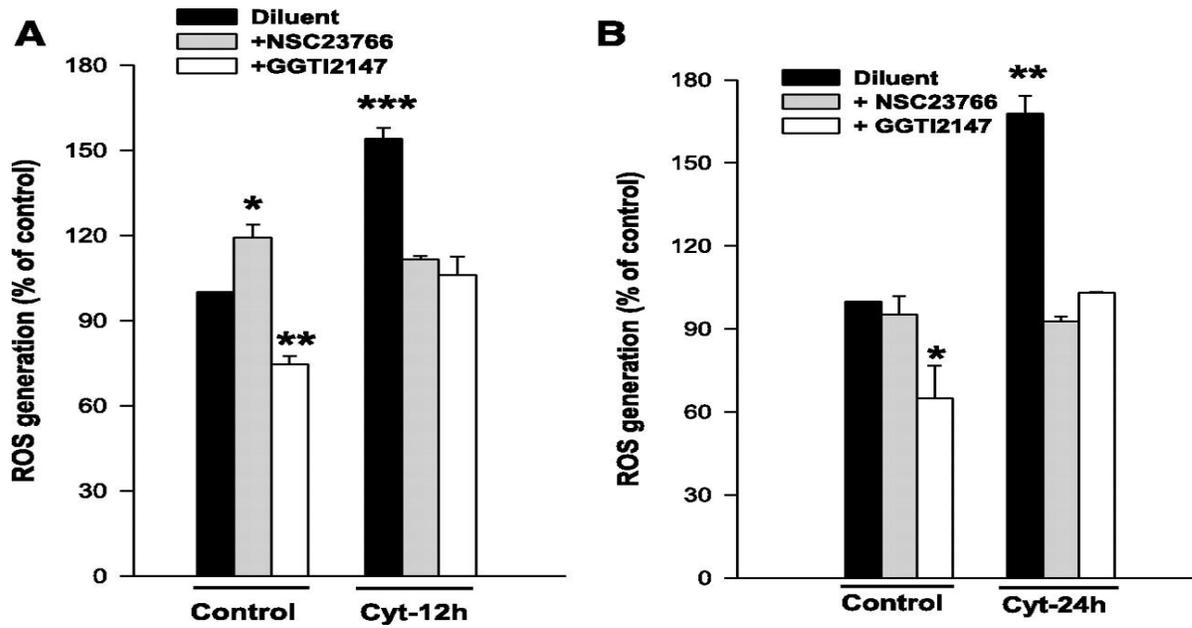


Figure 3-5: Cytomix-induced ROS generation is inhibited by NSC23766 and GGTI-2147.

INS-1 832/13 cells were treated with either diluent or cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] in the presence and absence of NSC23766 [20 μ M] or GGTI-2147 [10 μ M] for 12 h [Panel A] and 24 h [Panel B] as indicated in the figure, and intracellular levels of ROS was measured using DCHF-DA assay. Data are representative of three independent experiments, expressed as a percentage of control and represent means \pm SEM. Bars with different symbols (*, **, ***) are significantly different at $P < 0.05$ [47].

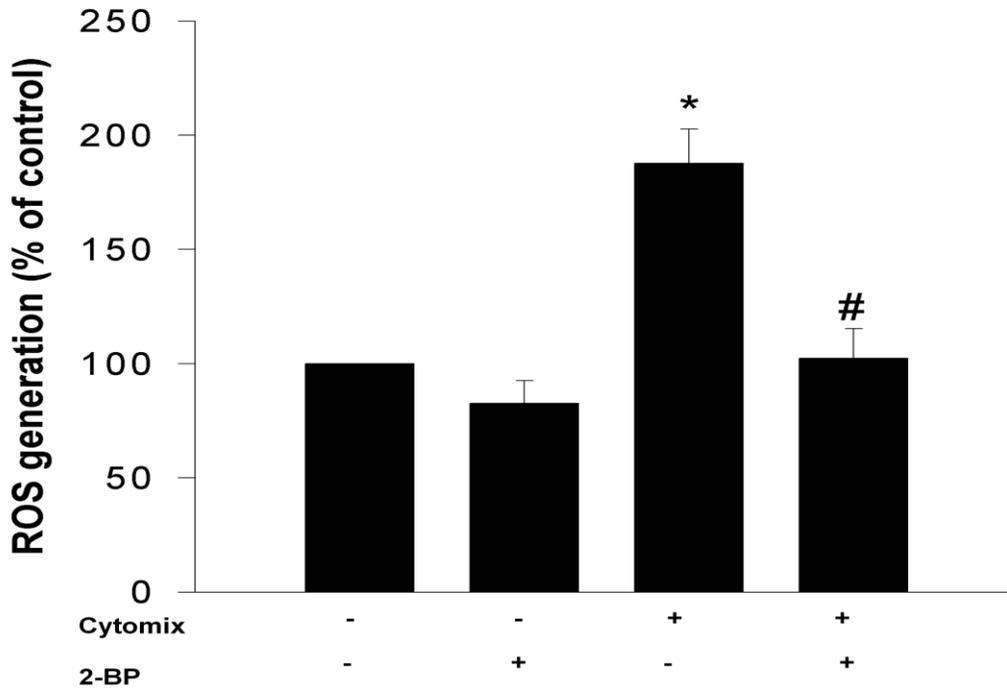


Figure 3-6: 2-BP attenuates cytomix-induced ROS generation in INS-1 832/13 cells.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] for 24 hrs in the presence and absence of 2-BP [100 μ M] and the amount of ROS generation was measured with DCHF-DA assay. Data represent mean \pm SEM from three independent experiments and expressed as % of control. * P < 0.05 versus control and # P < 0.05 versus cytomix.

2-Bromopalmitate inhibits cytomix-induced Rac1 activation

Rac1 is one of the components of the NADPH oxidase and its activation is required for assembly and activation of the holoenzyme. Recent study from our laboratory has shown that Tiam1 inhibitor [NSC23766] inhibited Rac1-GTP bound form [Figure 3-7] [47]. In the present study we hypothesize that in addition to geranylgeranylation Rac1 also undergoes palmitoylation, the addition of palmitate to a cysteine residue upstream to prenylated cysteine [Figure 1-6]. To address this, cytomix-induced Rac1 activation was quantitated in INS-1 832/13 cells incubated in the presence and absence of 2-BP [100 μ M; 15 min]. Data shown in Figure 3-8 indicated no significant effects of 2-BP on basal Rac1 activation [bar 1 vs. bar

2]. However, cytomix-induced Rac1 activation was blocked by 2-BP [bar 3 vs. bar 4]. These findings suggest that a palmitoylation-dependent signaling step is necessary for cytomix-induced Rac1 activation.

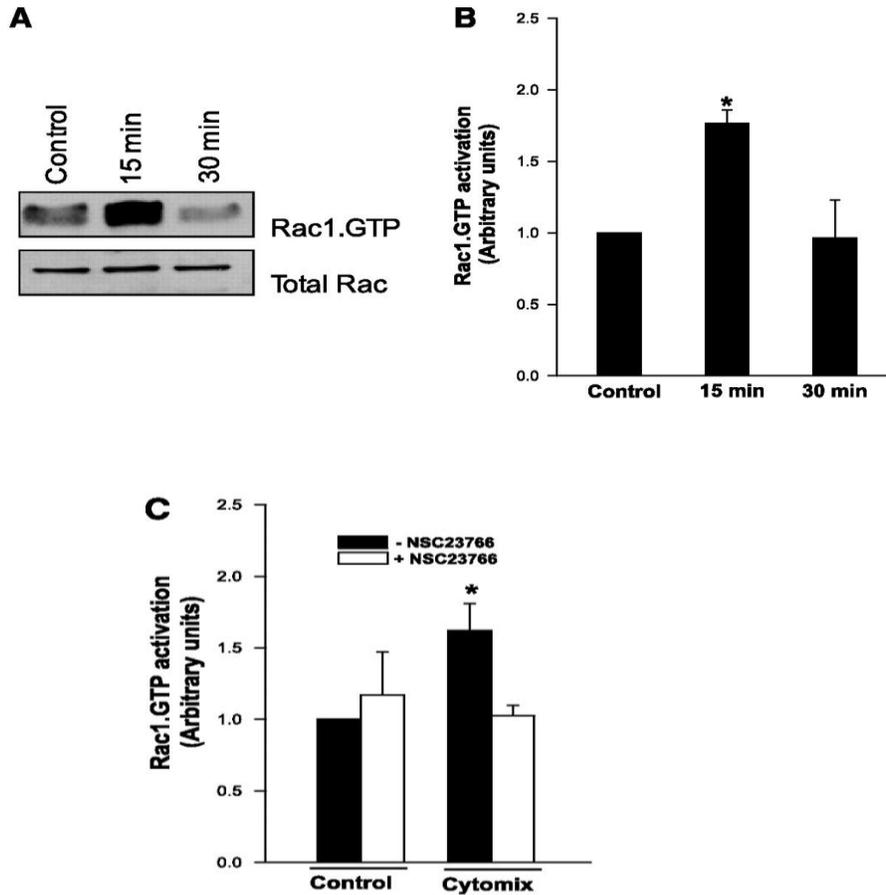


Figure 3-7: Cytomix induces transient activation of Rac1 in INS-1 832/13 cells: inhibition of this signaling step by NSC23766.

Cytomix causes transient activation of Rac1 in INS-1 832/13 cells, as determined by the pull-down assay followed by Western blot analysis, **Panel A**. Pooled activation data from three independent experiments are shown in **Panel B**. NSC23766 inhibition of cytomix-induced activation of Rac1. Pooled data from three independent studies are depicted in **Panel C**. *, represent the values that are significantly different from control at $P < 0.05$ [47].

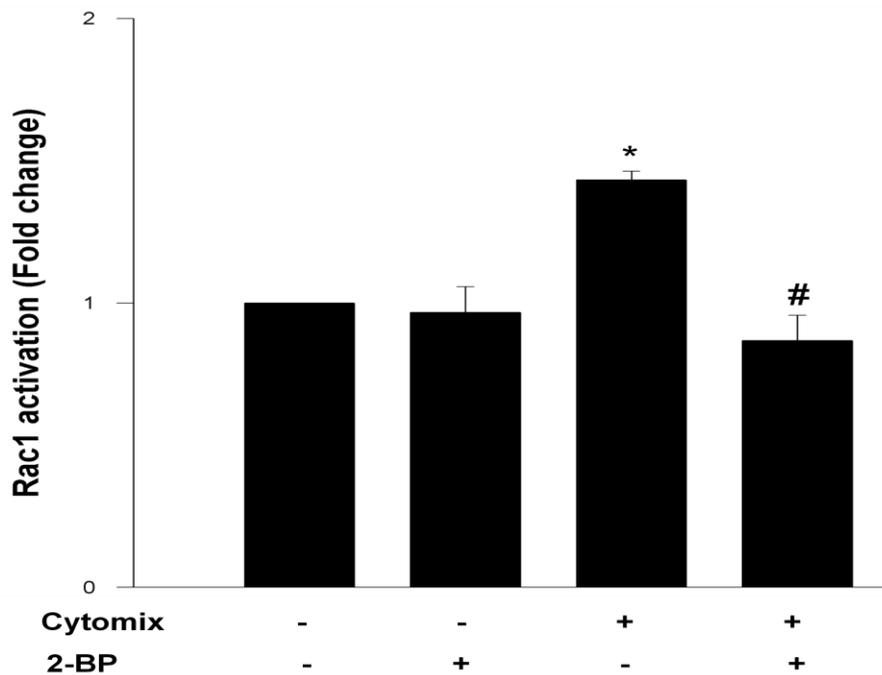


Figure 3-8: 2-BP attenuates cytomix-induced Rac1 activation.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] for 15min in the presence and absence of 2-BP [100 μ M] and Rac1 activation was measured with GLISA. Data represent mean \pm SEM from three independent experiments and expressed as fold change. * P < 0.05 versus control; # P < 0.05 versus cytomix.

2-Bromopalmitate inhibits cytomix-induced NO release

It is widely felt that both oxidative [ROS] and nitrosative [NO] stress could contribute to impairment of the overall health of the islet β -cell [25, 56, 73, 74]. Such concerns were raised indeed by investigators in the area warranting additional studies to investigate potential targets which could regulate both ROS and NO generation signaling steps under the duress of cytokines. To this end, we have previously reported that a palmitoylation-mediated signaling step may be necessary for cytokine-induced iNOS gene expression and NO release [42]. Herein, we revisited those earlier studies to demonstrate that inhibition of protein palmitoylation by 2-BP inhibits cytomix-induced NO release in INS-1 832/13 cells. Data in **Figure 3-9** suggested no direct effects of 2-BP on basal NO release [bar 1 vs. bar 2].

However, as in the case with ROS generation [Figure 3-6], 2-BP treatment also attenuated cytomix-induced NO release [bar 3 vs. bar 4]. Together, our findings highlight the importance of palmitoylation as a unifying mechanism involved in the induction of NOX2-mediated oxidative stress and iNOS-mediated nitrosative stress in pancreatic β -cells.

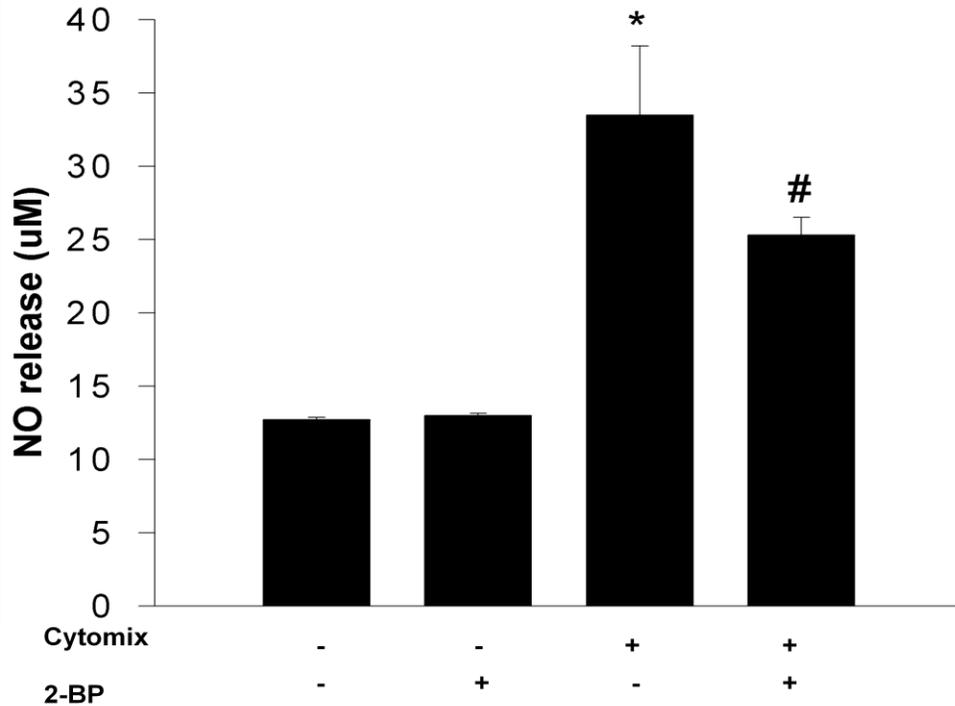


Figure 3-9: Cytomix-induced NO release is inhibited by 2-BP

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] for 24 hrs time point in the presence and absence of 2-bromopalmitate [100 μ M] and the amount of nitric oxide release was measured using Griess assay. 2-Bromopalmitate significantly decreased cytomix-induced nitric oxide release. Data represent mean \pm SEM from three independent experiments. * $P < 0.05$ versus control and # $P < 0.05$ versus cytomix.

2-Bromopalmitate inhibits high glucose-induced Rac1 activation

Studies from our laboratory have shown that Rac1 has both positive as well as negative regulatory roles in pancreatic β -cell function. Inhibiting the Rac1 function with pharmacological and molecular approach affects glucose stimulated insulin secretion. Inhibiting the Rac1 function also protected pancreatic β -cell from oxidative stress due to

chronic exposure to glucose, palmitate and proinflammatory cytokines [39]. In the present study we determined the role of palmitoylation on glucose-induced Rac1 activation. Similar to cytomix effect, incubation of INS-1 832/13 cells with high glucose induces Rac1 activation. Data shown in **Figure 3-10** indicated no significant effect of 2-BP on Rac1 activation [bar 1 vs. bar 2]. However, glucose-induced Rac1 activation was inhibited by 2-BP [bar 3 vs. bar 4] indicating that both cytomix- [Figure 3-8] and glucose-induced Rac1 activation is dependent on palmitoylation.

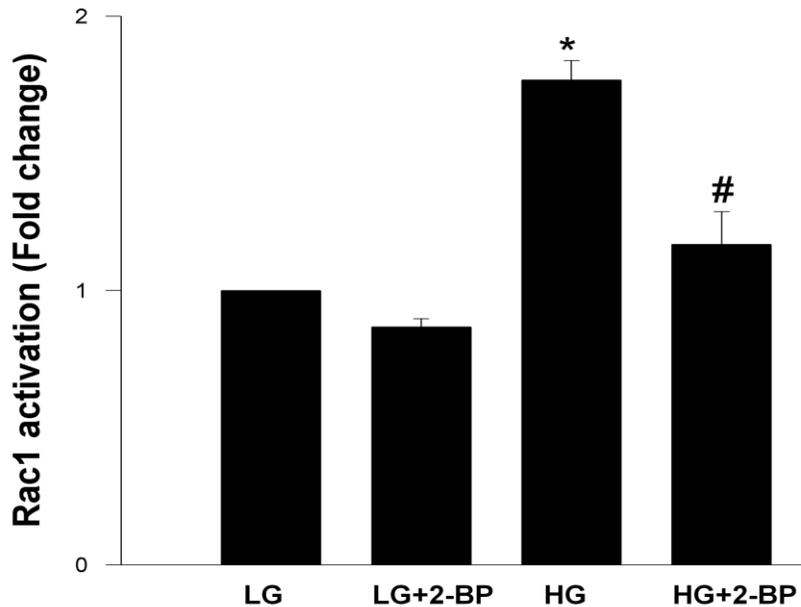


Figure 3-10: 2-BP attenuates high glucose-induced Rac1 activation.

INS-1 832/13 cells were treated with low glucose [LG; 2.5 mM] and high glucose [HG; 20 mM] for 15 min in the presence and absence of 2-bromopalmitate [2-BP; 100 μ M] and Rac 1 activation was measured with GLISA. Data represent mean \pm SEM from three independent experiments and expressed as fold change. * $P < 0.05$ versus LG; # $P < 0.05$ versus HG.

High glucose increases apocynin-sensitive NOX2 activity

Emerging evidence from *in vitro* and *in vivo* studies provides strong support to the hypothesis that chronic exposure of β -cells to elevated glucose [i.e., glucotoxicity], lipids [i.e., lipotoxicity], or glucose plus lipids [e.g., glucolipotoxicity] results in a significant metabolic dysregulation eventually leading to cell demise [103]. Published evidence also suggests a marked increase in the generation of ROS, which manifests in increased oxidative stress in cells under the conditions of glucotoxicity [25, 44]. Several mechanisms have been put forth in this context, including depletion of intracellular redox state via the oxidation of reducing equivalents [e.g., reduced glutathione] and activation of superoxide-generating enzymatic machinery [25, 103].

One of the enzymatic steps involved in the increased generation of ROS and associated induction of intracellular oxidative stress in the pancreatic β -cell includes activation of the phagocytic NADPH-oxidase [NOX2] system [25, 44]. NOX2 is a highly regulated membrane-associated protein complex that catalyzes the one electron reduction of oxygen to superoxide anion involving oxidation of cytosolic NADPH. The phagocytic NOX is a multicomponent system comprised of membrane as well as cytosolic components. The membrane-associated catalytic core is a complex comprising gp91^{phox}, p22^{phox}, and the small G protein Rap1. The cytosolic regulatory components include p47^{phox}, p67^{phox}, and the small G protein Rac. After stimulation, the cytosolic components of NOX translocate to the membrane fraction for association with the catalytic core for holoenzyme assembly. Available evidence suggests that a protein kinase C ζ -sensitive phosphorylation of p47^{phox} leads to its translocation to the membrane fraction. It has also been shown that functional activation of Rac [i.e., Rac-GTP] is vital for the holoenzyme assembly and activation of NOX [25].

Several recent studies have demonstrated localization and functional activation of the NOX in clonal β -cells, normal rat islets, and human islets under the duress of various stimuli

known to cause metabolic dysregulation [44, 94]. Some of these stimuli include, but are not limited to, high glucose, saturated fatty acids, and proinflammatory cytokines [47, 95]. Recently, our laboratory has reported increased ROS generation in islets from the diabetic Zucker Diabetic Fatty [ZDF] rat and Type 2 DM human islets. In that study, we also provided evidence to implicate Rac1-NOX2-ROS-JNK1/2 signaling cascade in glucose-induced metabolic dysfunction of the islet. However, we have not directly quantitated NOX2 activity in isolated β -cells exposed to hyperglycemic conditions to conclusively demonstrate that NOX2-derived ROS generation is critical for high glucose-mediated effects on β -cell dysfunction. Therefore, a brief study was undertaken to quantitate NADPH oxidase activity in INS-1 832/13 cells exposed to hyperglycemic conditions. To further demonstrate that such an activity represents NOX2, we included apocynin, a selective inhibitor of NOX2, in the assay.

Data in **Figure 3-11** suggested no clear effects of apocynin on NADPH oxidase activity in INS-1 832/13 cells under basal [low-glucose] conditions [bar 1 vs. bar 2]. However, exposure of these cells to high glucose resulted in a significant increase [~ 2-fold] in the enzyme activity [bar 1 vs. bar 3]. Furthermore, coprovision of apocynin to these cells markedly attenuated the ability of glucotoxic conditions to stimulate NADPH oxidase activity. These data suggest that glucotoxic conditions promote activation of apocynin-sensitive NADPH oxidase activity in isolated β -cells.

Our findings provide further support to recently published evidence to suggest increased generation of ROS in cell culture models of glucotoxicity and in islets from Type 2 DM animals and humans. Biochemical and cell biological studies have also demonstrated increased expression of p47^{phox}, a member of the cytosolic core of NOX2 in these model systems [25, 89]. Furthermore, activation of Rac1 [GTP-bound conformation] has also been demonstrated in cells exposed to hyperglycemic conditions or in islets from animal models of Type 2 DM [89]. Lack of no effects of apocynin on basal [LG] NADPH oxidase activity

suggests very little activation of this enzyme under basal conditions. Along these lines, recent studies by Koulajian and associates have demonstrated significant activation of NADPH oxidase activity in rat islets following prolonged exposure to non saturated fatty acids, such as oleate. Furthermore, they have demonstrated a significant reduction in the activation of NADPH oxidase by apocynin [104], thus affirming critical regulatory roles for this enzyme in metabolic dysfunction induced by elevated glucose and lipids.

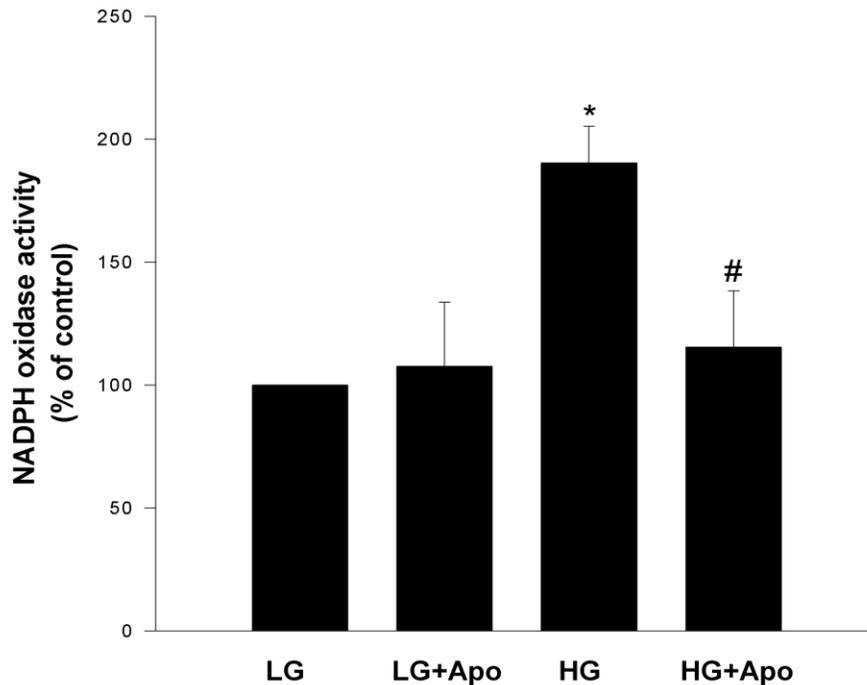


Figure 3-11: Exposure of INS-1 832/13 cells to high glucose leads to apocynin-sensitive NOX2 activity.

INS-1 832/13 cells were incubated with low glucose [LG; 2.5 mM] and high glucose [HG; 20 mM] in presence and absence of apocynin [Apo; 100 μ m] for 48 hrs and NADPH oxidase enzyme activity was measured for 15 min. The enzyme activity was expressed as chemiluminescence units per mg lysate protein per minute. Data represent mean \pm SEM from three independent experiments and expressed as percent of control. * P < 0.05 versus LG; # P < 0.05 versus HG.

Together, findings in this chapter of my thesis highlight the importance of palmitoylation as a unifying mechanism involved in the induction of NOX2-mediated oxidative stress and iNOS-mediated nitrosative stress in pancreatic β -cell.

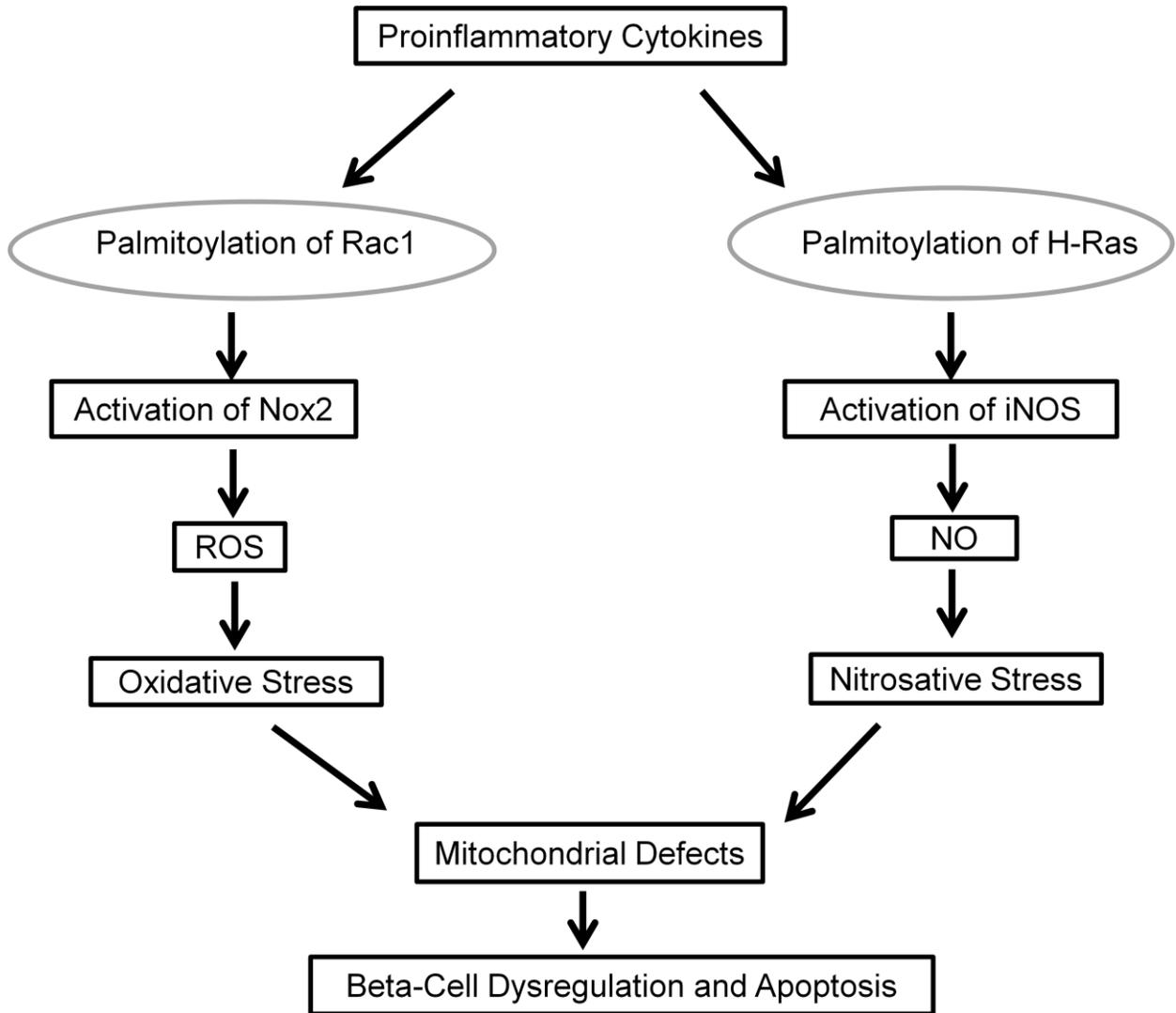


Figure 3-12: Model to implicate the role of palmitoylation in cytokine-induced metabolic dysregulation and apoptosis of the pancreatic β -cell.

Chapter 4: Studies of downstream signaling events involved in cytokine-induced dysfunction of the islet β -cell

- Portions of this work have been published [copy of the published manuscript is appended]

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):1-8.

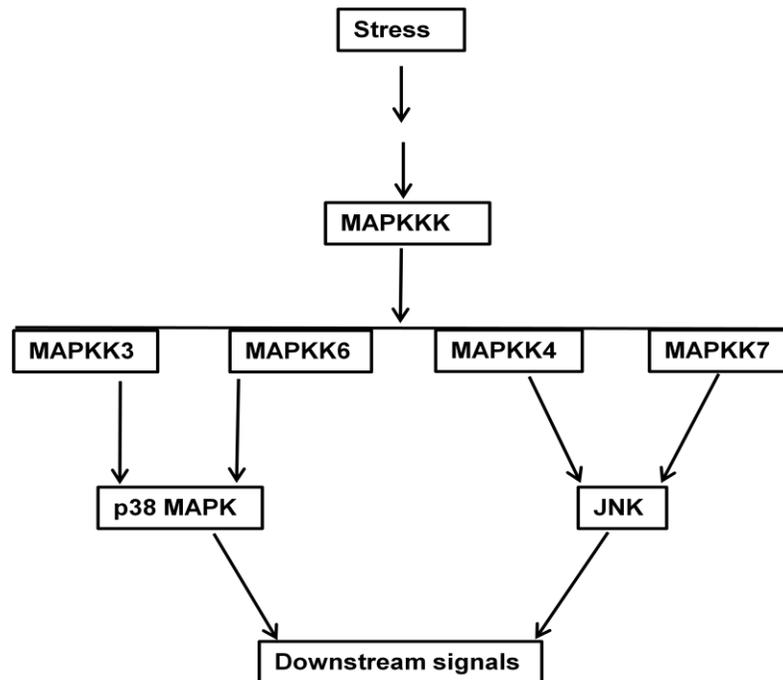


Figure 4-1: MAP kinase signaling cascade.

Cytomix activates JNK1/2 and p38 MAP kinase at 30 min

Previous studies have demonstrated a role for mitogen-activated protein [MAP] kinase signaling cascade in cytokine-induced dysfunction in multiple cell types including pancreatic β -cell [105-107]. Once the mitogen activated protein kinase kinase families

[MAPKKK] are activated by cytokines [stress], the immediate downstream MAP kinase kinase [MAPKK] get activated followed by phosphorylation and activation of either p38 MAP kinase by MAPKK3 and MAPKK6 or JNK by MAPKK4 and MAPKK7 at the threonine and tyrosine residues which lead to the downstream signaling pathways such as activation of mitochondrial apoptotic protein, transcription factors and ER stress **[Figure 4-1]**. Studies have shown that the stress [proinflammatory cytokines] signals are delivered to the protein kinases through small GTPase such as Rac1 and Ras proteins **[107-109]**.

2-Bromopalmitate inhibits cytomix-induced JNK1/2 activation

Recent study from our laboratory has shown that high glucose and palmitate activate JNK1/2 in INS-1 832/13 cells and this result has also confirmed in Zucker Diabetic Fatty [ZDF] rat islets, an animal model for type II diabetes **[89]**. To study the effect of cytokines on JNK1/2 and p38 MAP kinase and the role of protein palmitoylation in the signaling pathway, INS-1 832/13 cells were incubated with cytomix [0-24 hrs] in the presence and absence of 2-bromopalmitate [100 μ M]. Short term [30 min] incubation of INS-1 832/13 cells with cytomix significantly increased JNK1/2 and p38 MAP kinase activation **[Figure 4-2 and Figure 4-7]**. However, long term incubation [12 and 24 hrs] with cytomix had no notable effect on JNK1/2 activation **[Figure 4-3]**. 2-Bromopalmitate significantly inhibited cytomix-induced JNK1/2 activation at 30 min **[Figure 4-2]** and had no effect on p38 MAP kinase activation **[Figure 4-7]** indicating that p38 MAP kinase pathway is not regulated by palmitoylation. Interestingly, unlike short term incubation, long term incubation [12 and 24 hrs] of 2-BP increased JNK1/2 activation at the basal level **[Figure 4-3]** implying that there is/are palmitoylated proteins which suppress JNK1/2 activation in the absence of stress inducing agents.

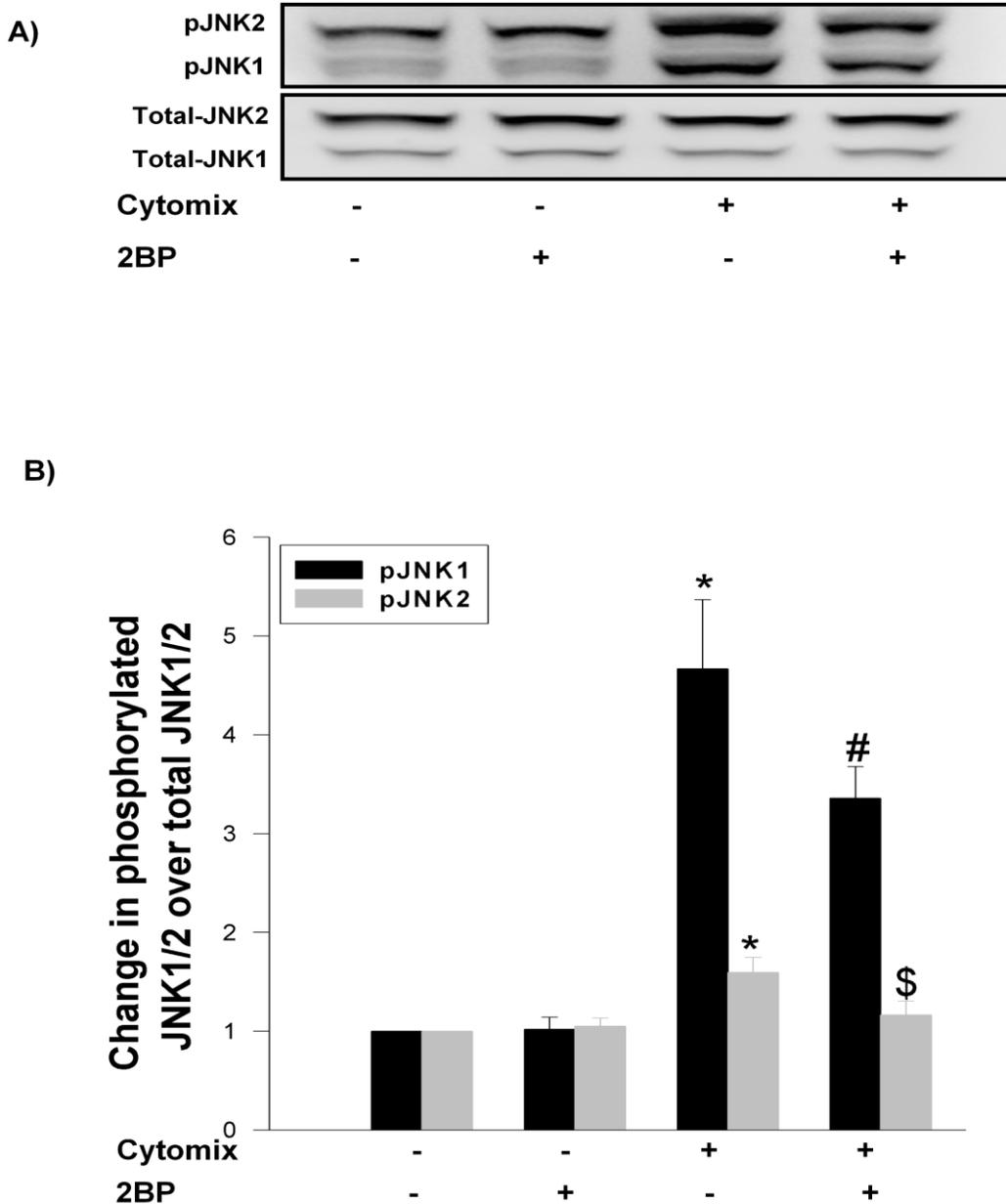


Figure 4-2: Cytomix-induced JNK1/2 activation is inhibited by 2-BP.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 30 min in the presence and absence of 2-BP [100 μ M]. JNK1/2 activation was determined by Western blot [Panel A] followed by densitometry [Panel B]. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between pJNK1/2 and total-JNK1/2. Cytomix significantly increased JNK1/2 activation [$*P < 0.05$ versus control] and it is inhibited by 2-BP [$\#P < 0.05$ versus cytomix for pJNK1 and $\$P < 0.05$ versus cytomix for pJNK2].

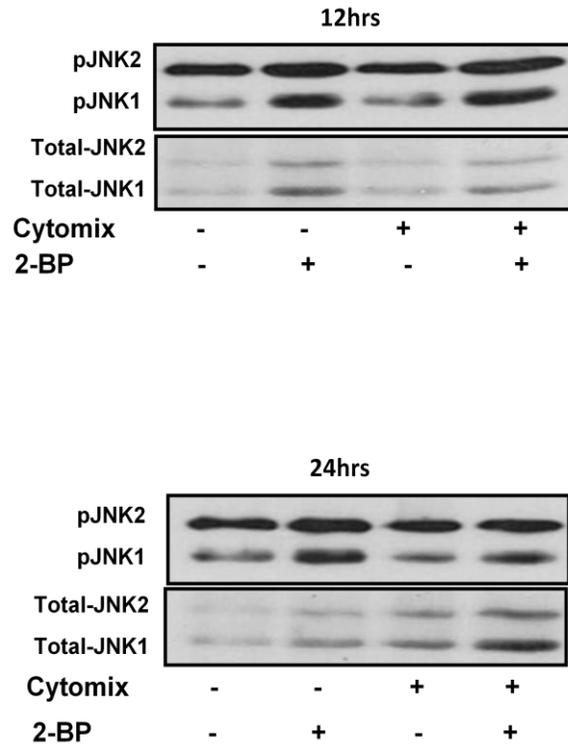


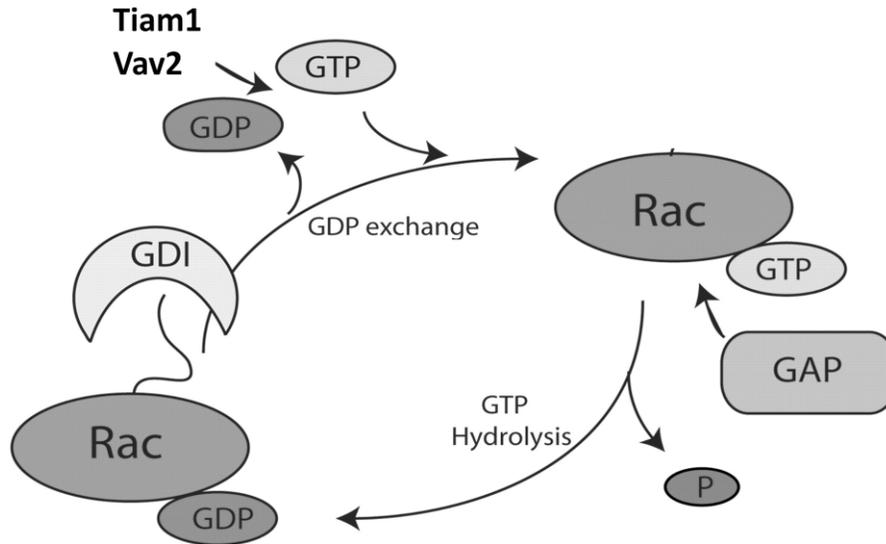
Figure 4-3: Long term incubation of INS-1 832/13 cells with cytomix and 2-BP.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 12 and 24 hrs in the presence and absence of 2-BP [100 μ M] and JNK1/2 activation was determined by Western blot. Both at 12 and 24 hrs, there is no difference in JNK1/2 activation between control and cytomix treatment [lane 1 vs. lane 3; 12 and 24 hrs]. However, 2-BP increased basal JNK1/2 activation [lane 1 vs. lane 2; 12 and 24 hrs].

EHop-016 and NSC23766 inhibit cytomix-induced JNK1/2 activation

Rac1 is regulated by three major types of proteins. These include GEFs [Guanine nucleotide Exchange Factors], GDIs [GDP Dissociation Inhibitors] and GAPs [GTPase Activating Proteins]. During the inactive state, Rac1 binds to GDI in the cytoplasmic compartment. Up on arrival of appropriate stimuli, GDI dissociates from the Rac1 and GEF namely Tiam1 and/or Vav2 facilitate the exchange of GTP for GDP causing the Rac1 to be in active form and regulates its effector proteins. Once the downstream events are activated by Rac1, the intrinsic GTPase of Rac1 and/or GAP hydrolysis the GTP loaded on Rac1 returning it to inactive state **[Figure 4-4]**.

We utilized two pharmacological agents that inhibit Rac1 function. The first being EHop-016 which is more potent inhibitor than NSC23766 [25, 94].



Gregg D et al. Am J Physiol Cell Physiol. 2003; 285(4):C723-C734

Figure 4-4: Proteins that regulate Rac1 function.

To study the role of Rac1 activation in cytomix-induced JNK1/2 and p38 MAP kinase activation, INS-1 832/13 cells were incubated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 30 min in the presence and absence of EHop-016 [5 μ M] and NSC23766 [20 μ M]. Both compounds had no effect on basal JNK1/2 and p38 MAP kinase activation where as cytomix consistently increased JNK1/2 [Figure 4-5 and Figure 4-6] and p38 MAP kinase activation [Figure 4-8 and Figure 4-9]. While cytomix induced JNK1/2 activation is inhibited by EHop-016 and NSC23766 [Figure 4-5 and Figure 4-6], their effect on cytomix-induced p38 MAP kinase activation has not been observed in this study [Figure 4-8 and 4-9] indicating that p38 MAP kinase activation does not require Rac1 activation.

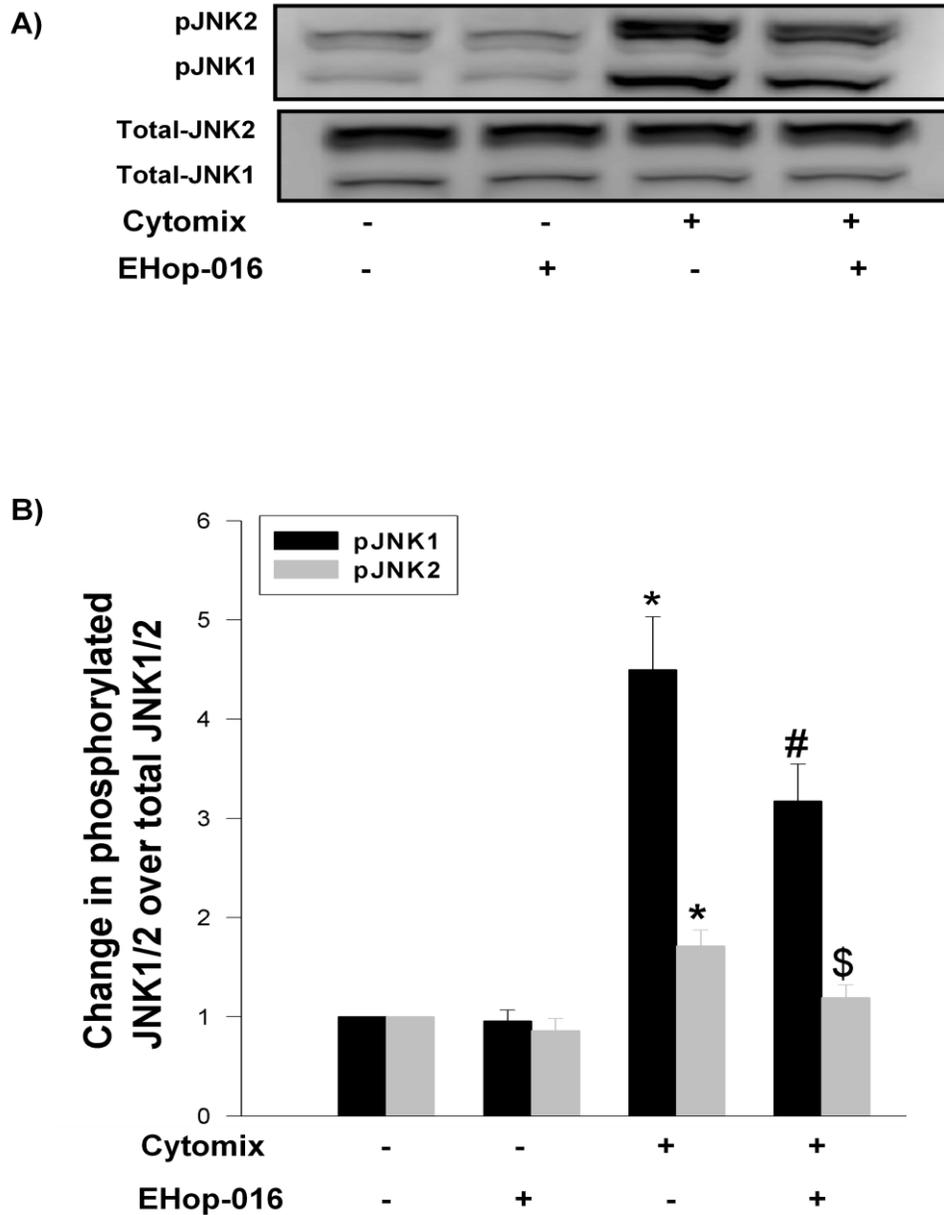


Figure 4-5: Cytomix-induced JNK1/2 activation is inhibited by EHop-016.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 30 min in the presence and absence of EHop-016 [5 μ M]. JNK1/2 activation was determined by Western blot [**Panel A**] followed by densitometry [**Panel B**]. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between pJNK1/2 and total-JNK1/2. Cytomix significantly increased JNK1/2 activation [$*P < 0.05$ versus control] and it is inhibited by EHop-016 [$\#P < 0.05$ versus cytomix for pJNK1 and $\$P < 0.05$ versus cytomix for pJNK2].

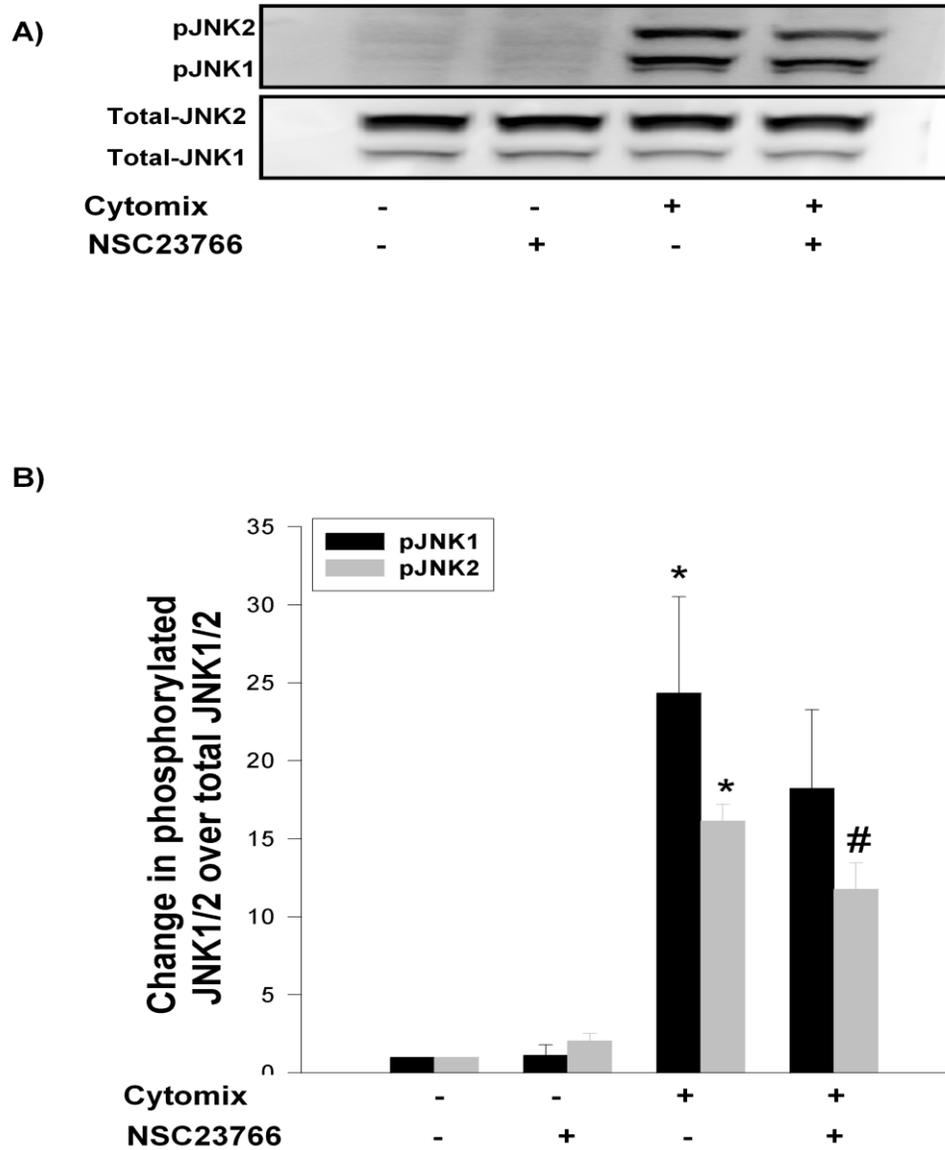


Figure 4-6: Cytomix-induced JNK2 activation is inhibited by NSC23766.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 30 min in the presence and absence of NSC23766 [20 μ M]. JNK1/2 activation was determined by Western blot [Panel A] followed by densitometry [Panel B]. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between pJNK1/2 and total-JNK1/2. Cytomix significantly increased JNK1/2 activation [$*P < 0.05$ versus control] and cytomix-induced JNK2 activation is inhibited by NSC23766 [$\#P < 0.05$ versus cytomix for pJNK2].

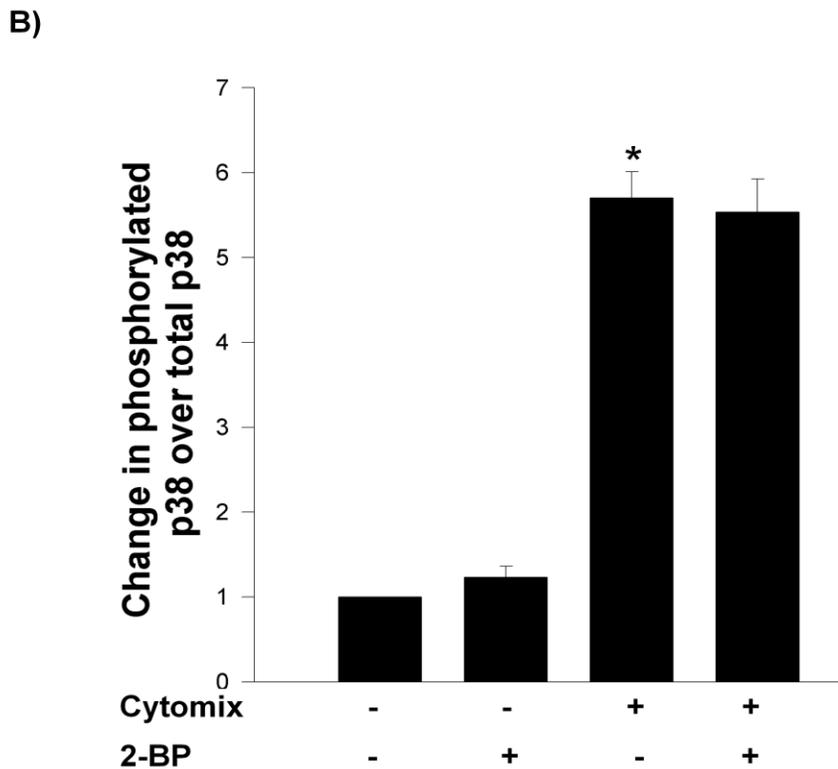
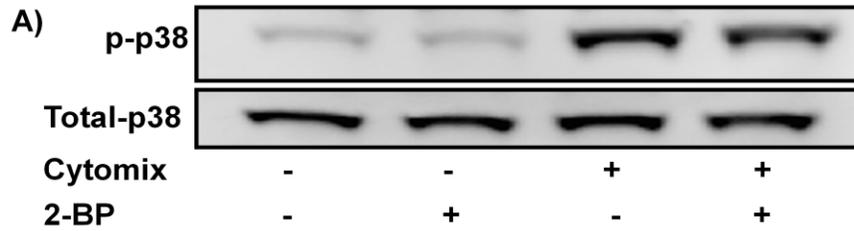


Figure 4-7: Cytomix-induced p38 MAP kinase activation is not inhibited by 2-BP.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 30 min in the presence and absence of 2-BP [100 μ M] and p38 MAP kinase activation was determined by Western blot **[Panel A]** followed by densitometry **[Panel B]**. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between p-p38 over total-p38. Cytomix increased p38 MAP kinase activation [$*P < 0.05$ versus control]; however, the activation is not inhibited by 2BP.

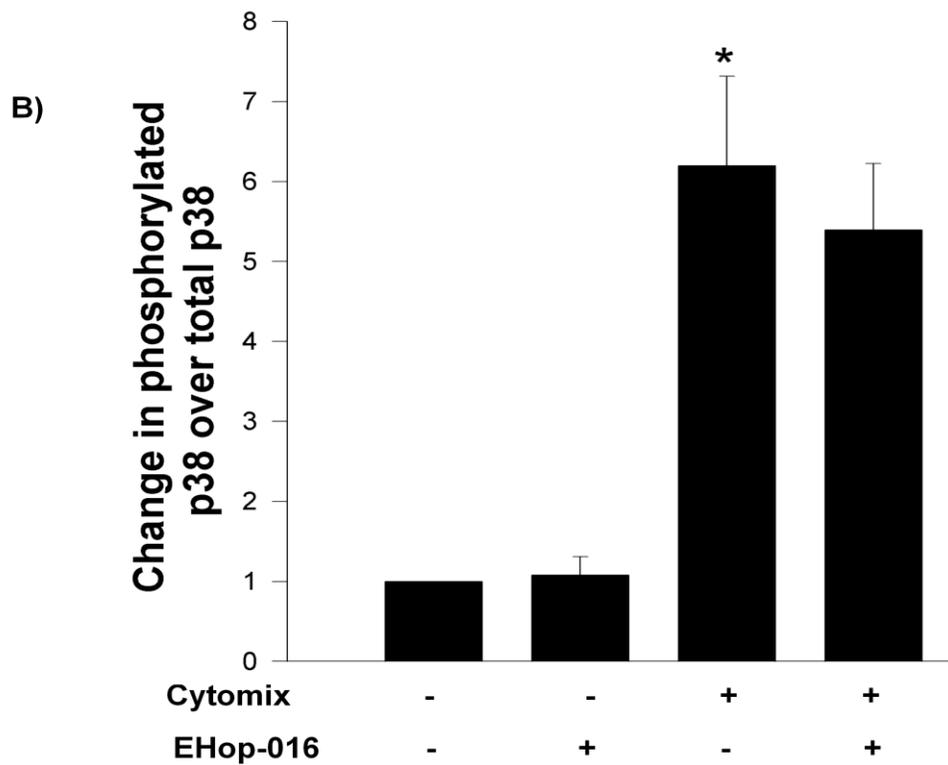
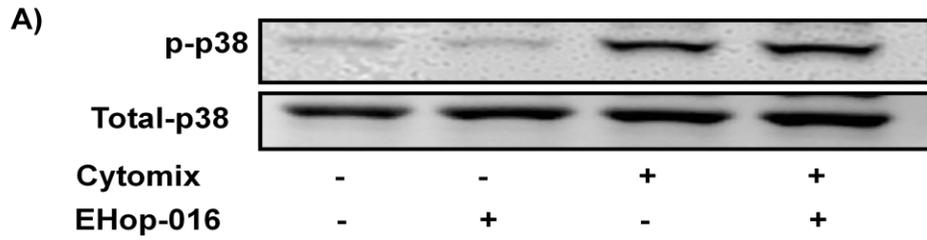


Figure 4-8: Cytomix-induced p38 MAP kinase activation is not inhibited by EHop-016.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 30 min in the presence and absence of EHop-016 [5 μ M] and p38 MAP kinase activation was determined by Western blot **[Panel A]** followed by densitometry **[Panel B]**. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between p-p38 over total-p38. Cytomix increased p38 MAP kinase activation [$*P < 0.05$ versus control]; however, the activation is not inhibited by EHop-016.

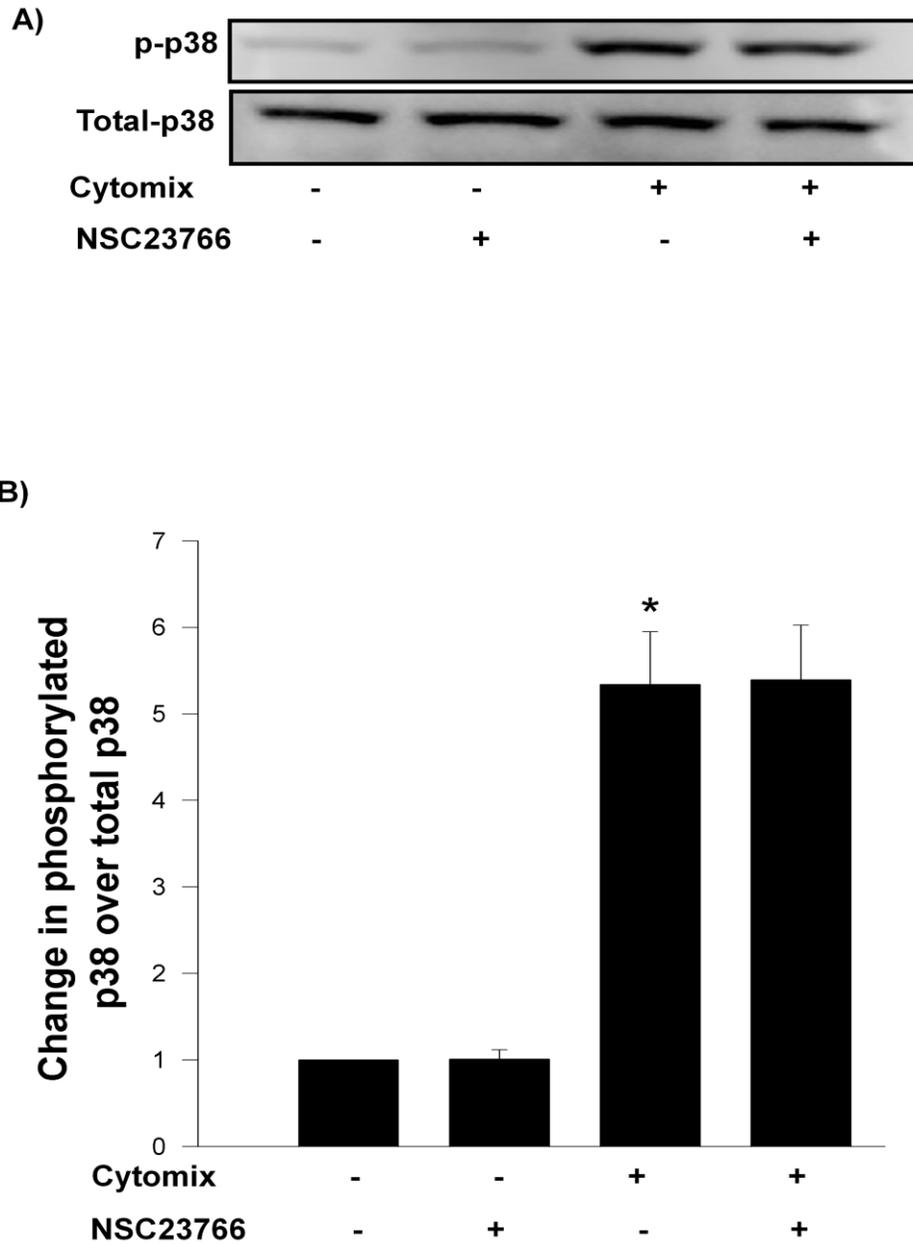


Figure 4-9: Cytomix-induced p38 MAP kinase activation is not inhibited by NSC23766.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 30 min in the presence and absence of NSC23766 [20 μ M] and p38 MAP kinase activation was determined by Western blot [Panel A] followed by densitometry [Panel B]. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between p-p38 over total-p38. Cytomix increased p38 MAP kinase activation [$*P < 0.05$ versus control]; however, the activation is not inhibited by NSC23766.

Cytomix-induced ER stress is not inhibited by 2-bromopalmitate

It is well established that ER stress underlies cytokine-mediated metabolic dysregulation of the islet β -cell [66, 75, 76]. Furthermore, recent studies by Baldwin and associates have suggested a critical requirement for protein palmitoylation in palmitate-induced CHOP expression [a marker for ER stress] in that, 2-BP significantly attenuated palmitate-induced CHOP expression in insulin-secreting RINm5F cells [111]. Therefore, we investigated if cytomix-induced CHOP expression in INS-1 832/13 cells is sensitive to 2-BP. To address this, cytomix-induced CHOP expression was measured in cells incubated in the presence of diluent alone or 2-BP.

Data depicted in **Figure 4-10** represents a Western blot for the expression of CHOP. It suggested no clear effects of 2-BP on CHOP expression under basal conditions [lane 1 vs. lane 2]. Exposure of these cells to cytomix significantly increased CHOP expression [lane 1 vs. lane 3]. However, provision of 2-BP to cytomix-treated cells did not exert any significant effect on CHOP expression [lane 3 vs. lane 4] suggesting that protein palmitoylation is not necessary for cytomix-induced CHOP expression.

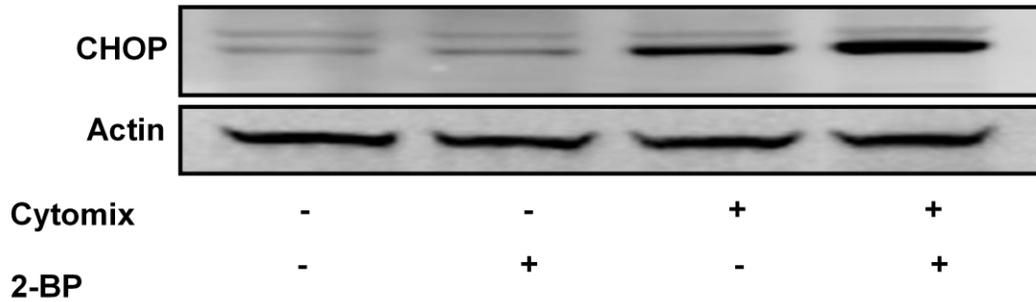


Figure 4-10: Cytomix-induced CHOP [C/EBP homologous protein] expression is not inhibited by 2-BP.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml each] for 24 hrs time point in the presence and absence of 2-bromopalmitate [100 μ M]. The level of CHOP expression, a marker for endoplasmic reticulum [ER] stress, was measured by Western blot. Equal protein loading was confirmed by β -actin content in individual lanes.

Cytomix induces caspase-3 activation and FTase/GGTase α degradation

Apoptosis, a genetically encoded programmed cell death, plays major role in cytokine-induced pancreatic β -cell dysfunction and death. The release of cytochrome C from mitochondrial intermembraneous space causes the initiation and activation of caspase family of cysteine proteases [112]. Studies from our laboratory have shown that palmitate, ceramide and proinflammatory cytokines cause loss of mitochondrial membrane potential and caspase-3 activation [47, 95]. In the present study, the downstream effects of cytomix-induced damage on mitochondrial membrane have been examined. Incubation of INS-1 832/13 cells with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 24 hrs significantly increased caspase-3 activation [Figure 4-11]. Cytomix also caused a significant degradation of FTase/GGTase α [Figure 4-12], one of the substrate protein for active caspase-3.

To further elucidate that caspase-3 activation leads to FTase/GGTase α degradation, studies have been carried out using caspase-3 inhibitor and etoposide, a known genotoxic agent that causes a robust activation of caspase-3. Incubation of INS-1 832/13 cells with etoposide [60 μ M; 6 h] significantly activated caspase-3 [Figure 4-13] and caspase-3 inhibitor, Z-DEVD-FMK, [25 μ M; 6 h] blocked the activation. Similarly, etoposide induces

significant amount of FTase/GGTase- α degradation [Figure 4-14] that was inhibited by the peptide inhibitor indicating that caspase-3 activation causes degradation and inactivation of FTase and GGTase that leads to defective activation of key G-proteins, defective nuclear assembly of lamins and loss of cell viability.

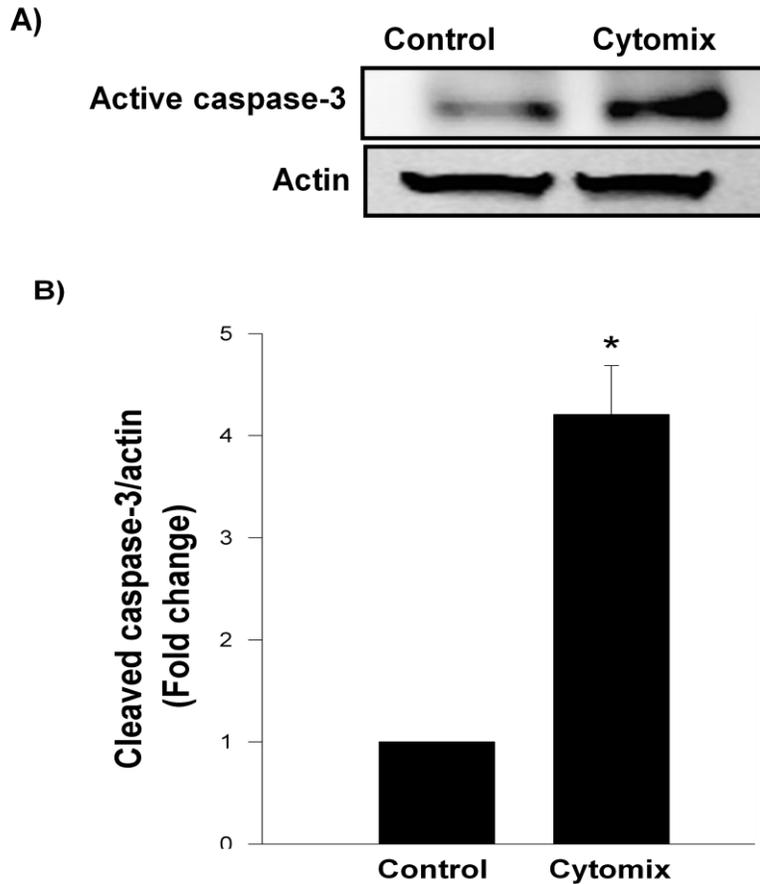


Figure 4-11: Cytomix induces caspase-3 activation.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 24hrs and the expression of cleaved caspase-3 was measured by Western blot [Panel A] followed by densitometry [Panel B]. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between cleaved caspase-3 and β -actin. Cytomix significantly induces caspase-3 activation. * $P < 0.05$ versus control.

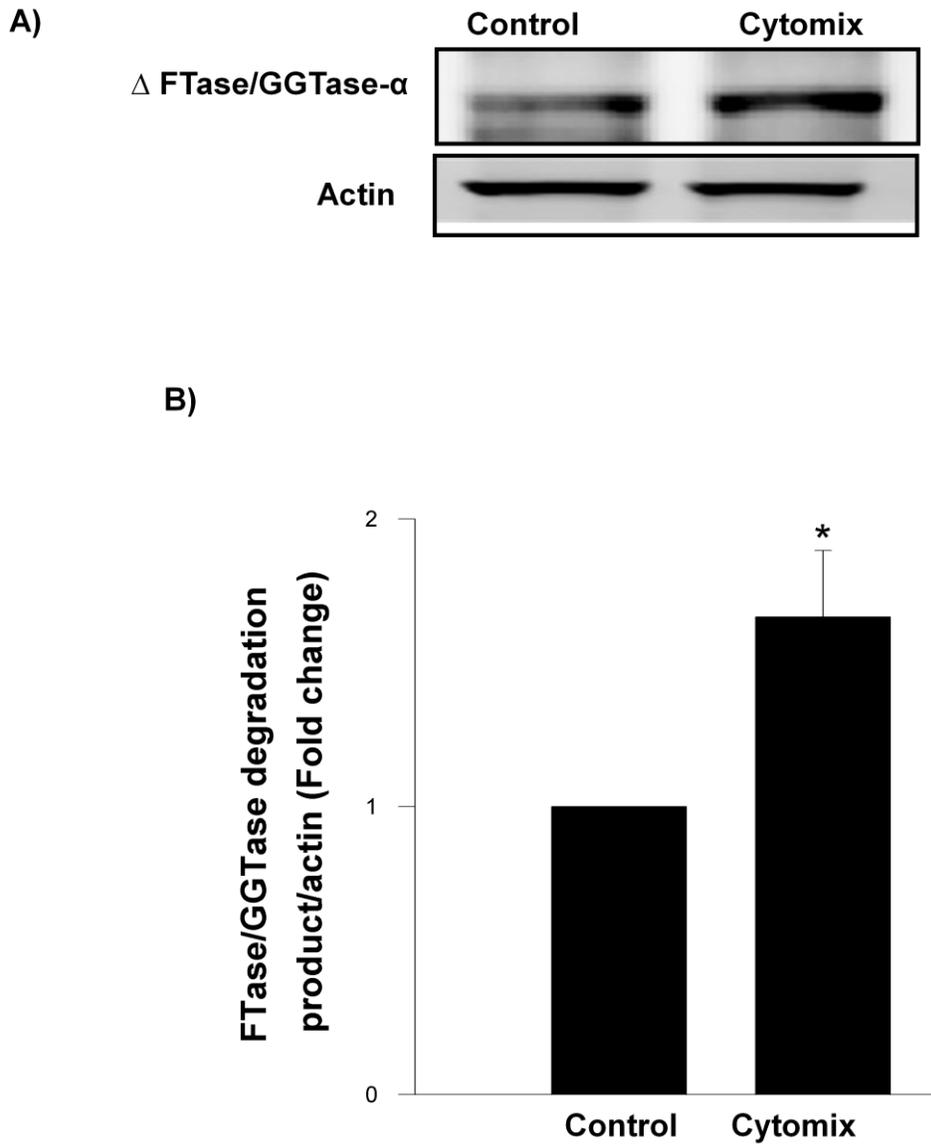
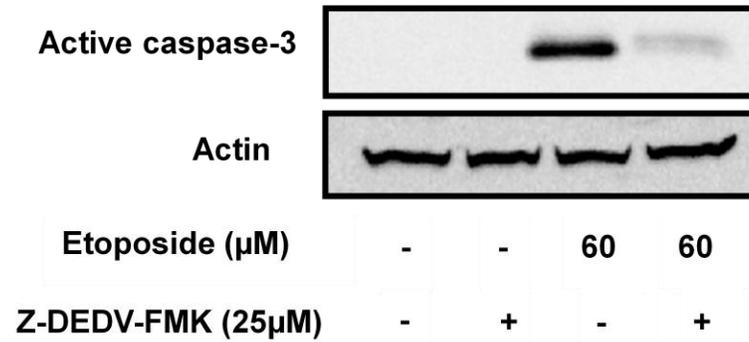


Figure 4-12: Cytomix increases FTase/GGTase α degradation.

INS-1 832/13 cells were treated with cytomix [IL-1 β , TNF α , and INF γ , 10 ng/ml each] for 24hrs time points and the expression of FTase/GGTase α degradation product was measured by Western blot [Panel A] followed by densitometry [Panel B]. Data represent mean \pm SEM from three independent experiment and expressed as fold change of the ratios between degraded FTase/GGTase α and β -actin. Cytomix significantly increases FTase/GGTase α degradation. * $P < 0.05$ versus control.

A)



B)

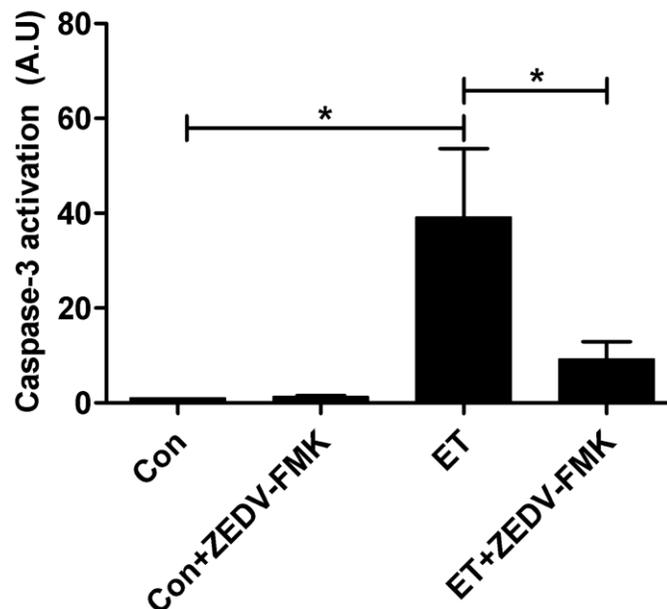
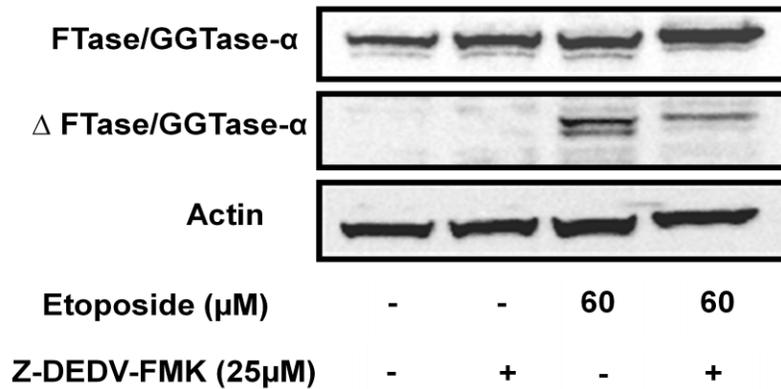


Figure 4-13: Etoposide induces caspase-3 activation: Protection of this signaling step by Z-DEVD-FMK, an inhibitor of caspase-3.

INS-1 832/13 cells were treated with either diluents or etoposide [60 μM] in the presence or absence of peptide inhibitor, Z-DEVD-FMK [25 μM ; 6 h]. Caspase-3 activation was determined by Western blotting. Equal amount of lysates protein were resolved by SDS-PAGE [10 %]. Protein loading was determined by β -actin content in individual lanes. Representatives blot indicating the caspase-3 activation [Panel A] is provided. Quantitative analysis of data obtained from three independent experiments for caspase-3 activation [Panel B] was carried out by densitometry. Results are shown as mean \pm SEM. * $P < 0.05$.

A)



B)

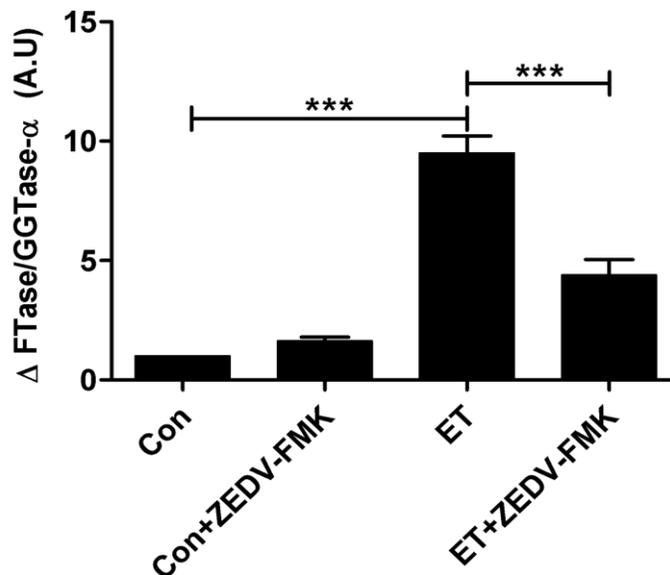


Figure 4-14: Etoposide induces FTase/GGTase- α degradation: Protection of this signaling step by Z-DEVD-FMK, an inhibitor of caspase-3.

INS-1 832/13 cells were treated with either diluents or etoposide [60 μ M] in the presence or absence of peptide inhibitor, Z-DEVD-FMK [25 μ M; 6 h]. FTase/GGTase- α degradation was determined by Western blotting. Equal amount of lysates protein were resolved by SDS-PAGE [10 %]. Protein loading was determined by β -actin content in individual lanes. Representatives blot indicating the FTase/GGTase- α degradation [Panel A] is provided. Quantitative analysis of data obtained from three independent experiments for FTase/GGTase- α degradation [Panel B] was carried out by densitometry. Results are shown as mean \pm SEM. *** P < 0.001.

In conclusion,

- I have studied the effect of cytokines on stress signals namely, JNK1/2, p38 MAP kinase, ER stress, NOX2 activation, NO release and downstream effect of mitochondrial dysfunction.
- I have assessed the effect of three pharmacological inhibitors namely, inhibitor of palmitoylation [2-BP], Vav2-Rac1 [EHop-016] and Tiam1-Rac1 [NSC23766] axis.

Stress Signals	2-BP	EHop-016	NSC23766
JNK1/2	Inhibition	Inhibition	Inhibition
p38 MAP kinase	No effect	No effect	No effect
ER stress	No effect	Not determined	Not determined
NOX2	Inhibition	Not determined	Inhibition
NO	inhibition	Not determined	No effect

Table 4-1: Summary of the effects of three pharmacological inhibitors on stress signaling kinases.

Chapter 5: Preliminary studies to determine potential difference, if any in NOX2 subunit expression and ROS generation in islets from pre-diabetic NOD mice and the control BALB mice.

Type I diabetes is an autoimmune disorder that results from the destruction of insulin secreting pancreatic β -cell. Although the exact causes of the disorder are still under scientific scrutiny, studies have shown that cytokines play a major role in the pathogenesis of the disease [12, 14, 16]. To reveal the pathophysiological mechanisms, non obese diabetic [NOD] mice, a well known animal model for type I diabetes, have been used for more than 30 years.

The NOD mice develop diabetes spontaneously around 12-14 weeks of age and the incidence rate is higher in the female mice than the male mice [23]. Based on the hypothesis that NOX2 hyperactivation plays a role in mediating cytokine-induced pancreatic β -cell dysfunction in the NOD mice, the present study examined the basal status of NOX2 subunit expression and activation in the islets of female NOD mice and age matched BALB control mice [7-8 weeks]. Preliminary results have shown that both islets of NOD and BALB mice express NOX2 subunits namely phospho-p47^{phox}, p67^{phox}, Rac1 and gp91^{phox}. There is no difference in the expression as well as activation of the NOX2 enzyme system between the two groups [Figure 5-1 and 5-2]. Incubation of the islets with cytomix [IL-1 β , TNF α and INF γ ; 10 ng/ml] for 24 hrs increased gp91^{phox} expression in both BALB and NOD mice islets preparation [Figure 5-3].

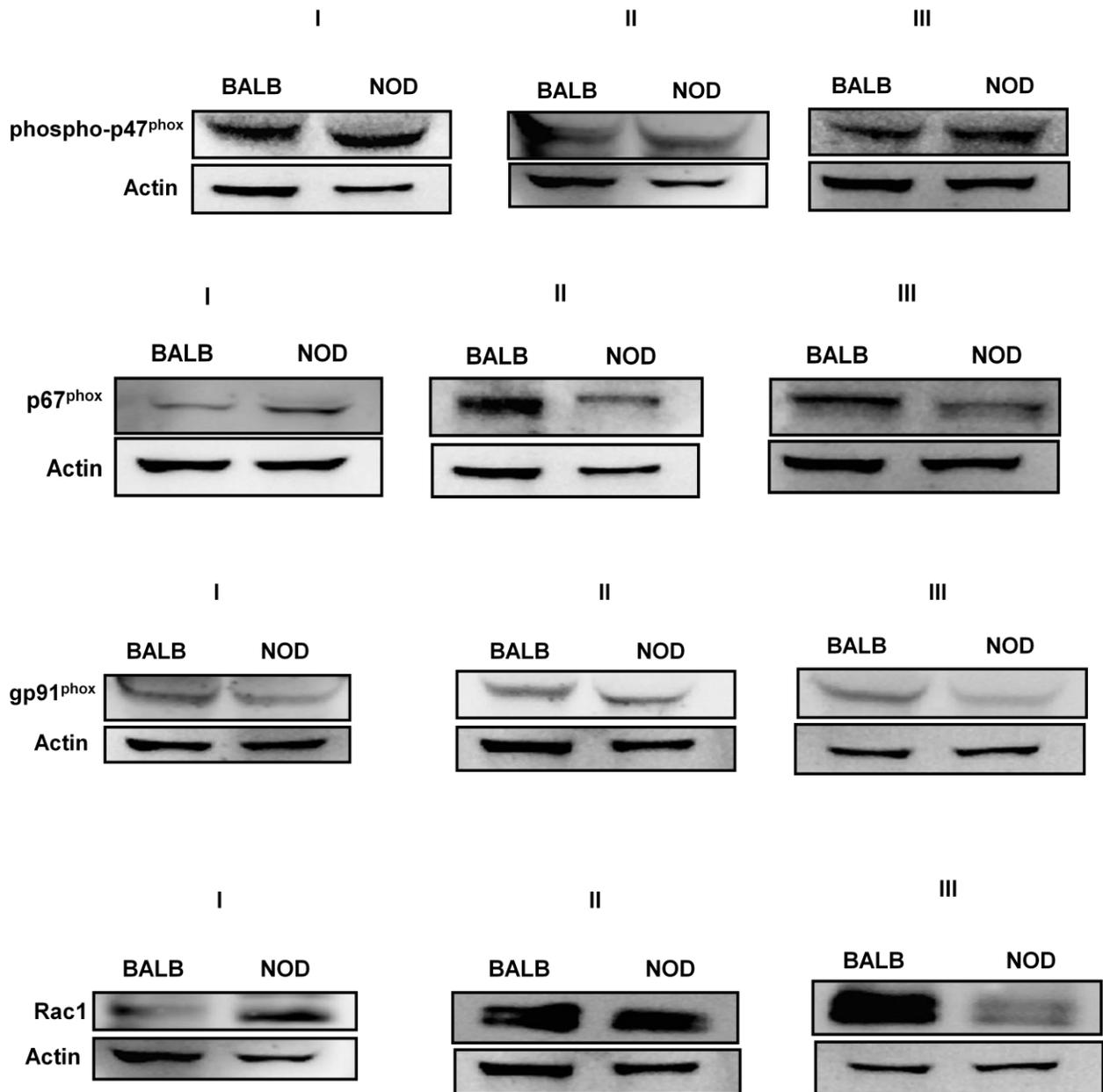


Figure 5-1: NOX2 subunit expression in the islets of NOD and BALB mice.

There is no difference in NOX2 subunit expression in the islets of NOD and control BALB mice. Pancreatic islets were isolated from female NOD and control BALB mice using collagenase digestion of the pancreas and the expression level of phospho-p47^{phox}, p67^{phox}, gp91^{phox} and Rac1 were determined by Western blot.

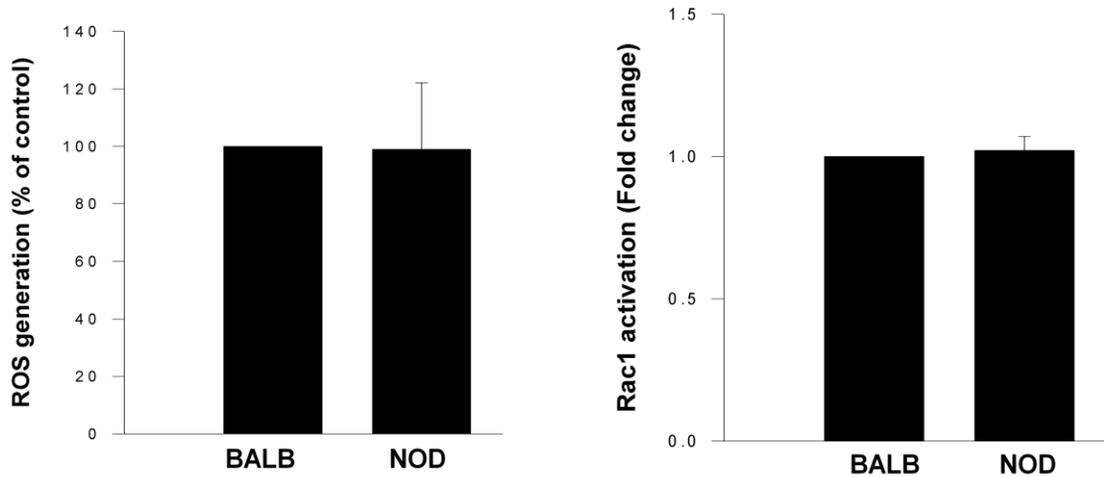
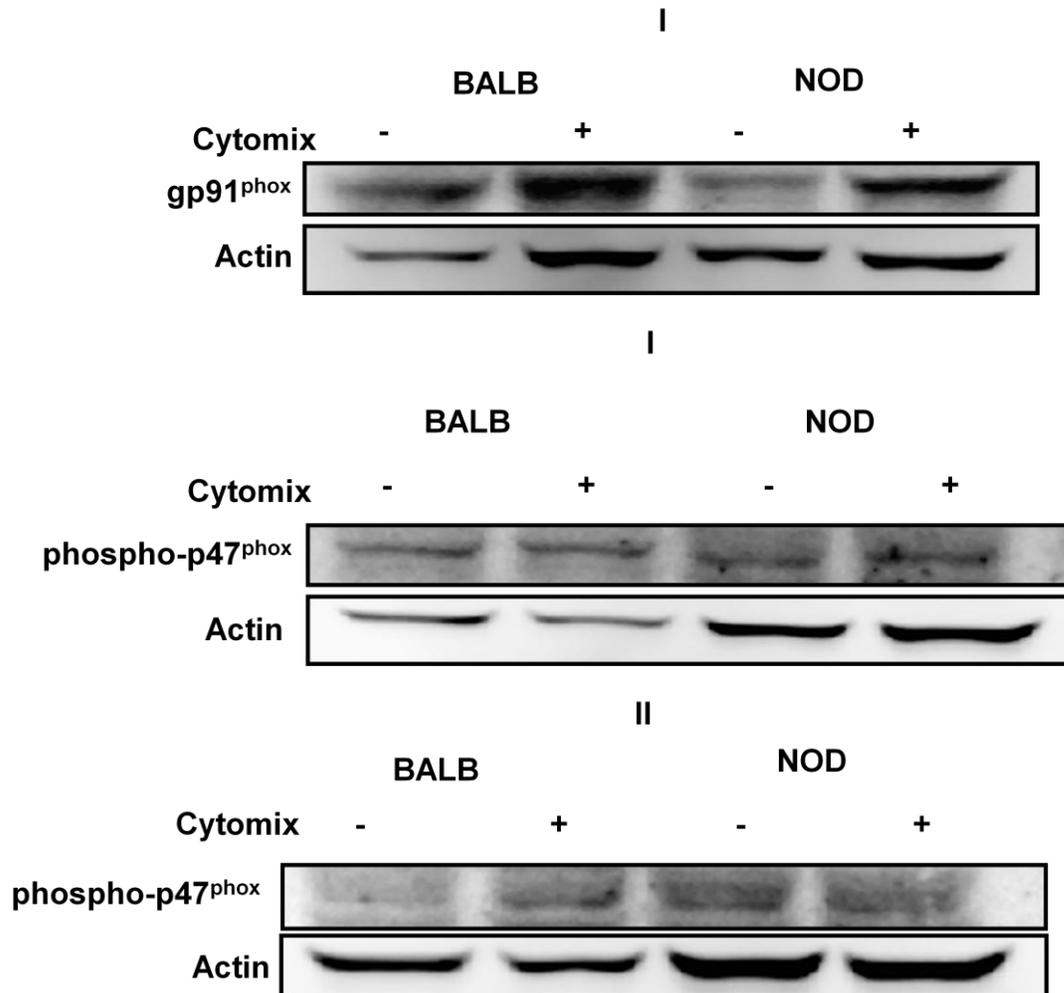


Figure 5-2: ROS generation and Rac1 activation in the islets of NOD and BALB mice.

There is no difference in ROS generation and Rac1 activation in the islets of NOD and control BALB mice. Pancreatic islets were isolated from female NOD and control BALB mice using collagenase digestion of the pancreas and the amount of ROS generation and Rac1 activation were determined using DCHF-DA assay and GLISA respectively. Data represent mean \pm SEM from three independent experiments and expressed as % of control for ROS generation and fold change for Rac1 activation.



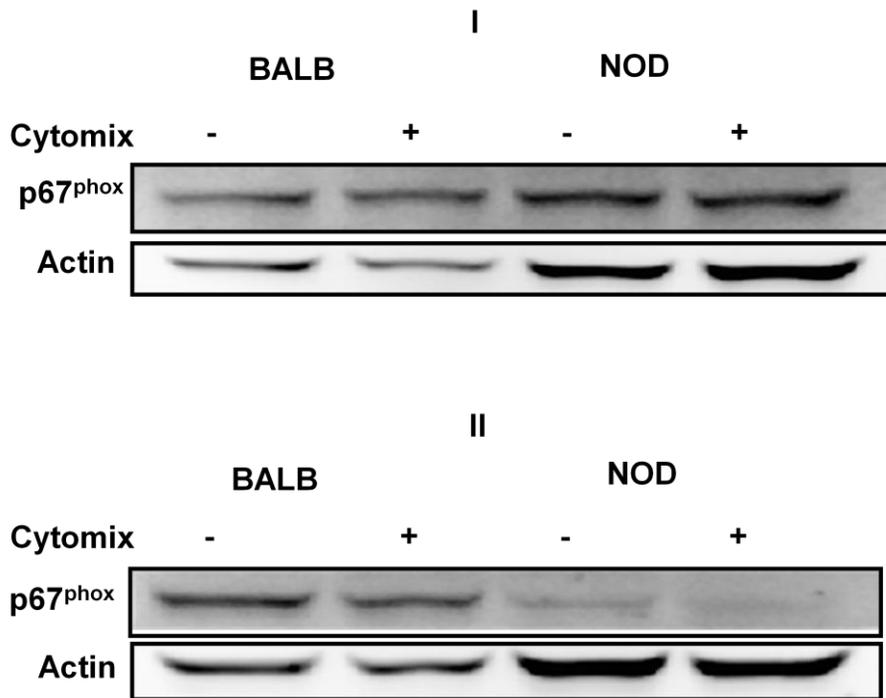


Figure 5-3: The effect of cytomix in the islets of NOD and BALB mice.

Cytomix induces gp91^{phox} expression in the islets of NOD and control BALB mice. Pancreatic islets were isolated from female NOD and control BALB mice using collagenase digestion of the pancreas. The isolated islets were incubated with cytomix [IL-1 β , TNF α , and INF γ ; 10 ng/ml each] for 24hrs and the expression level of gp91^{phox}, phospho-p47^{phox} and p67^{phox} were determined by Western blot.

In summary, the results obtained in this preliminary study are inconclusive and are yet to be confirmed. Future experiment will determine whether or not NSC23766, EHop-016 and 2BP prevent cytokine-induced damage *ex vivo* and delay or prevent the onset of diabetes in the NOD mice.

Chapter 6: Discussion

- Portions of this work have been published [copy of the published manuscript is appended]

Mohammed AM, Chen F, Kowluru A. The Two Faces of Protein Palmitoylation in Islet β -Cell Function: Potential Implications in the Pathophysiology of Islet Metabolic Dysregulation and Diabetes. *Recent Patents Endocrine Metabolic and Immune Drug Discovery*. 2013 [Epub ahead of print].

The uptake of glucose by the body cells is dependent upon the availability of insulin and the loss of functional pancreatic β -cell mass leads to a condition called “starvation amidst plenty” where there is an excess amount of glucose in the blood yet the body cells are starved. In type I diabetes, proinflammatory cytokines namely IL-1 β , TNF α and INF γ are involved in the loss of pancreatic β -cell mass. However the precise mechanisms underlying the loss of cytokine-induced pancreatic β -cells are relatively poorly understood. Therefore, the objective of my doctoral work is to study the putative mechanisms of cytokine-induced metabolic dysfunction of the pancreatic β -cell and identify a novel therapeutic target for the prevention of cytokine-induced metabolic dysfunction of the islet β -cell.

Studies have shown that phagocytic-like NADPH oxidase [NOX2] has a significant role in exerting detrimental effects on the pancreatic β -cells following exposure to high glucose [glucotoxicity], palmitate [lipotoxicity] or proinflammatory cytokines [25, 56, 72, 94]. Results from our laboratory have shown that incubation of INS-1 832/13 cells and normal rat islets with high glucose and palmitate increased Rac1 activation, p47^{phox} expression, ROS generation and mitochondrial damage. Follow up studies in islets from Zucker Diabetic Fatty (ZDF) rats, an animal model for type II diabetes, and diabetic human islets confirmed the

above results including an increase in expression of p47^{phox}, Rac1 and gp91^{phox} and activation of p47^{phox}, Rac1 and ROS generation [39, 51, 89].

Studies using proinflammatory cytokines and INS-1 832/13 cells have shown many similarities in identifying the NADPH oxidase [NOX2] as a common pathway for metabolic dysfunction of the pancreatic β -cell under gluco-lipo-toxicity and proinflammatory cytokine conditions. Those previous findings include, incubating INS-1 832/13 cells with cytomix [IL- β , TNF α and INF γ ; 10 ng/ml each] transiently [~15min] increase Rac1 activation, p47^{phox} expression, ROS generation and mitochondrial damage. The use of pharmacological and molecular biological approach indicated the role of Rac1 and p47^{phox} in cytomix-induced ROS generation and mitochondrial membrane damage. Inhibiting the Tiam1-Rac1 axis using NSC23766 and the use of GGTI-2147, a geranylgeranyl transferase inhibitor, attenuated Rac1 activation, ROS generation and mitochondrial damage. siRNA p47^{phox} transfection and apocynin also inhibited cytomix-induced ROS generation [47].

As a logical extension of these studies, we further explored the mechanism of NOX2 activation during the challenge with proinflammatory cytokines condition. To study the effect of individual cytokines [IL-1 β , TNF α and INF γ ; 25 ng/ml] on NOX2 activation, first, the amount of ROS generation was measured after 24 hrs incubation and the results showed that IL-1 β alone can activate the NOX2 system. As there are other cellular sources for ROS such as mitochondria and since NOX2 activation specifically produce superoxide we measured the NOX2 activity following cytokine [IL-1 β] and high glucose exposure by lucigenin [N, N'-dimethyl-9, 9'-biacridinium dinitrate] assay. The assay is based on detection of chemiluminescence signal that results from reaction of superoxide anion and lucigenin. The result showed that both glucose and IL-1 β activate NOX2. IL-1 β [25 ng/ml] activated the NOX2 as early as 30 min and the enzyme activity is completely blocked by apocynin, which

is relatively a selective NOX2 inhibitor, and the enzyme time kinetics coincide with activation of the cytosolic [p47^{phox}, Rac1] and membranous [gp91^{phox}] components of the Nox2 system.

The complex nature of human being cannot be explained only using the number of genes in our genome which is ~4-5 times higher when compared with the simple eukaryotic organisms such as yeast [113, 114]. Hence, post-translational modifications of proteins such as methylation, phosphorylation, prenylation and palmitoylation play major role in diversifying protein functions that make specific characteristics for a given species. The present study discloses that p47^{phox}, one of the cytosolic subunits of the NADPH oxidase enzyme system undergoes phosphorylation in INS-1 832/13 cells following cytomix exposure.

Similar results have been found in other cell types such as alveolar epithelial cell [97], endothelial cell [98], seabream head kidney [115] under different stimulatory conditions. The above studies also indicated that protein kinase C [PKC] might be responsible for the addition of phosphate group to p47^{phox} that participates in initiating the cascade for activation of the NOX2 enzyme system. Moreover, studies using protein kinase C [PKC] knockout mice have shown partial protection from multiple low dose of streptozotocin-induced hyperglycemia and cytokine mediated islet apoptosis *in vitro* [116]. Beside p47^{phox} phosphorylation, the present study also examined the effect of cytomix on gp91^{phox} expression, which is a membrane component of NOX2. The result showed acute regulation [~20 min] by cytomix along with other cytosolic components such as Rac1 [~15 min] and p47^{phox}.

In the context of the currently described studies, our laboratory has recently published evidence to suggest that exposure of isolated β -cells to proinflammatory cytokines results in increased expression of p47^{phox}, a member of the cytosolic core of NOX2, within 12-24 hr. It has also shown that Rac1, another cytosolic component of NOX2, gets activated transiently [~15 min] following exposure to cytokines [47]. More importantly, the data also suggested

that inhibition of p47^{phox} function via siRNA-p47^{phox} or Rac1 functions using inhibitors of Rac1 prenylation [i.e., GGTI-2147] or its GTP-exchange functions by Tiam1 [e.g., NSC23766] significantly attenuated cytokine-induced ROS generation and loss of mitochondrial membrane potential. Interestingly, neither GGTI-2147 nor NSC23766 prevented cytokine-induced NO generation [47] suggesting that NOX2 activation is not upstream to iNOS induction and NO release, and the two signaling steps are regulated by two distinct mechanisms.

Existing body of evidence clearly implicates novel regulatory roles for protein palmitoylation in cellular functions. Unlike isoprenylation, palmitoylation steps are subject to acute regulation at the level of the “on” steps [addition of palmitoyl group] as well as the “off” steps [removal of palmitoyl group]. The protein palmitoyltransferases [PATs] catalyze the transfer of palmitate into the cysteine residues of proteins containing DHHC [Asp-His-His-Cys] cysteine-rich domains [CRD] via a thioester linkage. Typically, these reactions are known to occur at the cytoplasmic face of membranes in the secretory pathway [e.g., endoplasmic reticulum and Golgi] and the plasma membrane [117, 118]. At least two types of PAT activities have been reported. The first group catalyzes palmitoylation of farnesylated proteins including Ras GTPases [e.g., H-Ras and N-Ras], whereas the second group of PATs mediates palmitoylation of Src family of tyrosine kinases. A distinct class of palmitoyl thioesterases, namely protein palmitoyl thioesterases 1 and 2 and acyl palmitoyl esterase 1 mediate depalmitoylation via hydrolysis of the ester bonds to complete the palmitoylation-depalmitoylation [i.e., activation-inactivation] cycle [Figure 6-1] [117, 118].

roles for these signaling steps in islet β -cell function. For example, cerulenin [CER] has been shown to inhibit insulin secretion induced by glucose, α -ketoiso-caproic acid, and long chain acyl CoAs [34, 134-136]. CER also inhibits glucose-induced incorporation of radio labeled palmitate into islet proteins and inhibition of palmitoylation of a 24 kDa protein [137], and palmitate-induced tyrosine phosphorylation of insulin receptor [138].

Interestingly, studies by Metz and associates have demonstrated that CER failed to inhibit insulin secretion facilitated by non-nutrient secretagogues, such as a membrane-depolarizing concentration of potassium, activators of protein kinase A, or mastoparan [34]. More recent studies by Abdel-Ghany and associates [139] have demonstrated significant incorporation of [3 H] palmitate into islet β -cell proteins, which was stimulated by glucose. Furthermore, 2-aminobicyclo heptane-3-carboxylic acid, a non-metabolizable analog of leucine and an insulin secretagogue, also promoted incorporation of labeled palmitate into β -cell proteins. Autoradiographic analysis of these proteins separated by gel electrophoresis demonstrated glucose-mediated increase in palmitoylation of at least four β -cell proteins with apparent molecular weights of 30, 44, 48 and 76 kDa. More importantly, CER inhibited incorporation of labeled palmitate into these proteins under basal as well as glucose-stimulated conditions indicating the role of protein acylation in islet function [139].

In addition to CER, some studies utilized 2-BP to understand the roles of acylation/lipid metabolism in islet functions including insulin secretion. For example, original studies by Warnotte and associates demonstrated partial inhibition of palmitate-induced potentiation of glucose-induced insulin secretion [140]. Studies by Parker and coworkers [141] have suggested significant inhibition, by 2-BP, of palmitate esterification into cellular lipids in isolated rat islets. Furthermore, they also demonstrated partial inhibition of palmitate-mediated potentiation of glucose stimulated insulin secretion [GSIS]. Cheng and associates [142] reported significant protection of distal inhibitory effects of norepinephrine on

physiological insulin secretion by CER and 2-BP. Based on these observations these investigators proposed novel roles for protein acylation signaling steps in exocytotic secretion of insulin. Together, pharmacological evidence suggests that protein acylation, specifically palmitoylation, plays critical regulatory roles in islet β -cell function, including insulin secretion.

Recent studies have implicated novel roles for small G-proteins [Arf6, Cdc42 and Rac1] in glucose stimulated insulin secretion [GSIS] [25, 27, 143, 144]. Furthermore, using various pharmacological and molecular biological approaches, our lab and others documented a requirement for post-translational prenylation and carboxymethylation of G-proteins [e.g., Cdc42, Rho, Rac1] for physiological insulin secretion [25, 27]. More importantly, despite the above described pharmacological evidence to indicate inhibition of GSIS by CER and 2-BP, very little is known if Rac1 and Cdc42 also require palmitoylation to facilitate GSIS. In this context, recently published evidence suggests that Rac1 and bCdc42 [a splice variant of Cdc42 with predominant localization in the brain] undergo palmitoylation.

For example, Navarro-Lerida and associates have recently demonstrated that Rac1 undergoes palmitoylation at cysteine 178 to facilitate its targeting for stabilization at actin cytoskeleton-linked ordered membrane regions [145]. Interestingly, they observed that palmitoylation of Rac1 requires its prior prenylation and the intact C-terminal polybasic region and is regulated by the triproline-rich motif. In addition, palmitoylation step appears to be required for Rac1 activation [i.e., GTP-binding] since non-palmitoylated Rac1 exhibited decreased GTP loading and lower association with detergent-resistant membranes. Further proof that palmitoylation of Rac1 is critical for cellular function was afforded in these studies since cells lacking Rac1 exhibited spreading and migration defects. Based on this compelling evidence, the authors concluded that palmitoylation of Rac1 is requisite for its role in actin cytoskeleton remodeling and regulation of membrane organization.

Along these lines, Kang et al [146] have demonstrated palmitoylation of a brain-

specific Cdc42 splice variant [bCdc42]. Additional studies by Nishimura and Linder have documented that bCdc42 undergoes classical CAAX processing as well as prenylation and palmitoylation at the CCAX motif [147]. Interestingly, the prenylated and palmitoylated bCdc42 interacted less efficiently with RhoGDI [a signaling step critical for Cdc42 activation-deactivation cycles] when compared to canonical Cdc42, which is only prenylated and carboxymethylated, but not palmitoylated. Additional studies are required to determine if palmitoylation of Rac1 and/or Cdc42 is necessary for GSIS to occur.

In addition to small G-proteins, heterotrimeric G-proteins also control islet function including GSIS [25, 27]. Along these lines, our laboratory has demonstrated non-receptor dependent activation of trimeric G-proteins by glucose. For example, we have reported phosphorylation of G_{β} subunits at critical histidine residues in clonal β -cells, normal rat islets and human islets, which, in turn, is transferred to $G_{\alpha.GDP}$ to yield biologically active GTP-bound G_{α} subunits [148, 149]. In addition, previous studies have also demonstrated glucose-mediated activation of the carboxymethylation of G_{γ} subunits [33]. Together, these observations provided the first evidence to indicate activation of trimeric GTPase via signaling steps generated during glucose metabolism culminating in insulin secretion. It is noteworthy that recent findings from the laboratory of Gautam [150, 151] have suggested critical roles for protein palmitoylation in the shuttling of trimeric G-protein subunits between the plasma membrane and intracellular membranes, which is inhibited by 2-BP. Potential involvement of protein palmitoylation in glucose-induced activation of trimeric-proteins in the islet β -cell remains to be verified experimentally.

In addition to their positive modulatory roles, accumulating evidence supports the view point that protein palmitoylation plays negative modulatory roles in the induction of metabolic dysfunction of the islet β -cell under the duress of noxious stimuli such as proinflammatory cytokines and saturated fatty acids. Original studies from our laboratory

suggested that a protein palmitoylation step controls IL-1 β -induced iNOS gene expression and NO release in insulin-secreting β -cells [40, 42]. In those studies, H-Ras, a small G-protein, was identified as one of the palmitoylated proteins, which is involved in IL-1 β -induced NO release. These conclusions were confirmed via the use of two selective inhibitors of protein palmitoylation, namely CER and 2-BP [42]. 2-hydroxymyristic acid, an inhibitor of protein myristoylation, failed to affect IL-1 β -induced NO release suggesting the relevance of palmitoylation, but not myristoylation, in these signaling steps.

It is also demonstrated that there is a significant accumulation of H-Ras in the cytosolic fraction in cells incubated with CER indicating that inhibition of palmitoylation leads to mis-targeting of G-proteins in islet β -cells [42, 152]. Furthermore, it has also been able to demonstrate that selective inhibitors of protein farnesylation [e.g., allylfarnesols, manumycin, damnacanthal, FTI-277] markedly attenuated cytokine-induced NO release suggesting that farnesylation as well as palmitoylation of H-Ras are necessary for IL-1 β -mediated effects on NO release [25]. Identity of H-Ras as one of the regulatory proteins involved in cytokine-induced NO release in β -cells was also confirmed through the use of bacterial toxins [40]. In addition to the H-Ras, iNOS also undergo palmitoylation [153] and the inhibitory effect of 2-BP and cerulenin on cytokine-induced NO release may be in part due to inhibition of iNOS palmitoylation.

Interestingly, the current findings demonstrated that 2-BP also inhibited cytokine-induced NOX2 activation and ROS generation suggesting that protein palmitoylation might be crucial to cytokine-mediated effects. To address the question of potential identity of the protein, cytomix-and glucose induced Rac1 activation was quantitated in the presence of palmitoylation inhibitor [2-BP]. 2-BP completely blocked Rac1 activation suggesting that for the Rac1 to become active and initiate NOX2 activation, it requires palmitoylation reaction. Along these lines, recent studies from Corbett's laboratory have demonstrated a requirement

for a palmitoylation step in palmitate-induced metabolic dysfunction of the islet β -cell [111]. Interestingly, they reported a significant inhibition in palmitate-induced CHOP expression and associated metabolic dysfunction of the islet β -cell in the presence of 2-BP. While the present results further validate such a model in the context of cytokines, we failed to observe any significant effects of inhibition of protein palmitoylation with 2-BP on cytokine-induced CHOP expression suggesting distinct mechanisms underlying palmitate and cytokine-induced ER stress and CHOP expression.

Evidences are out there indicating that c-Jun N-terminal kinase [JNK] and p38 which are part of the MAP kinase family mediate the downstream effect of cytokine-induced apoptosis in pancreatic β -cell [85-88]. Findings from the present study also confirmed that, short term exposure with cytomix increased JNK1/2 and p38 MAP kinase activation. To further explore the role of small G-proteins and their post-translational modification in cytomix-induced JNK1/2 and p38 MAP kinase activation, the effect of three pharmacological inhibitors namely, inhibitor of palmitoylation [2BP], Vav2-Rac1 [EHop-016] and Tiam1-Rac1 [NSC23766] axis were assessed on cytomix-induced JNK1/2 and p38 MAP kinase activation. The result showed that palmitoylation is required for short term cytomix induced JNK1/2 activation. Long term incubation with 2-BP increases JNK1/2 activation at the basal level and this may imply that a palmitoylated protein/s suppress JNK1/2 activation in the absence of stress inducing agents. In addition to the small G-proteins such as Rac1, Ras or bCdc42, there may be a condition that JNK1/2 undergoes palmitoylation following cytomix exposure although this needs to be verified experimentally in the β -cell. This is because palmitoylation of JNK3, one of the isoform of the JNK, regulate axonal branching and development in cortical neuronal culture [154].

The upstream role of Rac1 over JNK1/2 activation in gluco-lipotoxic conditions [89], cytokine-induced apoptosis of intestinal epithelial cell [107] and JNK1 mediated epithelial cell

migration during wound healing [155] have shown in this study using Vav2-Rac1 and Tiam1-Rac1 inhibitors. Lower concentration of EHop-016 inhibits both JNK1/2 indicating that EHop-016 is more potent than NSC23766 in inhibiting Rac1 activation. The absence of inhibitory effect of 2-BP, EHop-016 and NSC23766 on p38 MAP kinase activation implies that palmitoylation and Rac1 activation are not required for cytomix-induced p38 MAP kinase activation.

As stated in those previous paragraphs, small molecular mass and heterotrimeric G-proteins play important roles in cellular signaling events leading to glucose-stimulated insulin secretion [27, 156]. A growing body of evidence is also suggestive of novel roles for these signaling proteins in other β -cell functions including cell cycle progression, survival and apoptosis [25]. It is noteworthy that the majority of these proteins undergo post-translational modifications at their C-terminal cysteine residues [e.g., prenylation, methylation and acylation], which are essential for their trafficking to relevant cell membranes for optimal interaction with their respective effector proteins culminating in optimal regulation of β -cell function [25, 27, 34].

Despite the compelling evidence that FTase and GGTases are acutely regulated by glucose in the β -cell [156, 157], and that they are critical for insulin secretion [25, 31, 35], very little is known with regard to potential alterations in these enzymes under conditions of cellular apoptosis. Along these lines, studies by Kim and associates have demonstrated degradation of the FTase/GGTase α -subunit under conditions of caspase-3 activation [158]. With this in mind, we quantitated caspase-3 activation, FTase/GGTase degradation and cellular dysfunction in isolated β -cells exposed to cytomix and etoposide, a known inducer of apoptosis in pancreatic β -cells. Our findings suggested that both cytomix and etoposide induce caspase-3 activation and FTase/GGTase α degradation, and blocking the caspase-3 activation prevented the degradation of the common α -subunit of FTase/GGTase indicating

that caspase-3 activation causes degradation and inactivation of FTase and GGTase that leads to defective activation of key G-proteins, defective nuclear assembly of lamins and loss of cell viability.

As a logical extension of the *in vitro* findings of cytokine-induced pancreatic β -cell dysfunction to an *in vivo* animal model, preliminary results have shown that both NOD mice and BALB control mice islets express NOX2 subunits namely phospho-p47^{phox}[NCF1], p67^{phox}, Rac1 and gp91^{phox}; however, the results obtained in this study are inconclusive and yet to be confirmed.

Chapter 7: Conclusion and Future directions

NADPH oxidase [NOX2] has cytosolic and membranous components. p47^{phox}, p40^{phox}, p67^{phox} and Rac1 constitute the cytosolic components and the membranous core is comprised of gp91^{phox}/p22^{phox} and Rap1. It is well established that the cytosolic core translocates to the membrane for association with the membranous core to complete the holoenzyme assembly and catalytic activation. Several lines of evidence from multiple laboratories including our own implicated NOX2 in dysregulation of the islet β -cell exposed to a wide variety of pathological conditions, including chronic exposure to high glucose, palmitate and proinflammatory cytokines. The present study yielded some novel findings suggesting that: [i] cytokines induce p47^{phox} phosphorylation, gp91^{phox} expression, NOX2 activation, JNK1/2 and p38 MAP kinase activation under acute regulatory conditions; [ii] 2-BP, a classic inhibitor of protein palmitoylation significantly attenuated cytomix-induced Rac1 activation, NOX2-mediated ROS generation, iNOS-mediated NO release, JNK1/2 activation providing clues that protein palmitoylation step represents a novel therapeutic target for cytokine-induced pancreatic β -cell dysfunction; [iii] Cytokine-induced JNK1/2 activation requires Tiam1-Rac1 and Vav2-Rac1 axis.

Follow up studies to present findings will include the following:

- Evaluate if 2-BP, EHOp-016 and NSC23766 prevent NOX2 activation and ROS generation in pre-diabetic islets exposed to cytokines *in vitro*.
- Demonstrate if treatment of pre-diabetic NOD mice with 2BP, EHOp-016 and NSC23766 prevent the incidence or delay the onset of diabetes.
- Molecular biological studies [siRNA] of the regulatory role of palmitoyl transferase/esterase in cytokine-induced metabolic dysfunction of the pancreatic β -cell.

- Study the role of other isoforms of NADPH oxidase [NOX] in cytokine-induced pancreatic β -cell dysfunction.

APPENDIX A MOHAMMED ET AL 2013

Biochemical Pharmacology 85 (2013) 109–114



Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

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

Upregulation of phagocyte-like NADPH oxidase by cytokines in pancreatic beta-cells: Attenuation of oxidative and nitrosative stress by 2-bromopalmitate

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ARTICLE INFO

Article history:

Received 24 August 2012

Accepted 25 September 2012

Available online 23 October 2012

Keywords:

Pancreatic β -cell
NADPH-oxidase
2-Bromopalmitate
Cytokines
Oxidative stress
Nitrosative stress

ABSTRACT

Phagocyte-like NADPH oxidase (Nox2) has been shown to play regulatory roles in the metabolic dysfunction of the islet β -cell under the duress of glucolipotoxic conditions and exposure to proinflammatory cytokines. However, the precise mechanisms underlying Nox2 activation by these stimuli remain less understood. To this end, we report a time-dependent phosphorylation of p47phox, a cytosolic subunit of Nox2, by cytomix (IL-1 β + TNF α + IFN γ) in insulin-secreting INS-1 832/13 cells. Furthermore, cytomix induced the expression of gp91phox, a membrane component of Nox2. 2-Bromopalmitate (2-BP), a known inhibitor of protein palmitoylation, markedly attenuated cytokine-induced, Nox2-mediated reactive oxygen species (ROS) generation and inducible nitric oxide synthase (iNOS)-mediated nitric oxide (NO) generation. However, 2-BP failed to exert any significant effects on cytomix-induced CHOP expression, a marker for endoplasmic reticulum stress. Together, our findings identify palmitoyltransferase as a target for inhibition of cytomix-induced oxidative (ROS generation) and nitrosative (NO generation) stress in the pancreatic β -cell.

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1. Introduction

Type 1 diabetes is characterized by an absolute insulin deficiency arising from progressive autoimmune destruction of insulin-secreting pancreatic β -cells [1–3]. During the progression of this disease, several proinflammatory cytokines, including IL-1 β , TNF α and IFN γ are secreted into the islets by infiltrated, and activated T cells and macrophages, which, in turn, elicit damaging effects on pancreatic β -cells. However, putative mechanisms and the underlying signaling mechanisms involved in cytokine-induced loss of β -cells remain only partially understood [4,5]. Among numerous mechanisms proposed, IL-1 β has been demonstrated to mediate its effects via the induction of inducible nitric oxide synthase (iNOS), which, in turn, promotes cytotoxic NO

release culminating in β -cell demise. In addition, TNF α and IFN γ elicit regulatory effects on β -cell function via regulation of individual metabolic signaling steps leading to cell death [6–8]. Along these lines, previous studies have suggested that IFN γ sensitizes human islets to the effects of IL-1 β [9].

In addition to NO, several recent studies including our own have suggested that phagocyte-like NADPH oxidase (Nox2) contributes to loss of β -cell function, metabolic dysregulation and the loss of β -cell mass under the duress of pathological conditions such as glucolipotoxicity and exposure to cytokines. Specifically, we have demonstrated that chronic exposure of isolated β -cells to cytomix leads to Nox2 holoenzyme activation, ROS generation, mitochondrial dysfunction and cell death [10–14]. Moreover, using selective inhibitors of Rac1, a small G-protein, which is a member of Nox2 holoenzyme, we have been able to demonstrate that inhibition of Rac1 activation leads to prevention of ROS generation and mitochondrial dysregulation in isolated β -cells [14–16]. Interestingly however, we failed to see any clear effects of Rac1 inhibitors on cytomix-induced NO generation suggesting involvement of a Rac1-independent mechanism for cytomix-induced NO release in these cells.

Jastroch recently pointed out a need for the development of pharmacological probes/reagents that can block both ROS and NO generation in pancreatic β -cells exposed to cytomix as simultaneous release of both ROS and NO could lead to the formation of peroxynitrite, which may be more damaging to the β -cell

Abbreviations: 2-BP, 2-Bromopalmitate; BSA, Bovine serum albumin; CHAPS, 3-[3-cholamidopropyl] dimethylammonio]-1-propanesulfonate; CHOP, C/EBP homologous protein; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraacetic acid; FBS, Fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; iNOS, inducible nitric oxide synthase; NO, nitric oxide; Nox2, phagocyte-like NADPH oxidase; Na₂VO₄, Sodium orthovanadate; PIC, Protease inhibitor cocktail; PMSF, Phenylmethanesulfonylfluoride; RIPA, Radioimmuno-precipitation assay; and ROS, Reactive oxygen species.

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0006-2952/\$ – see front matter. Published by Elsevier Inc.
<http://dx.doi.org/10.1016/j.bcp.2012.09.024>

compared to ROS or NO alone [17]. Therefore, as a logical extension to our recently published evidence [14], we undertook an investigation to assess the role of protein palmitoylation in the cascade of events leading to cytomix-induced ROS and NO generation in INS-1 832/13 cells. We describe evidence to suggest that 2-bromopalmitate, a selective inhibitor of protein palmitoylation, markedly attenuates cytomix-mediated ROS and NO generation in these cells. Our findings identify palmitoyltransferase as a target for inhibition of cytomix-induced oxidative (ROS generation) and nitrosative (NO generation) stress in the pancreatic β -cell.

2. Materials and methods

2.1. Materials

IL-1 β , TNF α and IFN γ were purchased from R & D Systems (Minneapolis, MN). Antisera directed against p-p47phox (NCF1) and gp91phox were from Abcam (Abcam Inc, Cambridge MA). Anti-p47phox was from Santa Cruz Biotechnology (Santa Cruz, CA). 2',7-dichlorofluorescein diacetate (DCHF-DA), 2-bromopalmitate, Griess reagent were obtained from Sigma-Aldrich (St. Louis, MO). Anti-rabbit IgG-horseradish peroxidase conjugate and enhanced chemiluminescence (ECL) kits were from Amersham Biosciences (Piscataway, NJ).

2.2. Insulin-secreting INS-1 832/13 cells 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 μ M 2-mercaptoethanol and 10 mM HEPES (pH 7.4). The cultured cells were subcloned twice weekly following trypsinization and passages 53–61 were used for the study. INS-1 832/13 cells were incubated with cytomix (IL-1 β , TNF α and IFN γ ; 10 ng/ml each) or individual cytokines (25 ng/ml) for 0–24 h as indicated in the text. In select studies, INS-1 832/13 cells were incubated with cytomix (IL-1 β , TNF α and IFN γ ; 10 ng/ml each) in the presence and absence of 2-bromopalmitate (100 μ M) for 24 h. At the end of the incubation period the cells were harvested and lysed in RIPA buffer containing 1 μ g/ml protease inhibitor cocktail, 1 mM NaF, 1 mM PMSF, and 1 mM Na₃VO₄.

2.3. Isolation of membrane fraction

INS-1 832/13 cells were treated with cytomix for the designated time points as indicated in the text. At the end of the incubation period, cells were washed with ice-cold PBS, harvested by scraping and pelleted by centrifugation at 2000 rpm for 5 min at 4 °C. After re-suspending in the homogenizing buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 1 mM Na₃VO₄) and sonication, unbroken cells and nuclei were separated by centrifugation at 1300 rpm for 10 min. The cleared supernatant was further centrifuged at 40,000 rpm for 30 min (Optima™ MAX Ultracentrifuge). The cytosol fraction was separated and the membrane fraction was dissolved in 2% CHAPS.

2.4. ROS generation assay

INS-1 832/13 cells were plated in a 6 well plate and treated with cytomix (IL-1 β , TNF α and IFN γ ; 10 ng/ml each) or individual cytokines (25 ng/ml) for 24 h as indicated in the text. Subsequently, the media was removed and the cells were incubated further with 2',7-dichlorofluorescein diacetate (DCHF-DA) at 37 °C for 30 min. The cells were washed twice with ice-cold PBS and harvested, followed by loading equal amount of protein (50 μ g)

and reading the fluorescence using luminescence spectrophotometer (Ex:485 nm and Em:535 nm) (PerkinElmer, Waltham, MA).

2.5. NO release assay

INS-1 832/13 cells were plated in a 6 well plate and treated with cytomix (IL-1 β , TNF α and IFN γ ; 10 ng/ml each) for 24 h as indicated in the text. At the end of the incubation period, the media was collected and centrifuged at 1000 g for 5 min. Equal amount of media and Griess reagent were mixed and the absorbance (540 nm) was measured using a microplate reader (Molecular Devices, Sunnyvale, CA).

2.6. Western blotting

Proteins (~30–40 μ g/lane) were separated by SDS-PAGE on 10% (w/v) polyacrylamide mini gels and electro transferred to nitrocellulose membrane. The membranes were blocked with 5% BSA in 10 mM Tris-HCl, pH 7.6, 1.5 M NaCl and 0.1% Tween 20 followed by incubation with corresponding primary antibody and secondary polyclonal rabbit antibody conjugated to horseradish peroxidase. 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 and total p47phox to ensure equal loading and transfer of the proteins.

2.7. Statistical analysis

Results are expressed as mean \pm SEM. Statistical significant difference between groups was evaluated by ANOVA followed by SNK Post Hoc test where appropriate. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. IL-1 β , but not TNF α or IFN γ promotes ROS generation in insulin-secreting INS-1 832/13 cells

We recently reported a significant increase in Nox2-mediated generation of ROS in INS-1 832/13 cells. In the current set of experiments we examined effects of individual cytokines on ROS generation in order to determine which of the three cytokines exert stimulatory effects on ROS generation in INS-1 832/13 cells. Data in Fig. 1 indicated that of all the three cytokines tested, namely IL-1 β , TNF α or IFN γ (25 ng/ml each; 24 hr) only IL-1 β significantly augmented (~2.5 fold) ROS generation; these values were comparable to those demonstrated in the presence of all the three cytokines combined (referred to as cytomix throughout). A modest, but insignificant, effect of TNF α (bar 1 vs. 3) or IFN γ (bar 1 vs. 4), was demonstrated under these conditions. Therefore, we determined the effects of cytokines on Nox2 activation in all the studies described from here on since the cytomix represents an appropriate model to determine effects of cytokines on islet β -cell dysfunction.

3.2. Cytomix stimulates the phosphorylation of p47phox in a time-dependent manner

Recent studies from our laboratory and others have shown that cytokines induce the expression of p47phox, and siRNA-mediated depletion of endogenous pools of p47phox markedly attenuated cytokine-induced Nox2-mediated ROS generation in insulin-secreting cells [14]. Furthermore, evidence in other cell types indicates that p47phox is phosphorylated subsequent to agonist

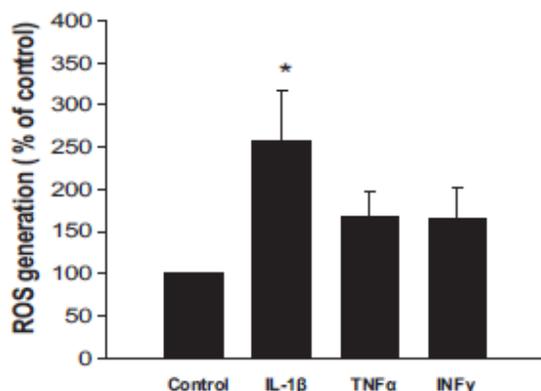


Fig. 1. IL-1 β , but not TNF α and INF γ , activates ROS generation in INS-1 832/13 cells. INS-1 832/13 cells were treated with individual cytokines (IL1 β , TNF α and INF γ ; 25 ng/ml each) for 24 h and the amount of ROS generation was measured with 2',7-dichlorofluorescein diacetate assay. Data represent mean \pm SEM from three independent experiments and expressed as % of control. * $P < 0.05$ versus control.

activation [18,19] and that the phosphorylation step is necessary for its translocation to the membrane fraction for association with other members of the Nox2 core proteins to complete holoenzyme assembly leading to the activation of Nox2. This has not been examined before in islet β -cells exposed to cytokines. Data in Fig. 2 (Panel A) suggested a time-dependent activation of p47phox (0–60 min) following exposure to cytokines. We observed nearly a 2-fold increase in the phosphorylation of p47phox by cytokines within an hour of incubation (Panel B). These data suggest that cytokines induce phosphorylation of p47phox.

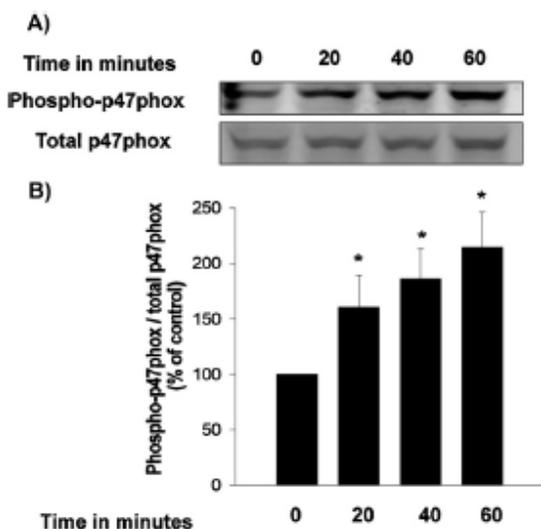


Fig. 2. Cytomix induces time-dependent phosphorylation of p47phox in INS-1 832/13 cells. INS-1 832/13 cells were treated with cytomix (IL1 β , TNF α , and INF γ , 10 ng/ml each) for different time intervals as indicated in the figure. Degree of phosphorylation of p47phox was determined by Western blot (Panel A) followed by densitometry (Panel B). Data are normalized to total p47phox content in corresponding lanes. Data represent mean \pm SEM from three independent experiments and expressed as percent control of the ratios between phosphorylated and total p47phox. * $P < 0.05$ versus control.

3.3. Cytomix stimulates the expression of gp91phox in a time-dependent manner

We next investigated alterations, if any, in the expression of gp91phox in cells following exposure to cytomix. To address this, INS-1 832/13 cells were incubated in the presence of cytomix for different time intervals (0–60 min) as above (Fig. 2). Relative abundance of gp91phox was determined in the total membrane fraction isolated by a single-step centrifugation method [see Methods] by Western blotting for gp91phox (Fig. 3; Panel A) and densitometry (Fig. 3; Panel B). These data indicated a time-dependent increase in the expression of gp91phox in INS-1 832/13 cells following exposure to cytomix. A significant increase in the expression was seen as early as 20 min (~2 fold), which appear to plateau with time. Together, these data are indicative of positive modulatory effects of cytomix on Nox2 in INS-1 832/12 cells. Such effects are comprised of increase in the activation of Rac1 [14,16], phosphorylation of p47phox and expression of gp91phox (this study); such conditions are essential for activation of Nox2 enzyme.

3.4. A protein palmitoylation step is necessary for cytomix-induced ROS generation in INS-1 832/13 cells

Our recent studies have demonstrated that activation of Rac1 is necessary for cytokine-induced Nox2 activation and ROS generation. In this context we reported a marked increase in Rac1-GTP (active conformation) formation in INS-1 832/13 cells following exposure to cytomix [14]. We also showed that prior incubation of INS-1 832/13 cells with GGTI-2147, an inhibitor of geranylgeranylation of Rac1 or NSC23766, a known inhibitor of Rac1 mediated by Tiam1, a guanine nucleotide exchange factor for Rac1 markedly attenuated cytomix-induced ROS generation [14]. Therefore, we determined the effects of 2-bromopalmitate, a known inhibitor of protein palmitoylation, on cytomix-induced ROS generation. These studies are based on the recent reports of palmitoylation of Rac1 at a cysteine residue upstream to the C-terminal cysteine. To address this cytomix-induced ROS

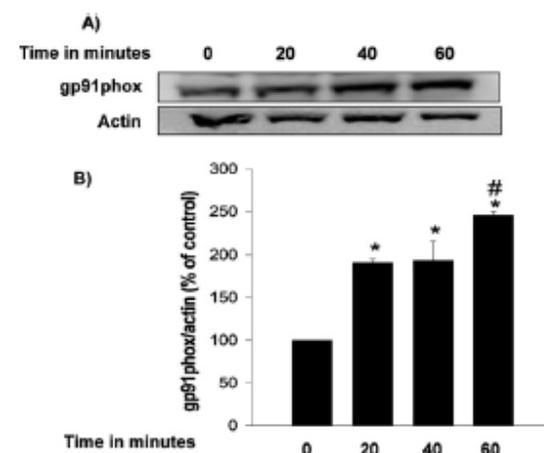


Fig. 3. Cytomix increases time-dependent expression of gp91phox in INS-1 832/13 cells. INS-1 832/13 cells were treated with cytomix (IL1 β , TNF α , and INF γ , 10 ng/ml each) for different time points as indicated in the figure. Degree of gp91phox expression was measured by Western blot (Panel A) followed by densitometry (Panel B). Data are normalized to β -actin content in individual lanes. Data represent mean \pm SEM from three independent experiments and expressed as % of control. * $P < 0.05$ versus control and # $P < 0.05$ versus 20 min and 40 min.

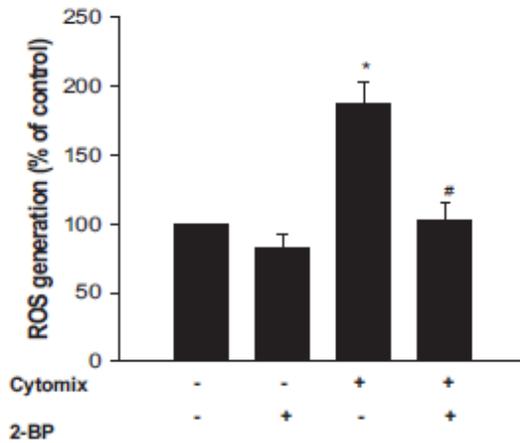


Fig. 4. 2-BP attenuates cytomix-induced ROS generation in INS-1 832/13 cells. INS-1 832/13 cells were treated with cytomix (IL1 β , TNF α and INF γ ; 10 ng/ml each) for 24 h in the presence and absence of 2-BP (100 μ M) and the amount of ROS generation was measured with 2,7-dichlorofluorescein diacetate assay. Data represent mean \pm SEM from three independent experiments and expressed as % of control. * $P < 0.05$ versus control and # $P < 0.05$ versus cytomix.

generation was quantitated in INS-1 832/13 cells incubated in the presence of diluent alone or 2-BP (100 μ M; 24 h). Data depicted in Fig. 4 indicated no significant effects of 2-BP on basal ROS generation (bar 1 vs. bar 2). However, cytomix-induced ROS generation was completely abolished by 2-BP (bar 3 vs. bar 4). These findings suggest that a palmitoylation-dependent signaling step is necessary for cytomix-induced ROS generation.

3.5. A protein palmitoylation step is necessary for cytomix-induced nitric oxide generation in INS-1 832/13 cells

It is widely felt that both oxidative (ROS) and nitrosative (NO) stress could contribute to impairment of the overall health of the islet β -cell [7,20–23]. Such concerns were raised indeed by investigators in the area warranting additional studies to investigate potential targets which could regulate both ROS and NO generation signaling steps under the duress of cytokines. To this end, we have previously reported that a palmitoylation-mediated signaling step may be necessary for cytokine-induced iNOS gene expression and NO release [24]. Herein, we revisited those earlier studies to demonstrate that inhibition of protein palmitoylation by 2-BP inhibits cytomix-induced NO release in INS-1 832/13 cells. Data in Fig. 5 suggested no direct effects of 2-BP on basal NO release (bar 1 vs. bar 2). However, as in the case with ROS generation (Fig. 4), 2-BP treatment also attenuated cytomix-induced NO release (bar 3 vs. bar 4). Together, our findings highlight the importance of palmitoylation as a unifying mechanism involved in the induction of Nox2-mediated oxidative stress and iNOS-mediated nitrosative stress in pancreatic β -cells (see Section 4).

3.6. A protein palmitoylation step is not necessary for cytomix-induced CHOP expression, a marker for endoplasmic reticulum stress

It is well established that ER stress underlies cytokine-mediated metabolic dysregulation of the islet β -cell [25–27]. Furthermore, recent studies by Baldwin and associates have suggested a critical requirement for protein palmitoylation in palmitate-induced CHOP expression (a marker for ER stress) in that 2-BP significantly attenuated palmitate-induced CHOP

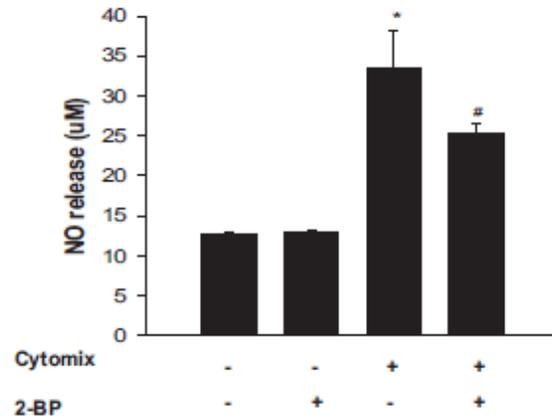


Fig. 5. Cytomix-induced NO release is inhibited by 2-BP. INS-1 832/13 cells were treated with cytomix (IL1 β , TNF α and INF γ ; 10 ng/ml each) for 24 h time point in the presence and absence of 2-bromopalmitate (100 μ M) and the amount of nitric oxide release was measured using Griess assay. 2-Bromopalmitate significantly decreased cytomix-induced nitric oxide release. Data represent mean \pm SEM from three independent experiments. * $P < 0.05$ versus control and # $P < 0.05$ versus cytomix.

expression in insulin-secreting RINm5F cells [28]. Therefore, we investigated if cytomix-induced CHOP expression in INS-1 832/13 cells is sensitive to 2-BP. To address this, cytomix-induced CHOP expression was measured in cells incubated in the presence of diluent alone or 2-BP. Data depicted in Fig. 6 represents a Western blot for the expression of CHOP. It suggested no clear effects of 2-BP on CHOP expression under basal conditions (lane 1 vs. lane 2). Exposure of these cells to cytomix significantly increased CHOP expression (lane 1 vs. lane 3). However, provision of 2-BP to cytomix-treated cells did not exert any significant effects on CHOP expression (lane 3 vs. lane 4) suggesting that protein palmitoylation is not necessary for cytomix-induced CHOP expression.

4. Discussion

Emerging evidence from multiple laboratories including our own implicates regulatory roles for Nox2 in the metabolic dysfunction under the duress of glucolipotoxicity and cytokines [10,14–16,21]. The overall goal of this study was to further understand Nox2 regulation in pancreatic β -cells exposed to cytomix. Our studies yielded some novel clues suggesting that: (i) cytokines induce p47phox phosphorylation and gp91phox expression under acute regulatory conditions; (ii) 2-BP, a classic inhibitor

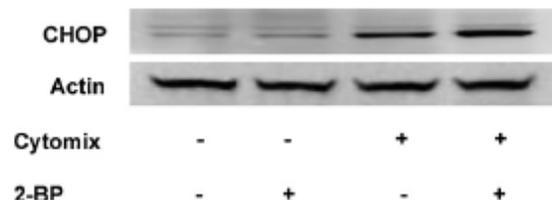


Fig. 6. Cytomix-induced CHOP (C/EBP homologous protein) expression is not inhibited by 2-bromopalmitate. INS-1 832/13 cells were treated with cytomix (IL1 β , TNF α and INF γ ; 10 ng/ml each) for 24 h time point in the presence and absence of 2-bromopalmitate (100 μ M). The level of CHOP expression, a marker for endoplasmic reticulum (ER) stress, was measured by Western blot. Equal protein loading was confirmed by β -actin content in individual lanes. A representative blot from three independent experiments is shown here.

of protein palmitoylation significantly attenuated cytomix-induced, Nox2-mediated ROS generation and iNOS-mediated NO generation. Interestingly, 2-BP failed to affect cytomix-induced ER stress (CHOP expression). We believe that our current data provide clues that protein palmitoylation step may represent a novel therapeutic target for cytokine-induced ROS and NO generation.

Nox2 holoenzyme is comprised of cytosolic and membranous cores. The cytosolic core is comprised of p47phox, p40phox, p67phox and Rac1 whereas the membranous core is comprised of gp91phox/p22phox and Rap1 [12,13,21]. It is well established that the cytosolic core translocates to the membrane for association with the membranous core to complete the holoenzyme assembly and catalytic activation. Several lines of evidence from multiple laboratories including our own implicated Nox2 in dysregulation of the islet β -cell exposed to a wide variety of pathological conditions, including chronic exposure to high glucose, palmitate and proinflammatory cytokines [10,11,14,15]. More recent studies from our laboratory have also suggested hyperactivation of Nox2 in islets derived from type 2 diabetic human donors and animal models such as the Zucker Diabetic Fatty rat [29]. Together, the above studies implicate novel regulatory roles for Nox2 in the pathophysiology of diabetes.

In the context of the currently described studies, we have recently published evidence to suggest that exposure of isolated β -cells to proinflammatory cytokines results in increased expression of p47phox, a member of the cytosolic core of Nox2, within 12–24 h. We have also shown that Rac1, another cytosolic component of Nox2, gets activated transiently (~ 15 min) following exposure to cytokines [14]. More importantly, our data also suggested that inhibition of p47phox function via siRNA-p47phox or Rac1 functions using inhibitors of Rac1 prenylation (i.e., GGTI-2147) or its GTP-exchange functions by Tiam1 (e.g., NSC23766) significantly attenuated cytokine-induced ROS generation and loss of mitochondrial membrane potential. Interestingly, neither GGTI-2147 nor NSC23766 prevented cytokine-induced NO generation [14] suggesting that Nox2 activation is not upstream to iNOS induction and NO release and the two signaling steps are regulated by two distinct mechanisms.

Original studies from our laboratory have suggested that a protein palmitoylation step controls cytokine-induced iNOS gene expression and NO release in insulin-secreting β -cells [8,24]. Therein, we identified H-Ras, a small G-protein, as one of the palmitoylated proteins, which is involved in cytokine-induced NO release. These conclusions were confirmed via the use of two selective inhibitors of protein palmitoylation, namely cerulenin and 2-BP [24]. Furthermore, we have also been able to demonstrate that selective inhibitors of protein farnesylation (e.g., allylfarnesols, manumycin, damnacanthal, FTI-277) markedly attenuated cytokine-induced NO release thereby suggesting that farnesylation as well as palmitoylation of H-Ras may be necessary for cytokine-mediated effects on NO release [21]. Identity of H-Ras as one of the regulatory proteins involved in cytokine-induced NO release in β -cells was also confirmed through the use of bacterial toxins [8]. Interestingly, our current findings demonstrated that 2-BP also inhibited cytokine-induced Nox2 activation and ROS generation suggesting that protein palmitoylation might be crucial to cytokine-mediated effects. While these data do not directly address the question of potential identity of the protein, it may be Rac1 since recent studies [30] suggest that Rac1 undergoes palmitoylation at cysteine residues upstream to the prenylated cysteine. This remains to be verified in the context of current studies. Along these lines, recent studies from Corbett's laboratory have demonstrated a requirement for a palmitoylation step in palmitate-induced metabolic dysfunction of the islet β -cell [28]. Interestingly, they reported a significant inhibition in palmitate-induced CHOP expression and associated metabolic dysfunction of

the islet β -cell in the presence of 2-BP. While our results further validate such a model in the context of cytokines, we failed to observe any significant effects of inhibition of protein palmitoylation with 2-BP on cytokine-induced CHOP expression suggesting distinct mechanisms underlying palmitate and cytokine-induced CHOP expression and ER stress. Additional studies are needed to further understand these signaling steps under these experimental conditions.

Our current findings also implicate alterations in the cytosolic and membranous core proteins under acute conditions in the presence of cytokines. For example, we have demonstrated that exposure of INS-1 832/13 cells to cytomix resulted in a time-dependent phosphorylation of p47phox and expression of gp91phox within 20 min of exposure. Interestingly, we have reported a significant activation of Rac1 by cytokines under short-term exposure conditions [14]. These kinetics are consistent with translocation of cytosolic components to the membrane fraction for holoenzyme assembly. Together, our studies further validate regulation of Nox2 function by cytokines under short- and long-term exposure conditions leading to metabolic dysregulation of the islet β -cell.

Based on the data accrued thus far we propose a working model (Fig. 7) for cytokine-induced metabolic dysregulation of the islet β -cell with an emphasis on Nox2 and iNOS signaling pathways. We propose that exposure of isolated β -cells to proinflammatory cytokines leads to generation of oxidative (increase in ROS) and nitrosative (increase in NO) stress mediated by Nox2 and iNOS, respectively. Previously published evidence from our laboratory suggested marked attenuation of cytokine-induced Nox2 activation and ROS generation by siRNA-p47phox, apocynin, NSC23766 and GGTI-2147. We have also demonstrated that the above manipulations prevented mitochondrial dysfunction induced by cytokines [14]. Published evidence from our laboratory also suggested a marked diminution in cytokine-induced iNOS gene expression and NO release by Clostridial toxins [8,31], farnesyl transferase inhibitors [21], and inhibitors of palmitoylation such as cerulenin and 2-BP [24].

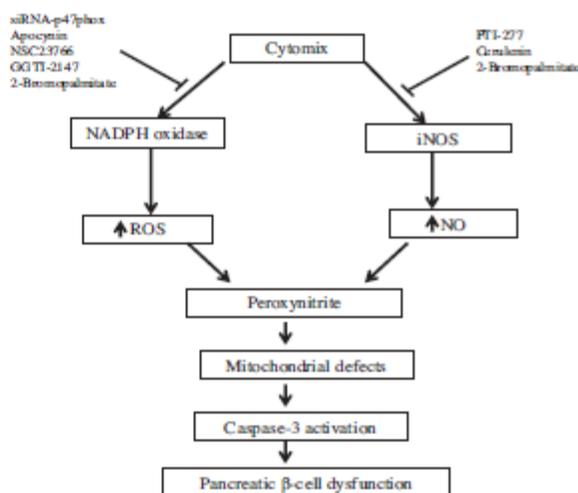


Fig. 7. Proposed model to halt cytokine-induced nitrosative and oxidative stress by 2-BP. A model depicting roles of Nox2-mediated ROS generation and iNOS-mediated NO generation in the cascade of events leading to the metabolic dysfunction of the islet β -cell is depicted herein. Data from our current investigation identify protein palmitoyltransferase as a novel therapeutic target for the prevention of cytokine-mediated effects on the islet β -cell (see Section 4).

Our current observations further implicate a palmitoylation-dependent signaling step in cytokine-induced Nox2-mediated ROS generation. Together, these data suggest that protein palmitoyltransferase might serve as a novel therapeutic target for the prevention of cytokine-induced oxidative and nitrosative stress leading to mitochondrial dysfunction and accelerated cell death. In addition, our current studies are also suggestive of acute regulation of Nox2 subunits by cytokines, which has not been demonstrated before in the islet β -cell. While our earlier studies identified H-Ras as the palmitoylated protein which regulates NO release induced by cytokines, the identity of the palmitoylated protein that regulates cytokine-induced ROS generation remains to be determined. It would be interesting to see such a protein that regulates Nox2 activation and ROS generation under the duress of various pathological stimuli including high glucose, saturated fatty acids and ceramide. These are being investigated in our laboratory currently.

Acknowledgements

This research was supported in part by a Merit Review Award (to AK; 1BX000469) from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development (Biomedical Laboratory Research and Development), an Innovative Grant from Juvenile Diabetes Research Foundation, and by the NIH/NIDDK (R01 DK74921). AK is also the recipient of a Senior Research Career Scientist Award from the Department of VA. We thank Prof. Chris Newgard for kindly providing INS-1 832/13 cells. AMM is the recipient of George C. Fuller Endowed Pharmacy Scholarship, Department of Pharmaceutical sciences, Wayne State University.

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Activation of apocynin-sensitive NADPH oxidase (Nox2) activity in INS-1 832/13 cells under glucotoxic conditions

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Keywords: islet β -cell, NADPH oxidase, reactive oxygen species, glucotoxicity

Submitted: 05/02/13

Accepted: 05/16/13

<http://dx.doi.org/10.4161/isl.25058>

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Addendum to: Syed I, Kyathanahalli CN, Jayaram B, Govind S, Rhodes CJ, Kowluru RA, et al. Increased phagocyte-like NADPH oxidase and ROS generation in type 2 diabetic ZDF rat and human islets: role of Rac1-JNK 1/2 signaling pathway in mitochondrial dysregulation in the diabetic islet. *Diabetes* 2011; 60:2843-52; PMID:21911753; <http://dx.doi.org/10.2337/db11-0809>

Several lines of recent evidence provided compelling evidence to suggest increased generation of reactive oxygen species (ROS) as causal for mitochondrial dysregulation and apoptosis in islet β -cells exposed to noxious stimuli including high glucose, lipids and pro-inflammatory cytokines. Studies along these lines are also suggestive of a significant contributory role for NADPH oxidase in the generation of ROS under the above conditions. We have recently reported a marked increase in the expression and activation of cytosolic components of NADPH oxidase (p47^{phox}, Rac1) in cell culture models of glucotoxicity and in islets from T2DM animals (Zucker Diabetic Fatty rat) and humans. In this communication, we provide further evidence indicating significant activation of NADPH activity (~2-fold) in INS-1 832/13 cells exposed to chronic hyperglycemic conditions (20 mM; 48 h). We also report marked attenuation of this activity, by apocynin, a selective inhibitor of phagocyte-like NADPH oxidase (Nox2) activity. Together, our findings implicate Nox2 as a source for ROS generation in β -cells exposed to glucotoxic conditions.

Introduction

Emerging evidence from in vitro and in vivo studies provides strong support to the hypothesis that chronic exposure of β -cells to elevated glucose (i.e., glucotoxicity), lipids (i.e., lipotoxicity) or glucose plus lipids (e.g., glucolipotoxicity) results in a significant metabolic dysregulation

eventually leading to cell demise [for a review see ref. 1]. Published evidence also suggests a marked increase in the generation of ROS, which manifests in increased oxidative stress in cells under the conditions of glucotoxicity [for a review see refs. 2 and 3]. Several mechanisms have been put forth in this context, including depletion of intracellular redox state via the oxidation of reducing equivalents (e.g., reduced glutathione) and activation of superoxide-generating enzymatic machinery.^{1,3}

One of the enzymatic steps involved in the increased generation of ROS and associated induction of intracellular oxidative stress in the pancreatic β -cell includes activation of the phagocytic NADPH oxidase (Nox2) system.^{2,3} Nox2 is a highly regulated membrane-associated protein complex that catalyzes the one electron reduction of oxygen to superoxide anion involving oxidation of cytosolic NADPH. The phagocytic NOX is a multicomponent system comprised of membrane as well as cytosolic components. The membrane-associated catalytic core is a complex comprising gp91^{phox}, p22^{phox} and the small G protein Rap1. The cytosolic regulatory components include p47^{phox}, p67^{phox} and the small G protein Rac. After stimulation, the cytosolic components of NOX translocate to the membrane fraction for association with the catalytic core for holoenzyme assembly. Available evidence suggests that a protein kinase C ζ -sensitive phosphorylation of p47^{phox} leads to its translocation to the membrane fraction. It has also been shown that functional activation of Rac (i.e., GTP-Rac) is vital for

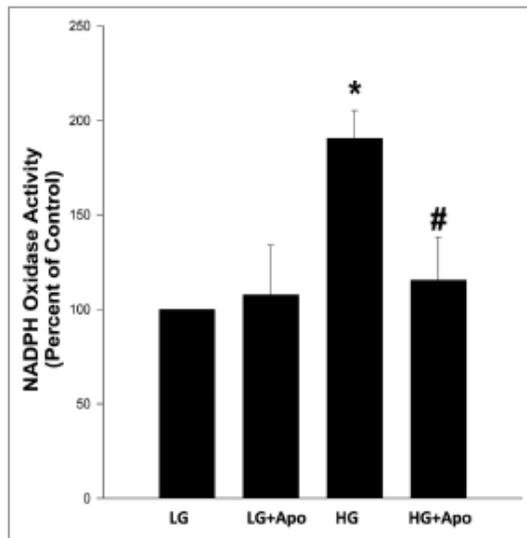


Figure 1. Exposure of INS-1 832/13 cells leads to activation of apocynin-sensitive NADPH oxidase activity. INS-1-832-13 cells were incubated with low glucose (LG) and high glucose (HG) in presence and absence of apocynin (Apo; 100 μ M) for 48 h and NADPH oxidase enzyme activity was measured for 15 min. The data are expressed as percent of control. Nox2 enzyme activity was expressed as chemiluminescence units per mg lysate protein per minute. * $p < 0.05$ vs. LG; # $p < 0.05$ vs. HG.

the holoenzyme assembly and activation of NOX.³

Several recent studies have demonstrated localization and functional activation of the NOX in clonal β -cells, normal rat islets and human islets under the duress of various stimuli known to cause metabolic dysregulation.^{2,4} Some of these stimuli include, but are not limited to, high glucose, saturated fatty acids and proinflammatory cytokines.^{5,6} Recently, we have reported increased ROS generation in islets from the diabetic Zucker Diabetic Fatty (ZDF) rat and type 2DM human islets.⁷ In that study, we also provided evidence to implicate Rac1-Nox2-ROS-JNK1/2 signaling cascade in glucose-induced metabolic dysfunction of the islet. However, we have not directly quantitated Nox2 activity in isolated β -cells exposed to hyperglycemic conditions to conclusively demonstrate that Nox2-derived ROS generation is critical for high glucose-mediated effects on β -cell dysfunction. Therefore, a brief study was undertaken to quantitate NADPH oxidase activity in INS-1 832/13

cells exposed to hyperglycemic conditions. To further demonstrate that such an activity represents Nox2, we included apocynin, a selective inhibitor of Nox2, in the assay. We provide evidence below to demonstrate a significant increase in apocynin-sensitive NADPH oxidase activity in INS-1 832/13 cells exposed to glucotoxic conditions.

Results and Discussion

As stated above, Nox2 is comprised of cytosolic and membrane core of proteins. Following stimulation, cytosolic components of the enzyme translocate to the membrane for association with the membranous components to form the holoenzyme culminating in the functional activation of the enzyme. Since earlier studies have demonstrated significant increase in ROS generation in β -cells exposed to high glucose,⁷ we aimed here to quantitate Nox2 activity in INS-1 832/13 cells, and then to determine potential regulatory effects of glucotoxic conditions. Data in Figure 1 suggested no clear

effects of apocynin on NADPH oxidase activity in INS-1 832/13 cells under basal (low-glucose) conditions (bar 1 vs. bar 2). However, exposure of these cells to high glucose resulted in a significant increase (~2-fold) increase in the enzyme activity (bar 1 vs. bar 3). Furthermore, coprovision of apocynin to these cells markedly attenuated the ability of glucotoxic conditions to stimulate NADPH oxidase activity. These data suggest that glucotoxic conditions promote activation of apocynin-sensitive NADPH oxidase activity in isolated β -cells.

Our findings provide further support to recently published evidence to suggest increased generation of ROS in cell culture models of glucotoxicity and in islets from T2DM animals and humans. Biochemical and cell biological studies have also demonstrated increased expression of p47phox, a member of the cytosolic core of Nox2 in these model systems.^{3,7} Furthermore, activation of Rac1 (GTP-bound conformation) has also been demonstrated in cells exposed to hyperglycemic conditions or in islets from animal models of T2DM.⁷ Lack of no effects of apocynin on basal (LG) NADPH oxidase activity suggests very little activation of this enzyme under basal conditions. Along these lines, recent studies by Koulajian and associates have demonstrated significant activation of NADPH oxidase activity in rat islets following prolonged exposure to non-saturated fatty acids, such as oleate. Furthermore, they have demonstrated a significant reduction in the activation of NADPH oxidase by apocynin,⁸ thus affirming critical regulatory roles for this enzyme in metabolic dysfunction induced by elevated glucose and lipids.

Materials and Methods

Materials. N,N'-Dimethyl-9,9'-biacridinium dinitrate (Lucigenin), 4'-Hydroxy-3'-methoxyacetophenone (Apocynin) and 50 mM phosphate buffer solution were from Sigma Aldrich Co. NADPH was from Calbiochem. IL-1 β (201-LB-005) was purchased from R & D Systems.

Cell culture and Nox2 activity measurement. INS-1 832/13 cells were plated in a 6-well plate and starved overnight

with low serum and low glucose media in the presence and absence of apocynin (100 μ M). Following this, cells were treated with either low (2.5 mM) or high glucose (20 mM) in the presence and absence of apocynin (100 μ M) for 48 h. The NADPH oxidase enzyme activity was measured using the method described by Hwang et al.⁹ Treated cells were homogenized using 50 mM phosphate buffer solution (pH 7.0) containing 1 mM EDTA and 1 mM PMSF. The homogenates were centrifuged at 3000 g for 10 min. The cleared lysates (250 μ g/ml of protein) were then incubated with N,N'-Dimethyl-9,9'-biacridinium dinitrate (Lucigenin) (5 μ M) for 2 min followed by the addition of NADPH (100 μ M). The chemiluminescence signal resulting from reaction of superoxide anion and lucigenin was recorded every 1 min for 15 min using BioTek Synergy HT, Gen5. Nox2 enzyme activity was expressed as chemiluminescence units per mg lysate protein per minute.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

This research was supported in part by a Merit Review award (to A.K.; 1BX000469) from the Department of Veterans Affairs, Office of Research and Development (Biomedical Laboratory Research and Development) and an Innovative Grant from the Juvenile Diabetes Research Foundation (to A.K.; 5-2012-257). A.K. is also the recipient of a Senior Research Career Scientist Award from the Department of VA. A.M. is the recipient of George Fuller Endowed Pre-Doctoral Fellowship from the Eugene Applebaum College of Pharmacy and Health Sciences.

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Apoptosis (2013) 18:1–8
 DOI 10.1007/s10495-012-0763-9

ORIGINAL PAPER

Nifedipine prevents etoposide-induced caspase-3 activation, prenyl transferase degradation and loss in cell viability in pancreatic β -cells

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Published online: 9 October 2012
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Abstract Emerging evidence implicates novel roles for post-translational prenylation (i.e., farnesylation and geranylgeranylation) of various signaling proteins in a variety of cellular functions including hormone secretion, survival and apoptosis. In the context of cellular apoptosis, it has been shown previously that caspase-3 activation, a hallmark of mitochondrial dysregulation, promotes hydrolysis of several key cellular proteins. We report herein that exposure of insulin-secreting INS 832/13 cells or normal rat islets to etoposide leads to significant activation of caspase-3 and subsequent degradation of the common α -subunit of farnesyl/geranylgeranyl transferases (FTase/GGTase). Furthermore, the above stated signaling steps were prevented by Z-DEVD-FMK, a known inhibitor of caspase-3. In addition, treatment of cell lysates with recombinant caspase-3 also caused FTase/GGTase α -subunit degradation. Moreover, nifedipine, a calcium channel blocker, markedly attenuated etoposide-induced caspase-3 activation, FTase/GGTase α -subunit degradation in INS 832/13 cells and normal rat islets. Further, nifedipine significantly restored etoposide-induced loss in metabolic cell viability in INS 832/13 cells. Based on these findings, we conclude that etoposide induces loss in cell viability by inducing mitochondrial dysfunction, caspase-3 activation and degradation of FTase/GGTase α -subunit. Potential

significance of these findings in the context of protein prenylation and β -cell survival are discussed.

Keywords Etoposide · Caspase-3 · Farnesyl transferase · Geranylgeranyl transferase · Nifedipine

Abbreviations

CSP3	Caspase-3
ET	Etoposide
FTase α	Farnesyltransferase α subunit
GGTase α	Geranylgeranyltransferase α subunit
Δ FTase/GGTase	Hydrolytic product of FTase/GGTase α -subunit
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
Nif	Nifedipine

Introduction

A variety of signaling proteins including small G-proteins (e.g., Ras, Cdc42 and Rac1), the γ -subunits of trimeric G-proteins and nuclear lamins (e.g., lamin-B) undergo a series of post-translational modifications at their C-terminal cysteine residues including prenylation (i.e., farnesylation and geranylgeranylation) and carboxylmethylation [1]. Earlier studies have also demonstrated that such modification steps are necessary for the trafficking of prenylated proteins to appropriate cellular compartments for optimal interaction with and activation of their effector proteins leading to cellular activation [2–5]. Prenylation represents the first modification step in which either a 15-carbon (farnesyl) or a 20-carbon (geranylgeranyl) derivatives of mevalonic acid are incorporated into the substrate proteins. The farnesyl transferase (FTase) catalyzes the transfer of farnesyl

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groups where as the geranylgeranyl transferase (GGTase) facilitates the incorporation of the geranylgeranyl groups in the presence of farnesyl pyrophosphate and geranylgeranyl pyrophosphate, respectively [6]. Examples of farnesylated proteins include H-Ras, lamin-B and geranylgeranylated proteins include small G-proteins such as Cdc42, Rac1, Rho and Rap1 [1]. FTase and GGTases are heterodimeric in nature comprising of a common α -subunit, but distinct β -subunits, and the later confer the substrate specificity for FTase and GGTases [7]. Using various pharmacological inhibitors of FTase and GGTases (e.g., FTI-277 and GGTI-2147), or dominant negative mutant of the FTase/GGTase- α subunit or siRNAs for FTase or GGTase β subunits, several recent studies have demonstrated novel roles for protein prenylation in nutrient-induced insulin secretion from clonal β -cells and normal rodent islets [8–12].

In the context of prenyltransferase-mediated regulation of cellular function, Kim et al. [13] have reported that the common α -subunit of FTase/GGTase undergoes caspase-3 mediated cleavage into a smaller peptide (~ 35 kDa) under conditions of cellular apoptosis. For example, they demonstrated caspase-3 activation and FTase/GGTase α -subunit degradation in a mouse lymphoma cell line (W4 cell expressing the Fas receptor) following exposure to anti-Fas antibodies. They also observed caspase-3 activation and FTase/GGTase α -subunit degradation in Rat-2/H-ras cells treated with an FTase inhibitor (LB42708) or in Rat-1 cells treated with etoposide. Based on these observations, these authors implicated key roles for caspase-3 mediated degradation of FTase/GGTase- α -subunit in cellular apoptosis [13].

Given the above mentioned regulatory roles of farnesylation and geranylgeranylation in islet function including insulin secretion and cell survival [1, 10], we undertook the current study to determine if FTase/GGTase α -subunit undergoes cleavage under conditions of caspase-3 activation and cellular apoptosis in pancreatic β -cells. Specifically, we have examined the effects of etoposide, a genotoxic agent, known to induce robust activation of caspase-3 in insulin-secreting cells on FTase/GGTase α -subunit cleavage and cell viability in isolated pancreatic β -cells [14]. Lastly, we also investigated potential roles of influx and intracellular accumulation of calcium as an intermediary signaling event in etoposide effects on caspase-3 activation, FTase/GGTase α -subunit degradation steps in the cascade of events leading to loss in cell viability in these cells.

Materials and methods

Materials

Z-DEVD-FMK, a caspase inhibitor, was from R&D Systems, Inc. (Minneapolis, MN). Antisera directed against

cleaved caspase-3 (active form) and β -actin were from Cell Signalling (Danvers, MA). The antiserum against the FTase/GGTase- α subunit was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-mouse or anti-rabbit IgG-horseradish peroxidase conjugates and Enhanced Chemiluminescence (ECL) kits were from Amersham Biosciences (Piscataway, NJ). All other reagents used in these studies were from Sigma Aldrich Co. (St. Louis, MO) unless stated otherwise.

Insulin-secreting cell culture and treatments

INS 832/13 cells were cultured in RPMI-1640 medium containing 10 % heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, and 10 mM HEPES (pH 7.4). The medium was changed twice, and cells were subcloned weekly. Islets from normal 6 week-old male Sprague-Dawley rats (Harlan Laboratories, Oxford, MI) were isolated by collagenase digestion method. All protocols, including isolation of pancreatic islets from rats, were reviewed and approved by our Institutional Animal Care and Use Committee. INS 832/13 cells or rat islets were incubated with diluents (DMSO and/or ethanol) or etoposide (60 μ M) in presence or absence of nifedipine (10 μ M) for 6–12 h as indicated in the text. In a separate experiment INS 832/13 cells were treated with either diluent or etoposide (60 μ M) in the presence or absence of peptide inhibitor Z-DEVD-FMK (25 μ M) for 6 h as stated in the text.

Recombinant caspase-3 studies

INS 832/13 cells were cultured to 80 % confluency and washed twice with PBS, harvested and resuspended in sample buffer [0.5 % Nonidet P-40, 20 mM HEPES (pH 7.4), 100 mM NaCl and 20 mM DTT]. 50 μ g of lysate proteins were treated with recombinant caspase-3 (0–0.1 unit/mg protein) and incubated at 25 °C for 1 h. Samples were processed and immunoprobed for caspase-3 and FTase/GGTase α .

Western blotting

Proteins from INS 832/13 cells or rat islets were separated by SDS-PAGE on 10 % (w/v) polyacrylamide mini gels and electrotransferred to nitrocellulose membrane. The membranes were blocked with 5 % non fat dry milk in TBS-T (10 mM Tris-HCl; pH 7.4, 8.8 g/l NaCl, and 0.1 % Tween 20) for 2 h at room temperature. The membranes were then incubated overnight at 4 °C with antisera raised against the cleaved (active fragment) caspase-3 (1:250) or FTase/GGTase- α subunit (1:400) in TBS-T containing 5 %

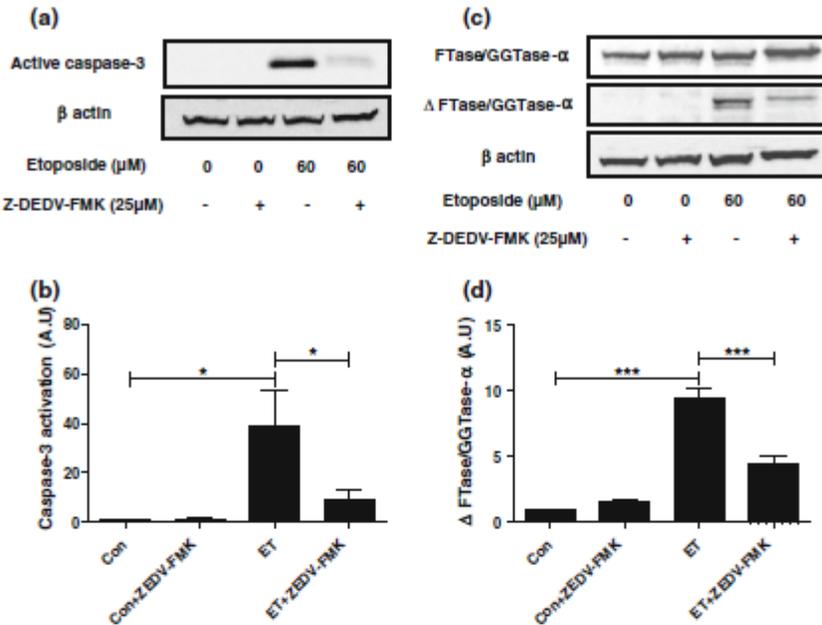


Fig. 1 Etoposide induces caspase-3 activation and FTase/GGTase- α degradation in INS 832/13 cells: Protection of these signaling steps by Z-DEVD-FMK, an inhibitor of caspase 3. INS 832/13 cells were treated with either diluents (DMSO and/or ethanol) or etoposide (60 μ M) in the presence or absence of peptide inhibitor Z-DEVD-FMK (25 μ M; 6 h). Caspase-3 activation and FTase/GGTase- α degradation were determined by Western blotting. Equal amount of

lysates protein were resolved by SDS-PAGE (10 %). Protein loading was determined by actin content in individual lanes. A representative blot indicating the caspase-3 activation (a) and FTase/GGTase- α degradation (c) is provided. Quantitative analysis of data obtained from three independent experiments for caspase-3 activation (b) and FTase/GGTase- α degradation (d) was carried out by densitometry. Results are shown as mean \pm SEM. * P < 0.05, *** P < 0.001

BSA. The membranes were washed 5 times for 5 min each with TBS-T and probed with appropriate horseradish peroxidase-conjugated secondary antibodies in 5 % non fat dry milk in TBS-T at room temperature for 1 h. After washing, the immune complexes comprising of the target proteins were detected using the ECL kit. The membranes then were stripped and re probed with β -actin. The band intensity was quantified and protein expression levels were calculated relative to β -actin in the same sample.

Metabolic cell viability assays

INS 832/13 cells were seeded in 96-well plates and treated as described above. After 6 h cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay which was carried out using manufacturer's instructions. The results are shown as relative optical density.

Statistical analysis

Results are expressed as means with their standard errors as indicated. Data are analyzed using one way ANOVA

followed by Bonferroni posthoc test (GraphPad Prism 5; GraphPad Software, Inc., La Jolla, CA, USA). Differences between control and treatment groups were considered significant if P < 0.05.

Results

Etoposide induces caspase-3 activation in INS 832/13 cells

At the outset, we quantitated etoposide-induced activation of caspase-3 in INS 832/13 cells. This was accomplished by incubating these cells with etoposide (60 μ M for 6 h) followed by identification of the cleaved peptide of caspase-3 (active form) by Western blotting method. Data in Fig. 1a show that etoposide significantly increased the emergence of the active form of caspase-3 (lane 3 vs. lane 1). Further, coprovision of Z-DEVD-FMK, a known inhibitor of caspase-3, markedly attenuated etoposide-induced activation of caspase-3 (lane 4 vs. lane 3). Data from multiple experiments are provided in Fig. 1b suggesting that etoposide significantly augments caspase-3 activity in INS 832/13 cells.

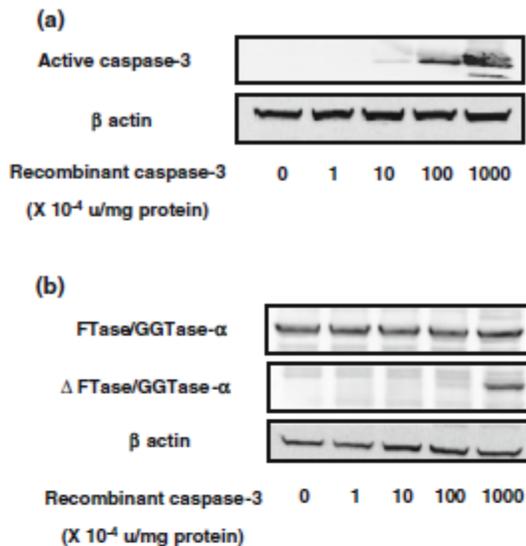


Fig. 2 Incubation of INS 832/13 cell lysates with human recombinant caspase-3 (CPP32) results in FTase/GGTase- α degradation in INS 832/13 cells. INS 832/13 cell lysates were treated with CPP32 (0–0.1 unit/mg protein) for 1 h as indicated in the figure followed by separation on SDS-PAGE (10 %). Separated proteins were transferred onto a nitrocellulose membrane and immunoblotted for Caspase-3 and FTase/GGTase- α . A representative Western blot for caspase-3 activation (a) and FTase/GGTase- α degradation (b) is shown here

Etoposide induces FTase/GGTase α -subunit degradation in INS 832/13 cells and normal rat islets

As stated above, earlier observations from Kim and associates have shown that the α -subunit of FTase/GGTase undergoes cleavage by caspase-3. We next determined if etoposide-induced caspase-3 activation culminates in the degradation of the FTase/GGTase α -subunit. To examine this, lysates from studies described under Fig. 1a, b were subjected to SDS-PAGE and relative abundance of the degraded product of FTase/GGTase α -subunit (~ 35 kDa) was determined by Western blotting. As depicted in Fig. 1c, d, a significant increase in the degradation of FTase/GGTase α -subunit was noticed in cells incubated with etoposide (Fig. 1c; lane 3 vs. lane 1). In a manner akin to caspase-3 activation (Fig. 1a, b), incubation of INS 832/13 cells with an inhibitor of caspase-3 markedly reduced etoposide-induced FTase/GGTase α -subunit degradation (Fig. 1c; lane 4 vs. lane 3). Data from multiple experiments are included in Fig. 1d.

Recombinant caspase-3 degrades FTase/GGTase α -subunit in INS 832/13 cells

To further demonstrate that caspase-3 mediates the hydrolysis of FTase/GGTase α -subunit, INS 832/13 cell lysates were incubated with human recombinant caspase-3 and the relative abundance of cleaved product of FTase/

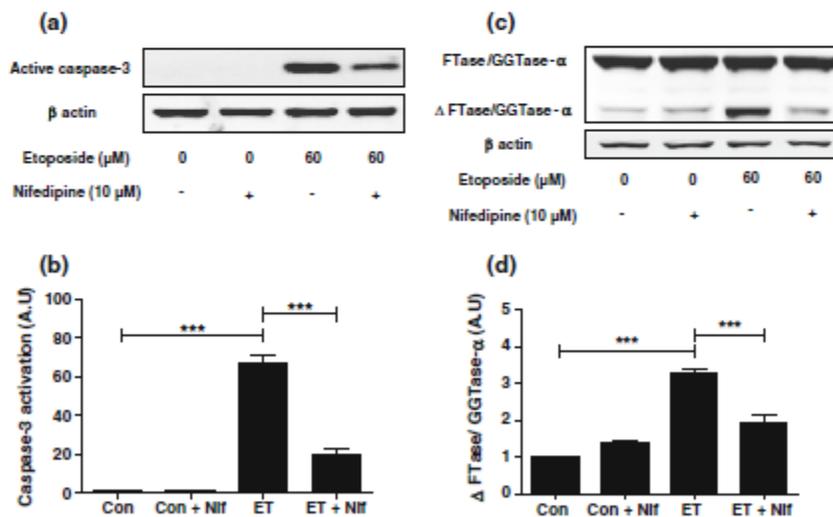


Fig. 3 Nifedipine prevents etoposide induced caspase-3 activation and FTase/GGTase- α degradation in INS 832/13 cells. INS 832/13 cells were treated with either diluents (DMSO and/or ethanol) or etoposide (ET) (60 μ M) in the presence or absence of Nifedipine (Nif; 10 μ M; 6 h). Caspase-3 activation and FTase/GGTase- α degradation were determined by Western blot analysis. Protein loading was

determined by actin content in individual lanes. Data are representative of four independent experiments. A representative blot indicating the caspase-3 activation (a) and FTase/GGTase- α degradation (c) is shown here. Quantitation of the active caspase-3 (b) and FTase/GGTase- α degradation (d) was carried out by densitometry. Results are shown as mean \pm SEM. ****P* < 0.001

GGTase α -subunit was determined as above. Indeed, data in Fig. 2 show the emergence of the degradative product of FTase/GGTase α -subunit under conditions of caspase-3 activation. These data further support our observations in Fig. 1 suggesting that etoposide induces caspase-3 activation leading to cleavage of FTase/GGTase α -subunit in pancreatic β -cells.

Nifedipine protects etoposide-induced caspase-3-mediated degradation of FTase/GGTase α -subunit in INS 832/13 cells and normal rodent islets

Existing evidence in other cell types implicates critical roles for intracellularly accumulated calcium in eliciting mitochondrial dysfunction and caspase-3 activation [15–18]. In addition, available data in many cell types indicated that etoposide-induced caspase activation may, in part, be due to accumulation of calcium intracellularly [19, 20]. Therefore, in the next set of experiments, we investigated if blocking the influx of extracellular calcium by nifedipine, a known blocker of voltage-gated calcium channels [21, 22], prevents etoposide-induced caspase-3 activation and FTase/GGTase α -subunit cleavage in INS 832/13 cells. Data depicted in Fig. 3a demonstrates a significant reduction in etoposide-induced caspase-3 activation in INS 832/13 cells following coprovision with nifedipine. Data from multiple experiments revealed

~70 % inhibition in etoposide-induced caspase-3 activation following incubation with nifedipine (Fig. 3b). We then investigated if nifedipine-treatment of pancreatic β -cells prevents etoposide-induced degradation of FTase/GGTase- α -subunit. Data in Fig. 3c, d demonstrate a marked reduction in etoposide-induced FTase/GGTase α -subunit hydrolysis by nifedipine.

In a manner akin to INS 832/13 cells, we also observed protective effects of nifedipine against etoposide induced caspase 3 activation and FTase/GGTase α -subunit degradation in normal rat islets. As shown in Fig. 4a, b coprovision of nifedipine markedly attenuated etoposide-induced caspase-3 activation in rat islets. Furthermore, nifedipine-treatment of rat islets resulted in marked reduction in etoposide-induced FTase/GGTase α -subunit hydrolysis (Fig. 4c, d).

Nifedipine protects etoposide-induced loss in cell viability in INS 832/13 cells

As mentioned above, several lines of evidence in multiple cell types including the islet β -cell implicate roles for prenylated G-proteins in cell survival [1, 10]. Therefore, in the last series of studies, we have examined if: etoposide induces loss in metabolic cell viability under conditions it induced caspase-3 activation and FTase/GGTase α -subunit degradation, and if so, whether nifedipine prevents such an

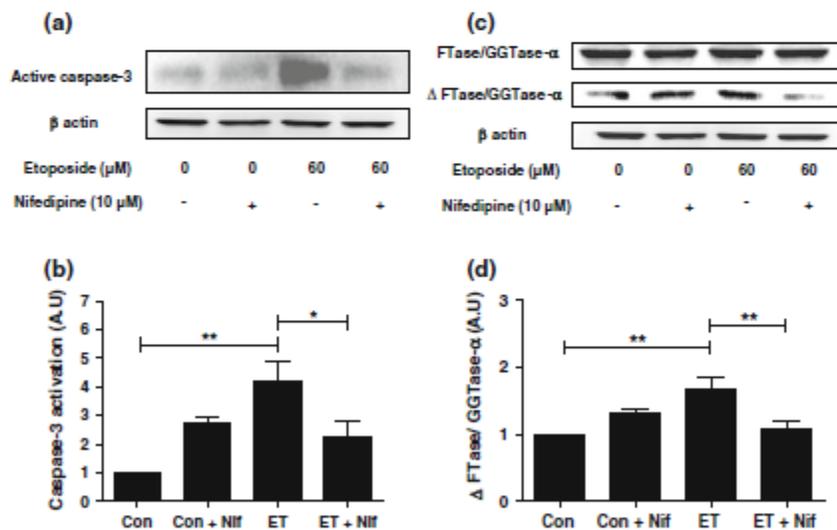


Fig. 4 Nifedipine prevents etoposide induced caspase-3 activation and FTase/GGTase- α degradation in rat islets. Rat islets were treated with either diluents (DMSO and/or ethanol) or etoposide (ET) (60 μ M) in the presence or absence of Nifedipine (Nif; 10 μ M; 12 h). Caspase-3 activation and FTase/GGTase- α degradation were determined by Western blot analysis. Protein loading was determined by

actin content in individual lanes. Data are representative of four independent experiments. A representative blot indicating the caspase-3 activation (a) and FTase/GGTase- α degradation (c) is shown here. Quantitation of the active caspase-3 (b) and FTase/GGTase- α degradation (d) was carried out by densitometry. Results are shown as mean \pm SEM. * P < 0.05, ** P < 0.01

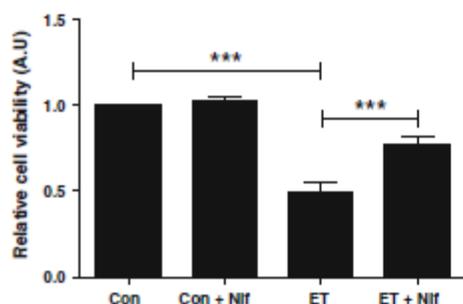


Fig. 5 Nifedipine prevents etoposide induced reduction in metabolic cell viability in INS 832/13 cells. INS 832/13 cells were incubated with either diluents or etoposide (ET; 60 μ M) in the presence or absence of Nifedipine (Nif; 10 μ M; 6 h) as indicated in the figure. Cell viability was measured by MTT assay. Data represent mean \pm SEM from four independent experiments. *** P < 0.001

effect. To test this, cells were incubated with diluents or etoposide and/or nifedipine in different combinations followed by quantitation of viability of these cells using the MTT method (see “Materials and methods” section for additional details). Data in Fig. 5 indicate no significant effects of nifedipine on cell viability (bar 1 vs. bar 2); these data are in agreement with lack of effects of nifedipine on caspase-3 activation and FTase/GGTase α -subunit degradation in these cells. In a manner akin to its effects on caspase-3 activation and FTase/GGTase α -subunit degradation, etoposide significantly reduced cell viability in INS 832/13 cells (bar 3 vs. bar 1). Coprovision of nifedipine to these cells alongside etoposide markedly prevented loss in cell viability (bar 4 vs. bar 3). Together, these data suggest that nifedipine affords protective effects against etoposide-induced caspase-3 activation, FTase/GGTase α -subunit degradation and loss in cell viability in pancreatic β -cells.

Discussion

It is well established that small molecular mass and heterotrimeric G-proteins play important roles in cellular signaling events leading to glucose-stimulated insulin secretion [5, 12]. A growing body of evidence is also suggestive of novel roles for these signaling proteins in other β -cell functions including cell cycle progression, survival and apoptosis [1]. It is noteworthy that the majority of these proteins undergo post-translational modifications at their c-terminal cysteine residues (e.g., prenylation, methylation and acylation), which are essential for their trafficking to relevant cell membranes for optimal interaction with their respective effector proteins culminating in optimal regulation of β -cell function [1, 4, 5]. Despite the compelling evidence that FTase and GGTTases are acutely regulated by glucose in the β -cell

[11, 12], and that they are critical for insulin secretion [1, 8, 9], very little is known with regard to potential alterations in these enzymes under conditions of cellular apoptosis. Along these lines, studies by Kim and associates have demonstrated degradation of the FTase/GGTase α -subunit under conditions of caspase-3 activation [13]. With this in mind, we quantitated caspase-3 activation, FTase/GGTase degradation and cellular dysfunction in isolated β -cells exposed to etoposide, a known inducer of apoptosis in pancreatic β -cells. Our findings suggested that: (i) etoposide induces caspase-3 activation, which in turn, degrades the common α -subunit of FTase/GGTase; these signaling events are blocked by a peptide inhibitor of caspase-3; (ii) nifedipine, a known inhibitor of plasma membrane-associated calcium channels, markedly attenuates etoposide-mediated effects on caspase-3 activation and FTase/GGTase α -subunit degradation in INS 832/13 cells and normal rat islets; and (iii) nifedipine protects pancreatic β -cells against etoposide-induced loss in metabolic viability. Based on these findings we speculate that influx and intracellular accumulation of calcium as one of the signaling events leading to etoposide-induced metabolic dysfunction of the islet β -cell, leading to the activation of caspase-3 and FTase/GGTase α -subunit degradation (see below). Given our observations, one might ask the question about the relevance and implications of these current findings in the context of β -cell survival and function. These aspects are discussed below.

Existing evidence suggests that inhibition of protein prenylation culminates in growth arrest, loss in cell viability and apoptosis, thus implicating regulatory roles for prenylated proteins, including small molecular mass and trimeric G-proteins as well as nuclear lamins in cell survival pathways [23, 24]. Furthermore, it has been demonstrated that functional inactivation of G-proteins or GTP-requiring signaling steps by depletion of intracellular GTP results in cell demise. For example, original studies by Li and associates [25] have suggested that prolonged depletion of intracellular GTP via inhibition of inosine monophosphate dehydrogenase using mycophenolic acid, results in the apoptotic demise of the islet β -cell. These authors have also demonstrated that functional inactivation of Rho G-proteins by clostridial toxins further potentiated mycophenolic acid-induced β -cell apoptosis. These data raise an interesting possibility that functional inactivation of islet endogenous G-proteins either by GTP-depletion or functional inactivation via Clostridial toxins or FTase/GGTase inhibitors results in impaired cell viability. Lastly, it was also demonstrated that inhibition of protein farnesylation using selective inhibitors of FTases (FTI-277 and FTI-2628) or siRNA-mediated knockdown of the β -subunit of FTase, markedly reduced glucose-induced ERK1/2 phosphorylation, Rac1 activation and insulin secretion in clonal

β -cells and normal rat islets [11]. Our current findings indicating a potential causal relationship between the degradation of FTase/GGTase α -subunit and loss in cell viability further validates the above experimental models and postulations.

It is also noteworthy that our current observations also suggested regulatory roles for accumulation of cytosolic calcium in etoposide-mediated effects on caspase-3 activation and FTase/GGTase α -subunit degradation. These findings are in concert with recent observations by Wang et al. [26] 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 a calcium-mediated mitochondrial dysregulation, caspase-3 activation and FTase/GGTase α -subunit degradation pathways leading to loss in cell viability. This possibility needs to be verified experimentally. Indeed, recent observations from Shalev's laboratory [27] 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 [27] and nifedipine (current study) appear to exert cytoprotective effects in β -cells against noxious stimuli and improve β -cell survival and function.

Our current findings form the basis for future investigations including determination of overall impact of degradation of FTase/GGTase α -subunit in the functional regulation of other key cellular proteins such as *Probin*, a novel farnesylated protein that has been shown to suppress the activation of survival proteins (e.g., Akt) in the islet β -cell [28]. Second, potential implications of our current findings on nuclear lamins and the assembly of nuclear envelope remains to be verified since inhibition of post-translational prenylation of lamins is known to increase their susceptibility to degradation leading to the collapse of nuclear assembly [29, 30]. 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 prelamins 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 [31]. Together, these findings implicate novel roles for post-translational prenylation steps in the functional regulation

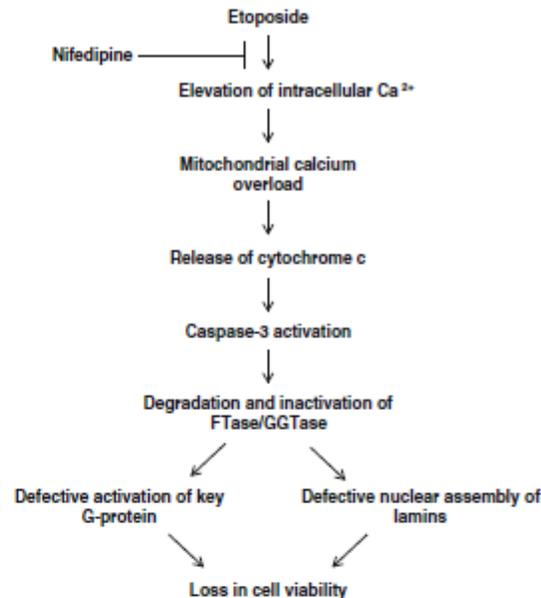


Fig. 6 Proposed model for etoposide-induced loss in viability of the β -cell. Based on the data accrued in the current study we speculate that exposure of pancreatic β -cells to etoposide leads to mitochondrial dysfunction mediated via intracellular accumulation of calcium, which is sensitive to nifedipine. Such a signaling step leads to increased activation of caspase-3, and peptidic cleavage of FTase/GGTase α -subunit. While our current data do not provide direct evidence, existing data from multiple laboratories including our own suggests that pharmacological and molecular biological inhibition of FTase/GGTases lead to functional inactivation of G-proteins and abnormal lamin metabolism leading to defective nuclear assembly and cell demise (see text for additional details). Lastly, we cannot rule out the potential possibility that the cleavage product of FTase/GGTase α -subunit could exert direct effects on inducing loss in metabolic cell viability in INS 832/13 cells. This remains to be verified experimentally

of lamins [23, 24]. Lastly, it is likely that the cleavage product of FTase/GGTase α -subunit could have direct effects on the induction of cell death. Indeed, findings by Kim and associates further substantiate this viewpoint. These investigators provided direct evidence to suggest that expression of p35, the cleavage product of FTase/GGTase α markedly increased cell death probably by interfering with the prenylase activities [13]. This remains to be confirmed in the β -cell.

In summary, we propose (Fig. 6) that etoposide-induced metabolic dysregulation and loss in cell viability may, in part, be due to calcium-mediated, caspase-3-induced degradation of FTase/GGTase α -subunit and associated downstream signaling events, which remain to be determined in the context of prenylation-dependent regulation of survival and function of the islet β -cell.

Acknowledgments This research was supported in part by a Merit Review award [to AK; 1BX000469] from the Department of Veterans Affairs, Veterans Health Administration, Office of Research and Development Biomedical Laboratory Research and Development, and by the NIH/NIDDK [RO1 DK74921]. AK is also the recipient of a Senior Research Career Scientist Award from the Department of VA. The authors would like to thank Prof. Chris Newgard for INS 832/13 cells. We thank Dr. Wasanthi Subasinghe for excellent technical assistance in these studies.

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APPENDIX D MOHAMMED ET AL 2013

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The Two Faces of Protein Palmitoylation in Islet β -Cell Function: Potential Implications in the Pathophysiology of Islet Metabolic Dysregulation and Diabetes

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Received: May 2, 2013; Accepted: June 28, 2013; Revised: July 1, 2013

Abstract: Several cellular proteins undergo post-translational lipidation, including prenylation, palmitoylation and myristoylation, which are felt to promote intracellular targeting, membrane association and interaction with effector partner proteins. Recent findings implicate definitive roles for isoprenylation in islet β -cell function including glucose-stimulated insulin secretion [GSIS]. Published evidence also suggests novel regulatory roles for protein palmitoylation not only in GSIS but also in the metabolic dysfunction induced by proinflammatory cytokines and lipotoxic conditions. Herein, we overviewed the existing evidence on the regulatory roles of protein palmitoylation in the metabolic [dys]regulation of the islet β -cell and highlighted the developments in this area, specifically on potential identity of palmitoylated proteins, and on the utility of two structurally distinct inhibitors of palmitoylation [e.g., cerulenin and 2-bromopalmitate] in halting the metabolic dysfunction of the islet β -cell known to occur following exposure to proinflammatory cytokines and lipotoxic conditions. Potential avenues for future research, including the immediate need for discovery novel small molecule compounds as inhibitors of palmitoyl transferases to attenuate deleterious consequences of proinflammatory cytokines and glucolipotoxicity are discussed. Furthermore, some relevant patents are also highlighted in this review.

Keywords: 2-bromo-palmitate, cerulenin, palmitoyl transferase, palmitoyl esterase, palmitoylation, pancreatic β -cells, proinflammatory cytokines, therapeutic targets, type 1 diabetes, and type 2 diabetes.

INTRODUCTION

Glucose-Stimulated Insulin Secretion: A Simplified Model

It is well established that glucose-stimulated insulin secretion [GSIS] is mediated largely due to the generation of soluble second messengers, such as cyclic nucleotides, hydrolytic products of phospholipases A₂, C and D [1, 2]. The principal signaling cascade involved in GSIS is initiated by Glut-2-mediated entry of glucose into the β -cell followed by an increase in the intra-islet ATP/ADP ratio as a consequence of glucose metabolism. Such an increase in ATP levels culminates in the closure of ATP-sensitive potassium channels localized on the plasma membrane resulting in membrane depolarization, and the influx of extra-cellular calcium through the voltage-sensitive calcium channels. A net increase in intracellular calcium has been shown to be critical for the transport of insulin-laden secretory granules to the plasma membrane for fusion and release of insulin [1-6].

Gtp-Binding Proteins In Gsis

In addition to the regulatory roles of ATP in GSIS, previous studies have examined possible contributory roles for

guanine nucleotides [i.e. GTP] in GSIS. For example, using selective inhibitors of GTP biosynthetic pathway (e.g., mycophenolic acid), a permissive role for GTP in GSIS was established [7,8]. Although the precise molecular and cellular mechanisms underlying the roles of GTP in GSIS remain to be defined, compelling evidence indicates that it might involve activation of one (or more) G-proteins endogenous to the islet β -cell. In this context, at least two major groups of G-proteins have been identified within the islet β -cell [9, 10]. The first group consists of heterotrimeric G-proteins, which are composed of α [39-43 kD], β [35-37 kD] and γ [5-10 kD] subunits. These signaling proteins are involved in the coupling of various G-protein-coupled receptors [GPCRs] to their intracellular effector proteins, including adenylate cyclase, phosphodiesterase and phospholipases. The second group of G-proteins is composed of small G-proteins [20-25 kD], which are involved in cytoskeletal remodeling and trafficking of secretory vesicles [10].

Original observations from multiple laboratories, including our own, demonstrated involvement of small G-proteins [e.g., Rac1, Cdc42, Rap1 and ARF6] in GSIS from normal rat islets, human islets and clonal β -cell preparations [9-11]. Such conclusions were drawn primarily based on data from three complementary experimental approaches. The first approach involved use of *Clostridial* toxins (e.g. toxin A or B), which mono-glucosylate and inactivate specific G-proteins. The second experimental manipulation involved molecular biological approaches, including expression of

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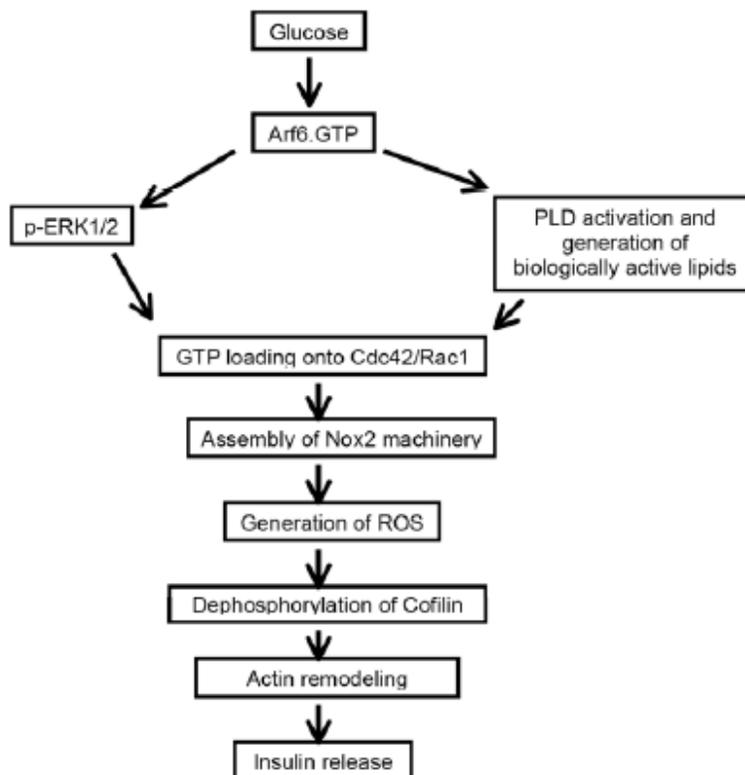


Fig. (1). G-protein-mediated regulation of GSIS

A schematic representation of potential involvement of small G-proteins in GSIS, based on the experimental evidence we propose that GSIS involves precise interplay between specific G-proteins [e.g., Cdc42, Rac1 and Arf6] and their regulatory factors [e.g., GEFs and GDIs]. Sequential activation of Arf6, Cdc42 and Rac1 leads to increased Nox2-mediated generation of ROS, which, in turn, facilitates cytoskeletal remodeling culminating in insulin secretion. In addition to regulation by GEFs and GDIs, some of these G-proteins undergo requisite post-translational modifications (see below; Fig. 2) including prenylation, carboxymethylation and palmitoylation, which are critical for their targeting to the respective membranous compartment for optimal interaction with their effector proteins. Not included in this schematic are other small G-proteins, including Rap1, Rab3A, Rab5, and Rab27A etc. which also exert direct regulatory roles in the signaling events leading to GSIS [9, 10]. In a manner akin to Cdc42 and Rac1, the Rap and Rab G-proteins are also modulated by their respective regulatory proteins and post-translational prenylation signaling steps [10 for reviews]. Additional details on this proposed working model can be found in [15]. Nox2: phagocyte-like NADPH oxidase and ROS: reactive oxygen species.

dominant negative mutants and/or selective knockdown [i.e., siRNA methodology] of the candidate G-proteins. The third approach involved the use of pharmacological inhibitors of G-protein activation to further decipher their regulatory roles in GSIS [9-12].

Several recent studies, most notably from the Thurmond and the authors' laboratories, have explored regulatory roles for small G-proteins [e.g., Cdc42, Rac1 and Arf-6] in GSIS [9-12]. Time course studies by Jayaram *et al* [11] have suggested that GSIS involves sequential activation of Arf-6 [~1 min], Cdc42 [~3 min] and Rac1 [~15 min]. Recent studies have also identified and characterized regulatory factors for these G-proteins [9, 10]. They include guanine nucleotide exchange factors [GEFs], which promote GTP/GDP exchange into candidate G-proteins. Studies by Kepner and coworkers have identified Cool-1/ β PIX as a GEF for Cdc42 in insulin-secreting cells [13]. Veluthakal and associates

have demonstrated Tiam1 as the GEF for Rac1 activation in the islet β -cell. Jayaram and colleagues reported ARNO as the GEF for Arf-6 in the islet [14]. In addition to GEFs, several recent studies have demonstrated expression and regulation of G-protein function by specific GDP-dissociation inhibitors [GDIs] in the pancreatic islet. These include Rho GDI for the regulation of Cdc42 and Rac1 and cavolin-1 as the GDI for Cdc42 [9, 10]. Based on the available evidence thus far, we propose a model for the regulation of GSIS by small G-proteins in the islet (Fig. 1). Glucose metabolism leads to ARNO-mediated activation of Arf6, which, in turn, regulates its downstream signaling events including phosphorylation and activation of ERK1/2. Arf6 activation also leads to activation of phospholipase D, leading to the generation of biologically-active lipids [15]. Evidence is also available to suggest increased dissociation of specific G-proteins [e.g., Rac1] from their GDIs by lipid second messengers leading to their functional activation [16]. Sequential activation of

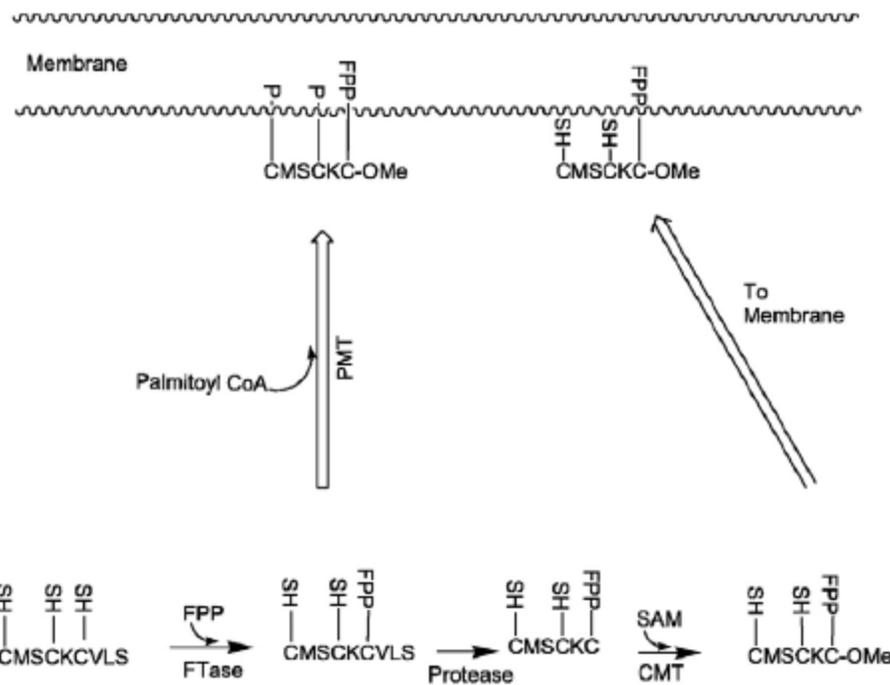


Fig. (2). Post-translational modifications of small G proteins:

The first of the four-step reaction is incorporation of either a 15 [farnesyl]- or a 20 [geranylgeranyl]-carbon derivative of mevalonic acid into the COOH-terminal cysteine *via* a thioether linkage. This reaction is catalyzed by either the farnesyl or geranylgeranyl transferases, respectively. Following this, the three amino acids after the prenylated cysteine are removed by a protease, thereby exposing the carboxylate anion. This site is then methylated by a carboxyl methyl transferase, which transfers a methyl group onto the carboxylate group using S-adenosyl methionine [SAM] as the methyl donor. Numerous studies from our laboratory have shown carboxyl methylation of specific G proteins (e.g., Cdc42, Rap1 and γ -subunits of trimeric G-proteins), which are critical islet function and insulin secretion [10]. In addition to these, a growing body of evidence suggests that certain G proteins [e.g., H-Ras, Cdc42 and Rac1] undergo palmitoylation at a cysteine residue, which is upstream to the prenylated cysteine. It is thought that palmitoylation provides a “firm” anchoring for the modified protein into the cell membrane for optimal interaction with its respective effector proteins. Unlike prenylation, the carboxylmethylation and palmitoylation steps are acutely regulated via regulation by carboxylmethyl and palmitoyl esterases [taken from 12]. FPP: farnesyl pyrophosphate; FTase: farnesyl transferase; CMT: carboxyl methyl transferase; SAM: S-adenosyl methionine; and PMT, palmitoyl transferase.

Arf6, Cdc42 and Rac1 leads to increased phagocyte-like NADPH oxidase [Nox2] resulting in a tonic increase in reactive oxygen species [ROS] in the glucose-stimulated islet [15]. This, in turn, leads to cytoskeletal remodeling and fusion of insulin granules with the plasma membrane and exocytotic secretion of insulin ((Fig. 1); see ref. 15 for additional details).

G-Proteins Undergo Post-Translational Modifications

The majority of small G-proteins and the γ -subunits of heterotrimeric G-proteins undergo post-translational modifications at their C-terminal cysteine residue (Fig. 2). The first of a four-step modification sequence involves incorporation of a mevalonic acid-derived 15-carbon [farnesyl] or 20-carbon [geranylgeranyl] isoprenoid moiety onto a cysteine residue toward the C-terminus of the candidate G-proteins. This is followed by proteolysis of several amino acids (up to a maximum of three) mediated by a protease of microsomal origin. A carboxyl methylation step then modifies the newly exposed carboxylate anion of the cysteine. In some cases, the covalent addition of a long-chain fatty acid, typically palmitoylation, at cysteine residues, which are upstream to the CAAX motif, completes the cascade [10, 12].

It is well established that the above mentioned modification steps increase the hydrophobicity to the modified G-proteins thereby enabling them to translocate to relevant membranous compartments for interaction with their respective effectors [10, 12]. Because the isoprenylation of G-proteins occurs shortly after their synthesis, and because “half-lives” of prenylated proteins are rather long, this is not likely to be an acute regulatory step. However, in many cases, prenylation is necessary to allow prenylated G-proteins to intercalate into the membrane [10, 12]. In contrast, the methylation and palmitoylation steps are subject to acute regulation at the level of addition or deletion of methyl or palmitoyl groups [9, 10]. The addition and removal of methyl groups are catalyzed by carboxyl methyl transferase and esterase, respectively. The addition and deletion of palmitoyl groups are facilitated by palmitoyl transferase and esterase, respectively. Studies from our laboratory and those of others have demonstrated the requisite nature and roles of posttranslational prenylation and carboxylmethylation of small G-proteins and the γ -subunits of trimeric G-proteins in the cascade of events leading to

GSIS [10, 12]. In the following section, we will highlight putative regulatory roles of protein palmitoylation-depalmitoylation steps in islet β -cell function.

Protein Palmitoylation–Depalmitoylation Cycle

Existing body of evidence clearly implicates novel regulatory roles for protein palmitoylation in cellular function [17, 18]. The protein palmitoyltransferases [PATs] catalyze the transfer of palmitate into the cysteine residues of proteins containing DHHC [Asp-His-His-Cys] cysteine-rich domains [CRD] via thioester linkage. Typically, these reactions are known to occur at the cytoplasmic face of membranes in the secretory pathway [e.g., endoplasmic reticulum and Golgi] and the plasma membrane [17, 18]. At least two types of PAT activities have been reported. The first group catalyzes palmitoylation of farnesylated proteins including Ras GTPases [e.g., H-Ras and N-Ras], whereas the second group of PATs mediates palmitoylation of Src family of tyrosine kinases [17, 18]. A distinct class of palmitoyl thioesterases, namely protein palmitoyl thioesterases 1 and 2 and acyl palmitolesterase 1 mediate depalmitoylation via hydrolysis of the ester bonds to complete the palmitoylation-depalmitoylation [i.e., activation-inactivation] cycle. As stated above, protein palmitoylation plays key regulatory roles in cellular function including subcellular localization of proteins, trafficking and stability [17-19]. It is widely felt that palmitoylation dictates interaction of peripheral membrane proteins. Potential regulatory roles of palmitoylation of integral membrane proteins still remain unclear. Several cellular proteins have been demonstrated to undergo palmitoylation. Some of these include, but not limited to, A-kinase anchoring protein 79/150 [20], phospholipase scramblase [21], ankyrin-g [22], glutamic acid decarboxylase [23], cytoskeletal-associated protein 4 [24], integrin $\alpha 6\beta 4$ [25], and calnexin [26]. In addition, several GPCRs, including μ -opioid receptor [27], protease-activated receptor-2 [28], $\beta 2$ adrenergic receptor [29], somatostatin receptor [30], regulator of G-protein signaling (RGS4) [31], neurotensin receptor [32], and p63Rho guanine nucleotide exchange factor [33] appear to be regulated by palmitoylation-depalmitoylation signaling steps.

Pharmacological Inhibitors of Protein Palmitoylation

Advancement in the area of regulatory roles of protein palmitoylation is hampered due to the unavailability of specific inhibitors for PATs. Original studies have utilized cerulenin [CER], an antifungal antibiotic, to decipher the roles of protein palmitoylation in cellular function in many cell types including the islet β -cell [see below]. However, use of CER is limited due to the fact that it has been shown to exhibit non-specific effects including inhibition of fatty acid and steroid biosynthesis as well as lipoprotein lipase [34]. Other studies have employed tunicamycin as an inhibitor to study roles of protein palmitoylation in cell function, but again, as in the case of CER, tunicamycin has been shown to inhibit fatty acid synthesis and N-glycosylation of proteins [35]. In addition, tunicamycin induces endoplasmic reticulum stress [36]. Several recent studies have utilized 2-bromopalmitate [2BP] to study roles of protein palmitoylation in cellular function [37, 38]. Even though 2BP appears to be a relatively

selective inhibitor of palmitoylation, it has been shown to inhibit several signaling steps/enzymes involved in intracellular lipid metabolism including inhibition of fatty acyl CoA ligase [39]. Therefore, data accrued from CER, tunicamycin and 2BP experiments should be interpreted with some degree of caution [see below]. Along these lines, more recent studies by Jennings and associates [40] compared inhibitory effects of 2BP and Compound V [2-(2-hydroxy-5-nitrobenzylidene)-benzo[b]thiophen-3-one] on DHHC-mediated palmitoylation *in vitro*. Data from these investigations have suggested that the inhibitory effects of Compound V on palmitoylation are reversible in contrast to the irreversible inhibitory effects of 2BP. Based on the above discussion it appears that additional studies, including the development of novel inhibitors of PATs, is necessary to further decipher the roles of protein palmitoylation in cellular function.

Positive Modulatory Roles of Palmitoylation in Islet Function

Despite considerable experimental evidence the identity and functional properties including subcellular distribution of PATs and palmitolesterases are described in a variety of cell types, very little is known about the identity and regulation of palmitoyl transferases and esterases in the islet β -cell. However, pharmacological evidence appears to support key roles for these signaling steps in islet β -cell function. For example, CER has been shown to inhibit insulin secretion induced by glucose, α -ketoiso-caproic acid, and long chain acyl CoAs [41-44]. CER also inhibits glucose-induced incorporation of radio labeled palmitate into islet proteins and inhibition of palmitoylation of a 24 kDa protein [45], and palmitate-induced tyrosine phosphorylation of insulin receptor [46]. Interestingly, studies by Metz and associates have demonstrated that CER failed to inhibit insulin secretion facilitated by non-nutrient secretagogues, such as a membrane-depolarizing concentration of potassium, activators of protein kinase A, or mastoparan [41]. More recent studies by Abdel-Ghany and associates [47] have demonstrated significant incorporation of [3 H] palmitate into islet β -cell proteins, which was stimulated by glucose. Furthermore, 2-aminobicyclo heptane-3-carboxylic acid, a non-metabolizable analog of leucine and an insulin secretagogue, also promoted incorporation of labeled palmitate into β -cell proteins. Autoradiographic analysis of lysate proteins separated by gel electrophoresis demonstrated glucose-mediated increase in palmitoylation of at least four β -cell proteins with apparent molecular weights of 30, 44, 48 and 76 kDa. More importantly, CER inhibited incorporation of labeled palmitate into these proteins under basal as well as glucose-stimulated conditions. Based on these findings the authors concluded that protein acylation plays regulatory roles in islet function [47].

In addition to CER, some studies utilized 2BP to understand the roles of acylation/lipid metabolism in islet function including insulin secretion. For example, original studies by Warnotte and associates demonstrated partial inhibition of palmitate-induced potentiation of glucose-induced insulin secretion [48]. Studies by Parker and coworkers [49] have suggested significant inhibition, by 2BP, of palmitate esterification into cellular lipids in isolated rat islets. Furthermore, they also demonstrated partial inhibition of palmitate-mediated potentiation of GSIS. Cheng and associates [50]

reported significant protection of distal inhibitory effects of norepinephrine on physiological insulin secretion by CER and 2BP. Based on these observations these investigators proposed novel roles for protein acylation signaling steps in exocytotic secretion of insulin. Together, pharmacological evidence suggests that protein acylation, specifically palmitoylation, plays critical regulatory roles in islet β -cell function, including insulin secretion.

As indicated above, recent studies have implicated novel roles for small G-proteins [Arf6, Cdc42 and Rac1] in GSIS [9-12]. Furthermore, using various pharmacological and molecular biological approaches, we and others documented a requirement for post-translational prenylation and carboxymethylation of G-proteins [e.g., Cdc42, Rho, Rac1] for physiological insulin secretion [10, 12]. More importantly, despite the above described pharmacological evidence to indicate inhibition of GSIS by CER and 2BP, very little is known if Rac1 and Cdc42 also require palmitoylation to facilitate GSIS. In this context, recently published evidence suggests that Rac1 and bCdc42 [a splice variant of Cdc42 with predominant localization in the brain] undergo palmitoylation. For example, Navarro-Lerida and associates have recently demonstrated that Rac1 undergoes palmitoylation at cysteine 178 to facilitate its targeting for stabilization at actin cytoskeleton-linked ordered membrane regions [51]. Interestingly, they observed that palmitoylation of Rac1 requires its prior prenylation and the intact C-terminal polybasic region and is regulated by the triproline-rich motif. In addition, palmitoylation step appears to be required for Rac1 activation [i.e., GTP-binding] since non-palmitoylated Rac1 exhibited decreased GTP loading and lower association with detergent-resistant membranes. Further proof that palmitoylation of Rac1 is critical for cellular function was afforded in these studies since cells lacking Rac1 exhibited spreading and migration defects. Based on this compelling evidence, the authors concluded that palmitoylation of Rac1 is requisite for its role in actin cytoskeleton remodeling and regulation of membrane organization. Along these lines, Kang *et al.* [52] have demonstrated palmitoylation of a brain-specific Cdc42 splice variant [bCdc42]. Additional studies by Nishimura and Linder have documented that bCdc42 undergoes classical CAAX processing as well as prenylation and palmitoylation at the CCAX motif [53]. Interestingly, the prenylated and palmitoylated bCdc42 interacted less efficiently with RhoGDI [a signaling step critical for Cdc42 activation-deactivation cycles] when compared to canonical Cdc42, which is only prenylated and carboxymethylated, but not palmitoylated. Additional studies are required to determine if palmitoylation of Rac1 and/or Cdc42 is necessary for GSIS to occur.

In addition to small G-proteins, heterotrimeric G-proteins also control islet function including GSIS [10, 12]. Along these lines, we have demonstrated non-receptor dependent activation of trimeric G-proteins by glucose. For example, we have reported phosphorylation of G^{β} subunits at critical histidine residues in clonal β -cells, normal rat islets and human islets, which, in turn, is transferred to $G_{\alpha, GDP}$ to yield biologically active GTP-bound G_{α} subunits [54, 55]. In addition, previous studies have also demonstrated glucose-mediated activation of the carboxymethylation of G_{γ} subunits [56]. Together, these observations provided the first

evidence to indicate activation of trimeric GTPase via signaling steps generated during glucose metabolism culminating in insulin secretion. It is noteworthy that recent findings from the laboratory of Gautam [57, 58] have suggested critical roles for protein palmitoylation in the shuttling of trimeric G-protein subunits between the plasma membrane and intracellular membranes, which is inhibited by 2BP. Potential involvement of protein palmitoylation in glucose-induced activation of trimeric-proteins in the islet β -cell remains to be verified experimentally.

Negative Modulatory Roles of Protein Palmitoylation in Islet Function

In addition to their positive modulatory roles, accumulating evidence supports the view point that protein palmitoylation plays negative modulatory roles in the induction of metabolic dysfunction of the islet β -cell under the duress of noxious stimuli such as proinflammatory cytokines and saturated fatty acids. Along these lines, we first reported that functional inactivation of H-Ras, a small G-protein, and results in significant reduction in IL-1 β -mediated effects on isolated β -cells [59]. Our findings in clonal β -cells [HIT-T15 and INS-1 cells] also showed a significant inhibition of IL-1 β -induced inducible nitric oxide synthase and NO release by CER and 2BP. 2-hydroxymyristic acid, an inhibitor of protein myristoylation, failed to affect IL-induced NO release suggesting the relevance of palmitoylation, but not myristoylation, in these signaling steps. Further, we demonstrated significant accumulation of H-Ras in the cytosolic fraction in cells incubated with CER; these data supported our original proposal that inhibition of palmitoylation leads to mistargeting of G-proteins in islet β -cells. Interestingly, we noted that depletion of membrane-bound cholesterol using methyl- β -cyclodextrin, which also disrupts caveolar organization within the plasma membrane, abolished IL-1 β -induced NO release suggesting that IL-1 β -mediated Ras-dependent signaling in these cells involves the intermediacy of caveolae and their key constituents [e.g. caveolin-1] in isolated β -cells. Confocal light microscopic evidence indicated significant co-localization of Ras with caveolin-1. Based on this evidence we concluded that palmitoylation of Ras is essential for IL-1 β -induced cytotoxic effects on the islet β -cell [59]. A follow-up study investigated [60] potential cross-talk between H-Ras and caveolin-1 in the signaling events leading to cytokine-induced metabolic dysfunction of the islet β -cell. Using immunologic and confocal microscopic approaches we demonstrated a transient but significant stimulation of tyrosine phosphorylation of Cav-1 in β -cells briefly [\sim 15 min] exposed to IL-1 β ; three structurally distinct inhibitors of protein tyrosine phosphorylation markedly attenuated tyrosine phosphorylation of Cav-1. Over expression of an inactive mutant of Cav-1 lacking the tyrosine phosphorylation site (Y14F) or siRNA-mediated Cav-1 knock down also resulted in marked attenuation of IL-1 β -induced iNOS gene expression and NO release from these cells, thus further implicating Cav-1 in this signaling cascade. IL-1 β treatment also increased (within 20 min) the translocation of H-Ras into lipid rafts. Based on this evidence we proposed that tyrosine phosphorylation of Cav-1 and subsequent interaction among members of the Ras signaling pathway within the membrane lipid microdomains

represent early signaling mechanisms of IL-1 β in β -cells [60]. It is noteworthy that earlier studies by Di Vizio and associates have proposed potential involvement of palmitoylation of caveolin-1 for its interaction with fatty acid synthase in the lipid rafts [61]. The reader is referred to a recent review by Eisenberg and associates [62] on contributory roles of palmitoylation in subcellular localization and function of Ras. It will be interesting to determine if CER or 2BP-mediated inhibition of iNOS gene expression and associated NO release is due to disruption in the interaction/cross-talk between Caveolin-1 and H-Ras.

More recent observations from our laboratory have suggested novel regulatory roles for phagocyte-like NADPH oxidase [Nox2] in the induction of metabolic dysfunction of the islet β -cell under the duress of glucolipotoxic conditions and exposure to proinflammatory cytokines [63, 64]. In an attempt to further understand the signaling mechanisms underlying the activation of Nox2 and the associated increases in superoxide generation and metabolic dysfunction in β -cells exposed to proinflammatory cytokines, we reported that 2-BP markedly attenuated cytokine-induced, Nox2-mediated ROS generation and iNOS-mediated NO generation. Although it needs to be confirmed experimentally, the observed inhibition of Nox2 activation and associated ROS generation by 2BP may, in part, be due to inhibition of palmitoylation of Rac1 [see above]. Based on these observations we conclude that cytokine-induced metabolic dysfunction of the β -cell involves 2BP-sensitive signaling steps that control iNOS-induced NO release and NOX2-induced ROS generation leading to mitochondrial dysregulation [caspase 3 activation] and apoptotic demise of the islet β -cell. We also conclude that palmitoylation of small G-proteins [e.g., H-Ras, Rac1] may represent an upstream signaling step in the cascade of events leading to the generation of nitrosative and oxidative stress induced by proinflammatory cytokines in the islet β -cell (Fig. 3).

Besides cytokine-induced dysfunction, recent evidence appears to support key roles for protein palmitoylation in free fatty acid [FFA]-induced dysregulation of the islet. For example, using RINm5F cells and normal rat islets, Baldwin and associates have proposed a role for palmitoylation in FFA-induced endoplasmic reticulum stress and β -cell death [69]. Data from their studies suggested concentration-dependent loss in β -cell viability by palmitate, which was attenuated by 2BP. The latter also inhibited incorporation of [³H]palmitate into RINm5F cell proteins. Lastly, 2BP also reduced palmitate-induced inhibition of insulin secretion, ER stress and caspase activation. Based on this evidence they postulated key roles for protein palmitoylation in FFA-induced ER stress activation and loss of β -cell viability [69].

CURRENT & FUTURE DEVELOPMENTS

From the above discussion, at least based on the pharmacological evidence [CER and 2BP], we conclude that palmitoylation plays a significant role in GSIS. Potential pleiotropic effects of the pharmacologic inhibitors remain a significant concern. Furthermore, potential identity of the palmitoylated proteins remains to be a subject of immediate investigation. This is critical, at least for Rac1 since it has been shown to play both positive and negative modulatory

roles in islet function [65]. For example, it has been shown to be involved in cytoskeletal rearrangement, vesicular transport and insulin secretion in clonal β -cells, normal rat islets and human islets [9, 10, 12]. Besides, it has been shown to play regulatory roles in the generation of oxidative stress, mitochondrial dysfunction and demise of the β -cell under the duress of glucotoxicity, lipotoxicity, and exposure to cytokines [63-67]. Additional studies are needed to conclusively demonstrate that palmitoylation of specific G-proteins [e.g., H-Ras, Rac1 and Cdc42] is critical for islet function and insulin secretion under normal physiological conditions [68], and pathophysiological conditions such as glucolipotoxicity and cytokine exposure [63-67].

In addition to proinflammatory cytokines, several recent studies have implicated novel regulatory roles for chemokines as mediators of apoptotic demise of the islet β -cell in diabetes. Chemokines are small molecular weight [~8-10 kDa] glycoproteins that chemo-attracts a variety of cells expressing appropriate GPCRs [70]. Accumulating evidence suggests increased expression of specific chemokines [e.g., CXCL10] in the serum and in tissues of models of autoimmune diseases like Type 1DM [70-72]. Recent studies by Sarkar and coworkers have demonstrated significantly high levels of CXCL10 expression in the islet environment of prediabetic animals as well as Type 1DM humans compared to other chemokines such as CCL5, CCL8, CXCL9 and CX3CL1 [72]. Recent studies by Burke *et al.* have suggested novel mechanisms for the increased expression of the CXCL10 gene by proinflammatory cytokines such as IL-1 β and IFN γ ; these include NF- κ B activation, tyrosine phosphorylation of STAT1 and acetylation of histones H3 and H4 [73]. Studies along these lines by Paroni and associates have identified the Toll like receptor 4 as the cognate receptor for CXCL10-induced cytotoxic effects on the islet β -cell [74]. Despite the compelling evidence on CXCL10-mediated regulation of islet β -cell death, very little is known thus far with regard to potential intracellular down-stream signaling steps, including G-protein activation. This represents a promising area of research in the future.

It should be kept in mind that while the pharmacological data are encouraging, it is necessary to confirm these observations in these model systems using molecular biological approaches [siRNAs and/or adenoviral] to specifically knockdown individual PATs to further deduce regulatory roles for these signaling steps in the onset of metabolic dysfunction of the β -cell. Furthermore, as discussed in the above section, there is a critical need for the development of more specific inhibitors of PATs, especially in light of the known pleiotropic effects of CER and 2BP that could negate other cellular functions. In addition to the development of specific inhibitors for PATs, it may be worthwhile to develop methodologies/probes to augment palmitoyl esterase activities in β -cells under the duress of chronic hyperglycemia, lipidemia or inflammatory cytokine exposure conditions.

Lastly, it is well established that glucotoxicity and lipotoxicity contribute significantly to the metabolic impairment of the islet [75]. Convincing observations from multiple laboratories have provided evidence to suggest significant defects in the transcription of genes [e.g., insulin gene] relevant to islet function. Such defects could come from exces-

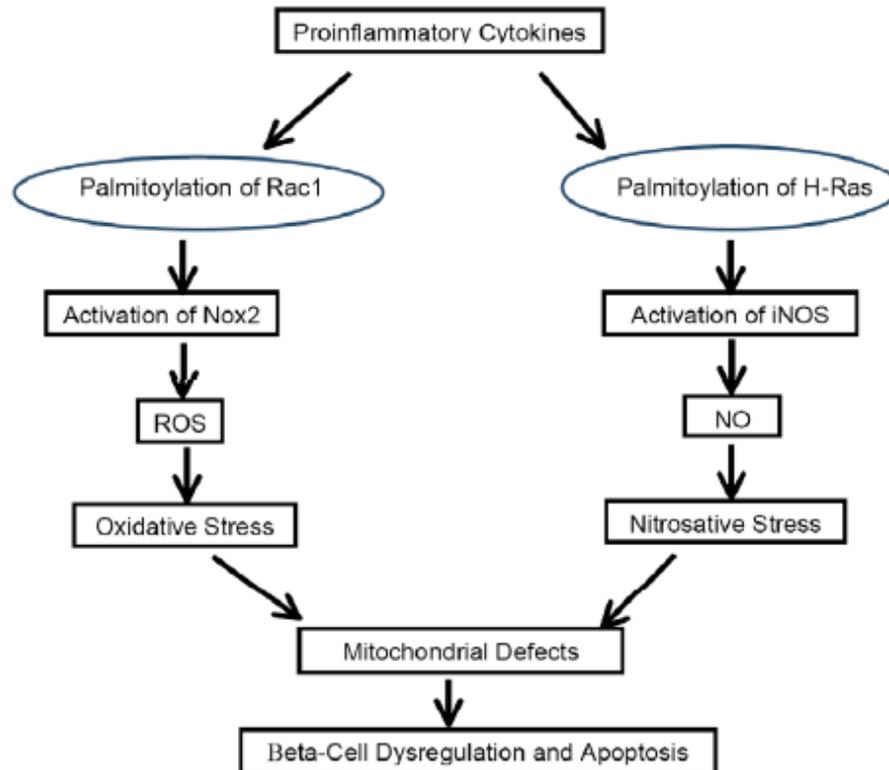


Fig. (3). Model depicting potential regulatory roles for protein palmitoylation in the metabolic dysregulation and demise of the islet β -cell under the duress of exposure to proinflammatory cytokines

Existing evidence suggests that exposure of isolated β -cells to proinflammatory cytokines [e.g., IL-1 β , TNF α and IFN γ] results in a significant increase in the oxidative and nitrosative stress. Increase in oxidative stress may, in part, be due to increased activation of phagocyte-like NADPH oxidase [Nox2]. The holoenzyme assembly and functional activation depends on *transient* activation of Rac1, which has been shown to be mediated via activation of Tiam1, a guanine nucleotide exchange factor for Rac1 [63]. Inhibition of activation of this pathway by NSC23766 has been shown to prevent Nox2 activation and ROS generation [63]. Recent findings from our laboratory demonstrate that cytokine-induced ROS generation is inhibited by 2-BP [64], indicating the role of protein palmitoylation in this signaling cascade. Based on above discussion, it is likely that palmitoylation of Rac1 may be necessary for cytokine-induced Nox2 activation. In addition, cytokine exposure leads to increased nitrosative stress, which is mediated via activation of iNOS. Earlier studies involving radiometric measurements and CER have identified H-Ras as one of the palmitoylated G-proteins involved in cytokine-induced NO release. Requirement for protein palmitoylation in this signaling cascade was confirmed using 2-BP [59]. Based on these observations, we propose that palmitoylation plays a key modulatory role in cytokine-induced β -cell dysregulation and demise. It must be noted that similar palmitoylation-mediated defects in islet β -cell function might be occurring in other pathophysiological conditions such as gluco- and lipotoxicity since increased Tiam1-Rac1-mediated activation of Nox2 and ROS generation have been reported in β -cells under these conditions [65-68]. Nox2: phagocyte-like NADPH oxidase; iNOS: inducible nitric oxide synthetase; ROS: reactive oxygen species; and NO: nitric oxide.

sive palmitoylation of cellular proteins/factors involved in transcriptional regulation of proteins. Along these lines, recent studies by Kang and associates [76] provided compelling evidence to suggest significant reduction in the expression of incretin receptors and associated signaling steps in islets under the duress of elevated glucose and lipids. More importantly, administration of lipid lowering drugs alongside DPP4 inhibitors or GLP-1 agonist to diabetic animals markedly improved glucose tolerance and prevented loss in β -cell mass. Based on these observations, the authors concluded that restoration of circulating levels of lipids to normal levels in T2DM diabetics could significantly augment the therapeutic efficiency of incretin hormones [76]. However, it remains to be seen if alterations [i.e., excessive] in palmitoylation of

candidate proteins under glucolipotoxic conditions contributes to reduced expression of incretin receptor expression.

It should be noted that inhibitors of HMG-CoA reductase [e.g., statins] have been used in many early studies to inhibit protein prenylation [77-80] since mevalonic acid is the precursor for the biosynthesis of farnesyl and geranylgeranyl pyrophosphates, which are incorporated into C-terminal cysteines of candidate proteins by farnesyl and geranylgeranyltransferases (Fig. 2). A growing body of evidence appears to establish a clear link between incident diabetes and statin therapy [81-83]. It is likely that the "diabetogenic" effects of statins may, in part, be due to inhibition of glucose metabolic events [e.g., inhibition of requisite prenylation of G-proteins]

leading to impaired insulin secretion ultimately leading to loss in β -cell viability culminating in the onset of diabetes. In the context of this article, HMG-CoA reductase inhibitors are not expected to inhibit affect islet function via inhibition of protein palmitoylation although it remains to be verified experimentally.

RECENT PATENTS FOR DEVELOPMENT OF PALMITOYLTRANSFERASES TO PREVENT DIABETES AND ITS COMPLICATIONS

It is important to note that a significant amount of work has been carried out in the development of inhibitors for palmitoyltransferases such as carnitine palmitoyltransferases e.g., patent publications USRE41226; USRE40861; US6444701; US6369073 [84] and serine palmitoyltransferases US20080287479; TW200831487; WO2008084300; CA2671138 [85, 86]. Inhibitors of carnitine palmitoyltransferase have been patented for the induction of apoptosis, inhibition of cell proliferation, for the treatment of type 2 diabetes, metabolic syndrome, impaired glucose tolerance, Cushing's disease, cardiovascular disease, hyperglycemia, obesity, hypertension, hyperlipidemia, fatty liver disease, renal failure, diabetes, inflammatory diseases, sepsis syndrome, malaria, Crohn's disease, cachexia, inflammatory bowel diseases, mycobacterial infection, retinitis pigmentosa and especially for the treatment of cancer [87-93]. However, very little has been done in the area of development of small molecule compounds with greater efficacy to inhibit palmitoylation of proteins at cysteine residues of DHHC class of proteins; such compounds could have potential utility including the prevention of loss of β -cell mass, onset of diabetes and its associated complications. In conclusion, we are hopeful that data reviewed in this article will form the basis for future investigations to conclusively demonstrate the therapeutic utility of PAT inhibitors and/or palmitoyltransferase activators in the prevention islet metabolic dysfunction in inflammation and diabetes.

CONFLICT OF INTEREST

A.M.M conducted some of the research work described herein. F.C. contributed to the scientific discussion and collaborated in studies conducted by A.M.M. A.K. contributed to the design, writing and editing of the manuscript. No conflict of interests is declared by the authors. A.K. is an employee of the U.S. Government [Department of Veterans Administration].

ACKNOWLEDGEMENTS

This research was supported in part by a Merit Review award [to AK; 1BX000469] from the Department of Veterans Affairs, Office of Research and Development [Biomedical Laboratory Research and Development], an Innovative Grant from the Juvenile Diabetes Research Foundation [to AK; 5-2012-257], NIH [R01 DK74921 to AK; R01 ES017217 and R01 ES020137 to FC]. AK is also the recipient of a Senior Research Career Scientist Award from the Department of VA. AM is the recipient of George Fuller Endowed Pre-Doctoral Fellowship from the Eugene Applebaum College of Pharmacy and Health Sciences.

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ABSTRACT**MECHANISMS OF CYTOKINE-INDUCED METABOLIC DYSFUNCTION OF THE PANCREATIC BETA-CELL**

by

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August 2013

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Type I diabetes is characterized by an absolute insulin deficiency due to loss of pancreatic β -cell mass by autoimmune aggression. During the progression of the disease proinflammatory cytokines such as IL-1 β , TNF α and INF γ are secreted by infiltrated and activated T-cells and macrophages which ultimately damage the pancreatic β -cell. However, the signaling mechanisms involved in cytokine-induced damage are only partially understood. Phagocyte-like NADPH oxidase [NOX2] has been shown to play regulatory roles in the metabolic dysfunction of the islet β -cell under the duress of glucolipotoxic conditions and exposure to proinflammatory cytokines. However, the precise mechanisms underlying NOX2 activation by these stimuli remain less understood. Herein, I determined some of the putative cellular mechanisms underlying proinflammatory cytokine-induced metabolic dysfunction and demise of the islet β -cell. Some of the novel findings of my study are: [i] cytokines induce ROS generation and oxidative stress *via* activation of phagocyte-like NADPH-oxidase [NOX2] such effects are comprised of Rac1 activation, p47phox phosphorylation, and gp91phox expression. I further confirmed that NOX2 is one of the sources for ROS generation under proinflammatory cytokines and glucotoxic conditions as

demonstrated by activation of NOX2 activity which is sensitive to apocynin under those conditions; [ii] 2-Bromopalmitate, a classic inhibitor of protein palmitoylation, markedly attenuated cytokine-induced Rac1 activation, NOX2-mediated reactive oxygen species generation and inducible nitric oxide synthase-mediated nitric oxide release indicating that palmitoylation of specific G-proteins [e.g., H-Ras and Rac1] is a key regulatory step involved in cytokine-induced nitrosative and oxidative stress.

In addition to oxidative and nitrosative stress, the effect of cytokines in other stress related signaling pathways were also examined. Cytokines activated JNK1/2 and p38 MAPK kinases. They also increased CHOP [C/EBP homologous protein] expression, a marker for endoplasmic reticulum stress, caused caspase-3 activation and FTase and GGTase degradation which leads to defective activation of key G-proteins, defective nuclear membrane assembly and loss in cell viability. Pharmacological inhibitors such as 2-bromopalmitate [inhibitor of palmitoylation], EHop-016 [inhibitor of Vav2-Rac1 axis] and NSC23766 [inhibitor of Tiam1-Rac1 axis] attenuated cytokine-induced JNK1/2 activation implying that Rac1 is upstream to cytokine-induced JNK1/2 activation. Based on the results obtained from my studies, I propose that protein palmitoyl transferase is a novel therapeutic target for the prevention of cytokine-induced metabolic dysfunction of the islet β -cell.

As a logical extension of the *in vitro* studies, a preliminary work has been done in the NOD [non obese diabetic] mice, an animal model of Type I diabetes, and the results showed that both the islets of NOD and control mice express the NOX2 subunits namely, p47^{phox}, p67^{phox}, Rac1 and gp91^{phox}.

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