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Mechanisms of regulation of islet function by nadph oxidase

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MECHANISMS OF REGULATION OF ISLET FUNCTION BY NADPH OXIDASE

by

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DEDICATION

Dedicated to my parents Janab Syed Ahmed and Beejan Sayeeda; my family members and Ms. Jasmine.

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A journey is easier when you travel together. Interdependence is certainly more valuable than independence. This dissertation is the result of four years of work whereby I have been accompanied and supported by many people. It is a pleasant aspect that I have now the opportunity to express my gratitude for all of them.

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LIST OF ABBREVIATIONS

ROS: Reactive oxygen species

DPI: Diphenylene iodonium chloride

Nox: NADPH oxidase

FTI: Farnesyl transferase

GGTI: Geranygeranyl transferase

MPA: Mycophenolic acid

CSA: Cyclosporin A

Ptx: Pertussis toxin

DCHFDA: 2`, 7`- dichlorodihydrofluorescein diacetate

IMP: Inosine monophosphate

MMS: Mono methylsuccinate

KIC: α-keto-isocaproic acid

C2-CER: C2-Ceramide

DHC: Dihydroceramide

FB-1: Fumonisin B-1

GEF: Guanine nucleotide exchange factor

MDA: Malondialdehyde

MMP: Mitochondrial membrane potential

NBT: Nitroblue tetrazolium

PA: Palmitic acid

Rac1: Ras-related C3 botulinum toxin substrate 1

Tiam1: T-lymphoma invasion and metastasis 1

ERK 1/2: extracellular mitogen regulated kinase 1/2

JNK 1/2: c-Jun N-terminal kinase 1/2

PIM: Prodo islet media

T2DM: type 2 diabetes mellitus

ZDF rat: Zucker Diabetic Fatty rat

ZLC rat: Zucker Lean Control rat

INTRODUCTION

According to the 2011 National Diabetes Fact Sheet [released in January, 2011] nearly 26 million children and adults in the United States [~8.3% of the population] have diabetes. In addition, recent estimates [using much stricter guidelines] suggest that ~ 79 million people are prediabetic [American Diabetes Association website; http://www.diabetes.org/diabetes-basics/diabetes-statistics/]. Diabetes is now considered as the fourth leading cause of death by disease in this country. There are two principal forms of diabetes. Type 1 diabetes [previously known as juvenile diabetes] is typically diagnosed in children and young adults. In type 1 diabetes, the body does not produce insulin since the insulin-producing β-cells are lost due to autoimmune aggression-mediated by cytokines released by macrophages infiltrating the islet. In type 2 diabetes, either the body does not produce adequate insulin or the cells that require insulin for glucose uptake become resistant to insulin [i.e., insulin resistance]. Chronic elevation in circulating glucose and lipids leads to diabetic complications, including retinopathy, nephropathy, neuropathy, hypertension and coronary heart disease. Therefore, efforts to understand the pathophysiology of this disease are highly relevant to future developments in care and therapeutics of this disease. In this context, it is becoming increasingly clear that the insulin-producing islet β-cell lesion in type 2 diabetes is, probably, largely functional involving one or more defects in signal transduction. A better understanding of specific changes and defects in the signaling pathways leading to impaired insulin secretion should be useful in designing novel strategies for the prevention and treatment of type 2 diabetes.

ROS signaling and β-cell

Mitochondria being the power house of the cell generate energy through TCA cycle and associated electron transport chain of the inner membrane. During the course of TCA cycle, the reducing equivalents [NADH and FADH2] formed are reoxidized *via* a process that involves transfer of electrons through electron transport chain [ETC] and associated translocation of protons across the mitochondrial inner membrane in generating the transmembrane electrochemical gradient. The generated gradient provides electrochemical potential to synthesize ATP from ADP and Pi. Under physiological conditions, the proton gradient is diminished by H^+ 'leak' to the matrix either *via* protein-lipid interfaces, or by uncoupling proteins [UCPs]. However, due to oxidative phosphorylation mitochondria can generate excessive reactive oxygen species [ROS] and reactive nitrogen species. Superoxide anion [O_2^-] produced as a byproduct of single electron reduction is considered to be the major contributor to other reactive species inside the mitochondrion **[1]**.

Phagocytic cells such as macrophages and neutrophils express plasma membrane/phagosome-associated enzyme complex, the NADPH oxidase [Nox], to facilitate generation of O_2^- for defense against pathogenic organisms. However, it is widely felt now that Nox is not confined only to immune system but alternative isoforms of this holoenzyme may be active in other cell types as an essential component in redox signaling. It is well established that the majority of cells are endowed with antioxidant systems/enzymes such as manganese superoxide dismutase [SOD], glutathione peroxidase, catalase to nullify the excessive intracellular ROS. Even though cells have

a number of antioxidant mechanisms available, it is likely that ROS might disturb the defense homeostasis, resulting in gradual cellular damage. Therefore, any imbalances in host oxidative defense mechanism will lead to cellular dysregulation and death resulting in oxidative stress-related diseases. However, the biochemical and molecular mechanisms that lead to oxidative stress-related defects and diseases remain relatively poorly understood.

The major source for cellular ROS generation is mitochondrial respiration and various oxidoreductases. Superoxide ($\bullet O_2^-$) generated during electron transport chain gets converted to a less reactive H_2O_2 by SOD, and finally to molecular oxygen and water by catalase, peroxiredoxin and glutathione peroxidases. Superoxide may even get converted to HO• by Fenton's reaction or may react with nitric oxide to generate peroxynitrite (ONOO-) which further contributes to additional oxidative stress. Unlike other ROS, H_2O_2 is an ubiquitous intracellular messenger **[2, 3]** as it is stable, uncharged, freely diffusible molecule that can be rapidly generated and degraded in response to external stimuli **[4]**. Even though pancreatic β-cells are equipped with reasonable defense machinery for conversion of $\bullet O_2^-$ to H_2O_2 in cytoplasm and mitochondria **[5]**, H_2O_2 inactivating enzymes like glutathione peroxidase and catalase levels are exceptionally low, approximately 1% of its expression level in the liver **[6]**. Such an imbalance between $\bullet O_2^-$ generation and H_2O_2 inactivating enzymes makes pancreatic β-cell more susceptible to oxidative stress and to H_2O_2 mediated signal transduction. The figure depicted below [taken from Pi and Collins; **7**] further highlights the mechanisms underlying ROS generation and its detoxification by antioxidant machinery.

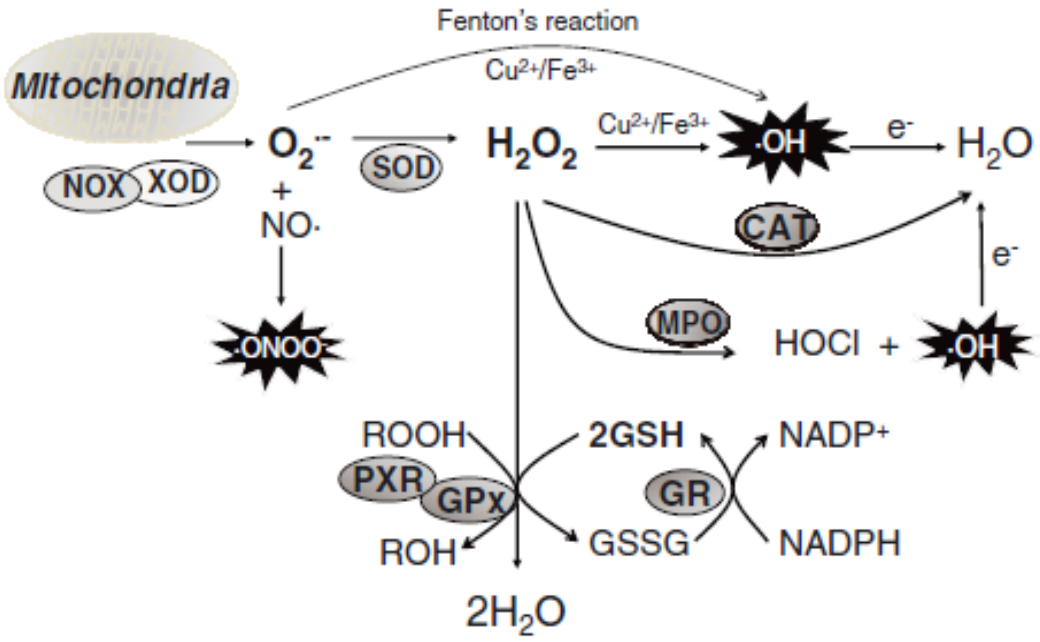


Figure 1: Schematic representation of ROS generated and antioxidant machinery. Oxidases like NADPH oxidase, Nox; or Xanthine oxidase, XOD and mitochondrial respiration are major sources of cellular ROS. Superoxide and its metabolites can be eliminated through several pathways, including superoxide dismutase, glutathione peroxidase, catalase, etc.

Endogenous source of ROS

Superoxides generated during the mitochondrial respiration upon increased substrate or decreased ADP concentration or increased intracellular Ca^{2+} concentration **[8, 9]** have been proposed to be a necessary stimulus for glucose stimulated insulin secretion [GSIS] **[10]**. On the other hand, ROS generated *via* Nox, is known for its role in the immune cell respiratory burst **[11, 12]**. Once activated, Nox takes an electron from donor NADPH and translocate it across the cell membrane to an extracellular O_2

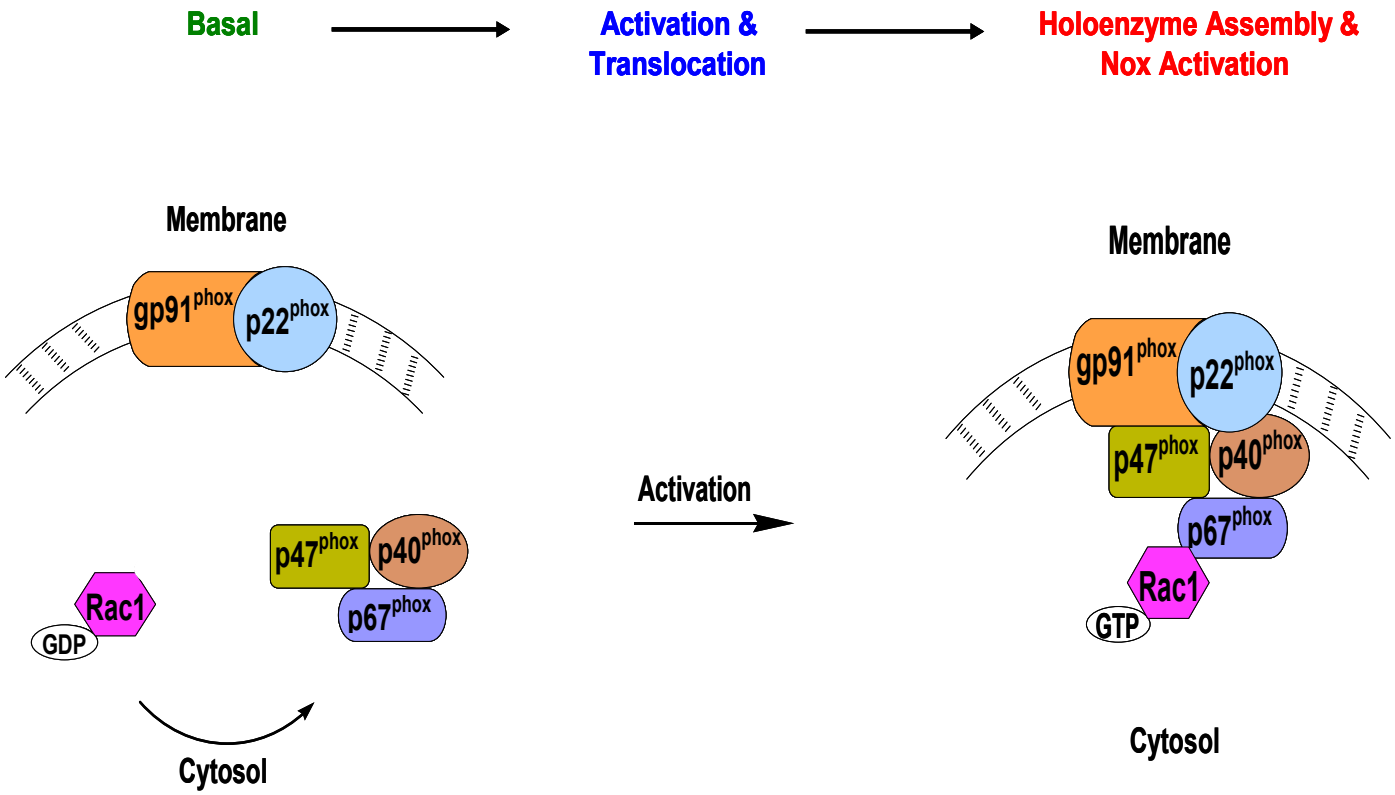
molecule generating $\bullet O_2^-$. Pancreatic islets express multiple Nox isoforms **[13]** which could possibly play a vital role in ROS generation during GSIS **[14]**.

NADPH oxidases represent a group of superoxide-generating enzymes which transport electrons through biological membranes and catalyze the cytosolic NADPH-dependent reduction of molecular oxygen to $O_2^{\bullet-}$ **[15]**. Till date, Nox family comprises of seven members *viz*., Nox1, Nox2, Nox3, Nox4, Nox5, DUOX1 and DUOX2 **[16]**. The members of Nox family differ in their membrane and cytosolic protein components but have a similar ability to produce $O_2^{\bullet-}$. According to structural and functional characteristics, the animal Nox family enzymes can be classified into three groups: (1) the Nox1 to Nox4 group, characterized by the requirement for interaction with $p22^{phox}$; (2) the Nox5 group, characterized by two calcium-binding EF-hand motifs in the cytosolic N-terminal region; and (3) the DUOX group, characterized by EF-hand calcium-binding domain and NH2-terminal peroxidase domain **[17, 18]**.

The Phagocytic Nox is a multicomponent enzyme complex, composed of two membrane components [catalytic glycosylated $gp91^{phox}$ and the regulatory non-glycosylated $p22^{phox}$], three cytosolic proteins [$p47^{phox,}$ $p67^{phox}$, $p40^{phox}$] and a small GTPase ras-related C3 botulinum toxin substrate, Rac 1/2 **[16]**. The catalytic glycosylated $gp91^{phox}$ has six transmembrane domains and is stable only in presence of $p22^{phox}$. These two membranic components stabilize one another in a tightly associated heterodimer, referred to as flavocytochrome b558 **[19]**. Activation of Nox2 requires translocation of cytosolic components to the membrane and association with $gp91^{phox}$ /$p22^{phox}$ complex **[20]**. It is evident that, upon stimulation protein kinase C promotes phosphorylation of the cytosolic $p47^{phox}$, which along with $p67^{phox}$ and $p40^{phox}$ interacts

with membranic flavocytochrome b558. Concomitantly, Rac dissociates from its RhoGDP-dissociation inhibitor and is triggered for exchange of GDP to GTP in regulating the activity of the oxidase by a two-step process, directly *via* contact with gp91^{phox} and by interaction with p67^{phox}. Upon interaction of the cytosolic components with the flavocytochrome b558, the electron transfer from NADH to flavin adenine dinucleotide [FAD] and subsequently to molecular oxygen is regulated by the activation domain of p67^{phox} **[21, 22]**. On the other hand, p40^{phox}, a non-glycosylated cytosolic component, interacts with p47^{phox} and p67^{phox} with a 1:1:1 stoichiometry **[23]**.

Figure 2:



Furthermore, Nox1 was the first homologue of $gp91^{phox}$ (Nox2) to be described and it might require small GTPase Rac in its activation **[24, 25, 26]**. Nox4, another homologue of $gp91^{phox}$ exhibits about 39% homology to Nox2 **[27]**. A characteristic feature of Nox4 is its high level of constitutive production of hydrogen peroxide, which is partially dependent on the presence of $p22^{phox}$ **[28, 29]**. In contrast to Nox1, 2 and 3, Nox4 is a constitutively active enzyme and is activated without the necessity for GTPase Rac or the cytosolic components **[29]**, and is regulated by protein expression level **[30]**. It has been demonstrated recently that Nox4 regulates cell differentiation in human and mouse pre-adipocytes suggesting a role for H_2O_2 production in adipocyte differentiation and maturation **[31, 32, 33]**.

In addition, the other endogenous sources of ROS consist of some cytochrome P-450 (CYP) enzymes and flavoproteins in endoplasmic reticulum, lipoxygenases, isoforms of nitric oxide synthase and prostaglandin synthase on plasma membrane, a diverse group of oxidases and flavoproteins in peroxisomes and Xanthine oxidases in cytoplasm. However, Nox-derived ROS and its implicated biochemistry in modifying the function of β-cell remain elusive. Despite the fact that Nox4 expression has been reported in pancreatic islets **[4]**, its role in β-cell has yet to be determined. Recently, mRNA for NADPH oxidase components Nox1, Nox2 and Nox4, and protein for NADPH oxidase subunits NoxA1, $p22^{phox}$, $p47^{phox}$ and $p67^{phox}$, have been detected in human and rat pancreatic stellate cells **[34, 35]**. The physiological role of Nox enzymes in pancreatic islets is poorly understood. On the basis of studies using the selective flavoprotein inhibitor DPI and the antioxidant/Nox inhibitor apocynin **[36]**, a role for Nox enzymes in insulin secretion **[13, 37, 38, 39]** has been suggested. Existing evidence

also suggests that a protein kinase C sensitive phosphorylation of $p47^{phox}$ is critical in its translocation to the membrane compartment. Further, recent data suggest that functional activation of Rac is important in the holoenzyme assembly and activation of Nox unit **[40, 41, 42]**.

G-proteins and pancreatic β-cell

Insulin secretion from the pancreatic β-cells is regulated by ambient concentrations of glucose. In pancreatic β-cells, GSIS is mediated largely through generation of soluble secondary messengers [cyclic nucleotides, hydrolytic products generated by phospholipases A2, C and D] **[43 - 55]** and changes in the intracellular calcium concentrations. These changes in intracellular calcium regulate various enzyme activities within the cell, including protein kinases, phosphodiesterases, adenylyl cyclases, and PLases **[44 - 61]** in insulin secretion. Even though many studies have shown the underlying mechanism[s] involved in stimulus-secretion coupling of GSIS, the precise molecular and cellular mechanism still remains profound. In addition, adenine nucleotides [e.g., ATP] and guanine nucleotides [e.g., GTP; **[62 - 65]**] regulate physiological insulin secretion.

Classification of G-proteins in β-cell

Till date three major classes of G-proteins have been identified in pancreatic β-cells **[66 - 70]**. The first class of G-proteins are heterotrimeric G-proteins, which are involved in coupling membrane-associated receptors to their intracellular effectors such as PLases, adenylyl cyclases, ion channels, and phosphodiesterases **[71 - 73]**. The

second class of G-proteins comprise of small monomeric G-proteins [17-30 kDa], which are ought to play a vital role in protein organization and trafficking of secretory vesicles in different cell types **[74]**. These small G-proteins undergo posttranslational modifications [isoprenylation and methylation] at their C-terminal residues (*CAAX* motif) **[74 - 78]** for their active confirmation. The third class of G-proteins are the elongation factors and Tau proteins, and are implicated in protein synthesis.

Posttranslational Modifications

The γ-subunits of the heterotrimeric G-proteins and most of the small G-proteins undergo a sequence of posttranslational alterations at their C-terminal (CAAX motifs, where C represents cysteine, A = aliphatic amino acid and X = terminal amino acid) **[67]** to attain the active confirmation. Incorporation of either a farnesyl [15 carbon derivative of mevalonic acid] or geranylgeranyl [20 carbon derivative of mevalonic acid] group to the C-terminal cysteine of proteins *via* a thioether linkage is the first step involved in the posttranslational modifications. Subsequently, the carboxylate anion of the prenylated cysteine is exposed once three amino acids after farnesylated/ geranylgeranylated cysteine are excised by Ras-converting enzyme 1 [Rec-1] mediated proteolysis **[67, 79]**. The exposed site is then methylated by the isoprenylcysteine-*O*-carboxyl methyltransferase. Several laboratories including ours have confirmed that carboxymethylation [CML] amplifies the hydrophobicity of the candidate protein. Besides these modifications, certain G-proteins undergo fatty acylation or palmitoylation at cysteine residues to have a firm anchoring into the cell membrane for optimal interaction of the candidate protein with their respective effectors **[67, 79, 80]**.

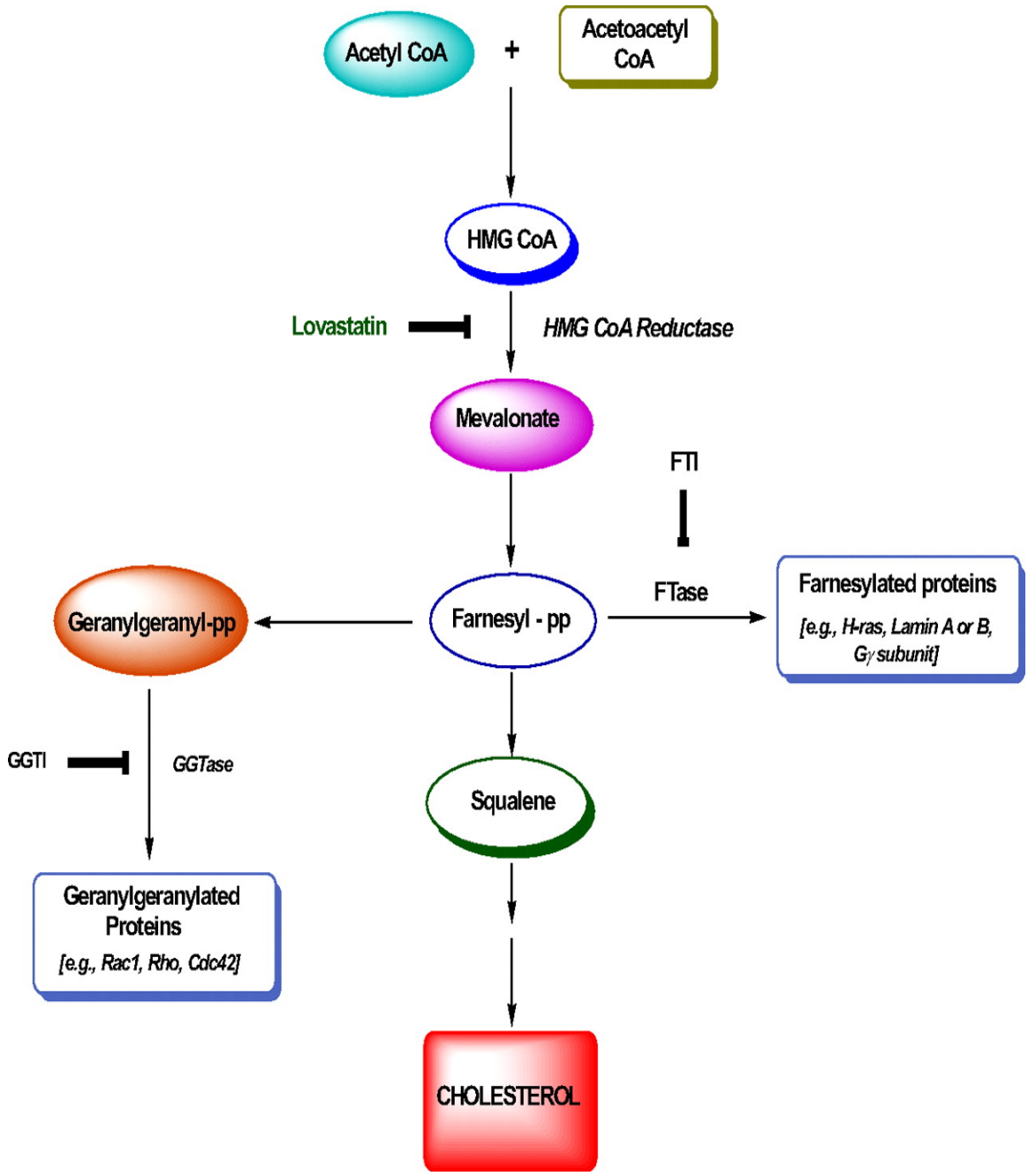


Figure 3: The above figure depicts the formation of farnesyl and geranylgeranyl pyrophosphates. Acetyl-CoA condenses with acetoacetyl-CoA to produce 3-hydroxy-3-methylglutaryl-CoA [HMG-CoA], which is catalyzed by HMG-CoA synthetase. In

presence of NADPH, HMG-CoA gets reduced to mevalonic acid [MVA] by HMG-CoA reductase. This reaction occurs in cytosol and is considered to be a rate limiting step in cholesterol biosynthesis. The generated MVA serves as a precursor for biosynthesis of farnesyl pyrophosphate (farnesyl-pp) and geranylgeranyl pyrophosphate (geranylgeranyl-pp). [Taken from Kowluru, Endocr Rev. 2010].

Both the farnesylated and geranylgeranylated groups are derived from acetyl-coenzyme-A and acetoacetyl-CoA of the cholesterol biosynthetic pathway **(Figure 3)**. These are integrated in the candidate protein by farnesyl transferases [FTases] and geranylgeranyl transferases [GGTases] respectively. Examples of farnesylated proteins include Ras, nuclear lamins A/B, and the γ-subunits of heterotrimeric G-proteins, whereas, geranylgeranylated proteins include Cdc42, Rac1 and Rho.

Till date three distinct prenylating enzymes *i.e.,* FTase, GGTase-I and GGTase-II have been depicted in the literature **[75 -78]** and are heterodimeric in nature consisting of α- and β-subunits. FTase and GGTase-I are considered as *CAAX* prenyl transferases as their substrate proteins share a preserved *CAAX* motif at their C-terminal region, whereas, GGTase-II is considered as a non-*CAAX* prenyl transferase as it prenylates the Rab subfamily of G-proteins at a different motif **[75 -78]**. Both the FTase and GGTase-I share a common regulatory α-subunit but have distinct substrate specific β-subunit. Krzysiak et al., explains that peptides having serine, glycine and methionine on their “X” residues of *CAAX* motifs are farnesylated, whereas, those bearing leucine are geranylgeranylated **[81]**. However, it has been shown that RhoB with a C-terminal CKVL CAAX box can be prenylated by either FTase or GGTase-I in mammalian cells

[82, 83]. Furthermore, involvement of this signaling cascade through the use of pharmacological inhibitors has been shown to play a vital role in GSIS in isolated β-cells **[79]**. Available evidence also shows the regulatory roles for carboxymethylation and palmitoylation in G-protein mediated effects on GSIS in pancreatic β-cells **[**see a recent review Kowluru, **84]**.

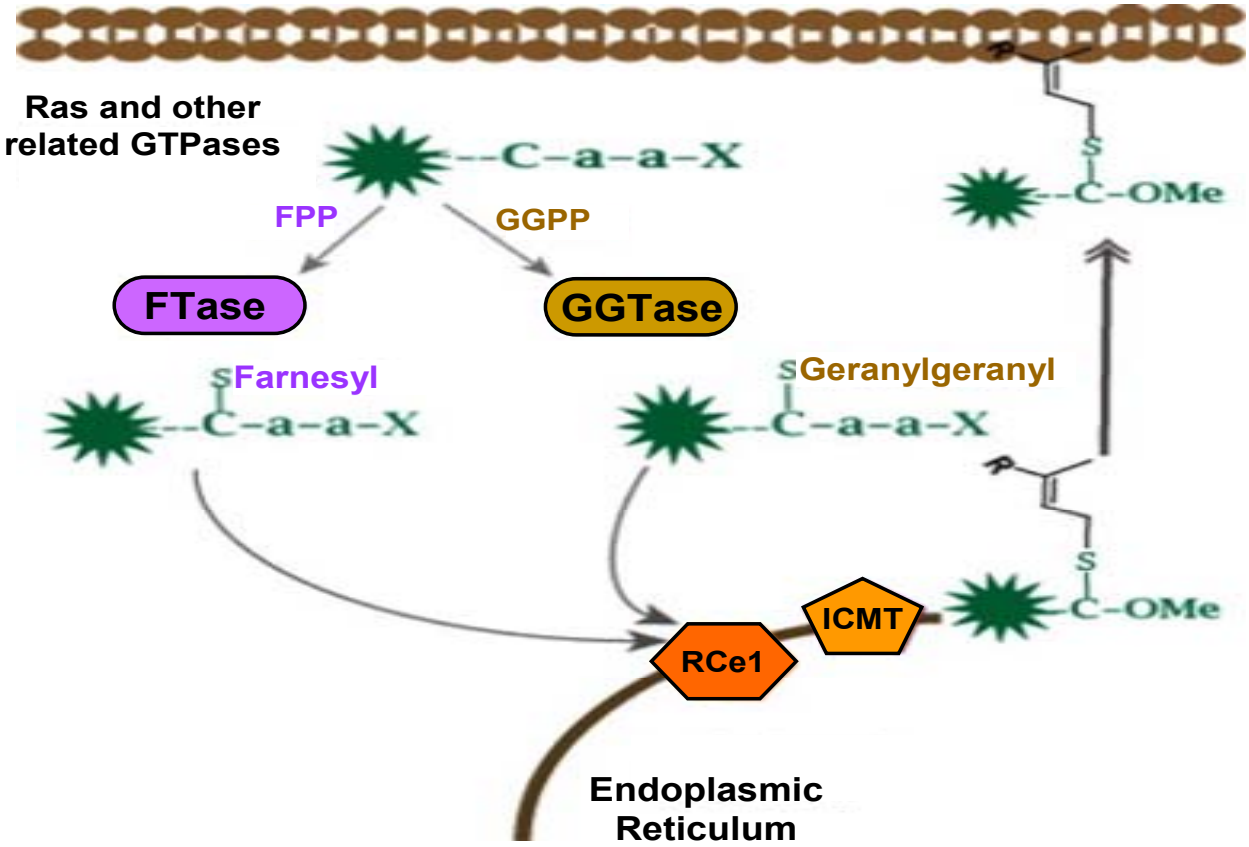


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Figure 4: The above figure explains prenylation i.e., either farnesylation by FTase or geranylgeranylation by GGTase-I of Ras and other-related proteins and consequential events.

Small G-proteins

Based on the substantial evidences on the regulation of pancreatic islet β-cell function, small G-proteins are categorized into three major groups. Rho, Rac1, Cdc42 and ADP-ribosylation factor-6 [Arf6] fall under the first category of small G-proteins and these play an important role in cytoskeletal remodeling and vesicular fusion **[85 - 102]**. The second category of small G-proteins comprises of Rap1 and Rab GTPases (Rab3A and Rab27) **[**see a recent review from Kowluru, **84]**. These Rab GTPases assists in priming and docking of insulin-laden secretory granules on the plasma membrane **[**see a recent review from Kowluru, **84]**. Unlike first category of small G-proteins, requisite for posttranslational modifications and mechanism[s] involved in the activation of Rab GTPases under the physiological insulin secretagogues remains elusive. However, Rap1 has been shown to get activated transiently by glucose **[66]** and undergoes carboxymethylation which is augmented by glucose and KCl **[66, 103]**. The third group of small G-proteins consists of Rab2, Rhes and Rem2 which are under-studied **[104 - 107]**, whereas, RalA appears to draw direct regulatory effects in exocytosis **[108]**.

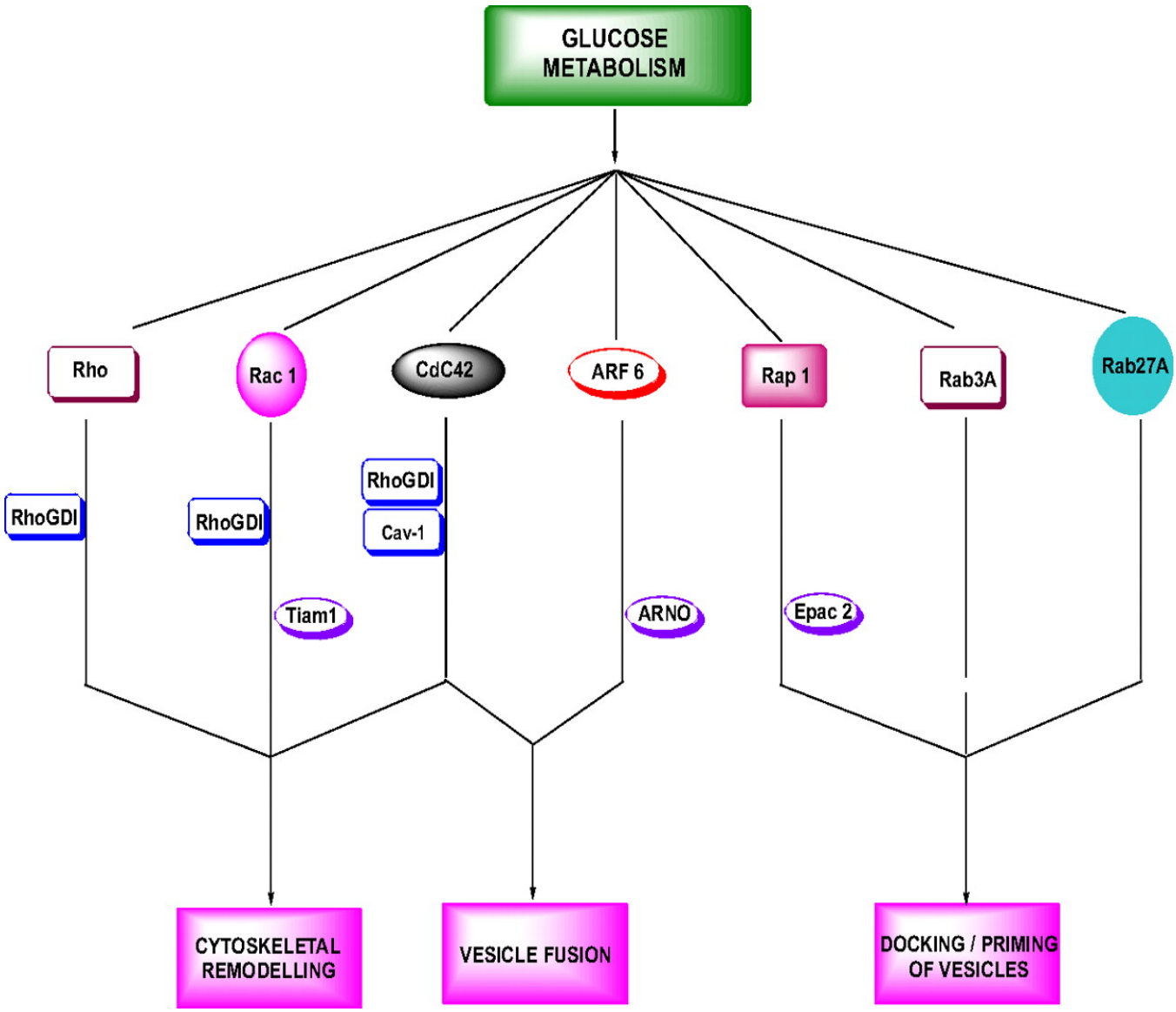


Figure 5: The above figure shows the functions of different classes of small G-proteins in the physiology of insulin secretion in pancreatic β-cells. [taken from Kowluru, Endocr Rev. 2010].

Activation and deactivation cycle of small G-proteins

Like heterotrimeric G-proteins, small G-proteins also shuttle between their inactive (GDP-bound) and active (GTP-bound) conformations, which are tightly regulated by various G-protein regulatory factors/proteins. Till date, three such types of regulatory factors have been identified for small G-proteins, *viz.,* guanine exchange nucleotide factors [GEFs], GDP-dissociation inhibitors [GDIs] and GTPase-activating proteins [GAPs]. GEFs facilitate the translation of the inactive GDP-bound forms of small G-proteins to their active GTP-bound forms, while, the GDIs avert the dissociation of GDP from the G-proteins, thereby keeping them in the inactive conformation. The final group of these regulatory factors for small G-proteins, GAPs, converts the active GTP-bound to their inactive GDP-bound conformation in the GTP hydrolytic cycle by inactivating the intrinsic GTPase activity of the candidate G-proteins.

The efficiency of the G-protein activation cascade *via* a GTPase depends on the relative amounts of active to inactive GTPase. The activity of GTPase can be altered either by accelerating GDP dissociation by GEFs or by inhibiting GDP dissociation by GDIs, or by accelerating GTP hydrolysis by GAPs. The figure below depicts the functions of each regulatory factors/proteins in the activation and deactivation cycle of the G-proteins. Any imbalance in either of the regulatory factors distorts the hydrolytic cycle and normal physiological functions in pancreatic β-cells.

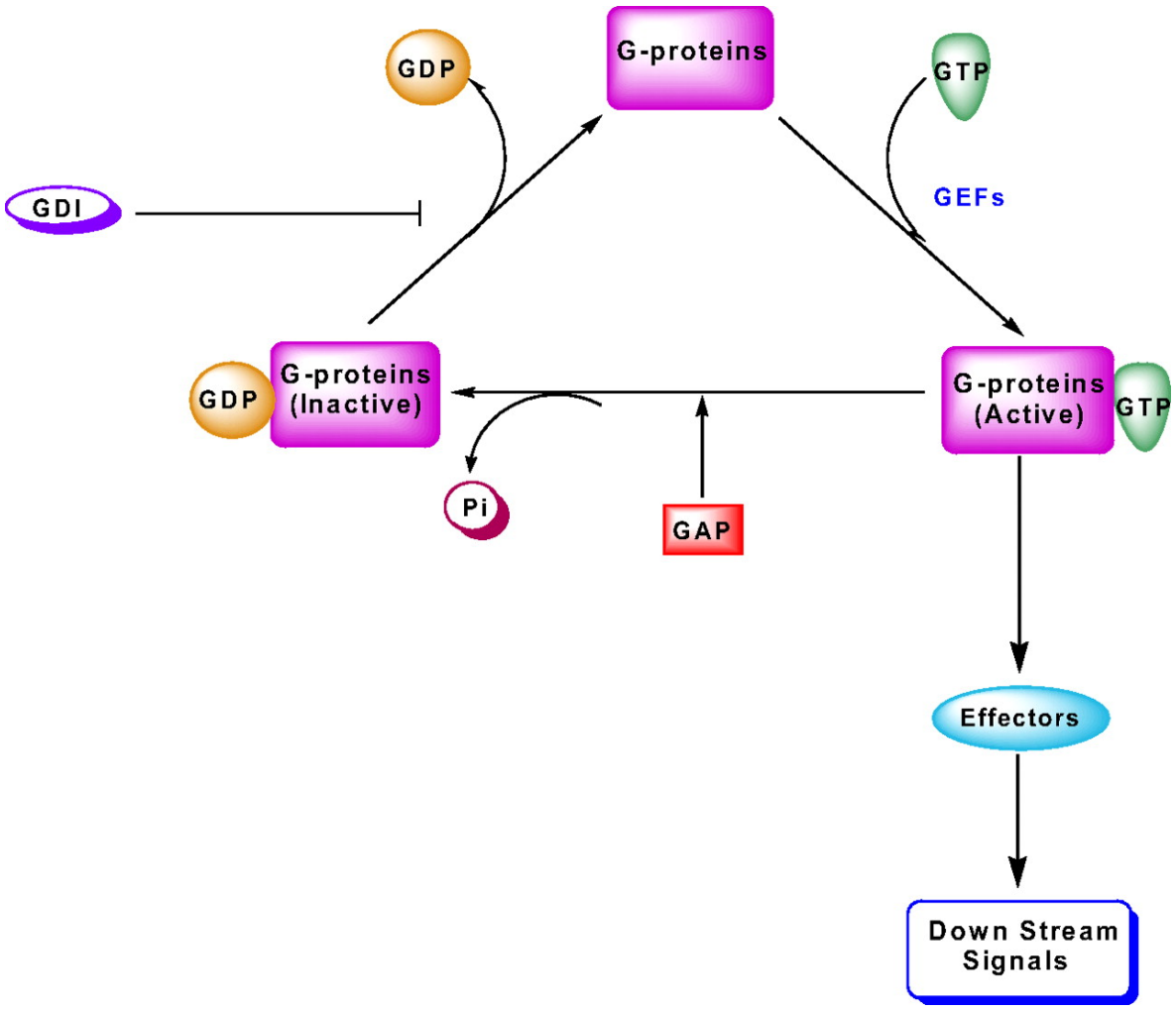


Figure 6: Regulatory factors/proteins involved in activation and deactivation cascade of G-proteins. [taken from Kowluru, Endocr Rev. 2010].

Small G-proteins and insulin secretion

Small G-proteins have been recognized as key regulatory molecules in vesicle trafficking and organelle dynamics coupled with proliferation, survival and demise of a cell. Recycling between GDP/GTP-bound forms, small G-proteins are coupled with their translocation between cytosol and membrane in carrying out their mechanistic roles. Published evidence from multiple laboratories has implicated small G-proteins [Cdc42, Rac1 and Arf6] in insulin secretion in clonal and islet β-cells.

A] Role of Cdc42 in insulin secretion

Regazzi and associates first reported **[87]** expression of Cdc42 in insulin-secreting clonal β-cells [RINm5F and HIT-T15 cells]. Follow-up studies from other laboratories have demonstrated localization of Cdc42 in clonal pancreatic β-cells, normal rat islets and human islets **[66, 88]**. Furthermore, Cdc42 remains associated with Rho-GDI in the cytosol and upon exposure to prenylation inhibitor prevented the association, substantiating the importance of posttranslational modification in functional regulation of Cdc42 **[87]**. It has also been demonstrated that, glucose-induced carboxymethylation of Cdc42 results in its translocation to the membranic fraction and the effect is very rapid and transient **[66]**. A series of recent investigations from Thurmond's laboratory have further substantiated novel regulatory roles for Cdc42 in islet function, including actin remodeling and insulin secretion **[91]**.

B] Role of Rac1 in insulin secretion

Like other small G-proteins, Rac also shuttles between inactive GDP and active GTP conformations to facilitate cellular function. Rac was first identified and implicated in cellular function by Didsbury et al **[109]**, where they isolated two cDNAs encoding proteins [Rac1 and Rac2] with ~92% homology. Interestingly, both Rac1 and Rac2 undergo ADP ribosylation by C3 component of botulinum toxin before their association with membrane. Potential role for Rac1 in GSIS was first demonstrated by using *Clostridium difficile* toxins A and B, which irreversibly monoglucosylate and inactivate specific G-proteins (Cdc42 and Rac1) **[86]**. Like Cdc42, Rac1 also undergoes posttranslational carboxymethylation and membrane translocation in the presence of stimulatory glucose concentrations **[86]**. Expression of an inactive mutant of Rac1 (N17Rac1) in INS-1 cells resulted in significant morphological changes including alterations in F-actin structures, leading to inhibition of GSIS. These findings confirmed the involvement of Rac1 in cytoskeletal remodeling and reorganization **[95]**. As stated above, Rac1 also requires prenylation for its function. Experiments involving pharmacological and molecular biological inhibition of Rac1 prenylation indicated marked reduction in GSIS in a variety of insulin-secreting β-cells. For an instance, GGTI-2147, a specific inhibitor for geranylgeranylation, significantly augmented accumulation of Rac1 in cytosol and inhibited GSIS in INS 832/13 cells. Over expression of the regulatory α-subunit of protein prenyltransferase also attenuated glucose-induced insulin secretion in INS 832/13 cells **[85]**. Furthermore, Rac1-null mice [βRac1−/−] exhibited impaired glucose tolerance and hypoinsulinemia, suggesting key

regulatory roles for Rac1 in normal insulin function **[97]**. Taken together, all these evidence suggests regulatory roles of Rac1 in islet function including GSIS.

i] Rac1-Nox signaling and insulin secretion

Recent evidence suggests that a tonic increase in generation is necessary for GSIS **[7, 110 - 113]**]. Rac1 being a cytosolic component of Nox holoenzyme, its functional activation [Rac1.GTP] has shown to be critical in holoenzyme assembly and activation of Nox **[16, 40 - 42, 114, 115]**. In support of this, Gorzalczany and associates **[116]** have shown the activation of Nox and subsequent generation of ROS by targeting Rac1 to the membrane fraction. They also demonstrated that its prenylated, but not the unprenylated, form of Rac1 binds to the phagocyte membrane more efficiently to facilitate the generation of superoxides. Along these lines, Pi and Collins have recently overviewed the existing evidence in supporting "secondary messenger" roles of ROS in physiological insulin secretion **[7]**. Based on this and other supporting evidence, it is concluded that a tonic increase in intracellular ROS is necessary for normal physiological insulin secretion and that Rac1 initiates subsequent signaling steps including Nox activation and insulin release **[**refer to a recent review from Kowluru, **117]**.

ii] Rac1-Nox signaling and metabolic dysfunction

Even though Rac1 has been shown to have positive modulatory effects in the normal cellular function, a growing body of evidence also implicates negative modulatory roles for Rac1 in the induction of metabolic dysfunction cells, particularly at the level of its activation of Nox holoenzyme **[118, 119]**. For example, a significant increase in Nox-mediated oxidative stress and caspase-3 activation was observed in

retinal pericytes, which was attenuated by overexpressing dominant-negative mutants of Rac1 and $p67^{phox}$. Furthermore, overexpression of constitutively active mutant of Rac1 [V12Rac1] augmented Nox and caspase-3 activation, thereby creating more oxidative stress environment and causing metabolic dysfunction of the retinal pericytes. Studies by Shen and associates in cardiac myocytes **[120]** have also suggested regulatory roles for Rac1 in the activation of Nox and associated generation of ROS in animal models of diabetes. However, very little is known thus far with regard to regulatory roles of Rac1 in the holoenzyme assembly and activation of Nox in islet β-cells following chronic exposure to glucose, saturated fatty acids or cytokines. This is the basis of studies that I have carried out for my doctoral work and the data from these studies are described in the following sections.

Pancreatic β-cells and Glucotoxicity

Several lines of evidence in *in vitro* and *in vivo* model systems clearly suggest that chronic exposure of the islet β-cell to elevated glucose concentrations leads to significant alterations in the function ultimately leading to cell demise *via* apoptosis and the onset of diabetes.

Glucose toxicity: Robertson and associates have defined glucose toxicity as non-physiological and potentially irreversible β-cell damage upon chronic exposure to supra-physiological concentrations of glucose. This is characterized by early defective insulin gene expression **[121, 122]** and a state of desensitization, which is referred to as a transient state of cellular refractoriness to glucose stimulation induced by repeated or prolonged exposure to elevated glucose levels. Interestingly, the later is reversed, in time-dependent fashion, usually within minutes following restoration of normal glucose levels. Glucose toxicity also affects other vital signaling steps including suppression of glucokinase gene expression, decreased mitochondrial function, compromised exocytotic mechanisms and accelerated apoptosis, impending from insulin gene expression to insulin release into the circulation **[123 - 125]**.

Pancreatic β-cell and oxidative stress

It has been shown that reactive oxygen species [ROS] are the basic ignition factors in the pathogenesis of diabetes and more importantly in the development of secondary complications. However, generation of ROS such as superoxide anion ($O_2^{\bullet -}$), hydroxyl radicals (•OH), hydrogen peroxide (H_2O_2) and associated generation of nitric

oxide (NO) **[126, 127]** have been implicated in pancreatic β-cell dysfunction and demise in both type I **[128]** and type II **[129]** diabetes. Generation of superoxide radical [$O_2^{\bullet-}$] was first demonstrated in insulin-producing cell lines by cytochrome c reduction method **[130]**.

Multiple lines of evidence in both *in vitro* and *in vivo* have confirmed continuous generation of free radicals under the persistent hyperglycemic state during diabetes **[131 - 134]**. Further, inhibition of ROS production using specific inhibitors of electron chain complex II, or by upregulating the expression of uncoupling protein-1 and mitochondrial SOD [MnSOD], confirmed the involvement of ROS in the complications of diabetes like retinopathy, neuropathy and nephropathy **[135]**. Many biochemical pathways like hexosamine pathway, polyol pathway, advanced glycation end products [AGEs] pathway and protein kinase-C pathway are activated under the conditions of excessive ROS generation during hyperglycemia.

In the hexosamine biosynthetic pathway, fructose-6-phosphate gets converted to N-acetylglucosamine-6-phosphate, which, in turn, is metabolized to N-acetylglucosamine-1, 6-phosphate and to uridine diphosphate (UDP)-GlcNAc. Newly formed UDP-Glc-NAc serves as a substrate for O-glycosylation of many cytosolic and nuclear proteins together with transcription factors involved in signal transduction, resulting in impairment of the activation of insulin receptor/substrates/PIK3 kinase survival pathways **[136]**. Along these lines Kaneto et al have shown that under the conditions of hyperglycemia, glucosamine increases H_2O_2 levels in the isolated rat islet β-cells causing oxidative stress and β-cell dysfunction **[137]**, which was reversed in the

presence of antioxidants like N-acetyl-l-cysteine. The polyol pathway is also triggered by elevated intracellular glucose, which is converted to sorbitol in the presence of aldose reductase (AR). In this signaling system, AR utilizes nicotinamide adenine dinucleotide phosphate (NADPH) as a cofactor for regenerating a critical intracellular pool of reduced glutathione, thereby increasing the cytosolic NADH:NAD+ ratio **[138]**. Consequently, a loss in the levels of reduced glutathione increases the vulnerability of β-cell to intracellular oxidative stress **[138]**. Along these lines, it has also been shown that AGEs produced as a result of the non-enzymatic protein glycation, and increased production of diacylglycerol [DAG] *via* protein kinase-C activation under hyperglycemic conditions, stimulate ROS generation and promote oxidative stress.

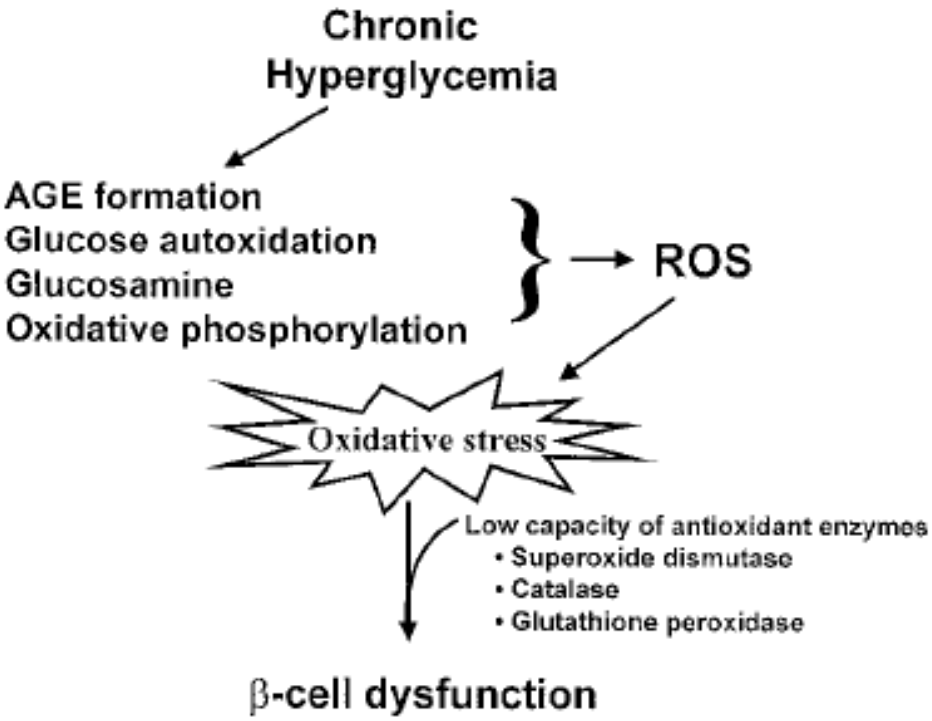


Figure 7: Diagramatic representation of different pathways which are sensitive to elevated glucose and generate ROS causing oxidative stress and ultimate pancreatic β-cell dysfunction [taken from Robertson et al., Diabetes Vol 52, 2003].

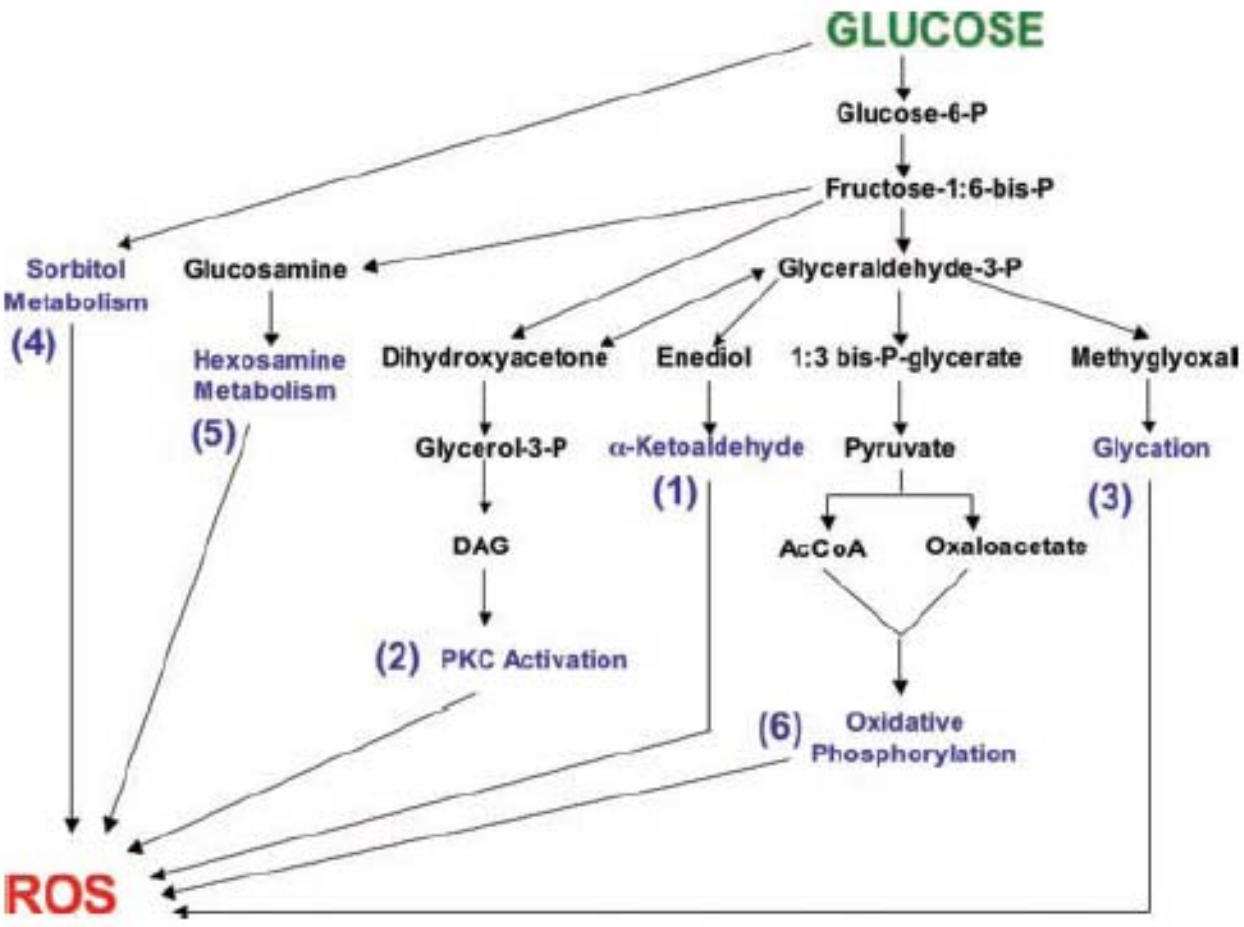


Figure 8: This figure [taken from Robertson, JBC, 2004] further depicts different pathways in glucose metabolism for the generation of ROS to induce oxidative stress leading to pancreatic β-cell dysfunction.

In summary, it is evident from the above discussion that chronic exposure of cells, including islet β-cells, to glucose leads to increased oxidative stress. Data from antioxidant studies are encouraging. Unfortunately, unlike the majority of cell types, the islet β-cell has an extremely low antioxidant capacity due to significantly low levels of antioxidant enzymes, thus making them vulnerable to oxidative damage and demise. In addition, relatively little is known with regard to roles of Nox as a potential source of ROS and oxidative stress in the islet β-cell. Therefore, I propose studies in the following sections to systematically evaluate this and to explore the underlying mechanisms involved in the regulation of Nox under the duress of glucotoxicity.

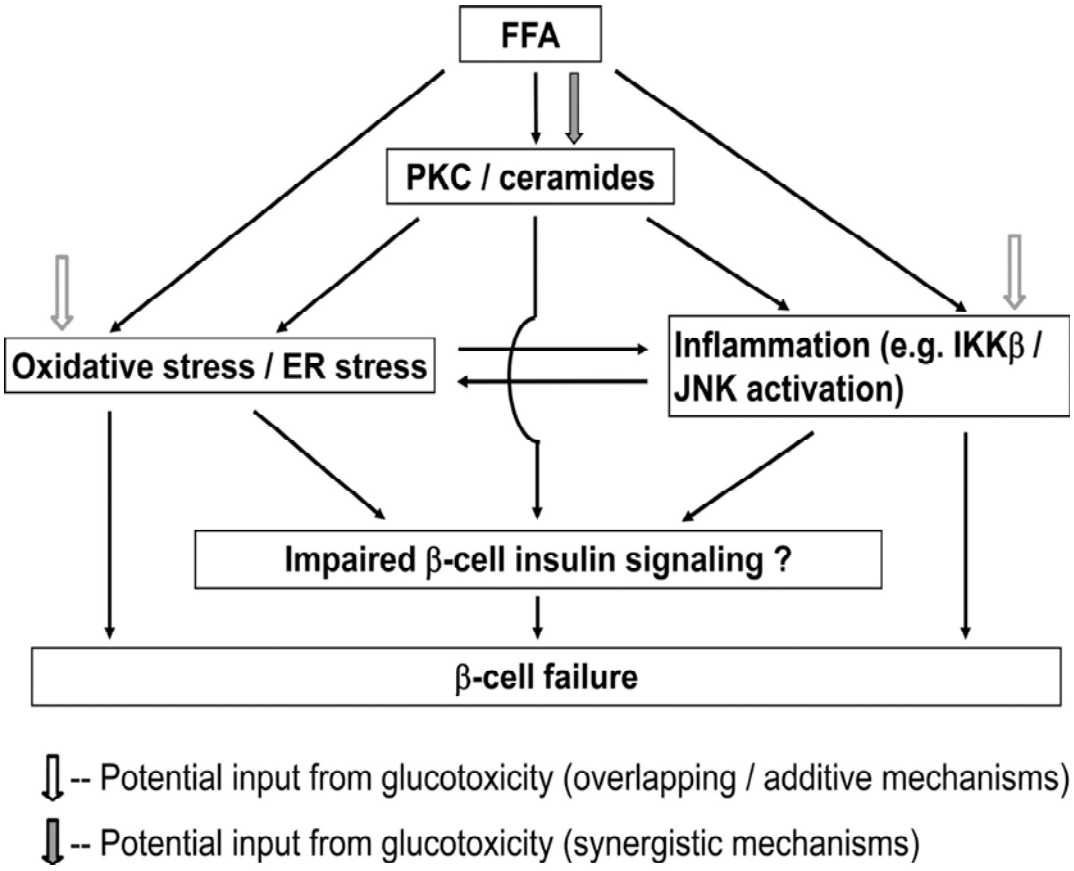
Pancreatic β-cells and free fatty acids

In addition to hyperglycemia, type II diabetic patients often have elevated free fatty acids (FFAs). Even though the characteristic increase in basal insulin secretion with reduced GSIS is well demonstrated following chronic exposure of pancreatic β-cells to high levels of FFAs, the precise mechanisms linking FFAs to dysregulation of β-cell function remain elusive **[139 - 142]**. In this context, it has been reported that short-term exposure of pancreatic β-cells to FFAs results in an increase of insulin secretion **[143 - 145]**, whereas, long-term exposure leads to attenuation of insulin secretion **[146, 147]**. In the presence of high glucose, short-term exposure of FFAs synergistically increases the insulin secretion as a result of accumulation of long chain CoA [LC-CoA] in the cytosolic fractions **[148]** due to malonyl-CoA inhibition of carnitine palmitoyl transferase I. The excessive formation of LC-CoA and several other lipid complexes which are

critical effectors of insulin secretion during inhibition of fatty acid oxidation induces protein kinase C activation, protein acylation **[149]**, calcium influx **[144, 150]**.

It is well established that high plasma levels of saturated fatty acids [e.g., palmitic acid] promote abnormal islet function and type II diabetes. Such effect has been proven to associate with increase lipid esterification, production of ceramides [see Figure 10], and oxidative stress **[151]**, This, in turn, results in increased lipid peroxidation leading to defective functions of proteins and DNA **[152]**. In addition, stimulating effects of FFAs on ROS generation has been demonstrated in several cell types suggesting that FFAs and their derivatives may modulate the cell function by increasing oxidative stress intracellularly **[153 - 155]**.

It has also been shown recently that elevated FFAs, over an extended period of time, cause damage to cells by a variety of mechanisms, and oxidative stress being the common link among all **[156, 157]**. Although, adverse effects of elevated FFAs on insulin secretion and the mechanism[s] underlying have been reported *in vitro* **[158]**, the *in vivo* translational impact of FFA supplementation on pancreatic islet function is far from being clear. In this context, based on the currently available information in *in vitro* and *in vivo* model systems, Giacca et al have summarized the mechanisms of lipotoxicity and glucolipotoxicity [Figure 9].



Giacca A et al. Am J Physiol Endocrinol Metab 2011;300:E255-E262

Figure 9: *In vivo* studies *via* lipid infusion or high-fat diet in animals and humans have shown to decrease pancreatic β-cell function and mass. The mechanistic insights on FFA leading to oxidative stress **[142, 159, 160]** / ER stress **[161]** / Inflammation **[162 - 165]** resulting in β-cell failure has been reviewed by Giacca et al. Also depicted in the figure are the potential inputs from glucotoxicity which cannot be separated from lipotoxicity (i.e., glucolipotoxicity) in animal models of Type II diabetes or Type II diabetic humans. In addition the role of ceramides in glucolipotoxicity has been explained by Shimabukuro et al **[166]**.

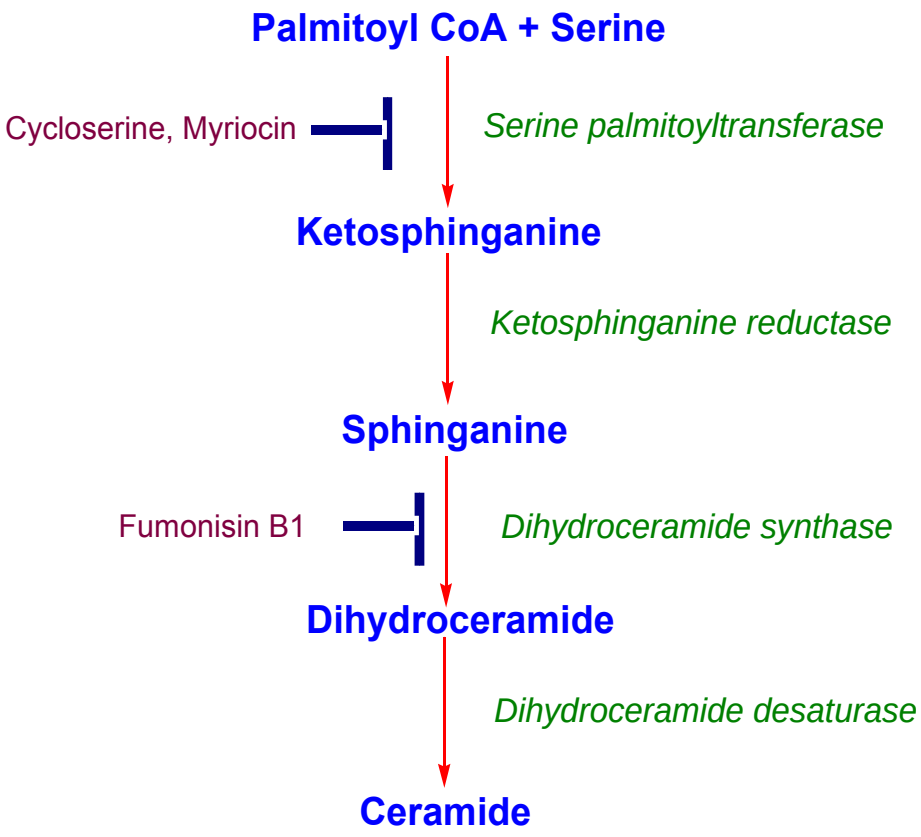


Figure 10: The above figure explains the *de novo* biosynthesis of ceramides from palmitic acid [taken from Kowluru 2010, Endocrine Reviews].

In summary, available evidence clearly suggests that saturated fatty acids exert deleterious effects on pancreatic β-cells at multiple levels. It also appears that generation of excessive ROS leads to increased oxidative stress culminating in mitochondrial dysfunction. These observations prompt further investigations to determine relative regulatory roles of Nox in the signaling mechanisms leading to palmitate or ceramide-induced metabolic dysfunction of the islet β-cell. I propose studies in the following sections to address this question.

Pancreatic β-cells and cytokines

Proinflammatory cytokines [e.g., IL-1β, TNFα and IFNγ] play a vital role in pancreatic β-cell dysfunction and in the development of both type I and II diabetes. Emerging evidence also suggests that high glucose levels can augment cytokine production in pancreatic islets **[124]**. Therefore, excessive cytokine levels not only exert damaging effects on β-cells in the pathogenesis of type I diabetes, but also contribute to the progressive decline in β-cell function typical feature of type II diabetes, as they promote accelerated β-cell apoptosis and demise.

During the progression of the disease, proinflammatory cytokines are released into islets of Langerhans by infiltrated, activated T-cells and macrophages **[167 - 169]**. However, the exact cellular mechanisms by which cytokines induce β-cell demise is only partially understood **[170]**. Though cytokines modulate the activity of several destructive signaling cascades (i.e., apoptosis, necrosis, and autophagic cell death), apoptosis is considered as the primary mode of cell death in human and mouse models of diabetes **[171 - 173]**. Apoptosis is a highly regulated, genetically encoded and energy-dependant cell death process activated by extracellular signals **[174 - 176]**. Caspases, a family of cysteine proteases, play a critical role in apoptosis. In the presence of apoptotic stimuli, caspase cascade is activated, in which activation of initiator caspases (i.e., Caspase 8 and 9) leads to the downstream activation of executioner caspase (i.e., Caspase 3) and once activated, caspase 3 cleaves ~40 different cellular substrates **[170, 174, 176, 177]**.

Recent studies indicate that cytokines may signal apoptosis *via* intrinsic apoptotic pathway, which involves destruction of mitochondrial membrane and subsequent release of cytochrome C from the inter-membranous space to cytosol, leading to the activation of caspase cascade **[169, 177]**. Emerging evidence also suggests that up-regulated oxidative stress from ROS and NO contributes to the damage in mitochondrial membrane eventually causing defects in membrane potential. Recently, members of the Nox family have been shown as one of the sources of ROS generation and oxidative stress in cells under the duress of cytokines **[118]**. Again, as in the case of gluco-, lipo-, or glucolipotoxicity, very little is known with regard to potential regulatory mechanisms underlying cytokine-induced, Nox-mediated and ROS-sensitive signaling pathways in β-cell dysfunction.

With this background information in mind, for my doctoral work, I have undertaken a series of investigations to methodically assess the friendly and non-friendly roles of Nox-derived ROS in islet β-cell function. Data accrued from these studies are described in the following sections.

HYPOTHESIS

The above review of literature clearly indicates that a tonic increase in intracellular ROS may be necessary for GSIS to occur. Further, it is also evident that chronic exposure of β-cells to high glucose [i.e., glucotoxicity], fatty acids [i.e., lipotoxicity], or both [i.e., glucolipotoxicity] results in increased oxidative stress leading to metabolic dysfunction and demise of the β-cell culminating in the onset of diabetes. Despite the above evidence, very little is known with regard to potential regulatory mechanisms underlying the holoenzyme assembly and functional activation of Nox by glucose, cytokines and/or palmitate under acute/chronic exposure conditions. Furthermore, putative mechanisms underlying ROS-mediated insulin secretion and cytotoxic effects on isolated β-cells remain largely unexplored.

The **overall objective** of my PhD work is to study the regulation of islet β-cell function by ROS, specifically generated *via* the catalytic activation of Nox signaling cascade in isolated β-cells exposed to acute and/or long-term incubation conditions [See Figure 11 for my Working Model]. My goal is to test short term effects in the presence of nutrient secretagogues such as glucose or mitochondrial fuels. I propose to utilize glucose, palmitate, ceramide and a mixture of cytokines in the long-term incubation studies. Lastly, I plan to confirm my findings in *in vitro* model systems in islets derived from diabetic rodents [Zucker diabetic fatty rat; ZDF rat] and diabetic human islets, if they become available. I will accomplish my goals by conducting studies described under the following three Specific Aims.

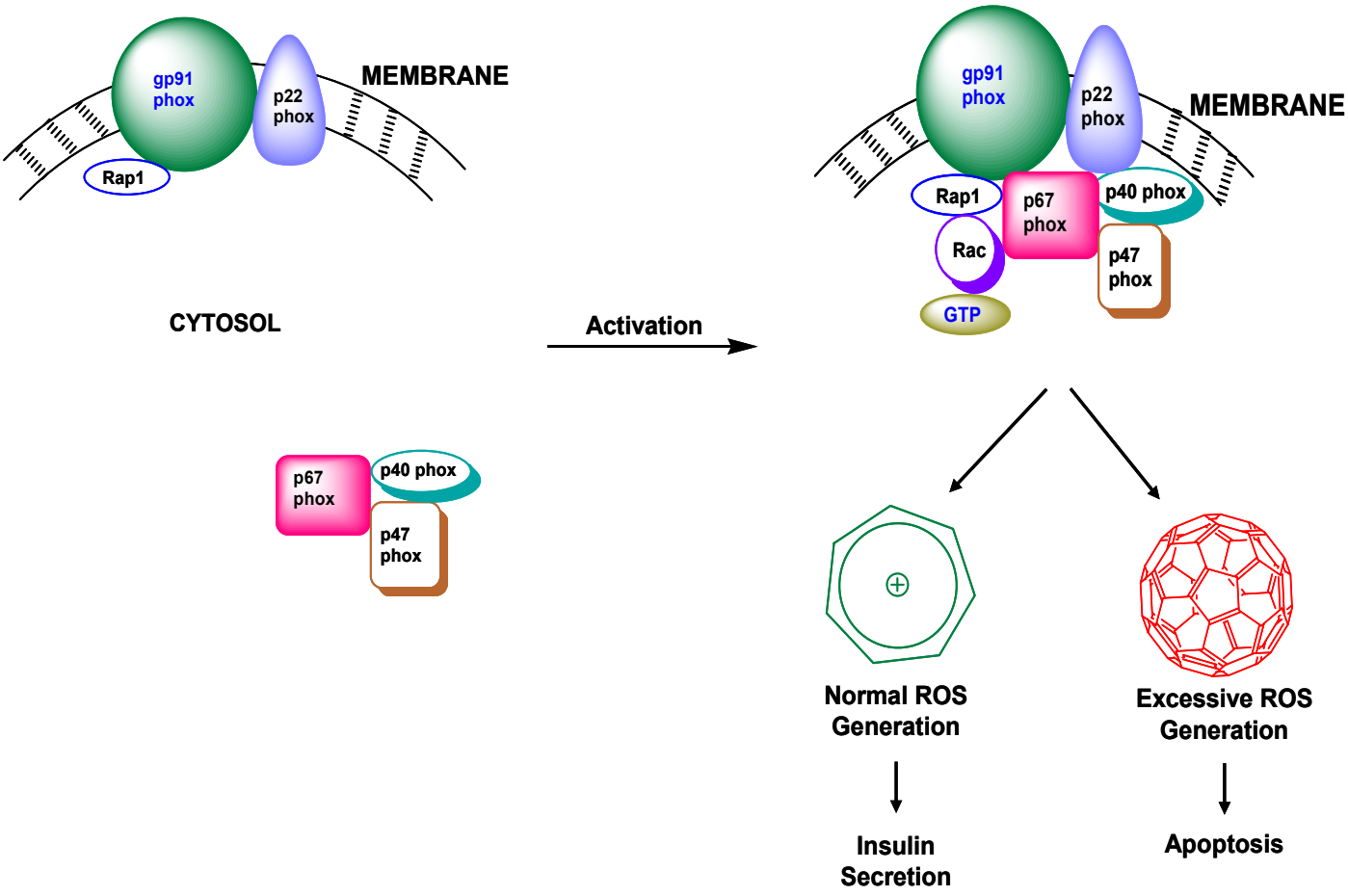


Figure 11: The above figure depicts both the positive and negative modulatory roles of ROS generation mediated *via* Tiam1/Rac1/Nox signaling axis.

Specific Aim 1: To determine contributory roles of Nox-derived ROS in the sequence of events leading to insulin secretion.

Specific Aim 2: To determine contributory roles of Nox-derived ROS in the onset of mitochondrial defects leading to the demise of the islet β-cell following exposure to elevated glucose, lipids, ceramide or a mixture of cytokines.

Specific Aim 3: To determine the functional status of Nox signaling cascade in islets from animal models of obesity and diabetes. Also, to assess these signaling steps in islets from human donors with type 2 diabetes.

I will use a number of biochemical, molecular and cell biological and immunological approaches in primary rat islets and glucose-responsive β [INS 832/13] cells to accomplish the above objectives. It is my hope that data derived from the proposed studies will provide fresh insights into regulatory roles of Nox signaling cascade in islet function following short term exposure to nutrient secretagogues. I also envision that data from my studies will provide much needed information on regulatory roles of Nox-derived ROS signaling steps in the onset of mitochondrial dysfunction leading to the demise of the β-cell under the duress of glucolipotoxic conditions. My **long-term** goal is to develop specific therapeutic modalities to prevent the establishment of these β-cell defects and the onset of diabetes.

Chapter I

Introduction

Glucose stimulated insulin secretion (GSIS) involves a series of metabolic and cationic events, leading to translocation of insulin-laden secretory granules from a distal site toward the plasma membrane for fusion and release of insulin into circulation. It is widely accepted that vesicular transport and fusion involves interplay between signaling proteins, including vesicle-associated membrane proteins on the secretory granule and docking proteins on the plasma membrane **(44, 45, 49)**. Furthermore, interaction between these proteins is widely felt to require cytoskeletal remodeling, which is under the fine control of small molecular mass G proteins belonging to the Rho subfamily (e.g., Cdc42 and Rac1; see **Ref. 84** for a recent review). Several effector proteins for these small G proteins have been identified in the islet β-cell, including phospholipases, p21-activated kinase-1 kinase, and ERK1/2 kinases **(68, 74, 84)**.

It is well established that G proteins undergo posttranslational modifications for optimal activation, membrane trafficking, and effector interactions. The majority of small G proteins undergo a series of modifications at their COOH-terminal cysteine residues, which include prenylation (i.e., farnesylation and geranylgeranylation), carboxylmethylation (CML), and palmitoylation. In addition to small G proteins, the γ-subunits of trimeric G proteins undergo prenylation and CML **(66, 74, 75, 79, 84)**. Indeed, using pharmacological and molecular biological approaches, several recent studies have confirmed the requisite nature of these modifications in GSIS in a variety

of insulin-secreting cells, including clonal β-cells, normal rodent islets, and human islets (see **Ref. 84** for a recent review).

A growing body of recent evidence implicated roles for reactive oxygen species (ROS) in metabolic dysfunction of the islet β-cell under the duress of glucolipotoxicity, cytokines, and ceramide **(178 - 180)**. It has been shown that increased ROS generation seen under the above experimental conditions is derived from the activation of phagocyte-like NADPH oxidase (Nox), since inhibition of this enzyme by selective inhibitors [e.g., Diphenylene iodonium chloride (DPI) or apocynin] or transfection of short interfering RNA (siRNA) against individual subunits of Nox (e.g., $p47^{phox}$) significantly attenuated deleterious effects of aforementioned Noxious stimuli (38, 39).

Despite the negative modulatory role(s) of ROS in cell function, recent evidence appears to indicate that a tonic increase in the ROS generation may be necessary for GSIS and fatty acid-induced insulin secretion **(7, 110 - 113)**. ROS have also been shown to modulate many physiological processes, including ion transport and protein phosphorylation **(181 - 184)**. As reviewed recently by Pi and Collins **(7)**, ROS plays "second messenger" role in modulating islet β-cell function. Along these lines, studies by Pi and coworkers **(113)** have demonstrated that glucose-mediated generation of H_2O_2 alters intracellular redox status, leading to augmented GSIS; such effects were attenuated by coprovision of antioxidants. These findings were further strengthened by Leloup and colleagues **(10)**, suggesting that generation of mitochondrial ROS is a requisite stimulus for GSIS to occur. Together, these data implicate an essential role for Nox-derived ROS as a signaling molecule involved in the regulation of β-cell function,

specifically at the level of insulin secretion. The present studies are undertaken to determine potential mechanisms underlying nutrient-induced elevation of ROS levels in INS 832/13 cells and normal rat islets. Specifically, I have determined the roles of G proteins in this signaling cascade; this was accomplished by selective inhibitors of protein prenylation (e.g., GGTI-2147 and FTI-277), which have been used to verify the roles for G proteins in GSIS **(84)**. In addition, I have examined permissive roles for endogenous GTP in nutrient-induced ROS generation. My findings implicate that prenylation-sensitive signaling steps are necessary for glucose- and mitochondrial fuel-induced intracellular generation of ROS in INS 832/13 cells and normal rat islets.

These findings have been published in Am J Physiol Regul Integr Comp Physiol. 2011; 300(3):R756-762. Reprint of this publication is included as Appendix A.

Materials and Methods

Materials

DPI, apocynin, pertussis toxin (Ptx), mycophenolic acid (MPA), cyclosporine A, rapamycin, mono-methylsuccinate, α-keto-isocaproic acid, and 2′, 7′-dichlorofluorescein diacetate (DCHFDA) were from Sigma (St. Louis, MO). $p47^{phox}$ siRNA and $p47^{phox}$ antiserum were from Santa Cruz Biotechnology (Santa Cruz, CA). FTI-277 and GGTI-2147 were from Calbiochem (San Diego, CA). Rac1 activation kit was from Cytoskeleton (Denver, CO).

Insulin-secreting cells

INS 832/13 cells were provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC) and 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 μM 2-mercaptoethanol, and 10 mM HEPES (pH 7.4). The medium was changed twice, and cells were subcloned weekly. Islets from normal Sprague-Dawley rats were isolated by collagenase digestion method described previously **(85)**. All animal experiments, including isolation of pancreatic islets from normal Sprague-Dawley rats, were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee.

Quantitation of ROS

This was carried out as our laboratory described recently in **179, 180**. In brief, INS 832/13 cells were seeded in six-well plate and treated with various insulin

secretagogues and inhibitors (or their respective diluents), as indicated in the text. Following incubation, the medium was removed, and cells were further incubated with DCHFDA (10 μM) at 37°C for 30 min in RPMI. DCHFDA, being a nonpolar compound, diffuses rapidly into the cells and hydrolyzes readily by cellular esterases into polar 2′, 7′-dichlorofluorescein. In the presence of ROS, 2′, 7′-dichlorofluorescein readily oxidizes to fluorescent dichlorofluorescein. The cells were washed with ice-cold phosphate-buffered saline and harvested, and equal amounts of protein were taken for fluorescence measurements (emission wavelength: 535 nm and excitation wavelength: 485nm) using luminescence spectrophotometer (PerkinElmer, Waltham, MA).

Inhibition of Nox activity via molecular biological or pharmacological approaches

INS 832/13 cells were seeded in a 24-well plate and at 50–60% confluence either mock transfected or transfected with antisense $p47^{phox}$ siRNA at a final concentration of 150 nM and cultured for 24 h. Following this, cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 1 h. At the end of stimulation, culture medium was removed; cells were incubated further with DCHFDA (10 μM) at 37°C for 30 min in RPMI, washed with ice cold PBS, and harvested; equal amount of proteins were taken; and fluorescence was measured (excitation wavelength: 485 nm, and emission wavelength: 535 nm) using luminescence spectrophotometer as described above. Alternatively, Nox activity was inhibited *via* a pharmacological approach by incubating INS 832/13 cells either with apocynin (100 μM; 12 h) or DPI (5 μM; 2 h) in low-serum, low-glucose-containing medium. Following incubation, cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 1 h in the continuous absence or presence of inhibitors, and NADPH activity was measured by DCHFDA assay, as

described above. The amount of fluorescence recorded is directly correlated with the amount of superoxide radicals generated due to Nox activity.

Rac1 activation assay

This was accomplished using a pull-down assay that our laboratory described recently **[185]**. Briefly, INS 832/13 cells were starved overnight in low-serum, low-glucose-containing medium in either the presence or absence of MPA (10 μM). At the end of incubation, cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 30 min in the continuous presence or absence of MPA. Lysates (~500 μg protein) were clarified by centrifugation for 5 min at 4,800 *g*, and p21-activated kinase-binding domain beads (20 μl) were added to the supernatant. The mixture was then rotated for 1 h at 4°C and pelleted by centrifugation at 4,000 g for 3 min. The pellet was washed once with lysis buffer followed by a rinse (3×) in wash buffer (25 mM Tris, pH 7.5, 30 mM MgCl2, 40 mM NaCl, and 150 mM EDTA). Proteins in the pellet were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and Western blotting method determined the relative abundance of activated Rac1.

Other assays and statistical analysis of data

Protein concentrations were determined by Bradford's dye-binding method using bovine serum albumin as the standard. Statistical significance of differences between diluent and experimental groups was determined by Student's t-test and ANOVA analysis. $p < 0.05$ was considered significant.

RESULTS

Pharmacological inhibitors or siRNA-p47^{phox} markedly attenuate glucose-induced ROS generation in insulin-secreting cells

At the outset, I determined whether stimulatory glucose promotes the generation of ROS, and whether selective inhibition of Nox attenuates such an effect in this model system. Data in Fig. 12 demonstrated a significant increase (~1.7-fold) in glucose-induced ROS generation in INS 832/13 cells, which was markedly attenuated by inhibitors of Nox holoenzyme (e.g., apocynin and DPI; Panel A). The above observations were further validated by knockdown of p47^{phox}, a cytosolic subunit of Nox. Data in Fig. 12; Panel B indicated ~50% inhibition in the expression of p47^{phox} subunit after siRNA transfection, and under these conditions I noticed a marked attenuation of glucose-induced ROS generation (Fig. 12; Panel C).

Selective inhibitors of protein prenylation markedly attenuate glucose-induced ROS generation in INS 832/13 cells and normal rat islets

Several earlier studies have demonstrated that posttranslational farnesylation and geranylgeranylation of specific G proteins are necessary for GSIS **[68, 84]**. With this in mind, using a pharmacological approach, I examined whether glucose-induced ROS generation in isolated β-cells is sensitive to inhibition of protein prenylation. Data in Fig. 13 demonstrated a significant reduction in glucose-induced ROS generation by selective inhibitors of farnesylation (e.g., FTI-277) or geranylgeranylation (e.g., GGTI-2147) in INS 832/13 cells (A) or rat islets (B). Together, these findings suggested

involvement of farnesylated and geranylgeranylated proteins in the signaling events, leading to glucose-induced ROS generation.

Protein prenylation is also necessary for mitochondrial fuel-, but not KCl-induced ROS generation

I next examined if a mixture of mitochondrial fuels (e.g., α-keto-isocaproic acid and mono-methylsuccinate), which elicits insulin secretion from INS 832/13 cells **[186]**, also promotes Nox-mediated generation of ROS in these cells. Data in Fig. 14 demonstrated that mitochondrial fuels increased ROS generation in a manner akin to glucose. Furthermore, I observed that such a signaling step was inhibited by FTI-277 and GGTI-2147, albeit to a lesser degree (Fig. 14) compared with glucose-induced ROS generation (Fig. 13). Together, data in Figs. 13 and 14 implicate protein farnesylation and geranylgeranylation in the cascade of events, leading to nutrient-induced generation of ROS in INS 832/13 cells. It should be noted that ROS generation appears to be specific for nutrient secretagogues, since a depolarizing concentration of KCl (40 mM), which facilitates insulin release *via* membrane depolarization and associated increase in cytosolic calcium, failed to promote ROS generation. (i.e., 109 ± 1.2% of control values; mean ± SE; n = 3).

Depletion of intracellular GTP inhibits glucose-induced Rac1 activation and ROS generation in INS 832/13 cells

Several previous studies have demonstrated a critical requirement for endogenous GTP in physiological insulin secretion by selectively inhibiting inosine monophosphate dehydrogenase (IMPDH) with MPA **[62, 63]**. Herein, using MPA, I

examined if endogenous GTP is required for glucose-induced Nox activation and associated ROS generation in INS 832/13 cells. Cyclosporine A and rapamycin were included as negative controls, which, like MPA, are endowed with immunosuppressive actions, but not GTP-lowering properties. Data in Table 1 suggested a marked attenuation in glucose-induced ROS generation by MPA, but not cyclosporine A or rapamycin. These data indicate a critical requirement for endogenous GTP for glucose to promote ROS generation in these cells. Together, data in Figs. 13, 14 and Table 1 indicate potential involvement of prenylated G protein, requiring newly synthesized GTP due to the catalytic activation of IMPDH in the signaling events leading to ROS generation.

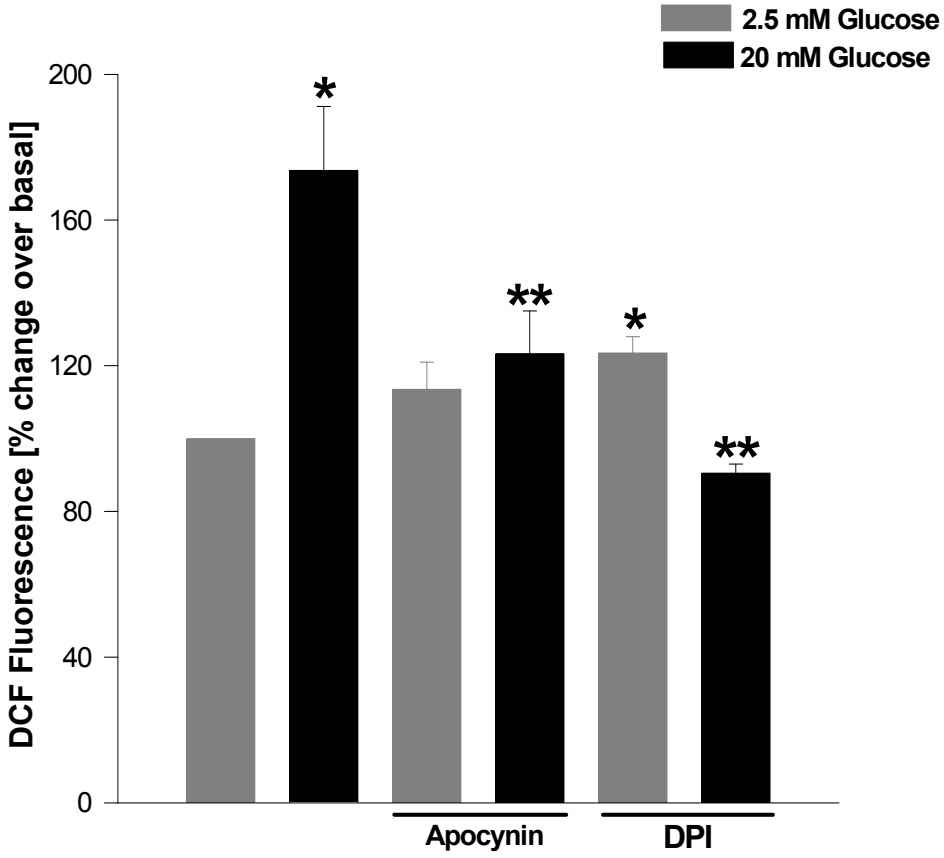
I next examined if GTP depletion impedes glucose-induced activation of specific G proteins involved in GSIS. To test this, I quantitated glucose-induced activation of Rac1 in MPA-treated (i.e., GTP-depleted) INS 832/13 cells. The premise underlying the selection of Rac1 in these studies is based on the evidence that 1) it has been shown to be activated by glucose and involved in GSIS; 2) it undergoes geranylgeranylation, and GGTI-2147 (above) inhibits glucose-induced Rac1 activation and GSIS; and 3) it is a member of the Nox holoenzyme. Data shown in Fig. 15 demonstrated that stimulatory concentration of glucose failed to activate Rac1 in INS 832/13 cells following depletion of endogenous GTP using MPA.

A Ptx-sensitive G protein mediates glucose-induced ROS generation in INS 832/13 cells

In the last series of studies, I determined the nature of the prenylated protein that might be involved in glucose-induced ROS generation shown in Figs. 13 and 14. In this context, our laboratory recently reported that coprovision of FTI-277 or FTI-2628 or siRNA-mediated knockdown of farnesyl transferase β-subunit resulted in a significant inhibition of glucose-stimulated activation of ERK1/2, Rac1, and insulin secretion, further ruling out the potential involvement of Ras in these signaling steps **[185]**. Based on these findings, I speculated a prenylated protein, most likely the γ-subunit(s) of trimeric G proteins, in the regulation of the above signaling cascade. Herein, I examined if a Ptx-sensitive trimeric G protein is involved in glucose-induced ROS generation. Data shown in Fig. 16 demonstrated marked attenuation of glucose-induced ROS generation in INS 832/13 cells (A) and normal rat islets (B) treated with Ptx.

Figure 12:

A]



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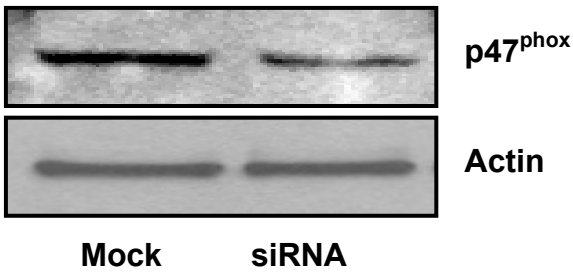
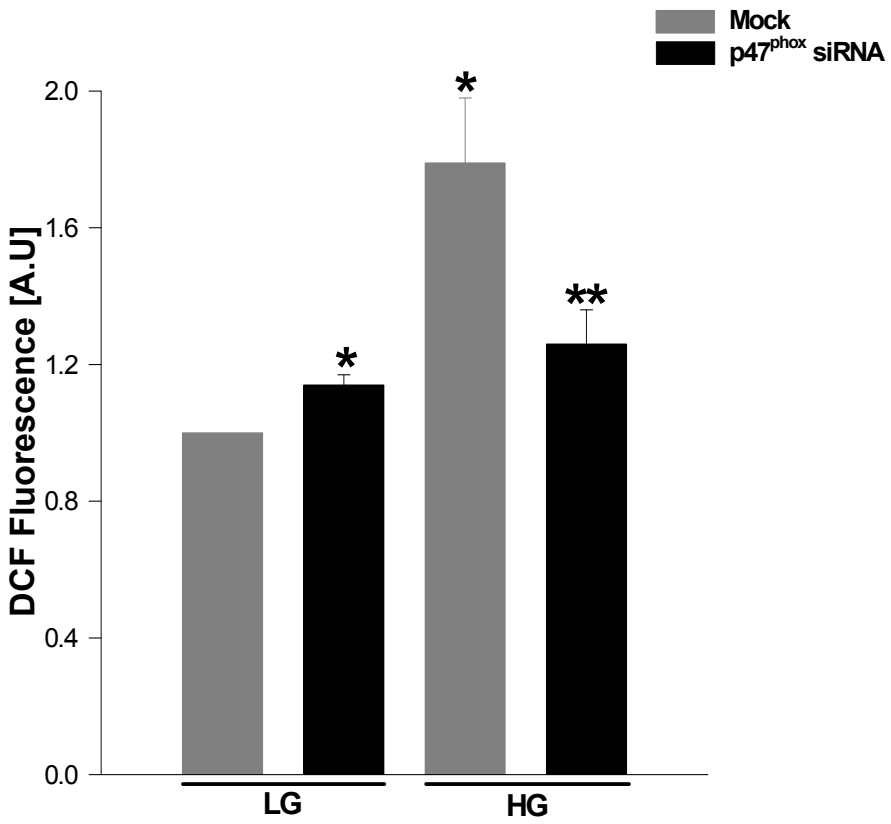


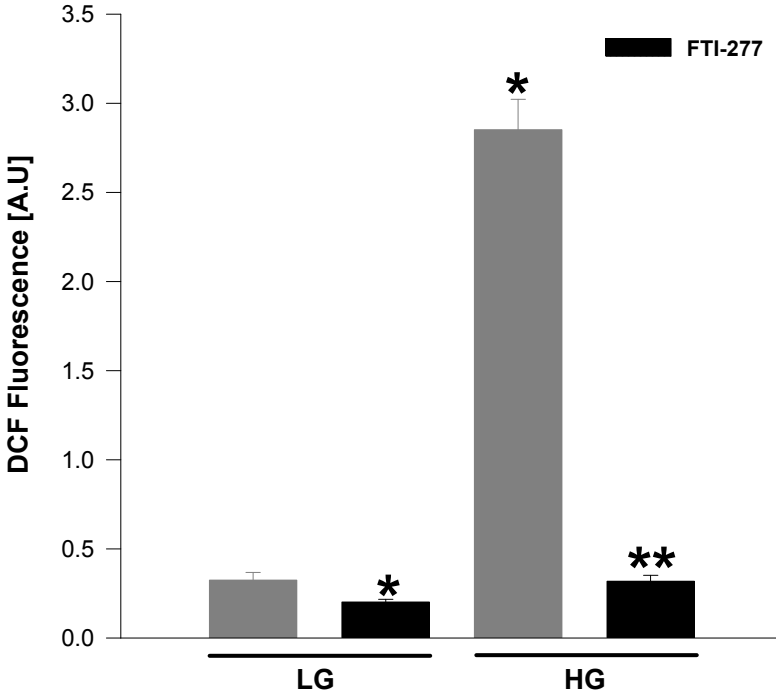
C]

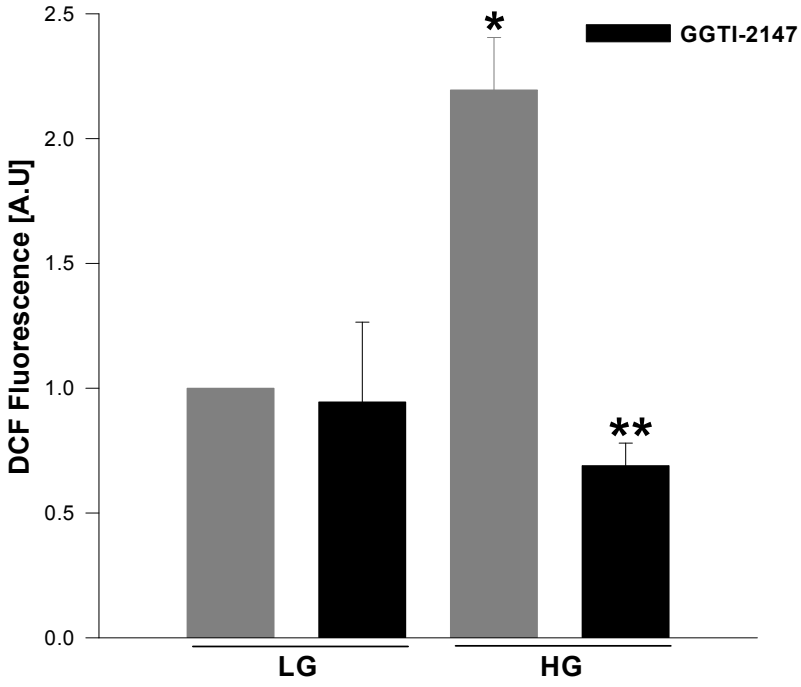


Selective inhibitor of NADPH oxidase or short interfering RNA (siRNA)-p47^{phox} inhibits glucose-stimulated reactive oxygen species (ROS) generation in insulin-secreting cells. INS 832/13 cells were incubated with either diluent or apocynin (100 μM, 12 h; A) or Diphenylene iodonium chloride (DPI; 5 μM, 2 h; A) or transfected with p47^{phox} siRNA (B and C), following which they were stimulated with either low (2.5 mM; LG) or high glucose (20 mM; HG) for 1 h. ROS generated was quantified as dichlorofluorescein (DCF) fluorescence and expressed as arbitrary units (AU). B: p47^{phox} knockdown efficiency was determined by immunoblotting. Values are means ± SE from three independent experiments done in triplicates in each case. *$p < 0.05$ *vs*. LG alone or mock transfected LG. **$p < 0.05$ *vs*. HG alone or mock transfected HG.

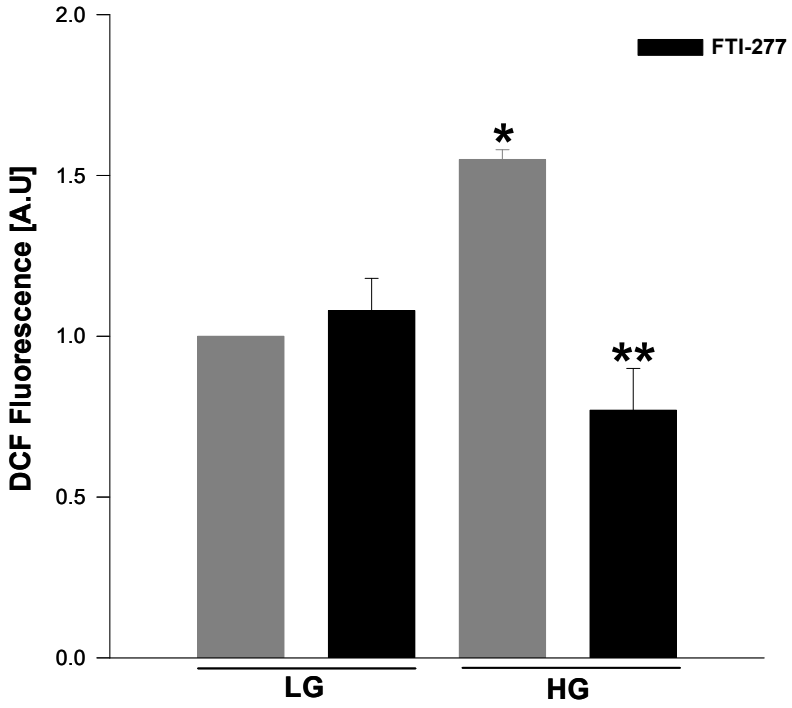
Figure 13:

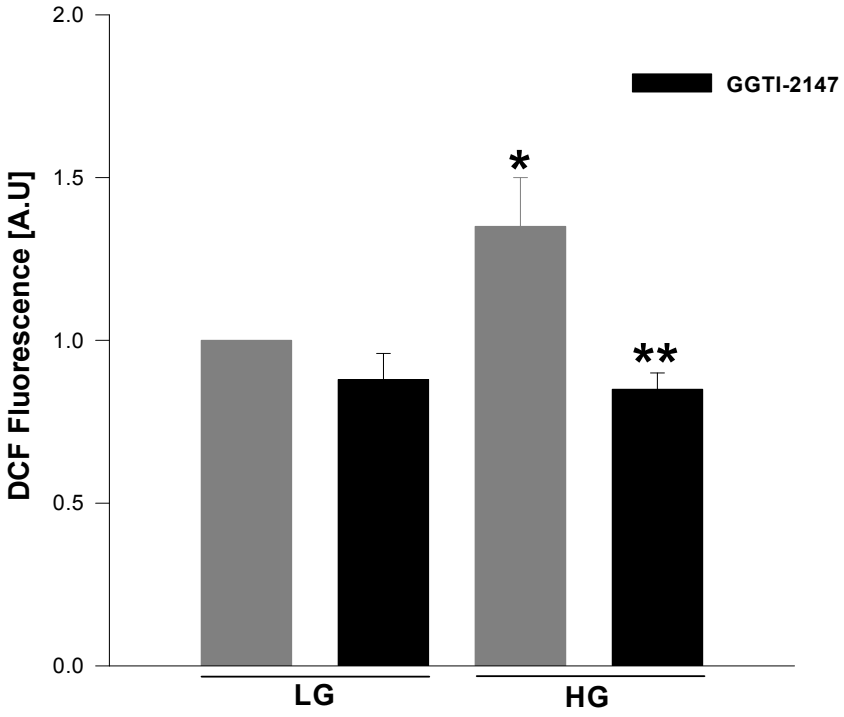
A]





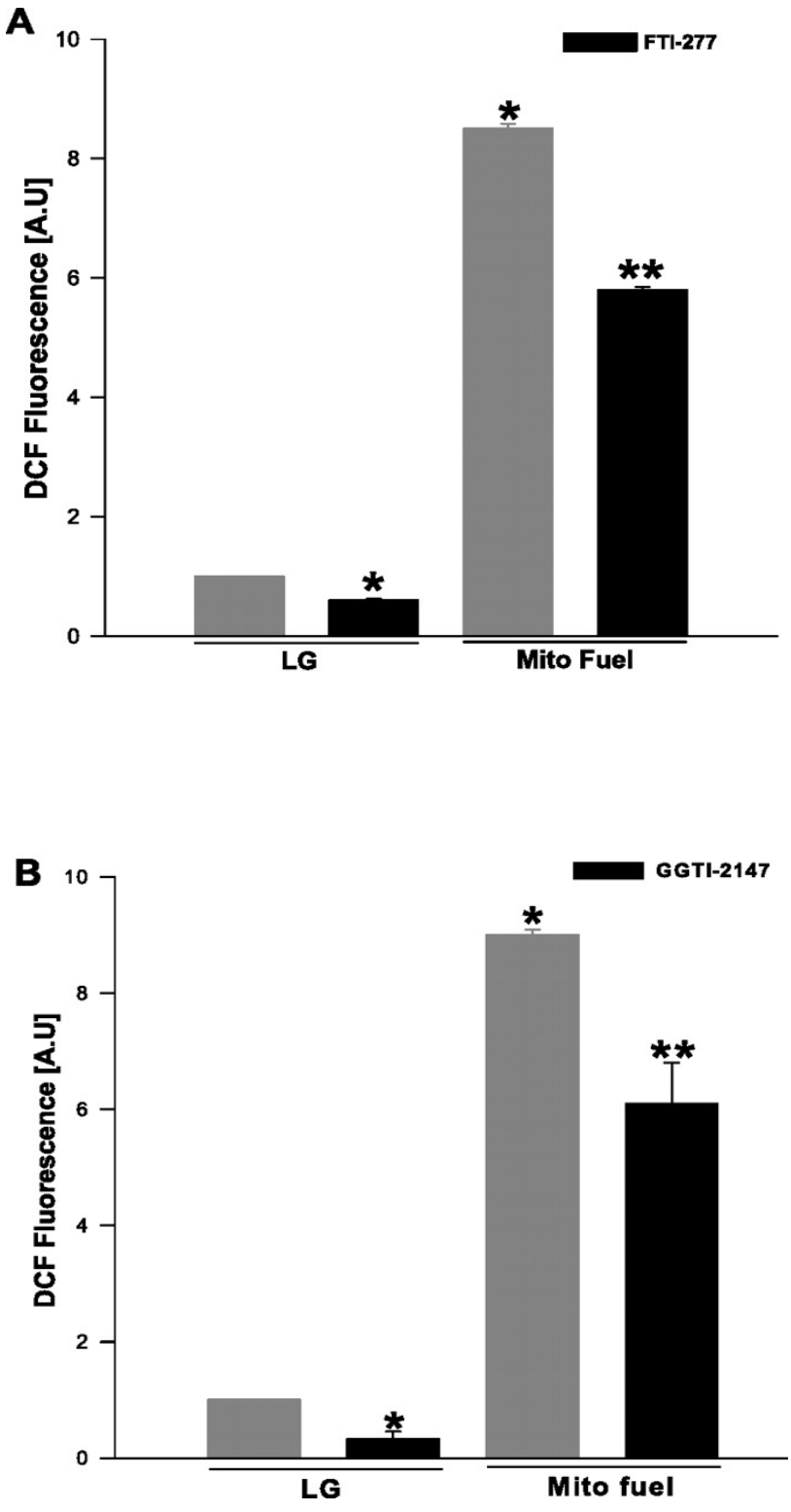
B]





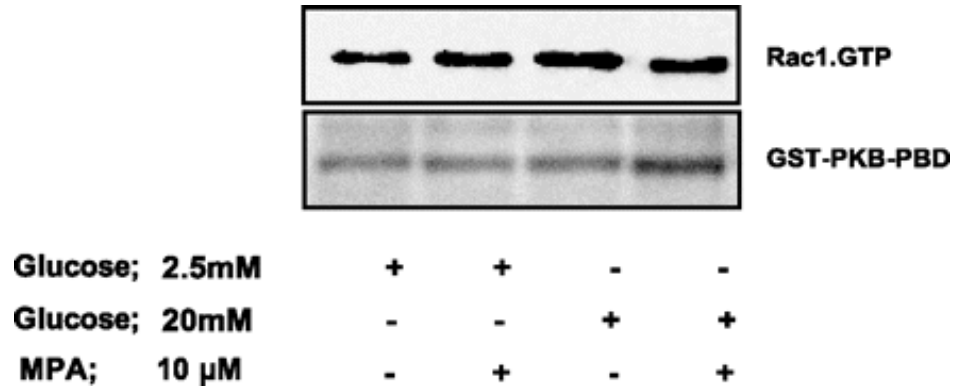
Selective inhibitors of protein farnesylation or geranylgeranylation markedly attenuate glucose-induced ROS generation in INS 832/13 cells and normal rodent islets. INS 832/13 cells (A) or normal rat islets (B) were incubated overnight in the absence or presence of FTI-277 (5 μM; left) or GGTI-2147 (10 μM; right), followed by stimulation with either LG (2.5 mM) or HG (20 mM) for 1 h. ROS generated was quantified as DCF fluorescence and expressed as AU. Values are means ± SE from three independent experiments done in triplicates (in INS 832/13 cells) and in duplicates (in islets) in each case. *$p < 0.05$ *vs*. LG alone. **$p < 0.05$ *vs*. HG alone.

Figure 14:



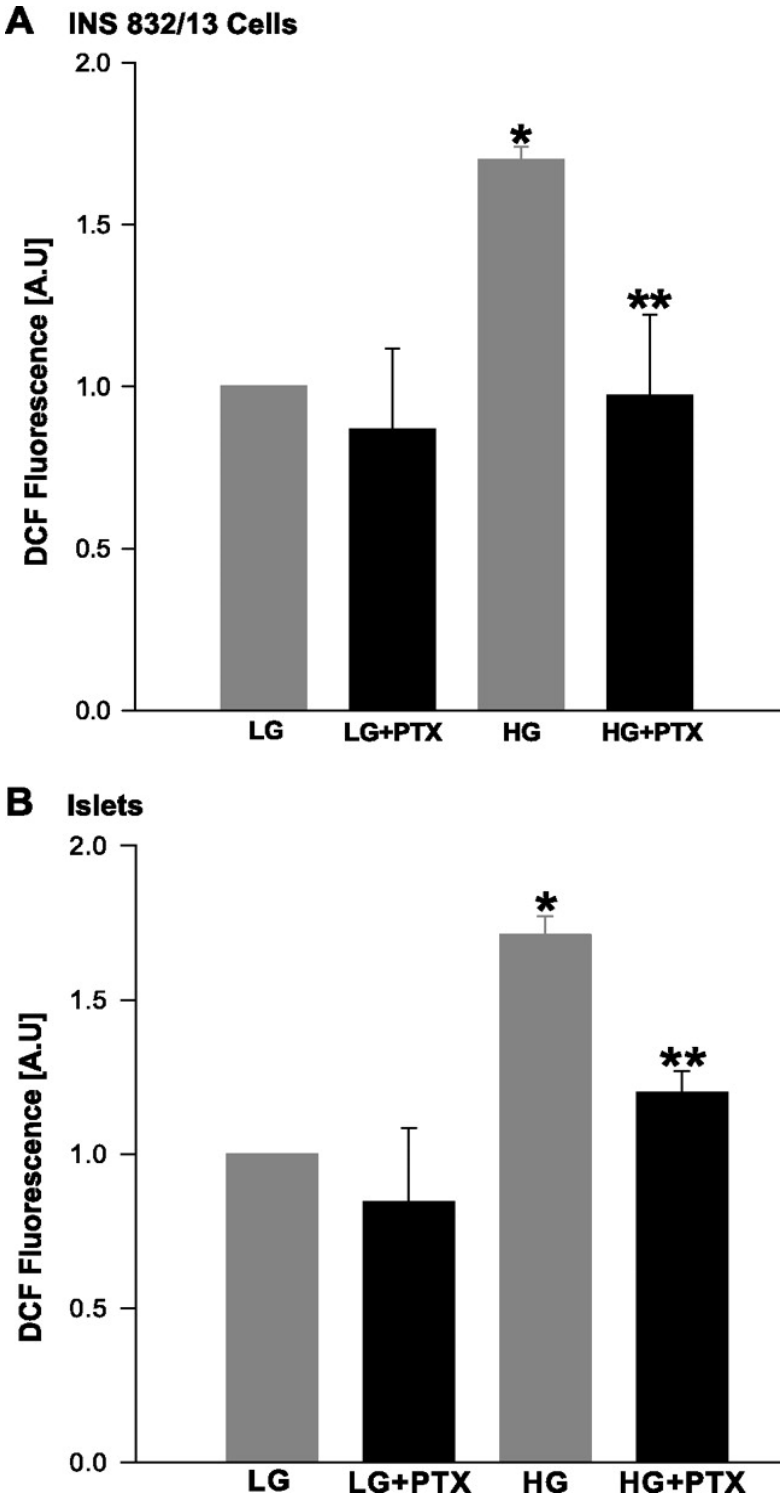
Selective inhibitors of protein prenylation inhibit ROS generation induced by a mixture of mitochondrial (Mito) fuels in INS 832/13 cells. INS 832/13 cells were incubated overnight in the presence or absence of FTI-277 (5 μM; A) and GGTI-2147 (10 μM; B), followed by stimulation with LG (2.5 mM) or a mixture of Mito fuels [monomethyl succinate (MMS) = 20 mM and α-keto-isocaproic acid (KIC) = 5 mM] for 1 h in continuous presence or absence of inhibitors. ROS generated was quantified as DCF fluorescence and expressed as AU. Values are means ± SE from three independent experiments done in triplicates in each case. *$p < 0.05$ *vs.* glucose alone. **$p < 0.05$ *vs.* Mito fuels alone.

Figure 15:



Endogenous GTP levels are required for glucose-induced Rac1 activation and subsequent ROS generation in pancreatic β-cells. INS 832/13 cells were incubated overnight with either diluent or mycophenolic acid (MPA; 10 µM), followed by stimulation with either LG (5 mM) or HG (20 mM) for 30 min. The degree of Rac1 activation was determined by p21-activated kinase-binding domain (PAK-PBD) pull-down assay, as described in materials and methods. A representative blot from two pull-down assays yielding similar data is depicted here.

Figure 16:



Pertussis toxin (Ptx) pretreatment attenuates glucose-induced ROS generation in INS 832/13 cells or normal rat islets. Untreated or Ptx-treated (100 ng/ml) INS 832/13 cells (A) or normal rat islets (B) were stimulated with either LG (2.5 mM) or HG (20 mM) for 1 h. ROS generated was quantified as DCF fluorescence and expressed as AU. Values are means ± SE from three independent experiments done in triplicates (in INS 832/13 cell) and in duplicates (in islets) in each case. *$p < 0.05$ *vs*. LG alone. **$p < 0.05$ *vs*. HG alone.

Table 1:

Conditions	Degree of ROS generation [fold over basal glucose]
Low glucose	1.00
High Glucose	1.58± 0.06 **
Low glucose + mycophenolic acid	1.08± 0.02*
High glucose + mycophenolic acid	1.16± 0.04 ***
Low glucose +cyclosporine A	1.06±0.09*
High glucose + cyclosporine A	1.46± 0.14 **
Low glucose + rapamycin	1.05± 0.08*
High glucose + rapamycin	1.42± 0.15 **

INS 832/13 cells were incubated with low glucose [2.5 mM] low serum in the presence or absence of mycophenolic acid [10 μM], cyclosporine-A [5 μM] and rapamycin [100 nM] for 24 h. Following, cells were stimulated either with low [2.5 mM] or high glucose [20 mM] for 1 h in continuous presence and or absence of the above inhibitors as indicated in the Table 1. At the end of stimulation, cells were incubated with DCHFDA [10 μM] for 30 min and harvested for DCF fluorescence. ROS generated was quantified as DCF fluorescence and expressed as arbitrary units. Data are mean ± SEM from three independent experiments in each case. * represents no significant difference *vs*. low glucose alone; ** p <0.05 *vs*. low glucose alone; *** p <0.05 *vs*. high glucose alone.

Discussion

The overall objective of the present study was to determine potential mechanisms underlying nutrient-induced generation of ROS in isolated β-cells. Salient features of my studies are as follows: 1) glucose and mitochondrial fuels, but not membrane depolarizing KCl, increase ROS generation significantly; 2) an increase in ROS seen under these conditions is derived from Nox, since pharmacological or molecular biological inhibition of Nox inhibited ROS generation; 3) such a regulatory effect of glucose requires the activation of farnesylated as well as geranylgeranylated proteins; 4) MPA, but not rapamycin or cyclosporine A, completely inhibits glucose-induced ROS generation, implying that endogenous GTP is necessary for such an effect; and 5) inactivation of Ptx-mediated ADP ribosylation of an inhibitory G protein(s) markedly attenuates glucose induced ROS generation. Taken together, these findings provide insights into potential G protein-mediated regulation of ROS in the islet β-cells under conditions in which they regulate physiological insulin secretion.

Nox is a highly regulated membrane-associated protein complex that facilitates the one electron reduction of oxygen to superoxide anion involving oxidation of cytosolic NADPH. The Nox holoenzyme is composed of membrane as well as cytosolic components. The membrane-associated catalytic core consists of $gp91^{phox}$, $p22^{phox}$, and the small G protein Rap1. The cytosolic regulatory components include $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small G protein Rac. Following 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-sensitive

phosphorylation of $p47^{phox}$ leads to its translocation to the membrane fraction **(187)**. It has also been shown that functional activation of Rac1 (Rac1.GTP) is vital for the holoenzyme assembly and activation of Nox in insulin-secreting cells **(179, 180)**.

Along these lines, Oliveira et al. **(112)** provided a detailed description of localization, expression, and functional regulation of Nox within the islet. More recent pharmacological and molecular biological observations by Morgan and coworkers **(14)** have provided compelling evidence for a regulatory role for Nox in glucose-stimulated insulin secretion in rat islets under static incubation and perifusion conditions. Follow-up studies from this group have demonstrated key roles for Nox-derived ROS in palmitate-induced insulin secretion in the presence of submaximal concentration of glucose in islets **(110)**. Under the above conditions, palmitate not only promoted translocation of $p47^{phox}$ to the membrane fraction, but also upregulated the protein content of $p47^{phox}$ and the mRNA levels of $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, proinsulin, and the G protein-coupled receptor 40. Essential role for Nox in palmitate-induced effects on β-cells was further strengthened by their observations to indicate a marked inhibition of fatty acid stimulation of insulin secretion in the presence of high-glucose concentration by inhibition of Nox activity. Based on these findings, it is evident that Nox plays key roles in islet function, including gene regulation and insulin secretion.

These observations also implicate roles for farnesylated and geranylgeranylated proteins in nutrient-induced Nox activation and associated ROS generation; the geranylated protein involved in nutrient-mediated activation of Nox might be Rac1, since it is one of the components of the Nox holoenzyme **(180)**. Pharmacological (i.e., generic

as well as more selective inhibitors of geranylgeranylation of Rac1), as well as molecular biological (i.e., dominant negative mutants of prenyltransferases; **Ref. 95**) studies from our laboratory have clearly implicated Rac1 in islet function, including insulin secretion **(84 - 86)**. The identity of the farnesylated protein, which is required for nutrient-induced ROS generation, remains to be determined. It is likely that it might represent the γ-subunit of a Ptx-sensitive G protein, since our laboratory has demonstrated earlier regulation of Ptx-sensitive G proteins by glucose in clonal β-cells, normal rat islets, and human islets **(88, 188 - 190)**. Several earlier studies by Seaquist et al **(191)**, Robertson et al **(69)**, and Sharp **(192)** have provided evidence for the expression of inhibitory (e.g., Gi or Go) class of Ptx-sensitive heterotrimeric proteins in the islet β-cell. Furthermore, studies from our laboratory **(188)** and those of Konrad and coworkers **(193)** have demonstrated functionally active heterotrimeric G proteins on the insulin granules in isolated β-cells. Lastly, using clonal β-cells, normal rat islets, and human islets, our laboratory has been able to demonstrate activation of the CML of γ-subunits by glucose; such effects of glucose were shown to be sensitive to Ptx, GTP, and extracellular calcium **(190)**.

Existing experimental evidence also implicates role(s) for trimeric G proteins, specifically the inhibitory Gi class of proteins in the regulation of NADPH-oxidase activity. For example, using human fat cells, Kreuzer and coworker's **(194)** demonstrate insulin-induced activation of NADPH-dependent H_2O_2 generation in human adipocyte plasma membranes is mediated by Gαi2, which is regulated *via* ADP-ribosylation by Ptx. Additional studies are needed to conclusively determine the identity of this protein. However, based on our laboratory's recently published evidence **(185)**, it is unlikely that

the farnesylated protein is Ras, since inhibition of Ras (a farnesylated protein) had no effects on glucose-induced ERK1/2 phosphorylation, Rac1 activation, and insulin secretion.

These findings also suggested that depletion of endogenous GTP by MPA results in a decreased activation of glucose-induced Rac1 and ROS generation. In this context, original studies by Metz and coworkers **(62, 63)** have documented permissive roles for endogenous GTP in physiological insulin secretion. MPA, which selectively inhibits GTP biosynthesis by inhibiting IMPDH, has been shown to inhibit GSIS and mastoparan-induced insulin secretion **(62, 65)**. Even though inhibition of G protein activation was speculated to be one of the underlying mechanisms in the inhibition of insulin secretion following GTP depletion by MPA, very little information is available to substantiate that speculation. In this context, our laboratory has described earlier the inability of glucose to increase the CML (and activation) of small G proteins in GTP-depleted cells **(66)**. The present studies identify Rac1 as one of the target proteins for glucose-mediated, endogenous GTP-dependent effects in β-cells. These present findings are also in agreement with observations of Krotz et al. **(195)**, demonstrating inhibition of endothelial Nox by MPA *via* a Rac1- dependent mechanism.

CHAPTER – II

Introduction

Several lines of evidence from multiple laboratories suggests that chronic exposure of isolated β-cells to elevated saturated fatty acids [e.g., palmitic acid; PA] leads to a significant metabolic dysregulation and eventual demise of the β-cell **[158, 196, 197]**. Multiple mechanisms have been put forth to explain PA-induced metabolic defects; one of these include generation of intracellular oxidative stress [e.g., reactive oxygen species; ROS; **118, 153, 198**], albeit recent studies by Moore et al. **[199]** appear to argue against fatty acid induced oxidative stress in the pancreatic β-cell. A signaling step involved in the increased generation of ROS and associated induction of intracellular oxidative stress in the pancreatic β-cell is the activation of the phagocytic Nox system, which 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 consisting of $gp91^{phox}$, $p22^{phox}$ and the small G-protein Rap1. The cytosolic regulatory components include $p47^{phox}$, $p67^{phox}$ and the small G-protein Rac1 **[16, 84, 111, 114, 115]**. Following stimulation, the cytosolic components of NADPH oxidase translocate to the membrane for association with the catalytic core for holoenzyme assembly. Available evidence also suggests that a protein kinase C ζ-sensitive phosphorylation of $p47^{phox}$ leads to its translocation to the membrane fraction **[187]**. It

has also been shown that functional activation of Rac [i.e., GTP-Rac] is vital for the holoenzyme assembly and activation of NOX **[40 - 42]**.

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 including elevated levels of glucose, saturated fatty acids and proinflammatory cytokines **[13, 112, 198, 200]**. It has also been demonstrated that pharmacological inhibition of Nox by Diphenylene iodonium chloride [DPI] or anti-sense oligonucleotides for $p47^{phox}$ markedly attenuated glucose-induced ROS production and oxidative stress, suggesting a critical involvement of Nox in the metabolic dysfunction induced by glucose **[14]**. These data implicate a significant contributory role for Nox in the metabolic dysfunction of the β-cell under conditions of oxidative stress **[31, 201, 202]**. Furthermore, existing evidence implicates apoptotic signaling cascade in cytokine-induced defects, which operate *via* an intrinsic pathway involving damage of the mitochondrial membrane and subsequent release of cytochrome C leading to caspase-3 activation **[177, 169]**. Unlike many other mammalian cell types, β-cells lack a strong defense mechanism, making them more vulnerable to oxidative damage **[31]**.

Despite the aforementioned compelling lines of evidence, very little has been studied with regards to the potential contributory roles of small G-proteins [e.g., Rac1] in the cascade of events leading to PA-induced Nox-mediated superoxides generation in β-cells. With this in mind, I undertook the current study to test the hypothesis that palmitate- or cytokine-induced ROS generation and subsequent oxidative stress in β-cells is mediated *via* functional activation of Rac1 [Rac1.GTP] since it is considered to

represent one of the signaling steps necessary for the functional regulation and activation of the Nox. To accomplish this, I investigated regulation of Nox activity in insulin-secreting cells following exposure to palmitate or a mixture of cytokines. I also examined potential contributory roles of ceramide, a biologically-active sphingolipid, which is biosynthesized from palmitate via the *de novo* pathway [Figure 10], in the regulation of Nox. Herein, I demonstrate that Nox signaling pathway plays a critical role in the generation of superoxides and lipid peroxides in palmitate- or cytokines-mediated effects on isolated β-cells. I also present the first evidence to suggest a modulatory role for Tiam1, a guanine nucleotide exchange factor for Rac1 **[207]**, in this signaling pathway leading to the onset of mitochondrial dysfunction. Lastly, I report that post translational modification of Rac1 is necessary for optimal Nox activation in insulin secreting cells.

Please note that my observations on palmitate and ceramide-induced regulation of Nox activation and metabolic dysfunction of the β-cell are published in Biochem Pharmacol 2010. Furthermore, the work I have carried out in collaboration with Dr. Wasanthi Subasinghe on cytokine-mediated Nox activation and mitochondrial dysfunction of the islet β-cell is published in Am J Physiol Regul Integr Comp Physiol 2011. Reprints of these two publications are included as Appendices B & C.

Materials and Methods

Materials

C2-Ceramide, Dihydroceramide, GGTI-2147 and NSC23766 were from Calbiochem [San Diego, CA]. Apocynin, Nitroblue tetrazolinium salt, malondialdehyde, thiobarbituric acid, diphenyleneiodonium chloride, butylated hydroxytoulene, oleic acid and palmitic acid were from Sigma [St. Louis, MO]. $p47^{phox}$ siRNA and antibodies directed against $p47^{phox}$, $p67^{phox}$, actin were from Santa Cruz Biotechnology [Santa Cruz, CA]. Rac1 activation kit was purchased from Cytoskeleton Inc. [Denver, CO]. JC-1 assay kit was from Cell Technology Inc. [Mountain View, CA]. Palmitate stock solutions were prepared as described in Ref. **[203]**. Interleukin-1β, IFN-γ, and TNF-α were obtained from R&D Systems (Minneapolis, MN). Caspase 3 antiserum was obtained from Cell Signaling Technology (Danvers, MA).

Insulin-secreting cells

INS 832/13 cells were provided by Dr. Chris Newgard [Duke University Medical Center, Durham, NC] and 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 μM 2-mercaptoethanol, and 10 mM HEPES [pH 7.4]. The medium was changed twice and cells were subcloned weekly. Islets were isolated from normal Sprague–Dawley rats using the collagenases digestion method described previously **[203]**.

Quantitation of superoxide generation by nitroblue tetrazolium [NBT] assay

INS 832/13 cells were plated in six-well plates and grown to subconfluence and then treated with PA [100 μM], C2-CER [30 μM], FB-1 [10 μM], DPI [5 μM] or NSC23766 [20 μM] in different combinations as described in the text. The medium was then removed and the cells were washed once with PBS and further incubated with 0.25% NBT for 30 min at 37 °C. Cells were then harvested and pelleted by low-speed centrifugation. The resulting pellet was resuspended in 50% acetic acid. The reduced NBT formazan product was quantified by measuring the absorbance at 510 nm using Beckman DU640 spectrophotometer.

Quantitation of superoxide generation by malondialdehyde [MDA] assay

INS 832/13 cell lysates derived from control or treated cells [100 μg protein] were treated with 10% trichloro acetic acid, 2% butylated hydroxytoulene, and freshly prepared 0.67% thiobarbituric acid. Following this, the samples were boiled for 15–20 min and then allowed to cool down at 4–8 °C for 15–20 min. The samples were then gently vortexed and centrifuged at 3500 rpm for 15 min. The resulting supernatant was used to measure the absorbance at 532 nm. A standard concentration curve was used to extrapolate MDA generated from various samples.

Nox assay

INS 832/13 cells were plated in six-well plates, grown to subconfluence and then treated with either diluent or PA [100 μM] or C2-CER [30 μM] for 6 h. After treatment the medium was removed and the cells were washed once with PBS and further incubated with 5 μM of 2′, 7′-dichlorodihydrofluorescein diacetate [DCHFDA] for 30 min at 37 °C.

Cells were then harvested and pelleted by low-speed centrifugation and the protein content of the pellet was determined using Bradford's assay. Following to this, equal amount of proteins were taken and fluorescence in each condition was recorded [excitation – 485 nm and emission – 530 nm]. The amount of fluorescence recorded is directly correlated to the amount of superoxide radicals generated due to Nox activity.

Molecular biological or pharmacological inhibition of Nox activity

INS 832/13 cells were seeded in a 24-well plate up to 50% confluence and transfected with mock or antisense siRNA-p47^{phox} (150 nM) and allowed to grow up to 80% or higher confluence. Then the cells were treated either with diluent or cytomix for a 12-h period. Following this, culture medium was removed, and cells were incubated further in DCHFDA (10 μM) at 37°C for 30 min, washed twice with ice-cold PBS, and harvested; equal amounts of proteins (50 μg) were taken, and fluorescence was measured (Ex: 485 nm and Em: 535 nm) using luminescence spectrophotometer (PerkinElmer, Waltham, MA). Alternatively, Nox was inhibited *via* a pharmacological approach by treating INS 832/13 cells with either diluent or cytomix for 12 or 24 h in the absence or presence of apocynin (75 μM), and Nox activity was measured with DCFHDA assay, as described above.

Rac1 activation assay

INS 832/13 cells were treated with either diluent or NSC23766 [20 μM] or C2-CER or PA or oleate or cytokines or GGTI-2147 [10 μM]. Before treatment, cells were incubated overnight with either NSC23766 or GGTI-2147 in a low serum–low glucose containing medium. Cells were further incubated with PA or C2-CER or cytokines as

indicated in the text in the continuous presence of either NSC23766 or GGTI-2147 or diluent. Lysates [500 µg protein] were clarified by centrifugation for 5 min at 4800 × g, and PAK-PBD [p21-activated kinase-binding domain] beads [20 µl] were added to the supernatant. The mixture was then rotated for 1 h at 4 °C and pelleted by centrifugation at 4000 × g for 3 min. The resulting pellet was washed once with lysis buffer followed by a rinse [3×] in wash buffer [25 mM Tris, pH 7.5, 30 mM MgCl2, 40 mM NaCl, and 150 mM EDTA]. Proteins in the pellet were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and Western blotting method determined the relative abundance of activated Rac1.

Assessment of mitochondrial dysfunction by JC-1 assay

Loss of mitochondrial membrane potential in cells has been estimated using JC-1 assay kit. Briefly, INS 832/13 cells were grown at 80% confluence on the cover slips and were incubated with and without NSC23766 [20 µM] or GGTI-2147 [10 µM] overnight in low serum–low glucose media. Cells were then treated with C2-CER [30 µM] or DHC [30 µM] for 6 h with or without NSC23766, or treated with cytomix for 12 or 24 h in the presence or absence of NSC23766 (20 µM) or GGTI-2147 (10 µM). At the end of incubation, cells were washed twice with assay buffer (provided with the kit) and were further incubated for 15 min with JC-1 dye [1×]. Cells were then washed twice with assay buffer and the cover slips were mounted onto a glass slide and images were taken at 40× magnification using Olympus IX71 microscope [Olympus America Inc., Center Valley, PA].

Other assays

Protein concentrations were determined by Bradford's dye-binding method using bovine serum albumin as the standard. Statistical significance of differences between diluent and experimental groups was determined by Student's t-test and ANOVA analysis. $p < 0.05$ was considered significant.

RESULTS

PA induces generation of lipid peroxides and ROS in insulin-secreting cells:

At the outset, I determined if exposure of isolated β-cells to PA results in the generation of superoxides and lipid peroxides. Data shown in Fig. 17 suggest that incubation of INS 832/13 cells with PA [100 mM; 6 h] significantly increased lipid peroxide levels [~2.5-fold; expressed as MDA equivalents; Panel A] and ROS levels [~2.7-fold; Panel B]. Furthermore, coprovision of DPI, a known inhibitor of Nox attenuated the PA-induced lipid peroxide levels [~37%] and ROS generation [~31%]. Together, these data suggest that PA-mediated generation of lipid peroxides and ROS in isolated β-cells may, in part, be due to activation of Nox.

PA induces activation of Nox in pancreatic β-cells:

Data described above prompted me to further investigate potential regulation of Nox activity by PA in insulin-secreting cells. As stated above, $p47^{phox}$ represents one of the subunits of the Nox holoenzyme which is subjected to regulation in cells under the duress of oxidative stress. It has been shown that small G protein Rac1, also a member of the Nox assembly, is also activated under conditions of oxidative stress leading to activation of Nox activity. Data described in Fig. 18 suggested that incubation of normal rat islets [Panel A; left] or INS 832/13 cells [Panel A; right] with PA significantly increased the activation [i.e., GTP-bound form] of Rac1 as determined by the PAK-pulldown assay [see Material and Methods]. In addition, I observed a marked increase in the expression of $p47^{phox}$ in these cells following exposure to PA [Fig. 18; Panel B]. Together, data in Panels A and B suggest upregulation of expression and function of key components of Nox holoenzyme in cells exposed to PA. I next quantitated the Nox

activity to determine if PA-induced activation of Rac1 [Panel A] and $p47^{phox}$ expression [Panel B] culminates in the functional activation of the enzyme. Indeed, findings described in Fig. 18 [Panel C] suggested a significant increase [~97%] in the catalytic activation of Nox in cells treated with PA. It should be noted that under these conditions, oleate exerted a modest effect on the Nox activity [Fig. 18; Panel C] without significantly affecting Rac1 activation in INS 832/13 cells [Fig. 18; Panel D]. Together, these findings suggest that PA, but not oleate, elicits stimulatory effects on Rac1 activation and Nox activity.

Tiam1, a GEF for Rac1, is involved in PA-induced Rac1 activation and generation of superoxides and lipid peroxides in pancreatic β-cells:

It has been demonstrated in many cells types, and more recently in pancreatic β-cells, that Rac1 activation is mediated by GEFs, such as Tiam1 **[204, 205]**. Recent studies from our laboratory have provided immunological evidence for Tiam1 in insulin secreting cells, and further indicated that NSC23766, a specific inhibitor of Tiam1, specifically inhibits GTP-loading onto Rac1, but not Cdc42 and Rho **[204]**. Therefore, I next investigated if pretreatment of isolated β-cells to NSC23766 prevents PA-induced Rac1 activation and associated increase in the generation of superoxides and lipid peroxides. Data shown in Fig. 19 [Panel A] demonstrated a near complete inhibition of PA-induced Rac1 activation by NSC23766 suggesting potential requirement for Tiam1 in PA-induced Rac1 activation. Furthermore, I observed that PA-induced generation of lipid peroxides [Panel B] and reactive oxygen species [Panel C] in INS 832/13 cells was also reduced [~20–30%] following inhibition of Tiam1-mediated activation of Rac1.

Together, these data implicate a novel regulatory role(s) for Tiam1/Rac1 signaling step(s) in PA-mediated generation of superoxides and lipid peroxides in isolated β-cells.

PA-induced generation of lipid peroxides and superoxides may, in part, be due to intracellular generation of CER via the de novo pathway:

Since PA is the precursor for the *de novo* biosynthesis of CER, in the next series of studies I investigated potential roles of intracellularly generated CER in aforementioned PA-induced effects on isolated β-cells. To address this, I quantitated PA induced generation of reactive oxygen species and lipid peroxides in cells pre-treated with or without FB-1, a known inhibitor of *de novo* biosynthesis of CER from PA **[207]**, incubation of isolated β-cells with 100 μM PA in presence of FB-1 significantly reduced PA-induced generation of ROS [~72%; Fig. 20; Panel A] and lipid peroxides [~62%; Fig. 20; Panel B] without significantly affecting these parameters in cells incubated with the diluent. I next quantitated PA-induced effects on Nox activity as a function of period of incubation and the concentration of PA. Data in Table 2 indicated that PA elicited significant stimulatory effects on Nox activity. Maximal effects were seen between 3 and 6 h of incubation. Interestingly, PA effects were not seen beyond 6 h time point as the Nox activity fell even below the control values. In addition, pre-incubation of these cells with FB-1 resulted in a significant inhibition in Nox activity at 6 h time point suggesting potential regulation of Nox activity by intracellularly generated CER [Table 2]. I next quantitated Nox activity in these cells as a function of PA concentration [0–200 μM] in the absence or presence of FB-1. Data in Fig. 20 [Panel C] suggested a concentration-dependent activation of Nox by PA. Further, a significant inhibition of PA-induced Nox activity by FB-1 was observed. Together, these data suggest that PA-induced effects on

lipid and superoxide levels and Nox activity may, in part, be due to the intracellularly generated CER.

A cell-permeable analog of CER mimics PA effects in isolated β-cells:

I next investigated if coprovision of a cell-permeable CER [e.g., C2-CER] mimics PA-induced oxidative stress in INS 832/13 cells, and if such an increase is mediated *via* activation of endogenous Nox. To address this, INS 832/13 cells were incubated with diluent or C2-CER, which has been effectively used to determine CER-induced metabolic dysfunction in isolated β-cells **[206, 207]** in the absence or presence of DPI to inhibit endogenous Nox. Data described in Fig. 21 showed a marked reduction in C2-CER-induced ROS levels [~71%; Panel A] or lipid peroxides [~69%; Panel B] in cells exposed to DPI. It should be noted that DPI exerted a modest increase in the generation of lipid peroxides in the absence of C2-CER without significantly affecting the basal superoxide generation [Panels A and B; lanes 1 *vs*. 3]. Taken together, these findings implicate Nox activity in C2-CER-induced generation of ROS and lipid peroxides in pancreatic β-cells.

C2-CER mimics PA effects in inducing p47^{phox} expression and Nox activity in isolated β-cells:

As a logical extension to the studies described in Fig. 21, I examined if C2-CER induces p47^{phox} expression and Nox activity in pancreatic β-cells. Data in Fig. 22 [Panel A] show that incubation of INS 832/13 cells with C2-CER significantly increased p47^{phox} expression. Moreover, in a manner akin to PA, C2-CER increased [more than 2-fold] the Nox activity in INS 832/13 cells [Fig. 22; Panel B]. Together, these data in Figs. 21 and

22 demonstrate that a cell-permeable analog of C2-CER mimics the effects of PA on isolated β-cells by increasing the Nox activity.

C2-CER-induced generation of superoxides and lipid peroxides is mediated by the Tiam1/Rac1 signaling pathway:

Herein, I have examined the possible involvement of Tiam1/Rac1 signaling cascade in C2-CER-induced oxidative stress in β-cells. Data shown in Fig. 23 suggested a significant activation of Rac1 by C2-CER in INS 832/13 cells [Panel A; left] and normal rat islets [Panel A; right]. Moreover, coprovision of NSC23766 substantially inhibited C2-CER-induced Rac1 activation in both cell types. These data clearly suggest that C2-CER-induced effects on isolated β-cells may, in part, be due to activation of a Rac1-dependent signaling mechanism. Furthermore, I have noticed that C2-CER induced generation of lipid peroxides [Fig. 23; Panel B] or superoxides [Fig. 23; Panel C] was reduced [~27–60%] by NSC23766, thus suggesting novel regulation of CER-mediated effects by a Tiam1/Rac1-dependent signaling mechanism [see below].

C2-CER, but not its inactive analogue, promotes mitochondrial dysfunction in INS 832/13 cells in a Tiam1/Rac1 signaling pathway:

Our laboratory has recently reported that exposure of isolated β-cells to C2-CER results in significant abnormalities in mitochondrial function including loss in membrane potential and leakage of cytochrome C into the cytosolic compartment **[207]**. Therefore, in the last set of experiments I verified if Tiam1/Rac1 signaling step might underlie the CER-induced mitochondrial dysfunction in INS 832/13 cells. To address this, mitochondrial membrane potential [MMP] was quantitated by the JC-1 staining method

in cells exposed to diluent or C2-CER in the absence or presence of NSC23766. To determine the specificity of CER effects, I have also utilized Dihydroceramide [DHC], an inactive analogue of CER, on MMP in INS 832/13 cells. Data in Fig. 24 indicated that exposure of these cells to C2-CER [lower left panel], but not DHC [middle left panel] significantly lowered the MMP as evidenced by staining of the majority of cells in green due to reduced MMP. Furthermore, NSC23766 prevented C2-CER-induced loss in membrane potential [as evidenced by a strong J-aggregation; red color] in these cells, further supporting the hypothesis that Tiam1/Rac1 signaling pathway contributes to CER-induced metabolic dysfunction in the pancreatic β-cells.

Cytomix induces phagocyte-like NADPH oxidase activation in INS 832/13 cell:

We quantitated NADPH oxidase activity in INS 832/13 cells exposed to Cytomix (i.e., IL-1β, IFN-γ, TNF-α; 10 ng/ml each). The amount of ROS generation and the degree of expression of NADPH subunits ($p47^{phox}$ and $p67^{phox}$) were determined following a 12-h or 24-h incubation of these cells with Cytomix. Data in Fig. 25 showed a significant increase in ROS generation at these time points (~60 and 85% above the control at 12 h and 24 h, respectively). Compatible with these findings are data presented in Fig. 26; Panel A and B, indicating a significant increase in the expression of $p47^{phox}$ in these cells following exposure to Cytomix. However, no effect of Cytomix on the expression of $p67^{phox}$ was demonstrable under these conditions (Fig. 26; Panel C and D).

To further assess whether the Cytomix-induced ROS are derived from NADPH oxidase, we quantitated Cytomix-induced ROS generation following inhibition of NADPH

oxidase *via* two independent approaches. In the first approach, we used apocynin, a selective inhibitor of NADPH oxidase. Data from these studies indicated a marked inhibition in Cytomix-induced ROS generation by apocynin. The values for Cytomix-mediated ROS generation represented 154.0 ± 3.9% and 167.8 ± 6.5% at 12 h and 24 h, respectively. The corresponding values in the presence of apocynin reached basal levels (i.e., 98.1 ± 5.2% and 106.6 ± 9.1% at 12 and 24 h, respectively; *n*=3 experiments in each case; $P < 0.05$). In the second approach, endogenous expression of the $p47^{phox}$ was knocked down by transfecting cells with siRNA-$p47^{phox}$. Under the current experimental conditions employed in the study (see MATERIALS AND METHODS), we were able to reduce $p47^{phox}$ expression by ~60–70% in siRNA-$p47^{phox}$-transfected cells. Furthermore, the ability of Cytomix to induce ROS generation (following 12 h of incubation) was completely abolished in siRNA$p47^{phox}$-transfected cells (i.e., 102.0 ± 5.5% of control; *n*=3 experiments), suggesting that NADPH oxidase might be the principal contributor in Cytomix-induced generation of ROS.

Cytomix transiently increases Rac1 activation in INS 832/13 cells: potential requirement for Tiam1 as a guanine nucleotide exchange factor for Rac1:

As stated above, Rac1, a small G protein, is one of the components of the NADPH oxidase holoenzyme assembly. Therefore, we next examined whether Cytomix-induced activation of NADPH oxidase is mediated via activation of Rac1. This was accomplished by quantitating the GTP-bound Rac1 (active configuration) by a pull-down assay (see MATERIALS AND METHODS for additional details). Data depicted in Fig. 27; Panel A and B suggested a significant (~1.7-fold), but transient, activation of Rac1 (within 15 min) in INS 832/13 cells following exposure to Cytomix. Rac1.GTP levels

reached basal levels at 30 min of exposure. These data implicate Rac1 activation as one of the signaling steps involved in Cytomix mediated effects on isolated β-cells.

Recently, we reported the expression and functional activation of GEFs for small G proteins in pancreatic β-cells **[204]**. The primary function of these proteins is to facilitate GTP/GDP exchange. Our findings in INS 832/13 cells and primary rat islets have indicated that Tiam1 serves as a GEF for Rac1 **[204]**. In the current study, we investigated whether Tiam1 is required for Cytomix-induced activation of Rac1 in INS 832/13 cells. This was accomplished using pharmacological inhibitor, NSC23766, which selectively inhibits Tiam1-mediated activation of Rac1, but not Cdc42 or Rho in insulin-secreting β-cells **[208]**. Data in Fig. 27; Panel C suggested a significant reduction in Cytomix-induced activation of Rac1 by NSC23766 in INS 832/13 cells. These findings support the viewpoint that Tiam1 plays a key regulatory role in Rac1 activation elicited by Cytomix in insulin-secreting cells.

It is well established that posttranslational geranylgeranylation is necessary for optimal activation of Rac1 in pancreatic β-cells (see **Ref 84**, for a review). Therefore, we examined whether geranylgeranylation of Rac1 is necessary for Cytomix-induced activation of Rac1. This was accomplished *via* a pharmacological approach, which involved quantitation of Cytomix-induced activation of Rac1 in cells exposed to diluent or in the presence of GGTI-2147, a known inhibitor Rac1 geranylgeranylation **[84, 85]**. Data in Fig. 27; Panel D, showed a marked reduction in Cytomix-induced Rac1 activation in cells exposed to GGTI-2147. Together, data depicted in Fig. 27 suggested

that Cytomix induces Rac1 activation in INS 832/13 cells, which is sensitive to inhibition of Tiam1 activation and posttranslational geranylgeranylation.

NSC23766 and GGTI-2147 markedly reduce Cytomix-induced ROS generation in INS 832/13 cells:

As a logical extension to the above studies, we asked whether inhibitors of Rac1 attenuate Cytomix-induced ROS generation. Data shown in Fig. 28 indicate a marked reduction in Cytomix-induced ROS generation at both 12- and 24-h time points by NSC23766 and GGTI-2147. It is noteworthy that GGTI-2147, but not NSC23766, also reduced the ROS generated under basal conditions (Fig. 28). Taken together, our findings establish a direct role for Tiam1-dependent, prenylation-sensitive Rac1activation in the signaling cascade leading to Cytomix-induced NADPH oxidase and ROS generation in INS 832/13 cells.

Inhibitors of Rac1 activation reduce Cytomix-induced mitochondrial defects in INS 832/13 cells:

It is widely felt that cytokine-mediated effects on pancreatic β-cells may, in part, be mediated *via* alterations in mitochondrial membrane properties, including loss of MMP leading to cytochrome-*c* release and caspase-3 activation **[171, 207, 217]**. Therefore, we examined whether inhibitors of Rac1 activation exert protective effects on Cytomix-induced loss in MMP. This was accomplished using JC-1 (5, 5`, 6, 6`-tetrachloro-1, 1`, 3, 3`-tetraethylbenzimidazolyl-carbocyanineiodide) assay. JC-1 is a lipophilic dye, which fluoresces red when aggregated above the critical concentration within mitochondria. In cells in which mitochondrial membrane is damaged, JC-1

remains in the cytoplasm, as a green fluorescence monomer. Data from these studies, which are depicted in Fig. 29, suggested a significant loss of MMP in INS 832/13 cells treated with Cytomix following a 12- or 24-h exposure. Coprovision of NSC23766, a Tiam1 inhibitor, and GGTI-2147, a prenylation inhibitor, modestly, but significantly protected these cells against Cytomix-induced damage to the mitochondrial potential (Fig. 29). Quantitation of fluorescence intensity ratios of red to green further confirmed these conclusions (Fig. 30). Further, these data also suggested that the protective effects were more prominent in the case of NSC23766 compared with GGTI-2147 (Figs. 29 and 30). It should be mentioned that NSC23766 exerted inhibitory effects on MMP in control (i.e., diluent-treated cells). Regardless of this inhibitory effect, it markedly prevented Cytomix-induced loss in MMP at both time points. Together, these data indicate that Rac1 activation might be requisite for Cytomix-induced mitochondrial defects in pancreatic β-cells.

Tiam1/Rac1 signaling axis is not necessary for Cytomix-induced caspase 3 activation in INS 832/13 cells:

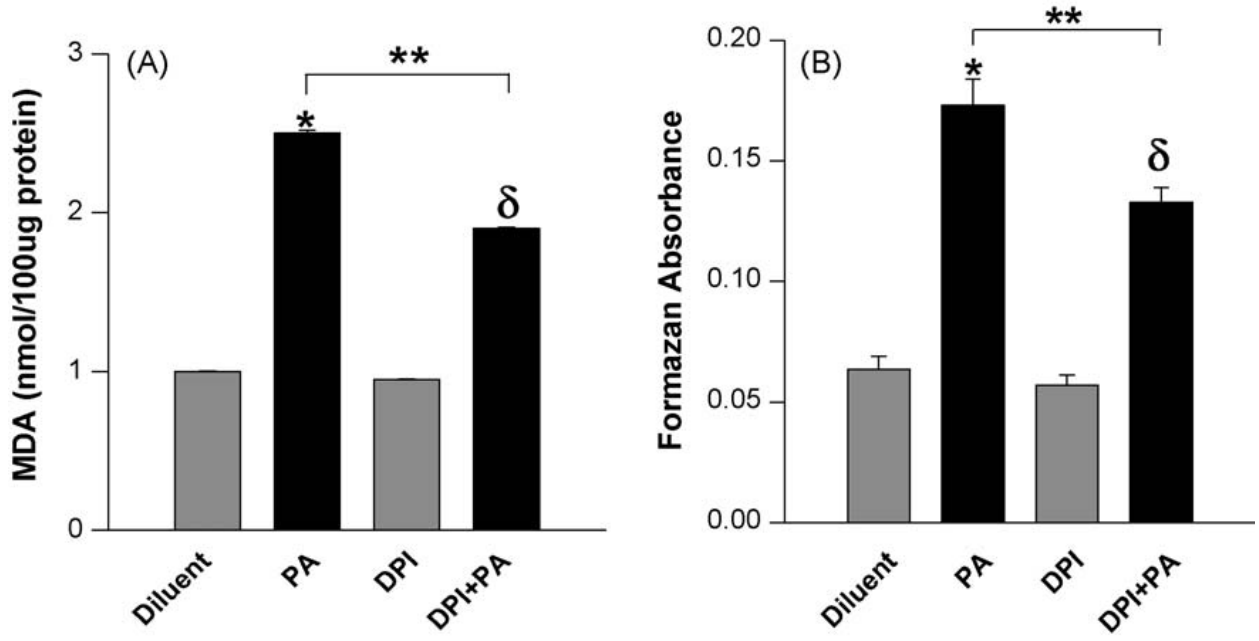
The observed protective effects of NSC23766 against Cytomix-induced loss in MMP (Figs. 29 and 30) prompted us to investigate whether caspase 3 activation, which is a hallmark of cellular apoptosis, is inhibited by Tiam1-mediated activation of Rac1. To accomplish this, INS 832/13 cells were treated with Cytomix (as above) or IL-1β alone (25 ng/ml) for 12 or 24 h. Activated caspase-3 in the lysates was determined by Western blot analysis using an antiserum that identifies both the native procaspase and degradative product of caspase-3. Under these conditions we noticed no significant effects of NSC23766 on either Cytomix-induced or IL-1β-mediated caspase-3 activation

at either time points. Cytomix-induced caspase-3 activation represented 1.55 ± 0.11 units and 1.83 ± 0.24 units at 12 and 24 h, respectively. The corresponding values in the presence of NSC23766 were 1.40 ± 0.14 units and 2.06 ±0.32 units, respectively (*n*=3 determinations in each case, not significantly different from each other). Likewise, IL-1β-induced caspase-3 activation represented 1.27 ± 0.10 units and 1.65 ± 0.23 units at 12 and 24 h, respectively. The corresponding values in the presence of NSC23766 were 1.23 ± 0.09 units and 1.71 ± 0.22 units, respectively (*n*=3 determinations in each case, not significantly different from each other). Together, these data indicate that additional mechanisms might underlie caspase-3 activation in these cells elicited by cytokines.

Evidence to further suggest that the Tiam1/Rac1 signaling step may not be required for cytokine-induced NO release from INS 832/13 cells:

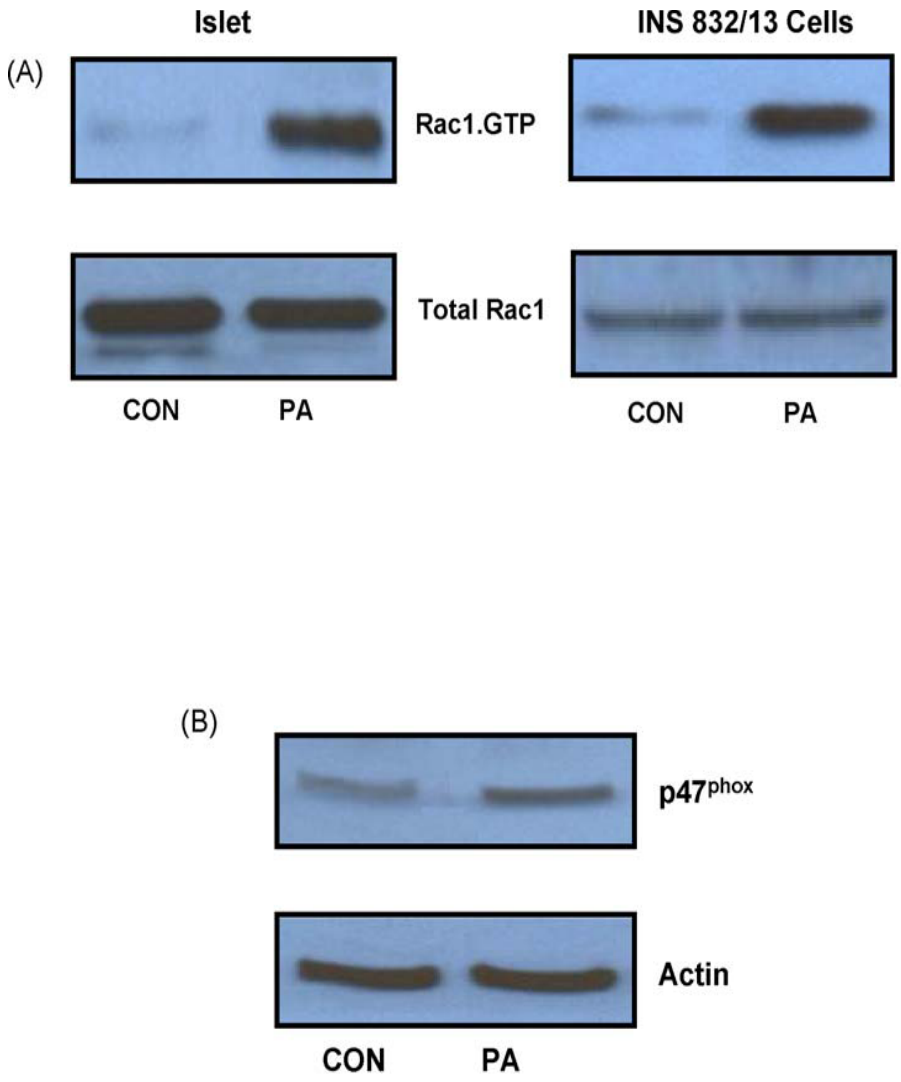
It is well established that cytokine-mediated effects on isolated β-cells are mediated *via* inducible nitric oxide synthase (iNOS) expression and associated NO release. It has also been suggested that NO exerts damaging effects on mitochondria leading to caspase-3 activation. Therefore, in the last set of studies, we investigated whether Tiam1/Rac1 activation is necessary for cytokine-induced NO release in INS 832/13 cells. Data in Fig. 31 demonstrated no significant effect of NSC23766 on either IL-1β or Cytomix-induced NO release in INS 832/13 cells either at 12 or 24 h. Together, the above data suggest that Tiam1/Rac1 signaling step is not involved in cytokine-induced NO release and caspase-3 activation and that additional regulatory steps might be necessary for these to occur.

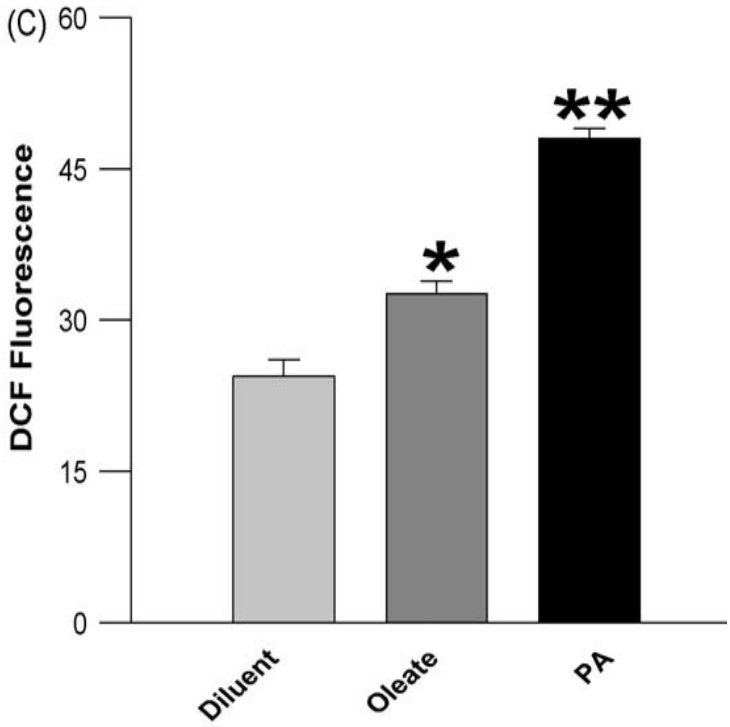
Figure 17:

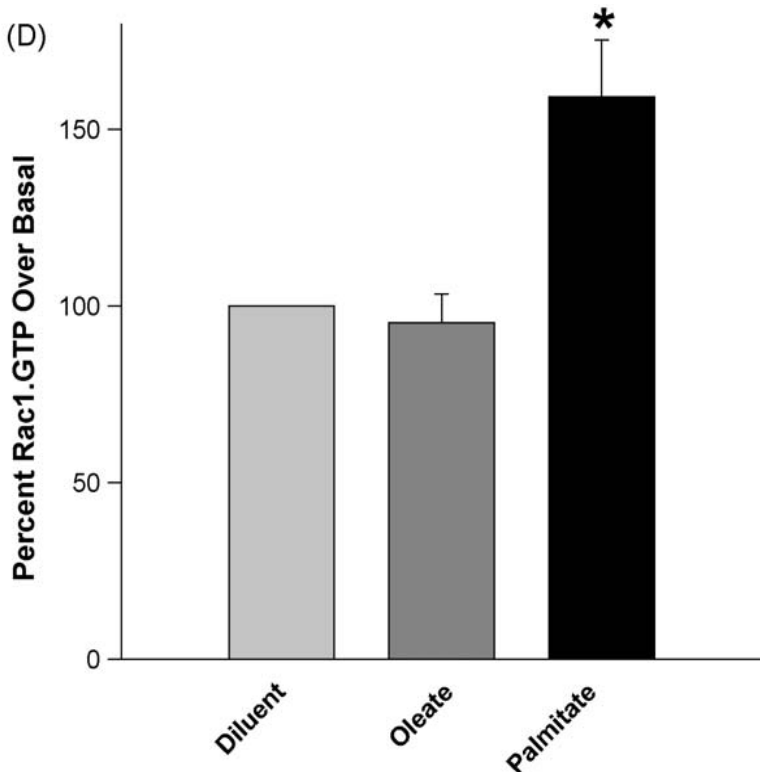


PA induces generation of lipid peroxides and superoxides in INS 832/13 cells: protection by DPI. INS 832/13 cells were incubated [6 h] with either diluent or PA [100 μM] and/or DPI [5 μM] as indicated in the figure. Lipid hydroperoxide levels were measured as MDA equivalents [Panel A] and superoxide levels [Panel B] were quantitated as formazan equivalents. Data are mean ± SEM from three independent determinations. Values were considered significant at $p < 0.05$. *Significant effect of PA to diluent. δ Significance between DPI and DPI + PA. **Significance between PA and DPI + PA.

Figure 18:

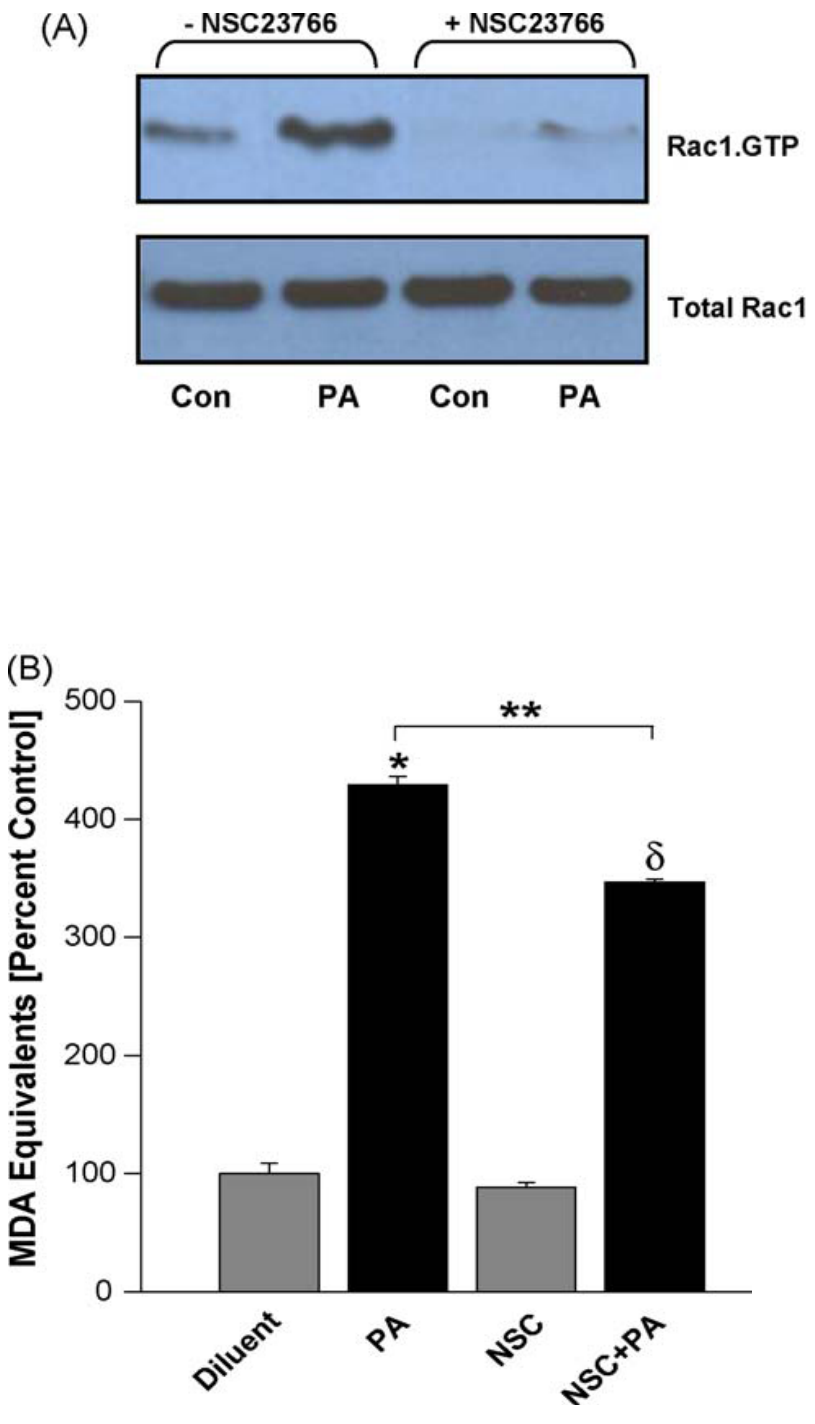


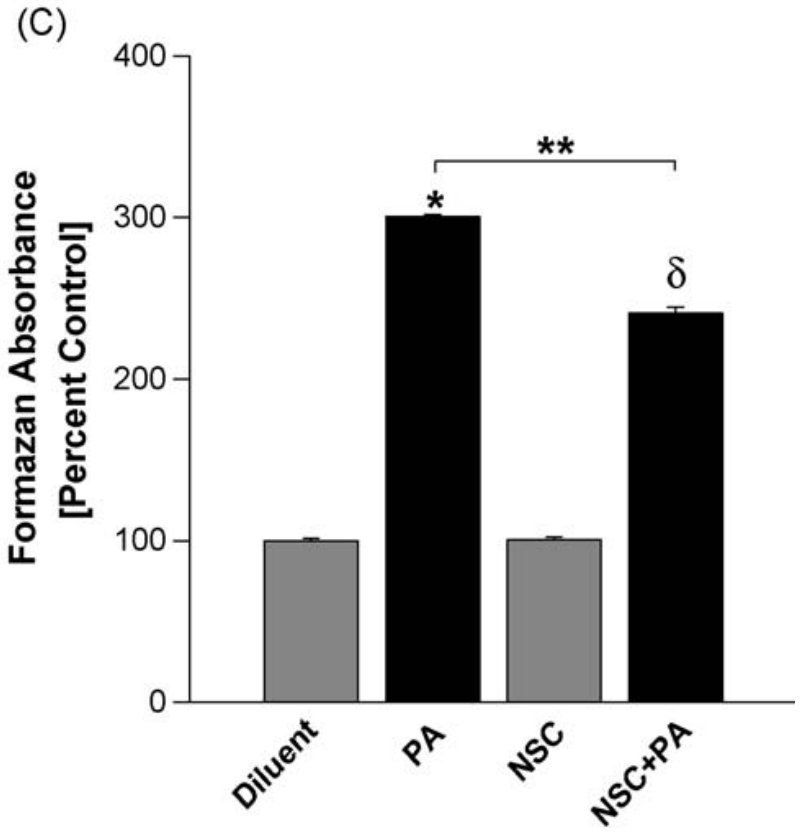




PA, but not oleate, induces Rac1 activation and Nox activation in β-cells. Normal rat islets and INS 832/13 cells were treated with diluent or PA [100 μM; Panel A]. The relative amounts of activated Rac1 [i.e., Rac1-GTP] were determined from these lysates by PAK-PBD pull down assay. Data are representative of two independent experiments. Panel B: lysates derived from INS 832/13 cells treated in the absence or presence of PA [100 μM] were separated by SDS-PAGE, and probed for $p47^{phox}$ and actin expressions. A representative blot from two independent experiments is shown here. Panel C: lysates derived from INS 832/13 cells treated in the absence or presence of PA or oleate [100 μM each] were processed for Nox activity and were quantitated by the DCHFDA assay and are expressed as DCF fluorescence units. Data are mean ± SEM from two individual measurements for DCF fluorescence. *,**$p < 0.05$ *vs*. diluent. Panel D: INS 832/13 cells were treated with diluent and/or oleate [100 μM] or PA [100 μM] and the relative amounts of activated Rac1 were determined by PAK-PBD pull down assay. Data presented in here are densitometric analysis of the blots and are mean ± SEM from four independent experiments. *$p < 0.05$ *vs*. diluent.

Figure 19:

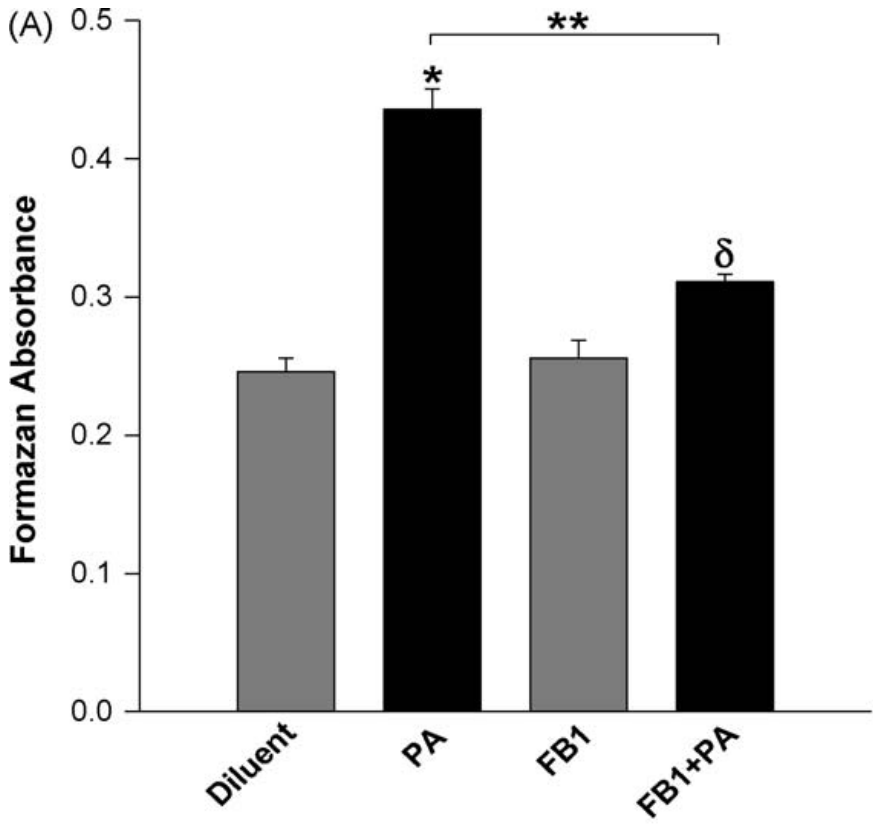


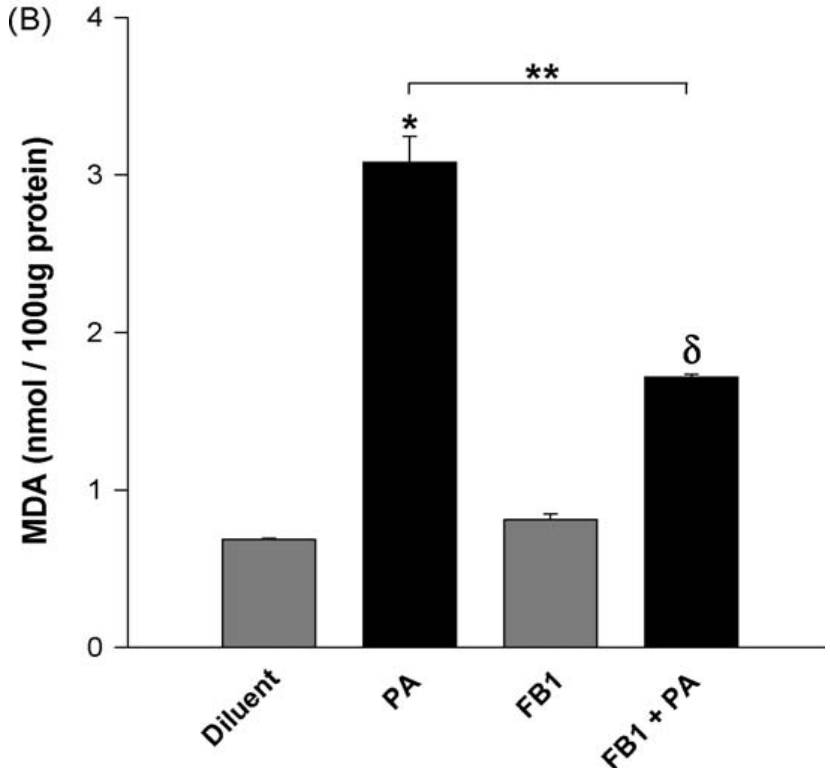


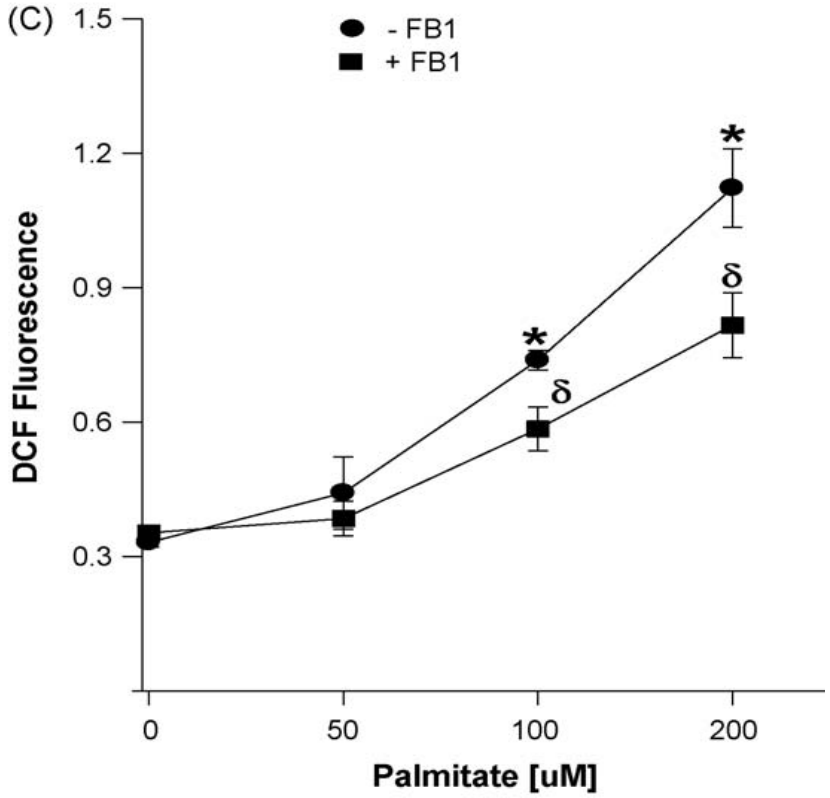
NSC23766, a specific inhibitor of Tiam1-mediated activation of Rac1, markedly attenuates PA-induced Rac1 activation in INS 832/13 cells. INS 832/13 cells were incubated overnight with either diluent or NSC23766 [20 μM]. The cells were further incubated [3 h] in the presence of either low glucose [5 mM] or PA [100 μM] in the continuous presence of NSC23766 or diluent. The degree of Rac1 activation was determined by PAK-PBD pull down assay. Panel A: Data are representative of two independent experiments. Levels of lipid hydroperoxides [Panel B] or ROS [Panel C] generated in PA or diluent-treated INS 832/13 cells in the absence or presence of

NSC23766 were measured as MDA equivalents or formazan equivalents, respectively. Data are mean ± SEM from three determinations. Values were considered significant at $p < 0.05$. *Significant effect of PA to diluent. δ Significance between NSC and NSC + PA. **Significance between PA and NSC + PA.

Figure 20:

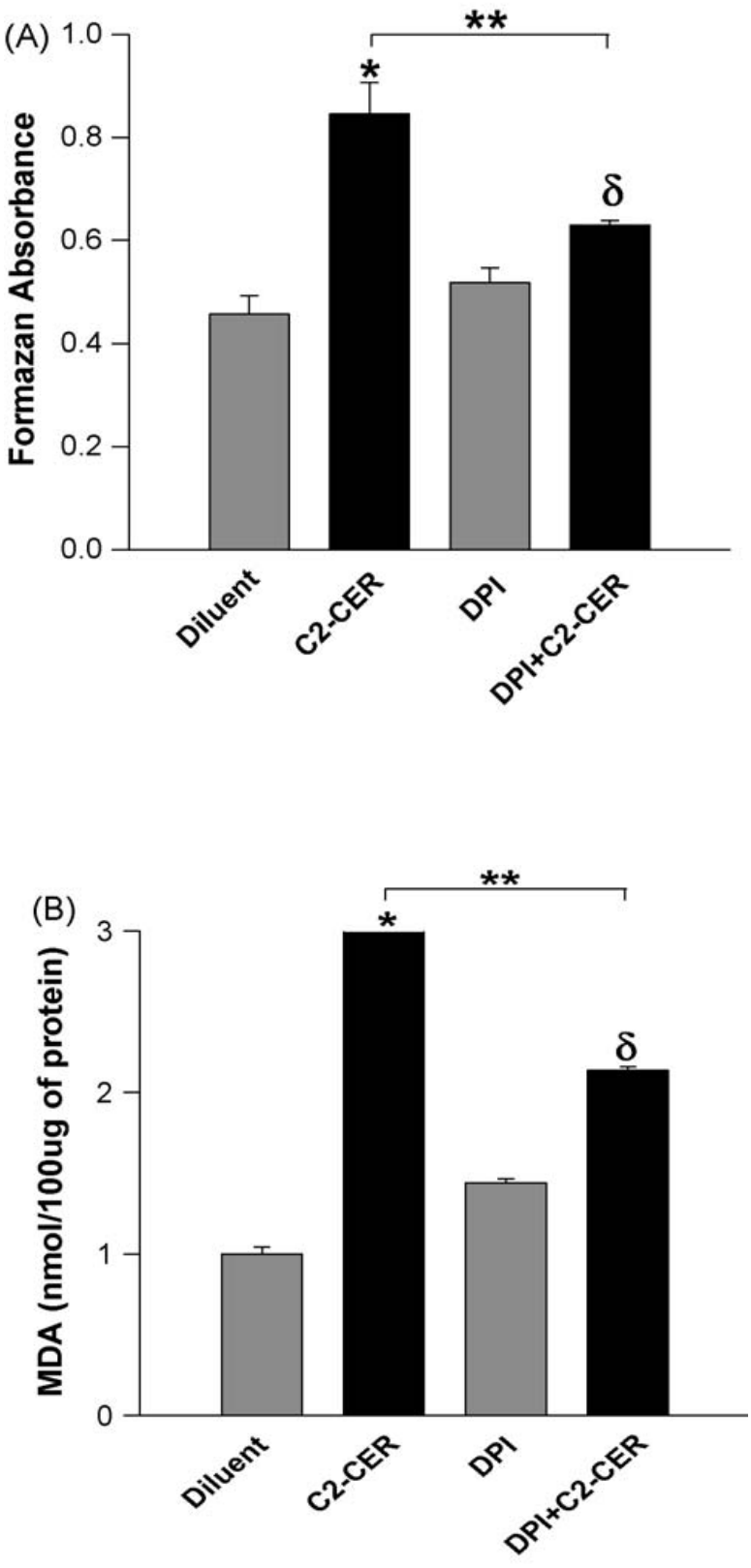






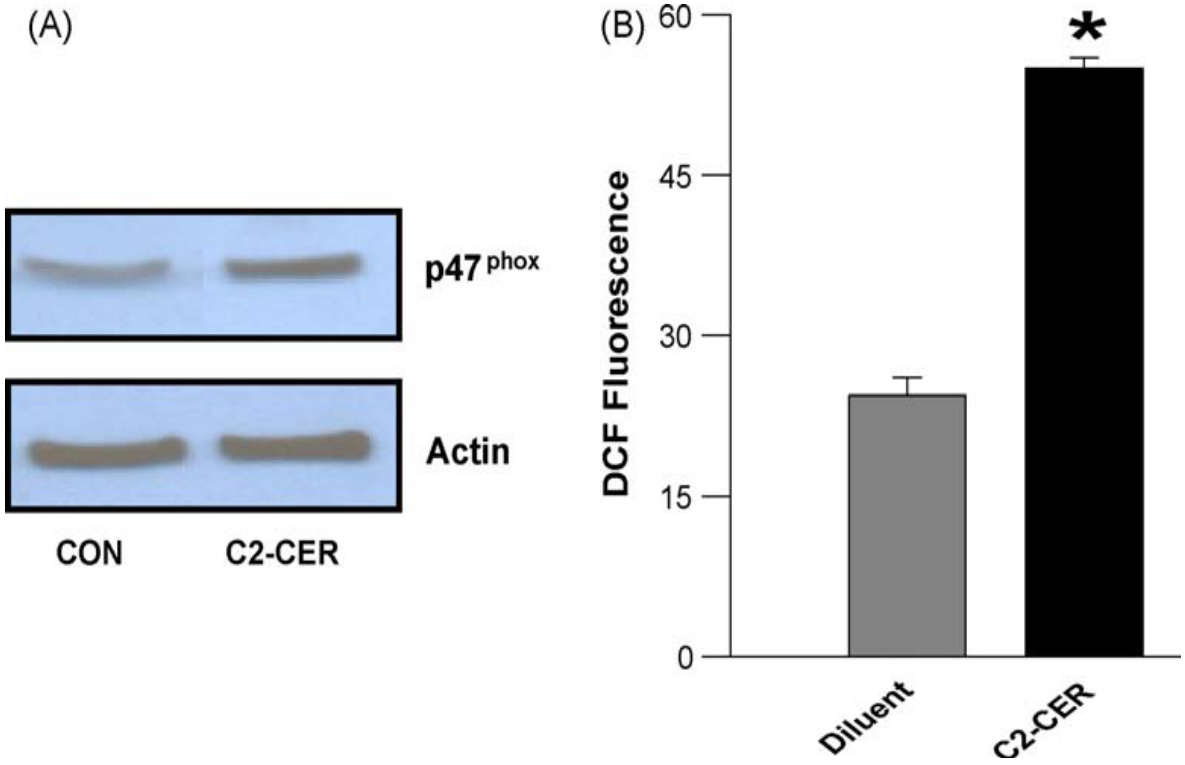
Fumonisin B-1, an inhibitor of *de novo* biosynthesis of CER from PA, markedly reduces PA-induced generation of lipid peroxides and superoxides in INS 832/13 cells. INS 832/13 cells were pre-treated in the presence or absence of FB-1 [10 μM] prior to the addition of PA [100 μM] and lysates derived from these cells were assessed for generation of superoxides and lipid peroxides. Superoxide generation was quantitated by NBT method and expressed as formazan equivalents [Panel A]. Lipid peroxide levels were quantitated by the MDA assay, and expressed as nmoles of MDA formed/100 μg protein [Panel B]. Data are mean ± SEM from three determinations. Values were considered significant at $p < 0.05$. *Significant effect of PA to diluent. δ Significance between FB1 and FB1 + PA. **Significance between PA and FB1 + PA. Furthermore, cells were pretreated in the presence or absence of FB1 [10 μM] prior to the addition of PA at different concentrations [0–200 μM]. Lysates derived were processed for Nox activity and were quantitated by the DCHFDA assay [Panel C] and are expressed as DCF fluorescence. Data are mean ± SEM from three determinations. Graph with different symbols is statistically significant at $p < 0.001$. * PA-induced ROS *vs*. diluent. δ PA + FB1 induced ROS *vs*. FB1.

Figure 21:



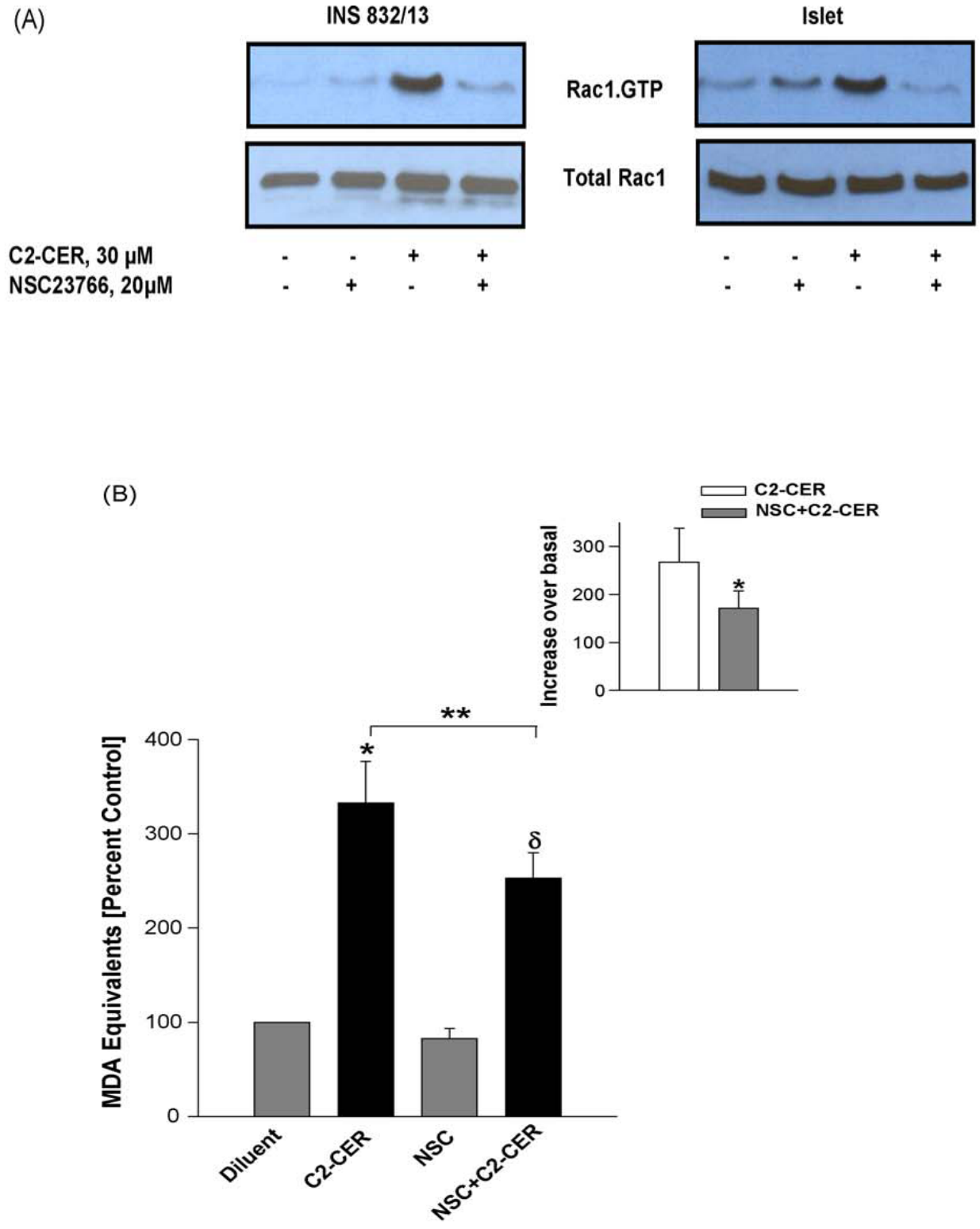
C2-CER promotes generation of lipid peroxides and ROS in INS 832/13 cells by activating endogenous NADPH oxidase activity. INS 832/13 cells were treated with either diluent or C2-CER [30 μM] and/or DPI [5 μM] in various combinations as indicated in the figure. The degree of ROS generation was quantitated by the NBT method and is expressed as formazan equivalents [Panel A]. The amount of lipid hydroperoxide generation was quantitated by the MDA assay and is expressed as MDA equivalents [Panel B]. Data are mean ± SEM from three determinations in each case. Values were considered significant at $p < 0.05$. *Significant effect of C2-CER *vs*. diluent. δ Significance between DPI and DPI + C2-CER. **Significance between C2-CER and DPI + C2-CER.

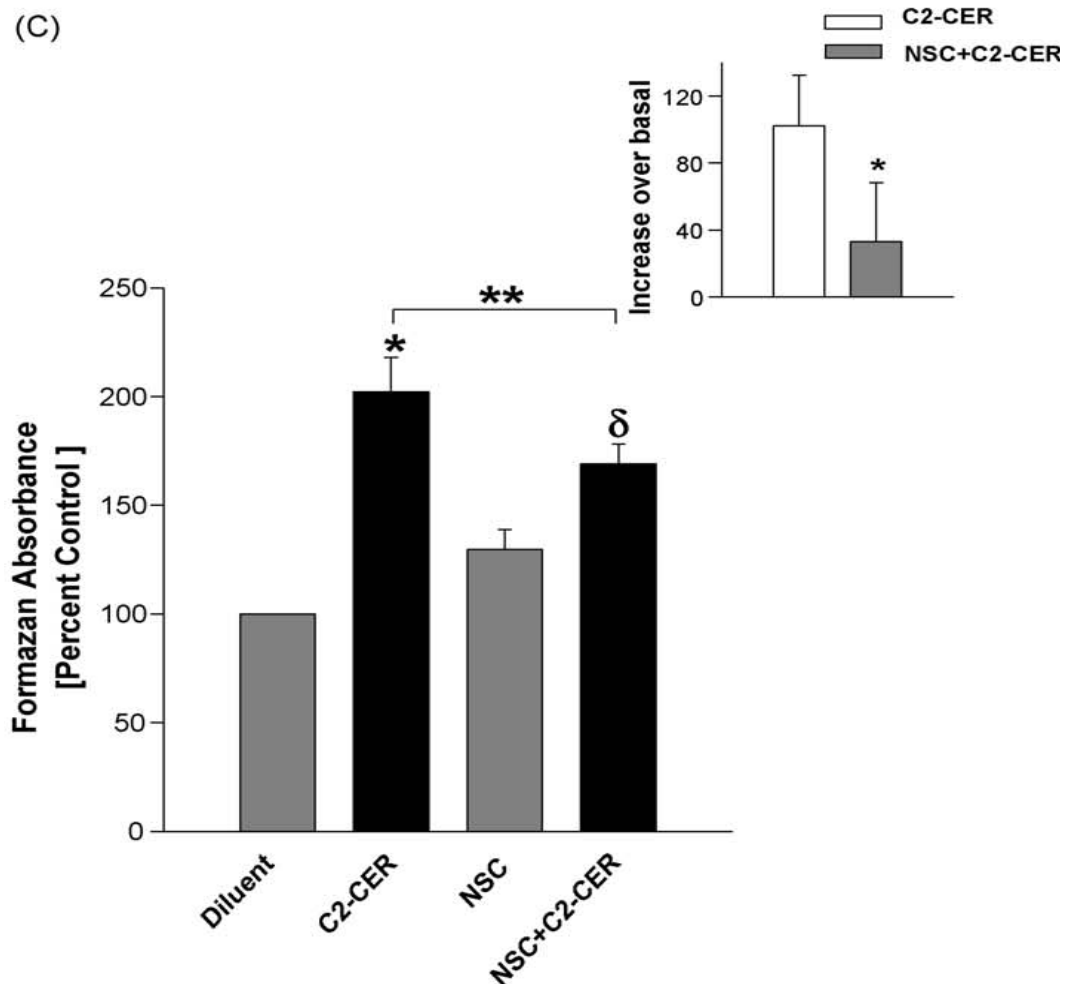
Figure 22:



C2-CER increases the expression of p47^{phox} and Nox activity in INS 832/13 cells. INS 832/13 cells were treated with either diluent or C2-CER [30 μM] and examined for relative increases in p47^{phox} expression and NADPH oxidase activity. Panel A: lysate proteins derived from diluent or C2-CER-treated cells were separated by SDS-page and probed for p47^{phox} and actin expression. A representative blot from two independent experiments is shown here. Panel B: Nox activity in diluent or C2-CER-treated cells was quantitated by the DCHFDA fluorescence assay and is expressed as DCF fluorescence. Data are mean ± SEM from two independent determinations. *$p < 0.05$ *vs.* diluent.

Figure 23:

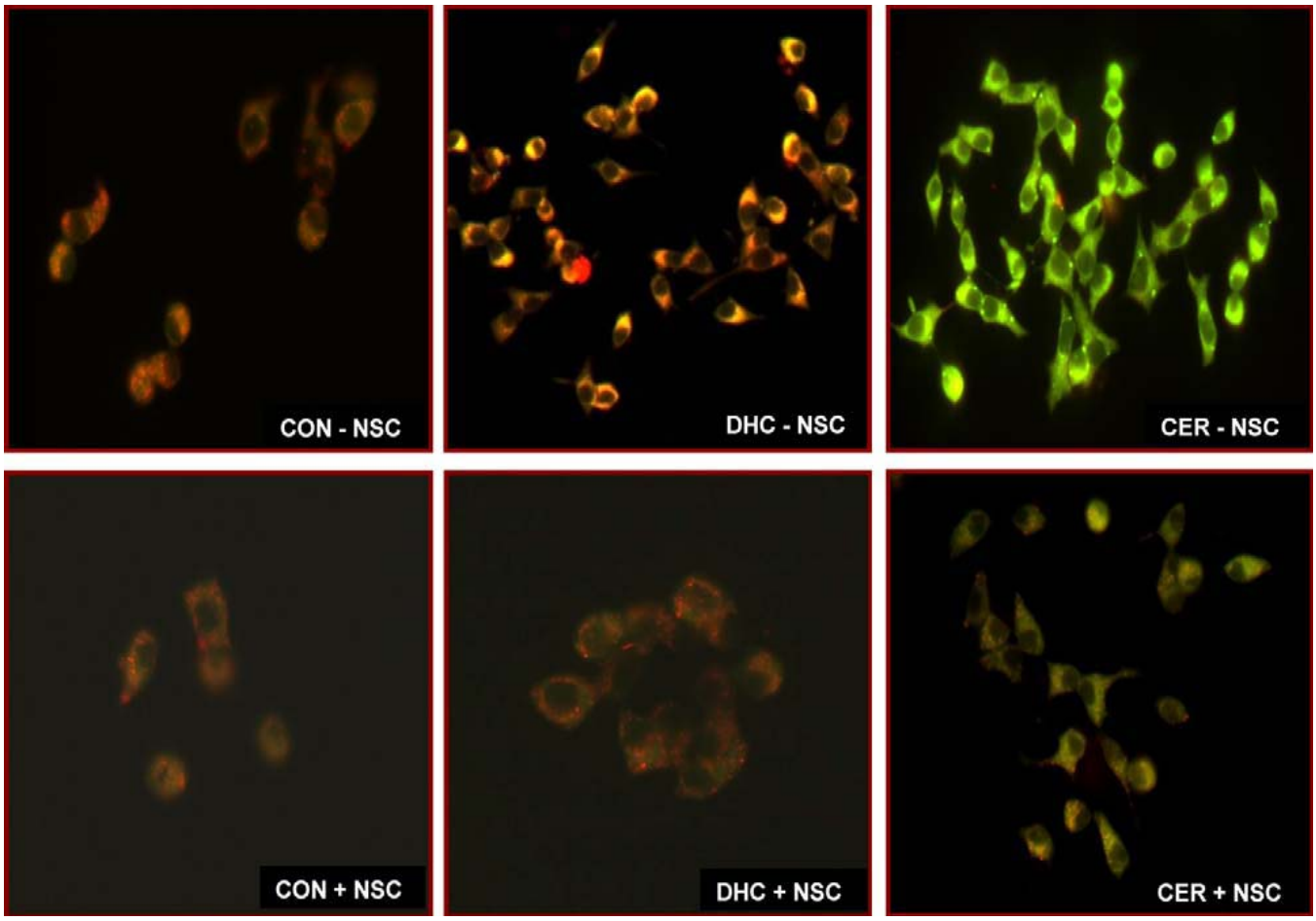




NSC23766 inhibits C2-CER-induced Rac1 activation and generation of lipid peroxides and superoxides in pancreatic β-cells. INS 832/13 cells and rat islet were treated with either diluent or NSC23766 [20 μM] and cultured overnight in low glucose/low serum media. Cells were further incubated in the presence of C2-CER [30 μM] for 30 min in INS 832/13 cells and 3 h in Islets in the continuous presence of NSC23766 or diluent. The relative amounts of activated Rac1 [i.e., Rac1-GTP] were determined by PAK-PBD pull down assay. Data are representative of two independent

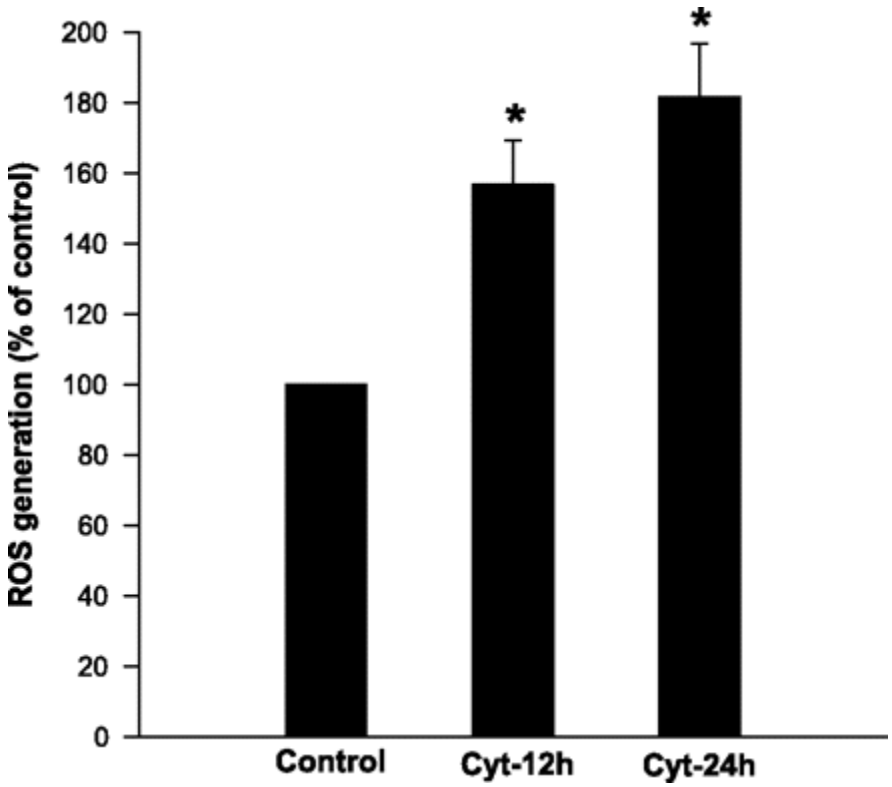
experiments [Panel A]. Panel B: INS 832/13 cells were incubated [6 h] with either diluent or with C2-CER [30 μM] or NSC23766 [20 μM; alone or in combination]. Lipid hydroperoxides were measured as MDA equivalents and plotted as increase over basal. Panel C: INS 832/13 cells were incubated [6 h] with either diluent or with C2-CER [30 μM] or NSC23766 [20 μM; alone or in combination as indicated in the figure]. Superoxide generation was measured as formazan equivalents and plotted as increase over basal. Data in the insets represent incremental response to C2-CER in the absence or presence of NSC23766. Data are mean ± SEM from three determinations in each case. Values were considered significant at $p < 0.05$. *Significant effect of C2-CER *vs*. diluent. δ Significance between NSC and NSC + C2-CER. ** Significance between C2-CER and NSC + C2-CER.

Figure 24:



NSC23766 inhibits C2-CER-induced mitochondrial dysfunction in pancreatic β-cells: INS 832/13 cells were treated with either diluent or NSC23766 [20 μM] and cultured overnight in low glucose and low serum media. Cells were further incubated in the presence of C2-CER [30 μM] and/or DHC [30 μM] for 6 h in the continuous presence of NSC23766 or diluent. Mitochondrial dysfunction was determined by JC-1 assay. Data are representative of two independent experiments.

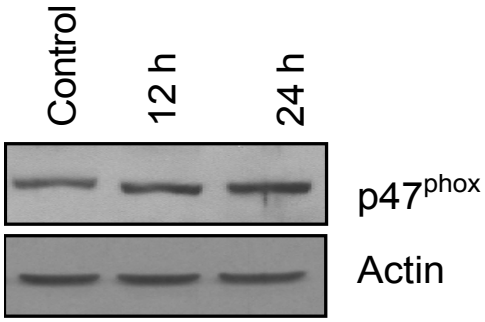
Figure 25:



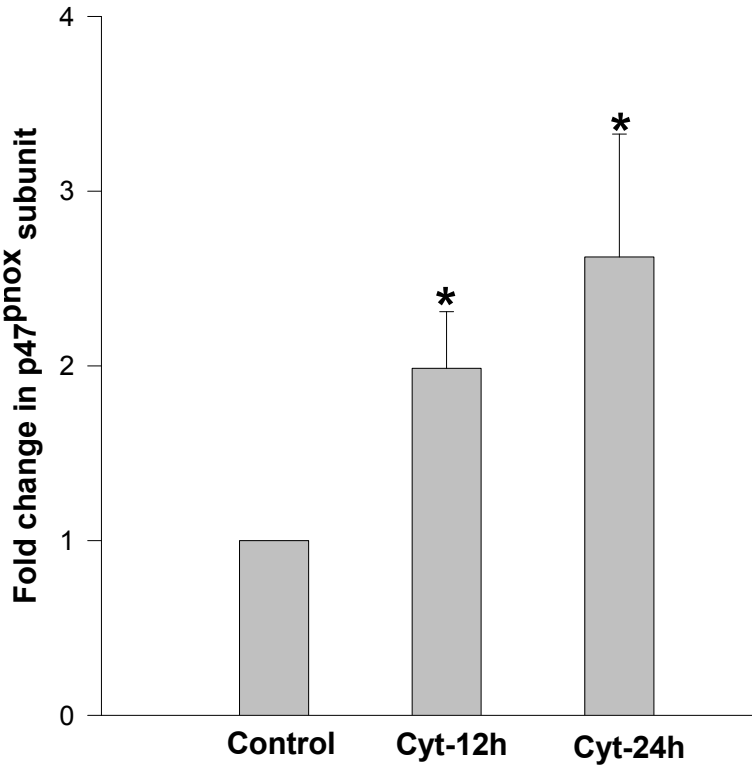
Incubation of INS 832/13 cells with Cytomix leads to a time-dependent increase in reactive oxygen species (ROS). INS 832/13 cells were incubated with either diluent or Cytomix for 12 or 24 h, as indicated in the figure, and ROS generation was measured using 2′, 7′-dichlorofluorescein diacetate (DCFHDA) assay. Intracellular levels of ROS in treated cells were expressed as a percent of control cells. Data are means ± SE from four independent experiments. * Significantly different ($P < 0.05$) from control.

Figure 26:

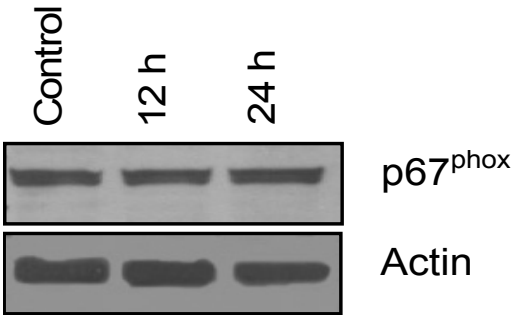
A]



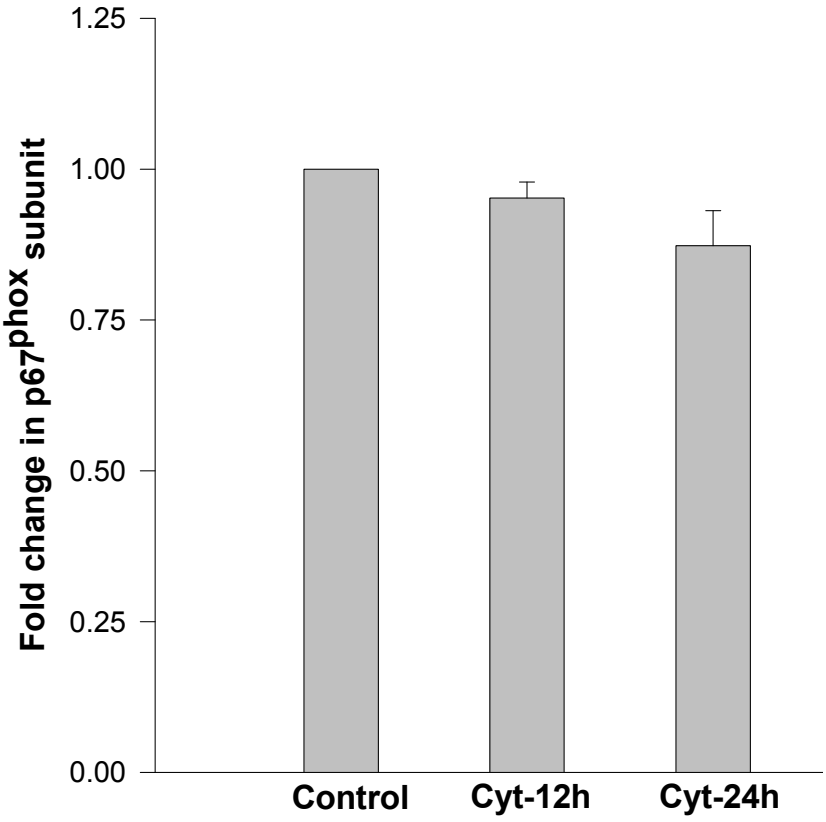
B]



C]



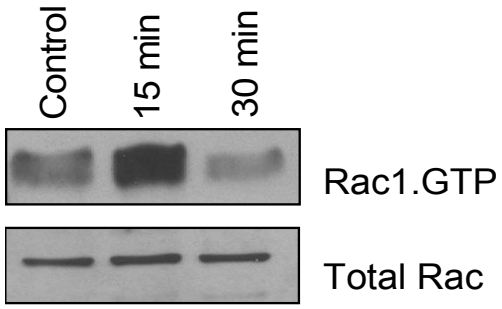
D]



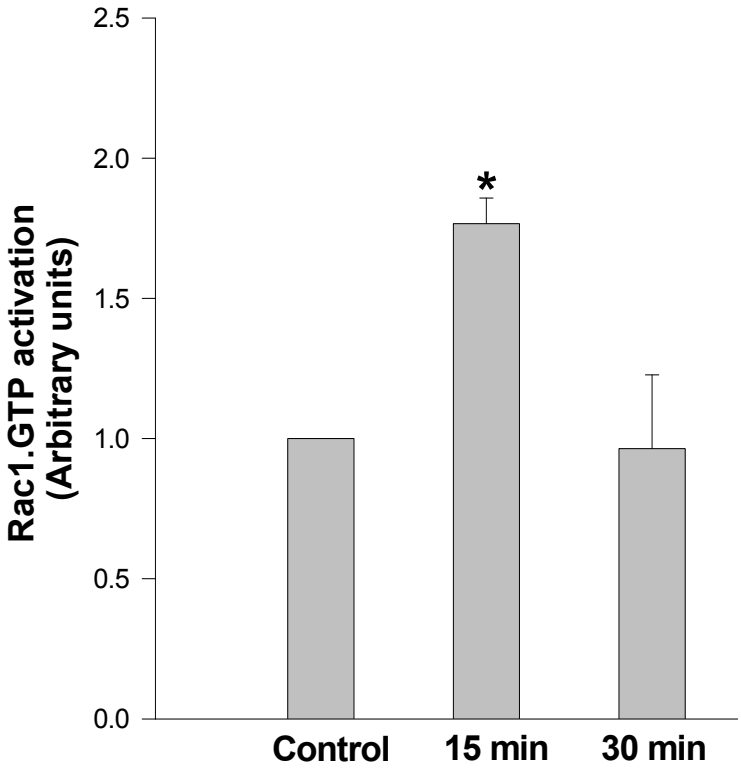
Panel A: INS 832/13 cells were exposed to Cytomix for 12 or 24 h as indicated. Relative degree of expression of $p47^{phox}$ was determined by Western blot analysis. $p47^{phox}$ expression was normalized to actin content in individual lanes. Pooled data from three independent experiments are provided in Panel B. * significantly different ($p < 0.05$) from control. Panel C: INS 832/13 cells were exposed to Cytomix for 12 or 24 h as indicated in the figure. Relative degree of expression of $p67^{phox}$ was measured by Western blot analysis. $p67^{phox}$ expression was normalized to actin content in individual lanes. Pooled data from three independent experiments are provided in Panel D.

Figure 27:

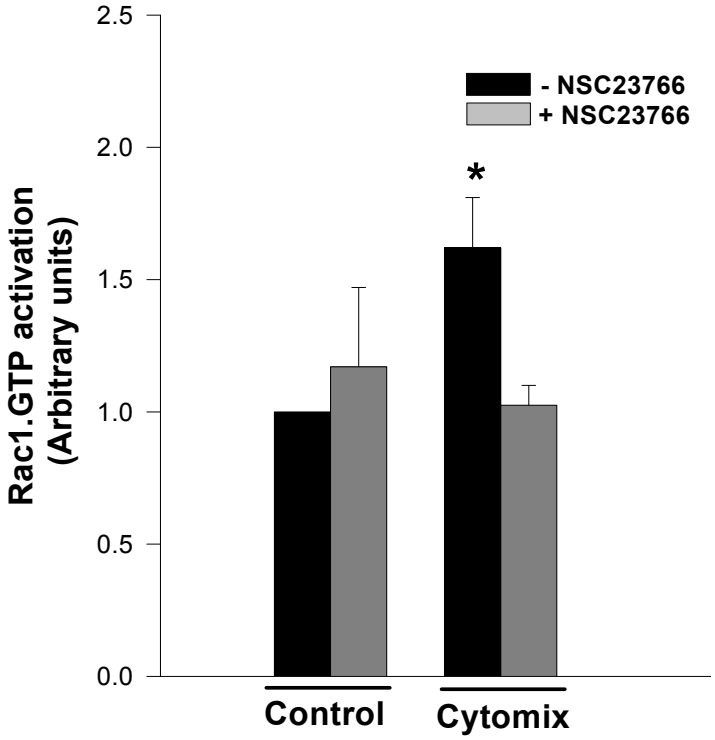
A]



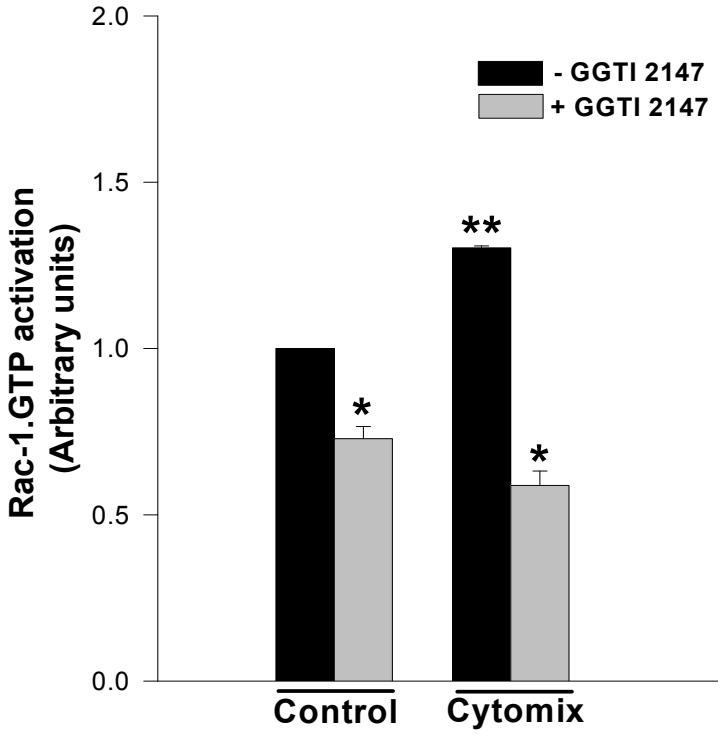
B]



C]



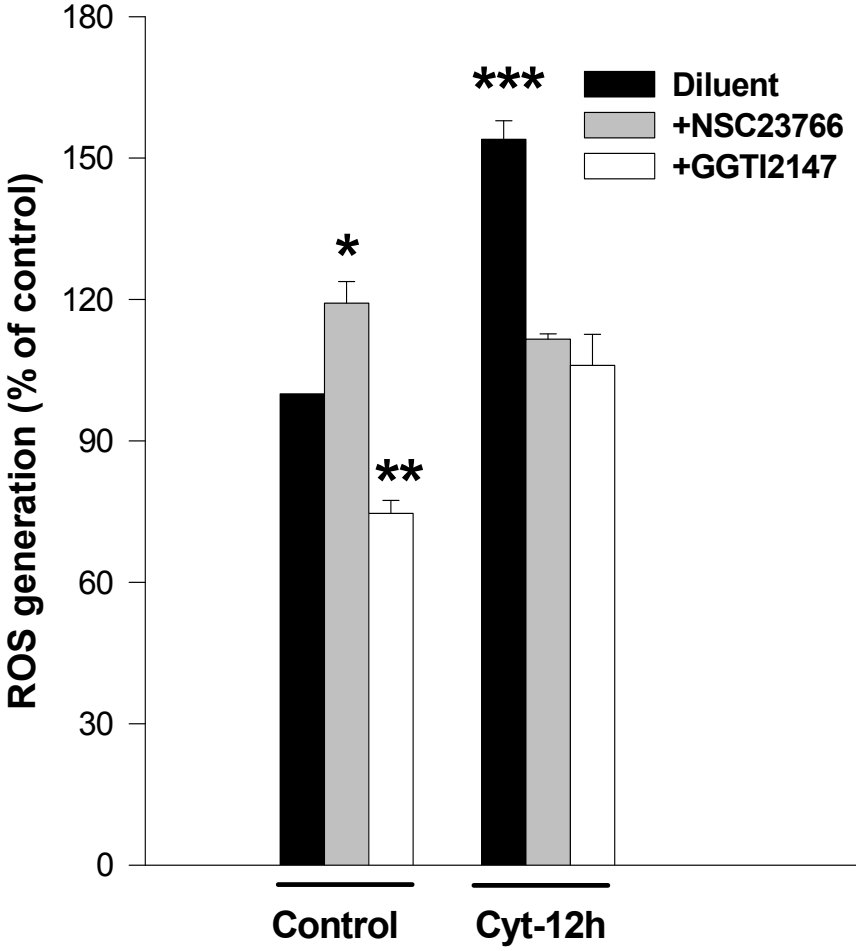
D]



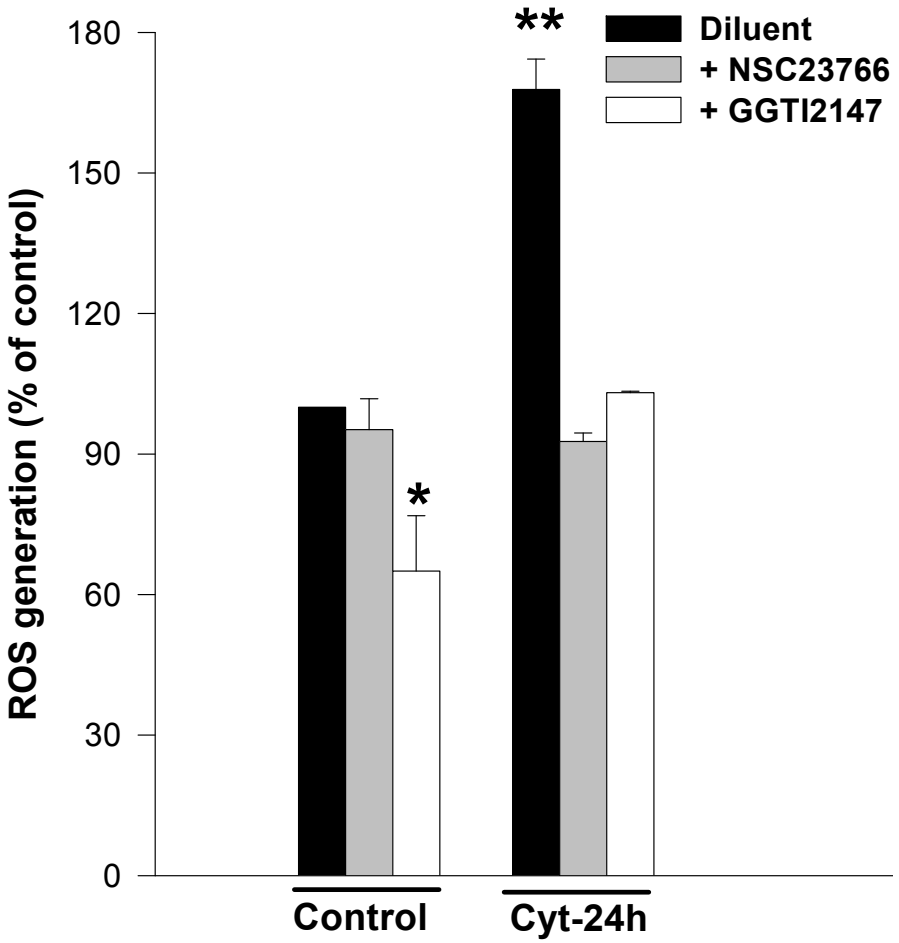
Cytomix induces transient activation of Rac1 in INS 832/13 cells: inhibition of this signaling step by NSC23766 and GGTI-2147. Panel A: Cytomix causes transient activation of small G-protein Rac1 in INS 832/13 cells, as determined by the pull-down assay followed by Western blot analysis (see materials and methods). Total Rac1 in the lysates is also provided as a loading control. A representative blot of three independent experiments is shown here. Panel B: pooled activation data from three independent experiments are shown here. Panel C: NSC23766 inhibition of Cytomix-induced activation of Rac1. Pooled data from three independent studies are depicted in the figure. Panel D: GGTI-2147 inhibits Cytomix-induced Rac1 activation in INS 832/13 cells. Pooled data from three independent studies are depicted in the figure. *Significantly different ($P < 0.05$) from control. *, **Different symbols represent the values that are significantly different at $P < 0.05$.

Figure 28:

A]

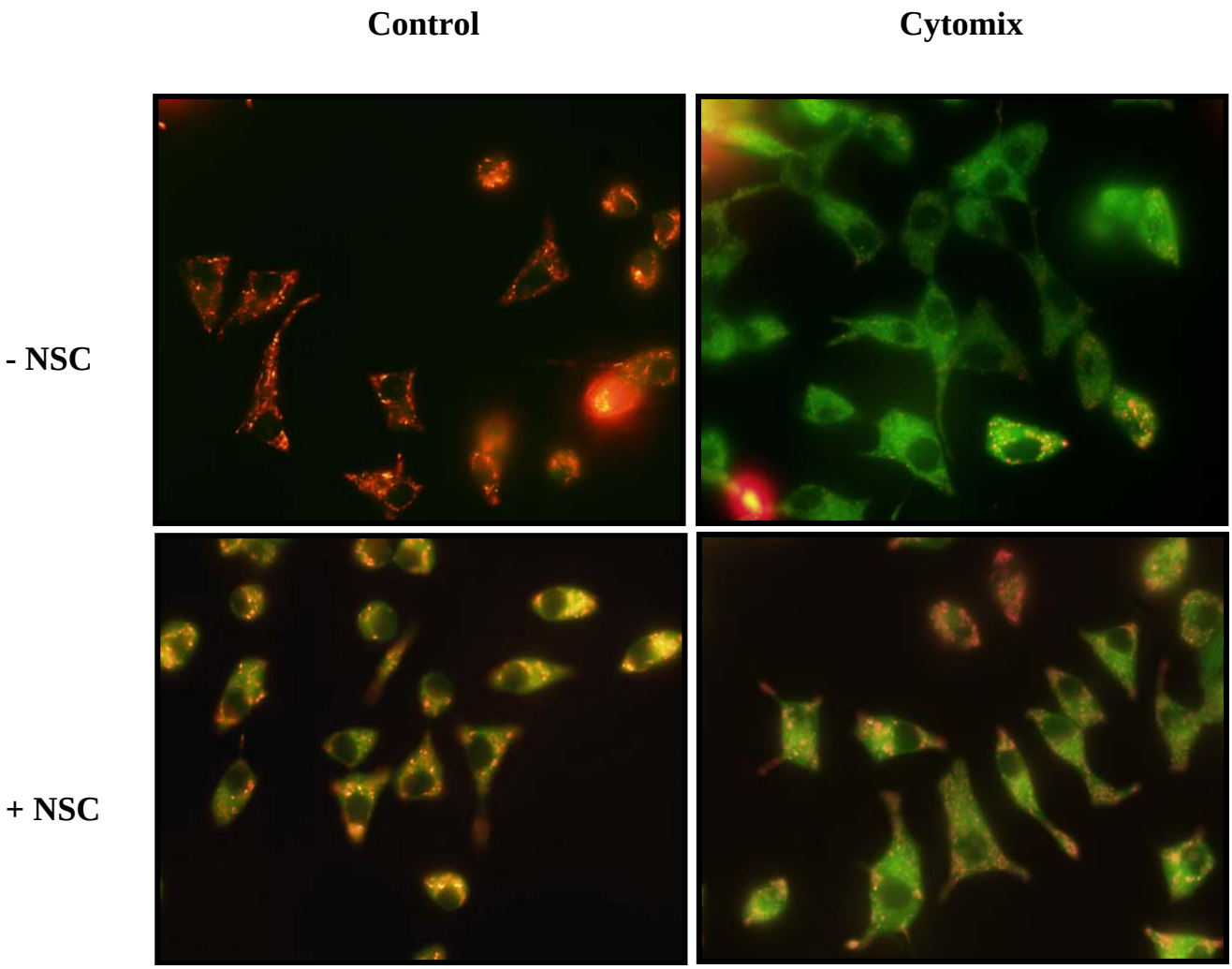


B]

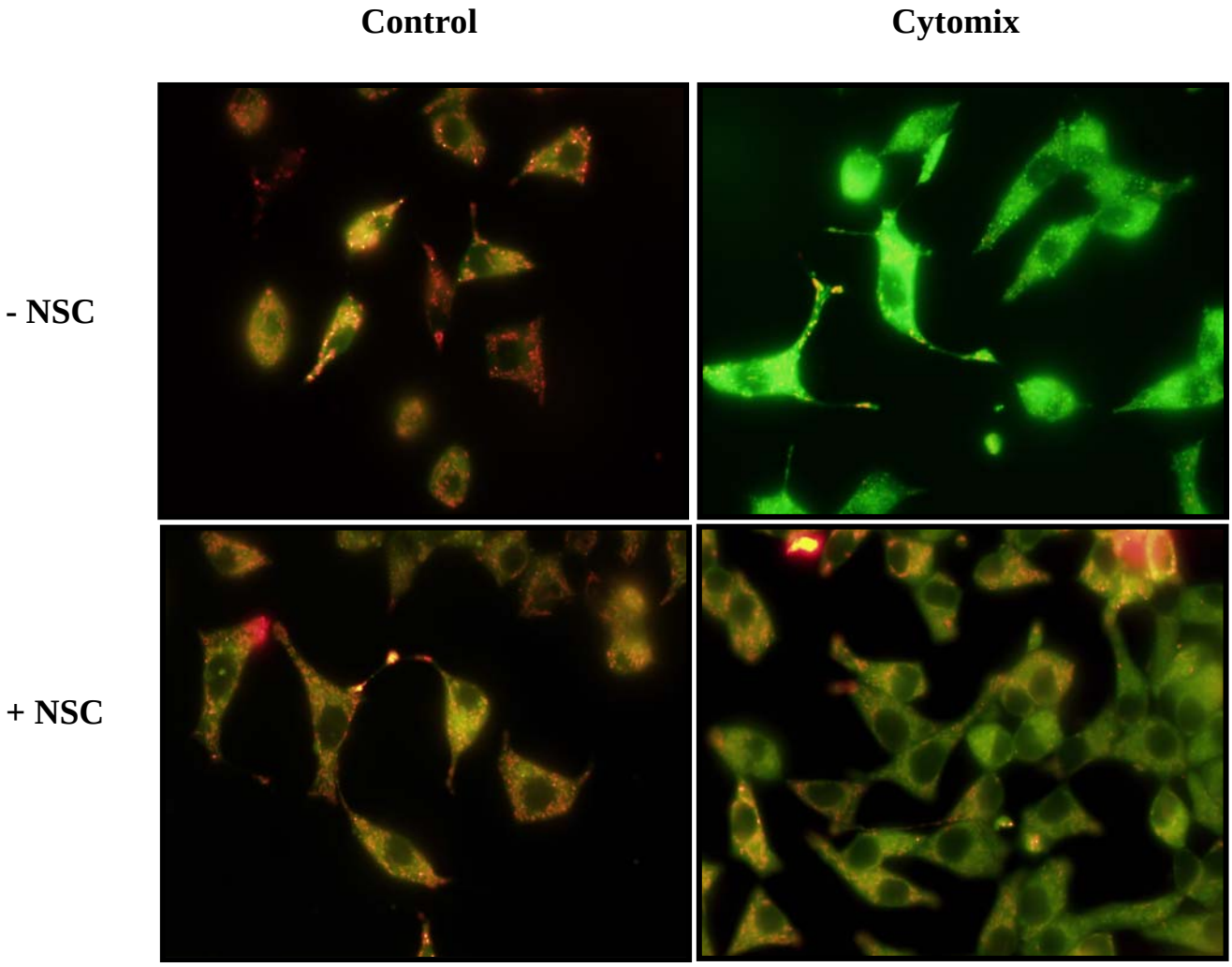


Cytomix-induced ROS generation is inhibited by NSC23766 and GGTI-2147 in INS 832/13 cells. INS 832/13 cells were treated with either diluent or Cytomix in the presence and absence of NSC23766 (20 μM) or GGTI-2147 (10 μM) for 12 h (Panel A) and 24 h (Panel B), as indicated in the figure and intracellular levels of ROS was measured using DCHF-DA assay. Data are representative of three independent experiments, expressed as a percentage of control cells and represent means ± SE. Bars with different symbols (*, **, ***) are significantly different at $p < 0.05$.

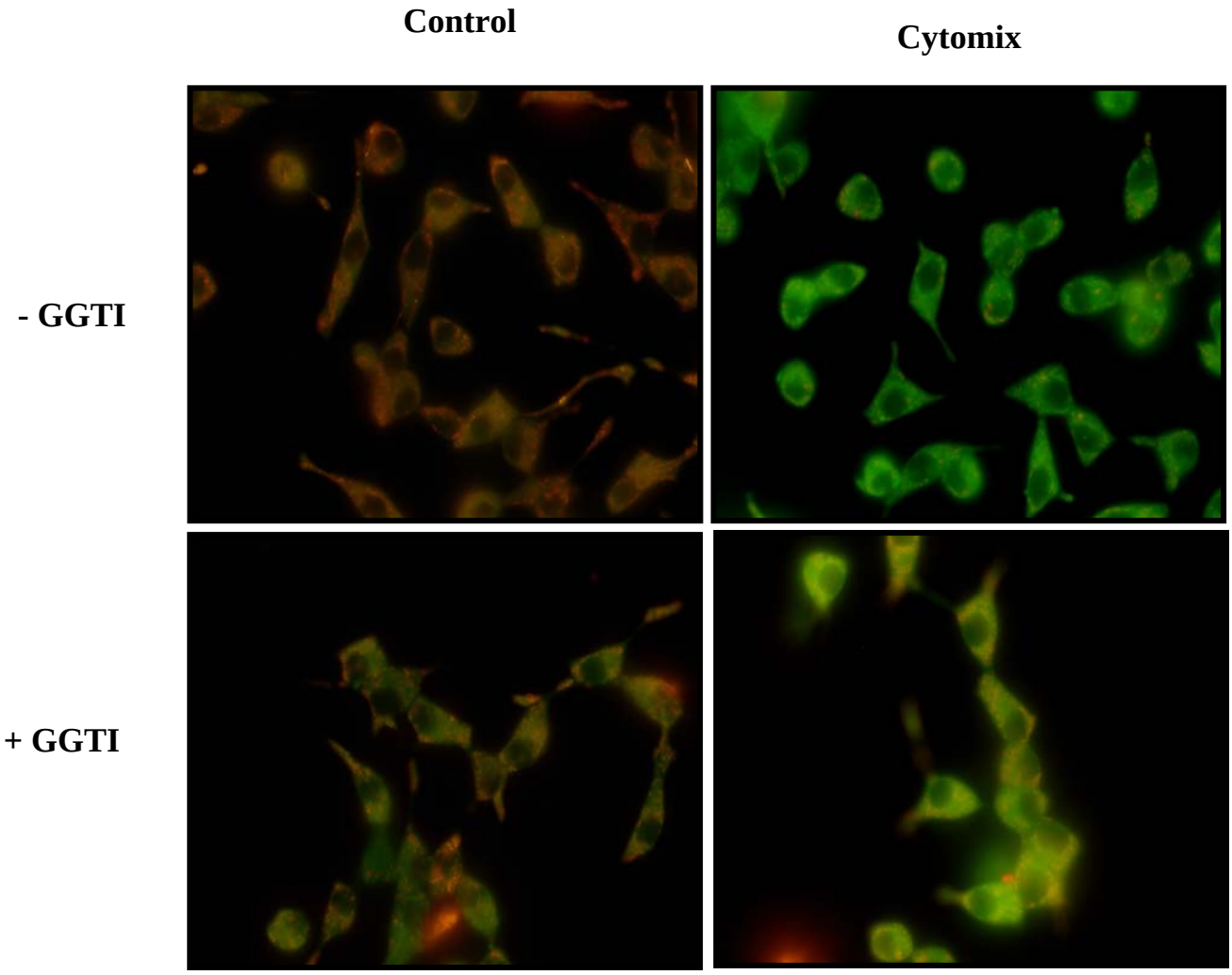
Figure 29:



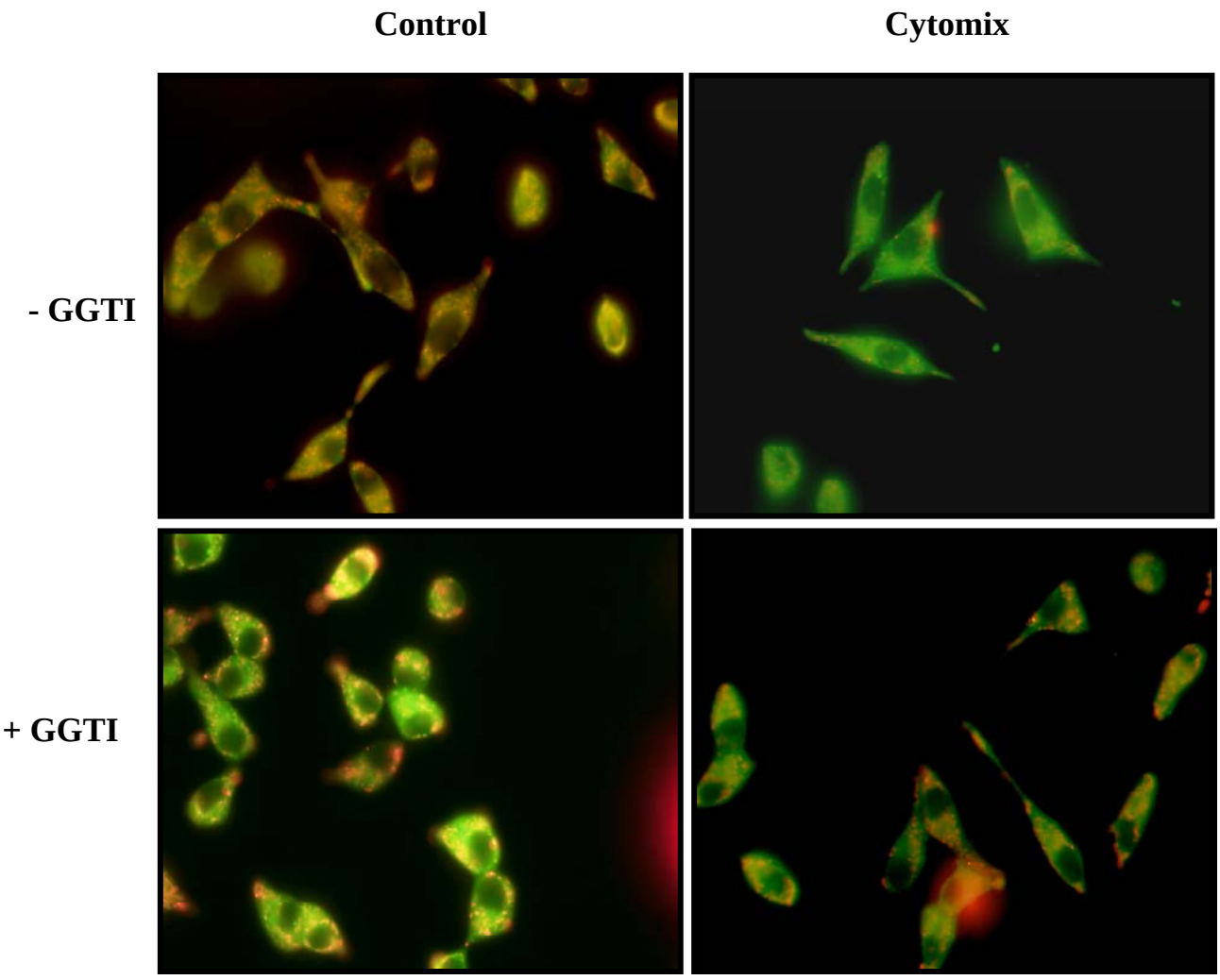
B]



C]



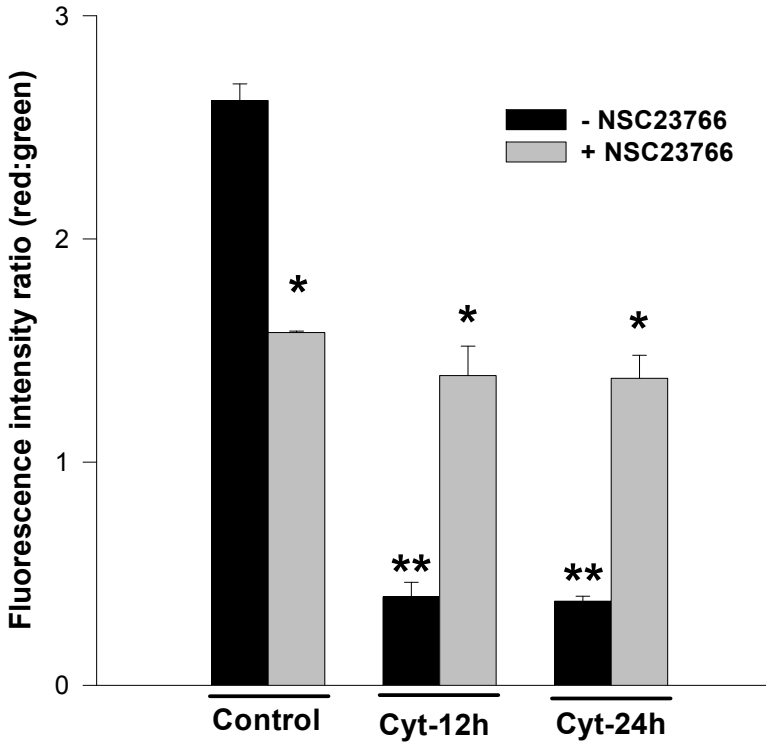
D]



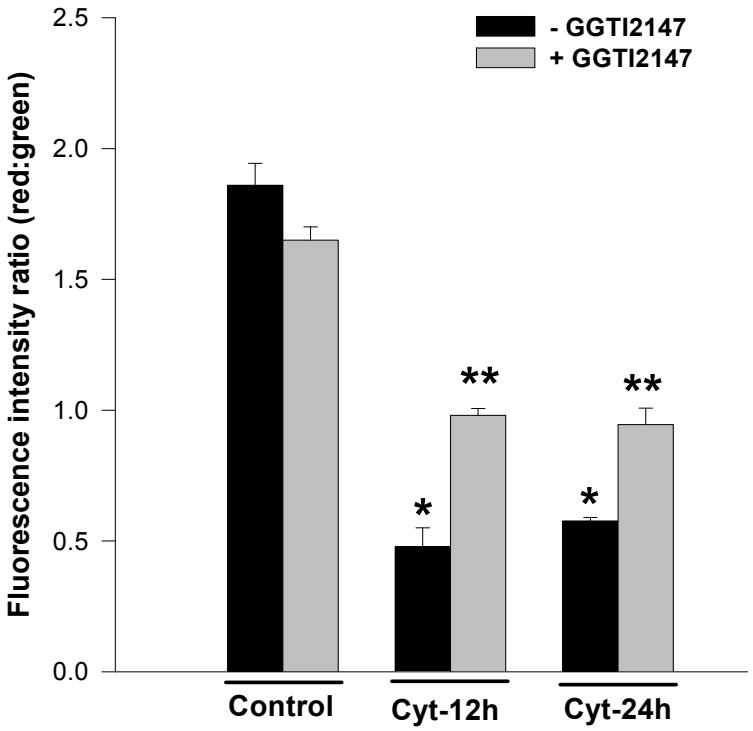
Cytomix-induced loss in mitochondrial membrane potential is partially prevented by NSC23766 and GGTI-2147. INS 832/13 cells were treated with either diluent alone or Cytomix for 12 (Panel A and C) and 24 h (Panel B and D) in the presence and absence of NSC23766 (20 μM) or GGTI-2147 (10 μM), as indicated in the figure. The mitochondrial membrane potential was measured with JC-1 assay kit. Data are representative of three independent experiments with comparable results.

Figure 30:

A]



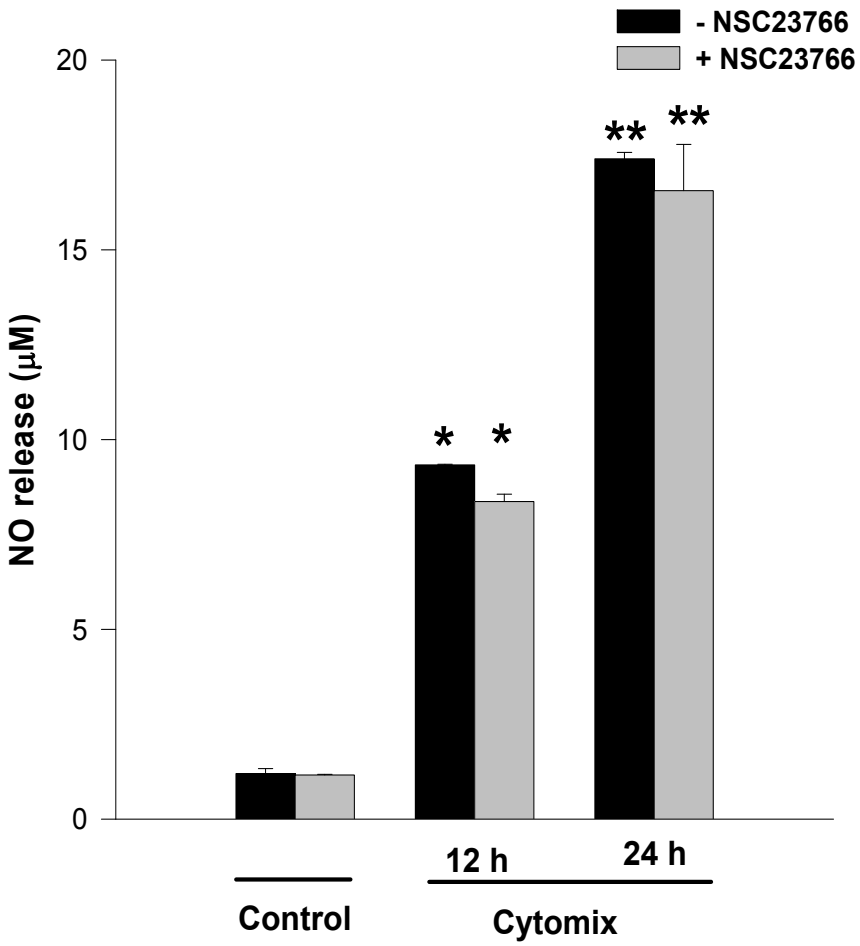
B]



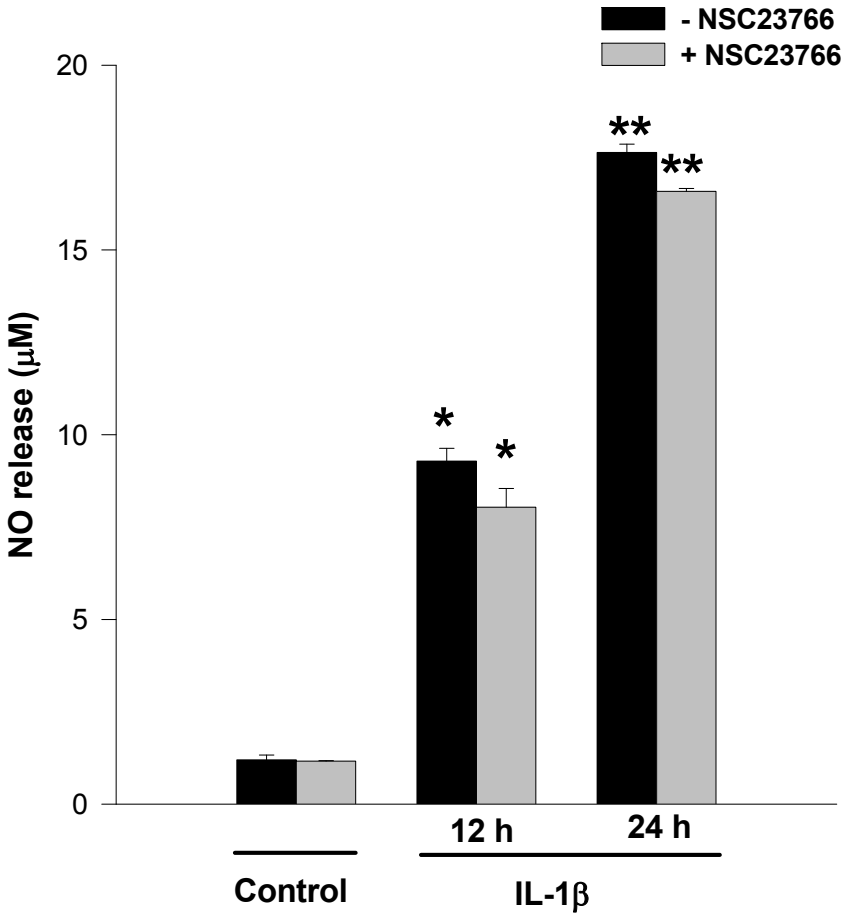
Cytomix-induced loss in mitochondrial membrane potential is partially prevented by NSC23766 and GGTI-2147. Cytomix induced changes in mitochondrial membrane potential was measured with JC-1 assay kit, as described in Fig. 29 and red: green fluorescence ratio was calculated by sampling (n = 10 data points per image) for three independent experiments with comparable results. *, **Bars with different symbols are significantly different $p < 0.05$. Panel A: data from cells treated with NSC23766 (20 μM). Panel B: data from cells treated with GGTI-2147 (10 μM).

Figure 31:

A]



B]



NSC23766 fails to inhibit Cytomix-induced NO release in INS 832/13 cells. INS 832/13 cells were treated with diluent, Cytomix (Panel A) or IL-1β (Panel B) for 12 or 24 h. NO released into the medium was measured using Griess assay. Data are expressed as means ± SE from three independent experiments. *, **Bars with different symbols represent the values that are significantly different.

Table 2:

Time (hr) / Treatment	0	3	6	12	24
PA	100 ± 3.11	157 ± 5.73 *	145 ± 2.99 *	72 ± 1.41 *†	62 ± 5.06 *†
PA + FB1	109 ± 2.27	156 ± 3.70 *	113 ± 1.84	65 ± 4.64 *†	60 ± 9.02 *†

DISCUSSION

Existing evidence from the literature suggest that damaging effects of elevated glucose, palmitate and cytokines on isolated β-cells are, in part, due to their ability to increase ROS-derived oxidative stress and mitochondrial dysfunction **[153, 178, 198]**. The main objective of this specific aim was to study the contributory roles for Nox-mediated ROS generation in the onset of mitochondrial dysfunction leading to demise of the islet β-cell, and that it requires activation of Tiam1/Rac1 signaling axis. Data accrued in my current studies suggested that: [i] exposure of isolated β-cells to palmitate and inflammatory cytokines leads to the generation of ROS, and this involves the intermediacy of Tiam1/Rac1 signaling axis; [ii] and that palmitate-induced effects are mediated through *de novo* synthesis of ceramide; [iii] inhibition of Tiam1/Rac1 signaling axis leads to restoration of mitochondrial membrane potential. Together, my findings provide the first evidence for Tiam1/Rac1 signaling cascade in palmitate- or cytokine-induced oxidative stress and metabolic dysfunction in pancreatic β-cells. I have also demonstrated that inhibition of Tiam1/Rac1 signaling axis leads to restoration of palmitate- or cytokine-induced mitochondrial dysfunction to a large degree.

As stated above, our current findings implicate the involvement of Tiam1 in PA- or C2-CER-induced activation of Rac1. In the context of potential regulation of Rac1, multiple GEFs have been identified in other cell types. These constitute the diffuse B cell lymphoma [Dbl] family of GEFs, including Trio and Tiam1. Recently, Zheng and co-workers have developed NSC23766, which is a soluble first generation small molecule inhibitor of Tiam1-mediated activation of Rac1 **[208]**. These investigators have reported

significant inhibition of Rac1-GTP-loading by NSC23766 without significantly affecting the GTP-loading onto other small G-proteins including Cdc42 and Rho A. Under these conditions, NSC23766 also attenuated cell proliferation induced by Tiam1, which is a Rac1-specific GEF. Based on these data, they concluded that NSC23766 represents a specific inhibitor of Tiam1-mediated activation of Rac1. Several other laboratories have utilized NSC23766 since then to decipher the potential contributory roles for Tiam1/Rac1 signaling pathway in cellular functions **[120, 204** and references therein]. Recently, we have confirmed the selectivity of NSC23766-mediated inhibition of Rac1 activation in insulin-secreting cells **[204]**. In the present study, I have demonstrated that NSC23766 not only attenuated PA or C2-CER induced Rac1 activation, but also markedly reduced PA or C2-CER induced generation of superoxides and lipid peroxides, implicating novel regulatory roles for Tiam1/Rac1 signaling pathway in the activation of phagocytic-like Nox in β-cells. Using molecular biological approaches Yi et al. **[209]** have recently demonstrated roles of Vav2, another GEF for Rac1, in homocysteine-induced Rac1/Nox activation in mesangial cells.

Several recent studies have demonstrated regulatory roles of Rac1 in high glucose-induced metabolic dysregulation and cell death. For example, Shen et al. **[120]** have recently reported a significant increase in cardiomyocyte apoptosis under hyperglycemic conditions. Using cultured myocytes, these investigators demonstrated a significant upregulation of Rac1 and Nox activity which was attenuated in cells overexpressing a dominant negative mutant of Rac1. Moreover, treatment of diabetic animals with NSC23766 significantly reduced Nox activity and cell demise followed by restoration of myocardial function **[120]**. These findings further support the involvement

of Tiam1/Rac1 signaling pathway in hyperglycemia-induced metabolic dysfunction and demise of myocytes. It may be germane to point out that unpublished observations from our laboratory have suggested similar regulatory roles of Rac1 in high glucose-induced activation of Nox activation and the associated increase in oxidative stress in INS832/13 cells and normal rat islets [Syed and Kowluru, unpublished].

Along these lines, studies by Cacicedo et al. in cultured retinal pericytes have demonstrated a role for NOX in PA-induced apoptosis **[119]**. A significant increase in Nox activity, oxidative stress and caspase-3 activity was demonstrable in cells exposed to PA. Overexpression of dominant negative mutants of $p67^{phox}$ and Rac1 [N17Rac1] markedly inhibited the increase in caspase-3 activation. Furthermore, overexpression of an active mutant of Rac1 [V12Rac1] increased caspase-3 activity suggesting that constitutive activation of Rac1 results in Nox activation culminating in the generation of oxidative stress and metabolic dysfunction in these cells.

In the first set of studies, using FB-1, a specific inhibitor of *de novo* synthesis of CER from PA, I have demonstrated that PA-induced effects may, in part, be due to intracellularly generated CER. Data accrued in studies using C2-CER further support this postulation. Published evidence along these lines suggests that CER-mediated effects are indeed mediated *via* activation of Rac1 in many cell types. For example, using C2-CER, Kim and Kim have reported activation of c-fos serum response element *via* the Rac1 signaling pathway in Rat-2 fibroblasts **[210]**. Interestingly, using NIH 3T3 cells, Embade et al. have demonstrated novel relationships between FasL generation and CER production in Rac1-induced apoptosis **[211]**. In another study, Deshpande et

al. **[212]** have demonstrated intermediacy of intracellularly generated CER in Rac1-induced mitochondrial oxidative stress and premature senescence in human umbellical vein endothelial cells. Together, these data appear to implicate CER/Rac1 signaling pathways in oxidative stress and metabolic dysfunction in multiple cells types. Therefore, based on these and other supporting evidence presented in this study, I believe that PA effects on lipid peroxides, superoxides and Nox activity are specific and that they require the intermediacy of Tiam1/Rac1 signaling pathway. It should be noted that I also observed modest effects of oleate on Nox activity without significantly affecting the Rac1 activation [Fig. 18] suggesting a clear distinction between the modes of action of these two fatty acids.

In second set of studies, we observed similar Tiam1/Rac1-mediated activation of Nox holoenzyme in cytokine-induced oxidative stress in pancreatic β-cells. However, it should be taken into account that, transient activation of Rac1 under cytokine stimulus is adequate to initiate Nox signaling. It appears that Rac1 activation during Cytomix treatment might be primarily due IL-1β present in the Cytomix, as we observed a significant Rac1 activation (2 ± 0.4 fold stimulation) in INS 832/13 cells when exposed to IL-1β (25 ng/mL) alone, whereas, no significant effects were observed with either TNF-α or INF-γ. And also, our findings suggests that prenylation of Rac1 is necessary for such mediatory effects, and incubation of INS 832/13 cells with geranylgeranylation inhibitor, GGTI-2147, markedly subdued Cytokines-mediated Rac1 and Nox activation. In this context, using molecular biological (e.g., dominant-negative Rac1 mutant or siRNA-Rac1) and pharmacological (e.g., GGTI-2147 and 3-allyl or vinyl geranyl geraniols) probes, our laboratory have shown that geranylgeranylation of Rac1 is necessary for its

optimal activation and membrane association in clonal β-cells and normal rats islets **[85]**. Furthermore, it should be considered that, small G-protein Rap1 is an integral part of the membrane component of Nox, and the inhibitory effects of GGTI-2147 on cytokine-induced Nox might be in part due to inhibition of geranylgeranylation of Rap1 too. However, our findings accrued from NSC23766 studies directly support the involvement for Taim1/Rac1 in this signaling cascade, since Tiam1 serves as a GEF for Rac1, but not other small G proteins. Taken together, on the basis of the current data amassed from NSC23766 and GGTI-2147 studies, we put forth that Tiam1-mediated and geranylgeranylation-sensitive Rac1 activation is necessary for cytokine-mediated effects of Nox and generation of oxidative stress in the islet β-cell.

In general, it is important to note that numerous recent studies have implicated physiological roles for a tonic increase in Nox activation and subsequent ROS generation in the stimulus-secretion coupling of GSIS **[113]**. Moreover, Newsholme et al demonstrated an increase in insulin secretion by fatty acids under acute conditions **[200]**. Therefore, one might ask if increase in Tiam1/Rac1 activation and Nox activation could contribute towards the physiological insulin secretion rather than inducing metabolic abnormalities in the isolated β-cell. Even though it appears likely, under definite experimental conditions, chronic activation of Nox by specific stimuli [e.g., high levels of glucose, fatty acids, CER or cytokines] leads to metabolic dysfunction and demise of the β-cell. For example, recent observations **[207]** from our laboratory have suggested significant abnormalities in mitochondrial function [i.e., loss in MMP] in cells exposed to C2-CER under acute conditions. In addition, it should be noted that our current observations [Figs. 24 and 29] indicate mitochondrial dysfunction in presence of

C2-CER and cytokines, and that NSC23766 and GGTI-2147 prevented it to a large degree, implicating the Tiam1-Rac1-Nox signaling in the onset of metabolic dysfunction. Therefore, we speculate that early biochemical and cellular changes that we reported herein might be paving way to metabolic dysfunction and demise of the islet β-cell.

However, NSC23766 affords a better protection in cytokines-induced mitochondrial dysfunction compared to GGTI-2147. Therefore, it appears that additional signaling mechanisms might be controlling mitochondrial membrane potential, which are distinct from Nox-derived ROS. Attuned with these observations are our findings that demonstrated relative lack of effects of NSC23766 on caspase 3 activation. As in the context of Rac1 activation mentioned above, such steps may be related to direct metabolic effects of IL-1β, but not TNF-α or IFN-γ (also present in the Cytomix), since IL-1β-mediated caspase 3 activation and NO release were not affected by Tiam1 inhibition. It may be germane to point out that recent studies by Moore et al. **[199]** have provided compelling evidence to argue against potential involvement of oxidative stress in fatty acid-induced metabolic dysfunction of the islet β-cell. It is, therefore, likely that additional regulatory mechanisms might underlie β-cell demise seen under the duress of lipotoxic conditions including those involving progressive alterations in the mitochondrial membrane permeability transition pore as suggested by recent studies of Koshkin et al. **[213]** in MIN6 and INS-1 cells.

In summary, we present the first evidence for a novel role of Tiam1/Rac1 signaling pathway in PA-induced, CER-sensitive and cytokine-mediated metabolic activation of Nox and associated production of superoxides and lipid peroxides in

pancreatic β-cells. It is likely that Tiam1 could serve as a novel drug target for inhibition of generation of superoxides and lipid peroxides in isolated β-cells under such duress. Based on these data we propose a working model [Fig. 39] to suggest that PA/CER and cytokines increase the Rac1 activation [GTP-bound active form] to generate signals that may be necessary for triggering cellular events leading to Nox activation, increased oxidative milieu, mitochondrial dysregulation in the pancreatic β-cell. It should be noted that while the proposed model principally addressed the roles of Tiam1–Rac1–Nox connection in PA/CER-mediated and cytokine-mediated effects, relative contributory roles of other sources of reactive oxygen species, including the glutathione peroxidase, manganese-sensitive superoxide dismutase, catalase signaling cascades must also be recognized as key contributors to the mitochondrial dysfunction in isolated β-cells under the duress of lipotoxic conditions **[158, 196, 197, 216]**. However, additional studies are needed to further understand these signaling steps in the islet β-cell.

Chapter III

Introduction

Glucose-stimulated insulin secretion [GSIS] involves a cascade of metabolic and cationic events leading to translocation of insulin-containing secretory granules toward the plasma membrane for fusion and release of insulin into circulation. It is well established that granule transport and fusion involves interplay between vesicle-associated membrane proteins on the insulin granules and docking proteins on the plasma membrane **[44, 45, 49]**. In addition, a significant cross-talk among multiple small G-proteins including Arf6, Cdc42 and Rac1 has been shown to be critical for GSIS **[84, 100, 218]**. Several effector proteins for these G-proteins have been identified in the islet β-cell, including phospholipases, Pak-1 and ERK1/2 kinases **[68, 74, 84]**. Recent evidence also implicates regulatory roles for G-proteins [e.g., Rac1] in the activation of phagocyte-like NADPH oxidase [Nox] and generation of reactive oxygen species [ROS] leading to GSIS **[219]**.

Excessive ROS generation is considered central to the development of diabetes and its associated complications. Under normal physiological conditions, generation of free radicals is relatively low, however increased levels of circulating glucose promote intracellular accumulation of superoxides leading to metabolic dysfunction. Although, mitochondria remain the primary source for free radicals, emerging evidence implicates Nox as one of the major sources of extra-mitochondrial ROS. Nox is a highly regulated membrane-associated protein complex that promotes one electron reduction of oxygen to superoxide anion involving oxidation of cytosolic NADPH. The Nox holoenzyme is

comprised of membrane and cytosolic components [Figure 2]. The membrane-associated catalytic core consists of gp91phox and p22phox and the cytosolic regulatory core includes p47phox, p67phox, p40phox and Rac1 [or Rac2]. Following stimulation, the cytosolic core translocates to the membrane for association with the catalytic core for functional activation of Nox. Immunological localization and functional regulation of Nox have been described in clonal β-cells, rat and human islets **[13, 14, 110, 112]**.

Findings from multiple laboratories suggest that ROS-derived from Nox play regulatory "second-messenger" roles in GSIS, a concept overviewed recently by Pi and Collins **[7]**. Along these lines, recent studies have highlighted roles for Nox in physiological insulin secretion. For example, Diphenylene iodonium [DPI], a selective inhibitor of Nox, inhibited glucose-induced Nox activity and GSIS **[112]**. These observations were further confirmed by Morgan and associates **[14]** suggesting that DPI or $p47^{phox}$ antisense-induced inhibition of Nox attenuated GSIS under static or perifusion conditions. Graciano and coworkers **[110]** demonstrated regulatory roles for Nox in palmitate-induced superoxide generation and insulin secretion in rat islets. Furthermore, my recent findings suggested that prenylation and activation of Rac1 are critical for glucose- and mitochondrial fuel-induced Nox-dependent ROS generation in INS 832/13 cells and rodent islets **[219]**. Together, the above observations support the overall hypothesis that Nox-mediated, Rac1-sensitive ROS generation is requisite for insulin secretion.

In addition to the above described “friendly” roles for ROS in islet function, recent evidence also suggests paradoxical “non-friendly” roles for ROS in the induction of oxidative stress and metabolic dysregulation of the islet β-cell under the duress of glucolipotoxicity, cytokines, and ceramide **[117]**. The generation of ROS seen under these experimental conditions appear to be largely due to the activation of Nox, since inhibition of Nox [e.g., DPI, apocynin or siRNA-$p47^{phox}$] or Rac1 activation [e.g., GGTI-2147, NSC23766] markedly attenuated deleterious effects of these stimuli **[179, 180]**. Despite these *in vitro* evidence, potential roles of Nox in islet dysfunction in animal models of type 2 diabetes remains unexplored. Therefore, I undertook the current study to systematically examine the functional status of Nox in islets from Zucker Diabetic Fatty [ZDF] rat, which develops obesity, hyperinsulinemia, hyperglycemia and a decline in β-cell function. Further, the ZDF rat is an excellent *in vivo* model for glucolipotoxicity-mediated metabolic dysfunction of the islets. Herein, I present evidence to suggest significant alterations in the Nox function in the diabetic islet, which promote ROS generation and mitochondrial dysregulation. Furthermore, these findings suggest similar metabolic defects in islets from a human donor with type 2 diabetes. I also present evidence to implicate roles for glucolipotoxicity in the induction of Nox-mediated cellular and metabolic defects in ZDF islets.

These findings have been submitted for peer review in Diabetes 2011.

MATERIALS AND METHODS

Materials

2`, 7`-dichlorofluorescein diacetate [DCHFDA] was from Sigma [St. Louis, MO]. Antisera for $p47^{phox}$ and phospho-$p47^{phox}$ were from Santa Cruz Biotechnology [Santa Cruz, CA] and Abcam [Cambridge, MA], respectively. $gp91^{phox}$ and Rac1 antisera were from BD Bioscience [Rockville, MD]. Antisera for Caspase-3, JNK1/2 and ERK1/2 were from Cell Signaling Technology [Boston, MA]. GLISA Rac1 activation kit was from Cytoskeleton [Denver, CO]. Horseradish peroxidase conjugates and ECL kits were from Amersham Biosciences [Piscataway, NJ].

Pancreatic islets and INS 832/13 β-cells

Male [9-11 wks] ZDF and ZLC rats were from Charles River laboratories [Wilmington, MA] and maintained in a 12-h light/dark cycle with free access to water and food [Purina Diet no. 5008; Charles River Laboratories]. All animal protocols were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee. Hyperglycemia in diabetic rats was confirmed prior to sacrifice by tail vein puncture using Glucometer Elite from Bayer [Leverkusen, Germany]. Body weights of ZLC and ZDF rats were 300 ± 6 g and 396 ± 12 g respectively [n=11; $p < 0.05$]. Islets were isolated by collagenase digestion method **[220]**. INS 832/13 cells [provided by Dr. Chris Newgard] were cultured and processed using protocols described in **[180]**.

Human islets from normal and diabetic donors were obtained from Prodo Laboratories, Inc. [Irvine, CA]. Control islets [from a 54 year old male donor; 85-90% purity] and diabetic islets [from a 45 year old male donor; ~60% purity] were

homogenized with Tris-HCl buffer [50 mM, pH 7.4] containing sucrose [250 mM], EDTA [1 mM], DTT [1 mM], and protease inhibitor cocktail. Lysate proteins were resolved on 12% SDS-PAGE, and used for Western blot analysis.

Quantitation of ROS

ZLC, ZDF or human islets were incubated with DCHFDA (10 μM) at 37°C for 30 min in RPMI-1640 media without serum and glucose **[219]**. Following incubation, islets were washed with ice-cold phosphate-buffered saline and sonicated. Equal amounts of protein were utilized for fluorescence measurements [λ_{em} 485 nm and λ_{ex} 535 nm] using PerkinElmer luminescence spectrophotometer.

Rac1 activation assay

Activated Rac1 was quantitated using a 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 islet lysate protein were incubated in the Rac1-GTP affinity plate for 30 min at 4 ˚C. The wells were washed twice with washing buffer and then incubated with antigen presenting buffer provided with the kit. The contents in Rac1-GTP affinity labeled plate were then successively incubated with anti-Rac1 primary antibody and secondary antibody for 45 min followed by additional incubation with HRP-detection reagent for 20 min. The reaction was terminated by adding HRP-stop buffer and the absorbance was measured at 490 nm using a microplate reader.

Other assays and statistical analysis of data

Western blot protein bands were visualized using a Kodak Imaging System and analyzed densitometrically using UN-SCAN-IT software [Orem, Utah]. Statistical significance of differences between control and experimental groups was determined by Student's *t*-test and ANOVA analysis. $P < 0.05$ was considered significant.

RESULTS

ROS levels, expression and phosphorylation of p47^{phox} are significantly increased in ZDF islets

The ZDF rats presented a four-fold increase in blood glucose levels compared to their age-matched ZLC rats [323 ±15 mg/dL *vs.* 85 ±1 mg/dL]. Quantitation of ROS by the DCHFDA fluorescence method, showed a significant increase [>60%] in superoxide generation in ZDF rat islets compared to the ZLC islets [Figure 32; Panel A]. Since recent evidence indicated a significant increase in Nox-derived ROS generation in isolated β-cells following exposure to high glucose, palmitate or cytokines **[179, 180]**, I next investigated functional regulation and involvement of Nox as a source of increased ROS in ZDF rat islet.

The Nox holoenzyme is comprised of membrane-associated and cytosolic components [Figure 2]. Evidence from multiple laboratories including our own suggests that the cytosolic components require post-translational modifications, including phosphorylation of p47^{phox} and prenylation of Rac1 for the holoenzyme assembly **[219, 221]**. Recent studies also demonstrated that the expression of p47^{phox} is significantly increased in isolated β-cells following exposure to high glucose, palmitate or cytokines **[117, 179, 180]**. Therefore, I have next determined the expression levels and the degree of phosphorylation of p47^{phox} in islets from ZLC and ZDF rats. Pooled data accrued from multiple islet preparations described in Figure 32 [Panels B-C] indicated a significant increase (~40%) in the expression of p47^{phox} in ZDF islets compared to ZLC islets. Furthermore, the levels of phosphorylated p47^{phox} were also increased

significantly [~50%] in ZDF islets [Figure 32; Panels D-E]. These findings of an increase in expression and phosphorylation of $p47^{phox}$ in ZDF islets are comparable to those accrued from *in vitro* studies of incubation of pancreatic β-cells with palmitate or glucose.

Rac1, a cytosolic component of Nox, is activated in ZDF islets

I next quantitated Rac1 expression and activation in ZLC and ZDF islets. The underlying premise here is that an increase in the Nox-derived ROS generation in the diabetic islet [Figure 32; Panel A] requires activation of Rac1. Data in Figure 33 [Panel A] indicated a marked increase [>60%] in the expression of Rac1 in ZDF islets compared to ZLC islets. I also observed that the abundance of activated Rac1 is significantly higher [~2.25 fold] in ZDF islets compared to ZLC islets [Figure 33; Panel B]. It should be noted that increase in Rac1 activation [Figure 33; Panel C] may not be a reflection of increased Rac1 expression in ZDF islet [Figure 33; Panel A] since the ratio of activated to total Rac1 also indicated a significant increase [>40%] in ZDF islet compared to ZLC rat islets [Figure 33; Panel D]. Together, data in Figures 32 and 33 indicate increase in the phosphorylation status of $p47^{phox}$ and activation of Rac1 in the ZDF islet, which are required for holoenzyme assembly and activation of Nox and subsequent increase in ROS generation [Figure 32; Panel A].

Increased expression of $gp91^{phox}$ in the ZDF islet

While numerous studies have focused on potential alterations in the expression of the cytosolic components of Nox in β-cells under the duress of glucolipotoxicity and cytokines **[118, 179, 180]**, relatively little is understood with regard to potential changes

in the expression of the membrane components of Nox under such conditions. Therefore, I next quantitated expression levels of $gp91^{phox}$ in islets from ZLC or ZDF rats. Data obtained from multiple islet preparations indicated an increase in the expression of $gp91^{phox}$ subunit in ZDF islets [Figure 34; Panel A] and densitometrical analysis showed more than 40% increase in $gp91^{phox}$ expression in ZDF islets compared to ZLC [Figure 34; Panel B]. Together, these findings support the overall hypothesis that an increase in the intracellular ROS in diabetic islet may, in part, be due to increased activation of Nox *via* increase in the expression and phosphorylation of individual subunits.

Assessment of mitochondrial damage in ZDF islets

Using *in vitro* model systems of glucolipotoxicity and chronic cytokine exposure, Subasinghe et al and I have recently proposed that Nox activation leads to loss of mitochondrial membrane potential and subsequent caspase-3 activation **[179, 180]**. We also demonstrated that inhibition of Rac1 activation either by attenuating the function of Tiam1, a guanine nucleotide exchange factor for Rac1 by NSC23766 or inhibition of prenylation of Rac1 by GGTI-2147 leads to partial restoration of mitochondrial dysfunction induced by a mixture of cytokines **[179]**. Therefore, I next quantitated caspase-3 activation, a hallmark of mitochondrial dysregulation, in ZLC or ZDF rat islets. Data from these studies [Figure 34; Panels C-D] indicated a significant activation of caspase-3 in islets from ZDF, but not from ZLC rats. These data are suggestive of mitochondrial defects in the ZDF islet at an age where significant changes in Nox activation are observed [see above].

Differential regulation of JNK 1/2 and ERK 1/2 in ZDF islet

It has been shown that stress-activated JNK activation lies upstream to mitochondrial dysfunction, including cytochrome C release and caspase-3 activation **[222]**. Further, constitutive activation of Rac1 promotes JNK phosphorylation and activation **[223, 224]**. Emerging evidence also implicates a significant cross-talk between ROS and JNK1/2 **[225]**. Therefore, I next determined the degree of expression and phosphorylation of JNK1/2 in islets from the ZLC and ZDF rats. In these studies, I utilized an antiserum that detects phosphorylated Tyr-185 [54 kDa; JNK-2] and phosphorylated Thr-183 [46 kDa; JNK-1]. Western blot analysis of islet lysates from ZLC and ZDF rats indicated consistently higher levels of phosphorylated JNK-1 and JNK-2 in ZDF rat islets [Figure 35; Panel A]. The ratios of phosphorylated to total JNK-1 and JNK-2, which are provided in Figure 35 [Panel B] indicated a significant increase [> 60%] in the activities in diabetic islets.

I next quantitated ERK1/2 phosphorylation in ZLC and ZDF islets to further determine if diabetic conditions elicit regulatory effects on this enzyme cascade, since it has been implicated in islet β-cell function at multiple levels, including insulin gene expression, GSIS and β-cell proliferation **[185, 226]**. Data shown in Figure 35 [Panels C-D] indicated a significant attenuation in ERK1/2 phosphorylation in ZDF islets compared to ZLC islets. Together, data described in Figure 35 suggest differential regulation of JNK1/2 and ERK1/2 in diabetic islets; such conditions might favor pro-apoptotic and non-proliferative events in the diabetic islets. Based on my recently published observations on increased Nox activity in β-cells under the duress of glucolipotoxic conditions **[180]** and current observations in the ZDF islets, I hypothesize

that glucolipotoxic distress may elicit such dual regulatory effects on JNK1/2 and ERK1/2 phosphorylation and activation. This hypothesis was further tested *via* studies described below in clonal β-cells.

***In vitro* exposure to high glucose or palmitate exerts differential effects on JNK1/2 and ERK1/2**

To assess if differential regulatory effects on JNK1/2 and ERK1/2 seen in the ZDF islets are due to gluco- or lipotoxicity, INS 832/13 cells were incubated with either high glucose [20 mM; 48 h] or palmitate [400 μM; 48 h] and relative abundance of total and phospho JNK1/2 and ERK1/2 was determined by Western blotting. Pooled data in Figure 36 [Panel A] indicated a marked increase [~40-87%] in JNK-1 and JNK-2 phosphorylation in high glucose- [lanes 3 and 4] or palmitate- [~ 30-34%; lanes 5 and 6] treated β-cells compared to their counterparts under basal conditions [lanes 1 and 2]. However, total levels of JNK 1/2 remained unchanged under these conditions. Further, I observed a significant reduction [~22-48%] in ERK1/2 phosphorylation in INS 832/13 cells treated with high glucose or palmitate [~60%]; such conditions did not affect the abundance of total ERK1/2 in INS 832/13 cells [Figure 36; Panel B]. Together, these *in vitro* findings in INS 832/13 cells, which are comparable to what I have observed in the ZDF islet [Figure 35], suggest differential regulation of JNK1/2 and ERK1/2 under the duress of gluco- or lipotoxic conditions.

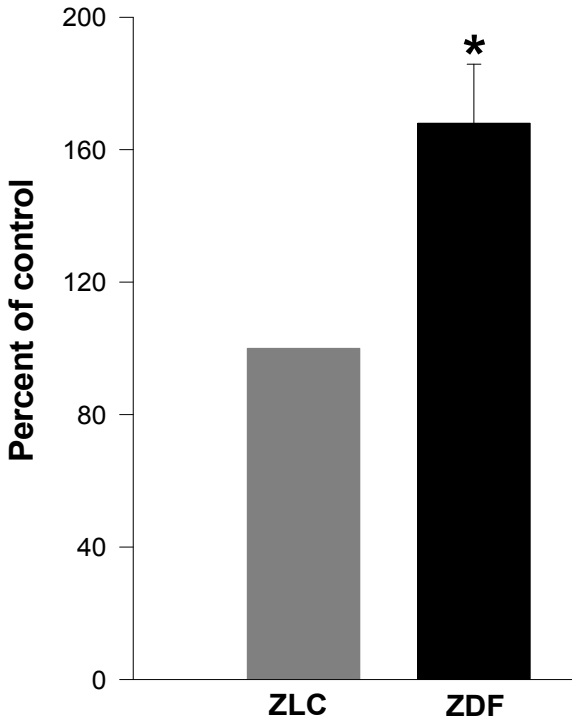
Regulation of Nox in human islets

I next studied regulation of Nox under glucotoxic conditions in human islets. First, ROS generation and Rac1 activation were quantitated in normal human islets incubated

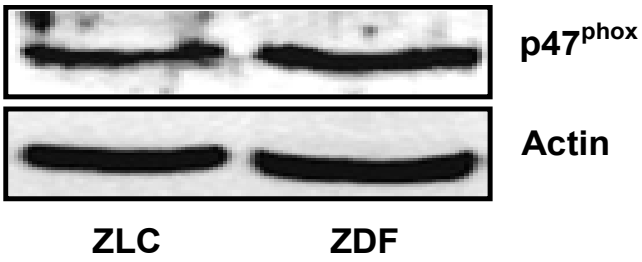
with either 5.8 mM or 30 mM glucose for 48 h. Data depicted in Figure 37 [Panel A] indicated ~2.2 fold increase in ROS generation in human islets following incubation with high glucose, which is compatible with my earlier observations in INS 832/13 cells and normal rat islets **[219]** and ZDF rat islets [current studies]. I have also observed that incubation of human islets with high glucose resulted in a significant [~1.5 fold] activation of Rac1 [Figure 37; Panel B]. Interestingly, I also noticed a marked increase in Rac1 expression, JNK1/2 activation and caspase-3 degradation in islets from a diabetic donor [Figure 37; Panel C, D and F, respectively]; findings are compatible with ZDF islet data. However, relative abundance of phosphorylated or total $p47^{phox}$ and $gp91^{phox}$ [Figure 37; Panel C and E, respectively] was comparable between normal and diabetic human islets. Limited availability of diabetic human islets precluded me from quantitation of Nox and Rac1 activities. Nonetheless, these preliminary data in human islets support my current findings in the ZDF islets and in INS 832/13 cells following exposure to glucolipotoxic conditions.

Figure 32:

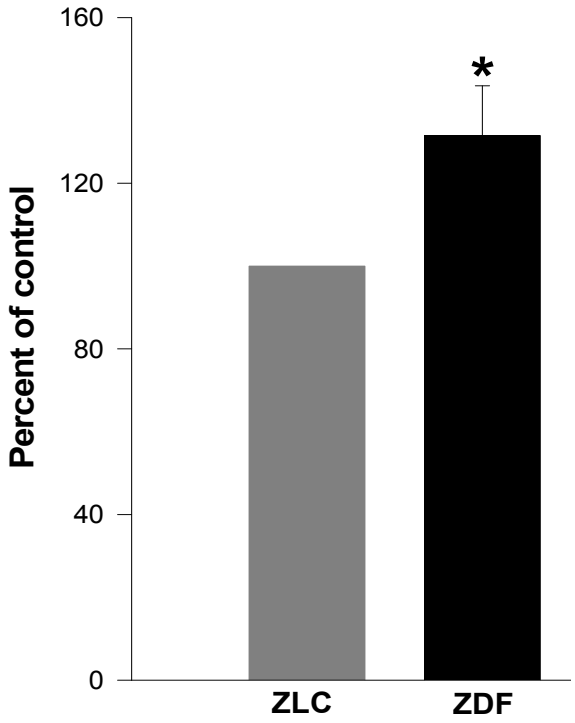
A]



B]



C]



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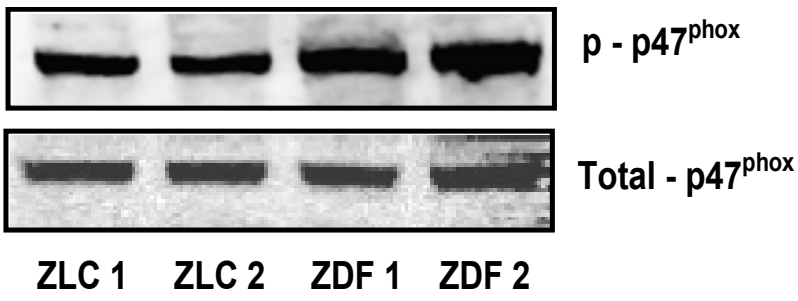
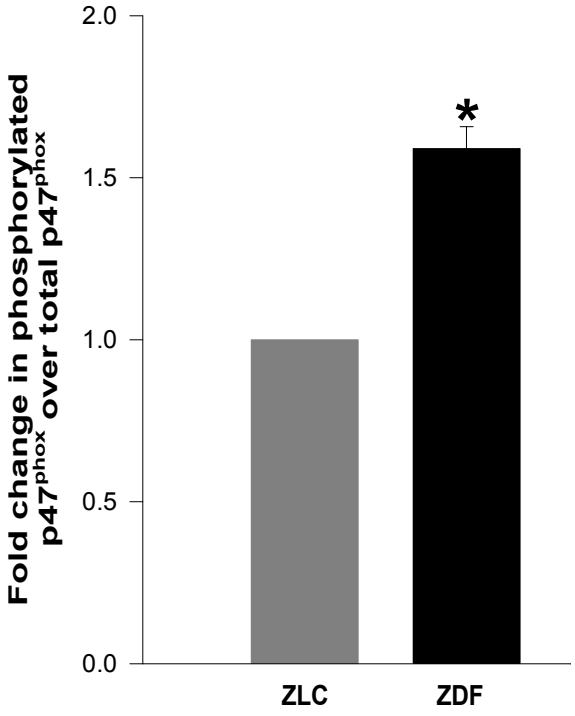


E]

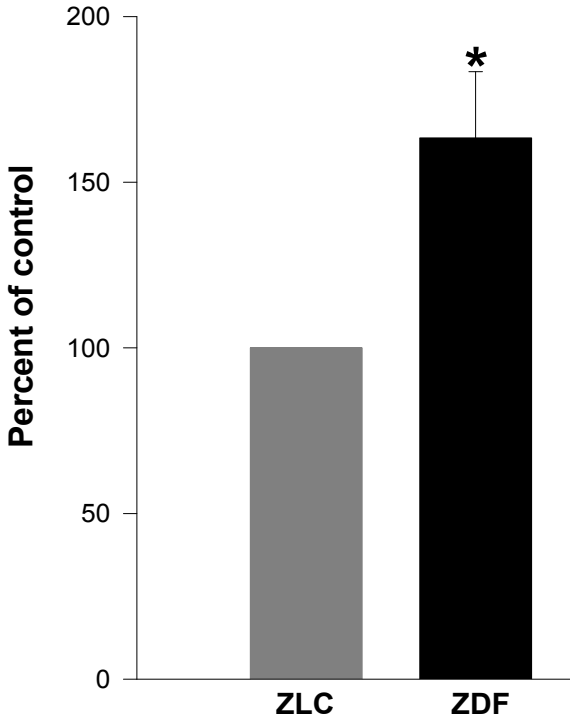


ROS levels were measured in isolated islets from ZLC and ZDF rats following incubation with DCHFDA [10 μM] for 30 min. Islets were washed with ice-cold PBS and sonicated. An equal amount of protein was used to quantitate DCF fluorescence. Data are expressed as percent control [Panel A] and are mean ± SEM from islets from four rats in each group.* p< 0.05 *vs*. ZLC islets. In a separate experiment, islets from ZLC or ZDF rats were lysed using RIPA buffer. Equal amount of lysate proteins were resolved by SDS-PAGE. Expression of phosphorylated and total $p47^{phox}$ was determined by Western blotting. A representative blot is provided in Panel B for total $p47^{phox}$ and Panel D for phospho-$p47^{phox}$. Densitometric quantitation of total $p47^{phox}$ and phosphorylated $p47^{phox}$ is provided in Panels C and E, respectively. Data are mean ± SEM from islets from four rats in each group. * p< 0.05 *vs*. ZLC rat islets.

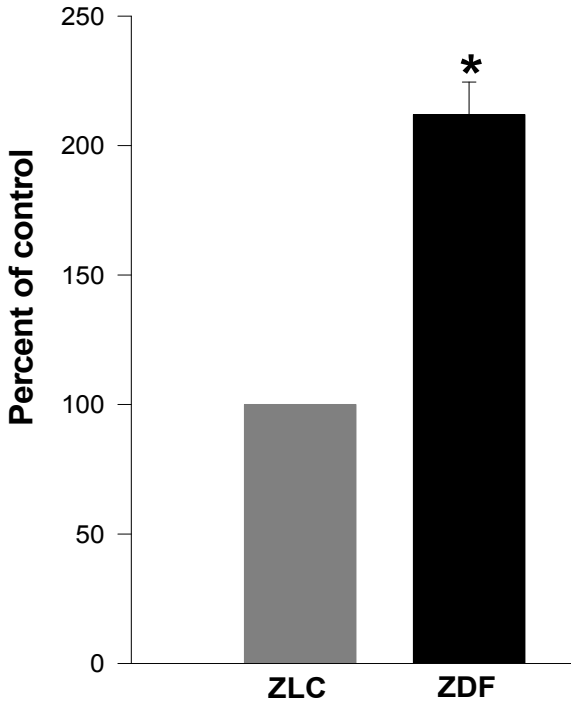
Figure 33:

A]

B]



C]



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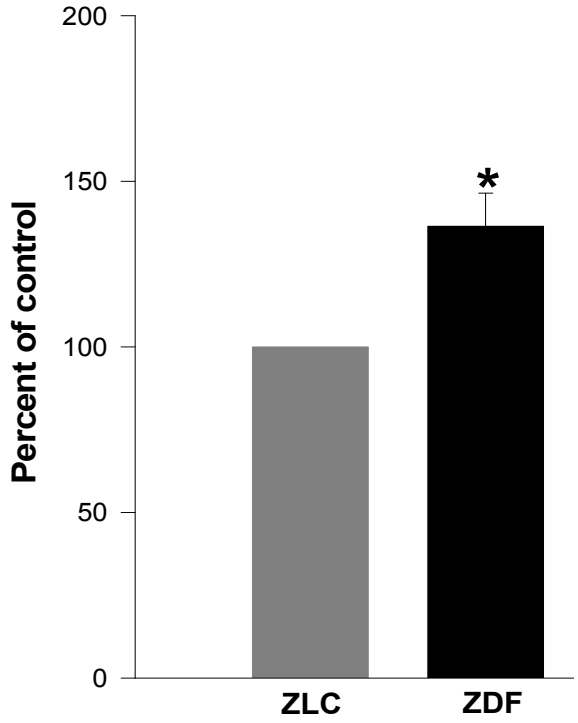
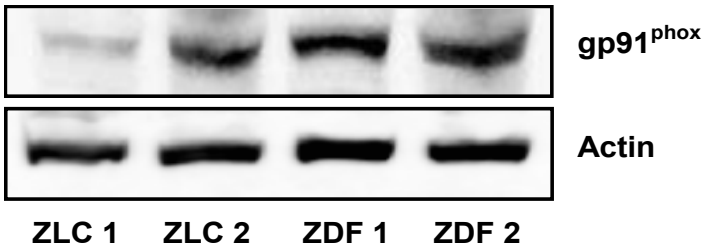


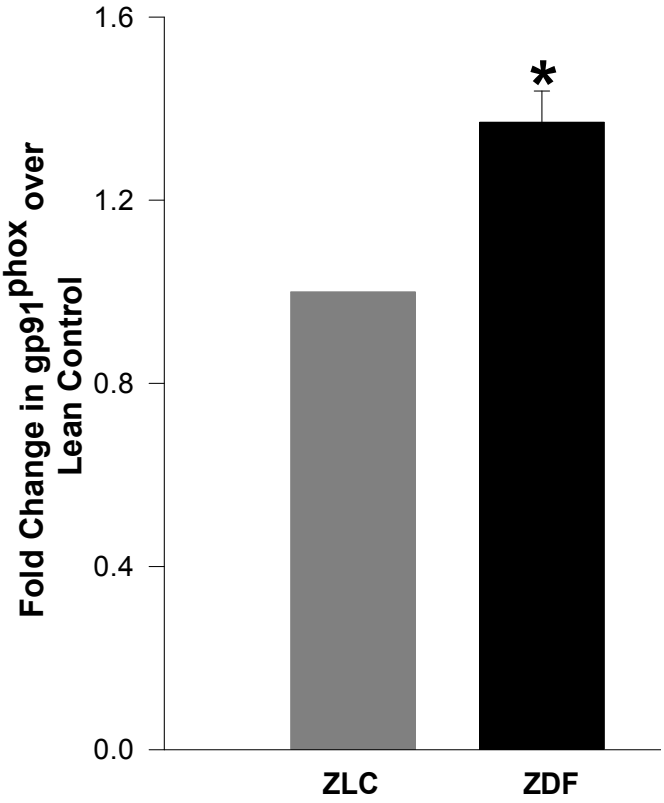
Total Rac1 expression in islets from ZLC and ZDF rats was determined by Western blotting [Panel A] and quantitated densitometrically [Panel B]. The degree of Rac1 activation [Panel C] was quantitated by GLISA method. Data are expressed as percent change in Rac1 activation over total Rac1 [Panel D] and are mean ± SEM from islets from six rats in each group. * $p < 0.05$ *vs*. ZLC rat islets.

Figure 34:

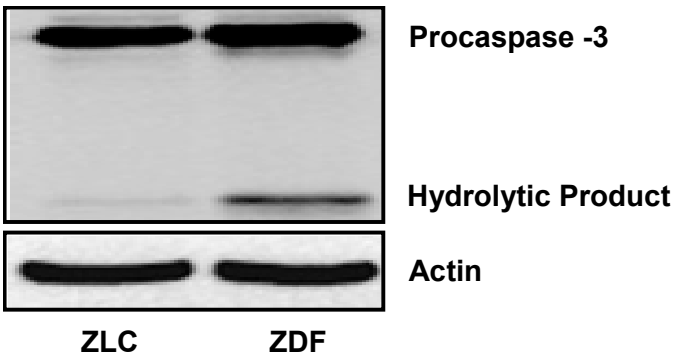
A]



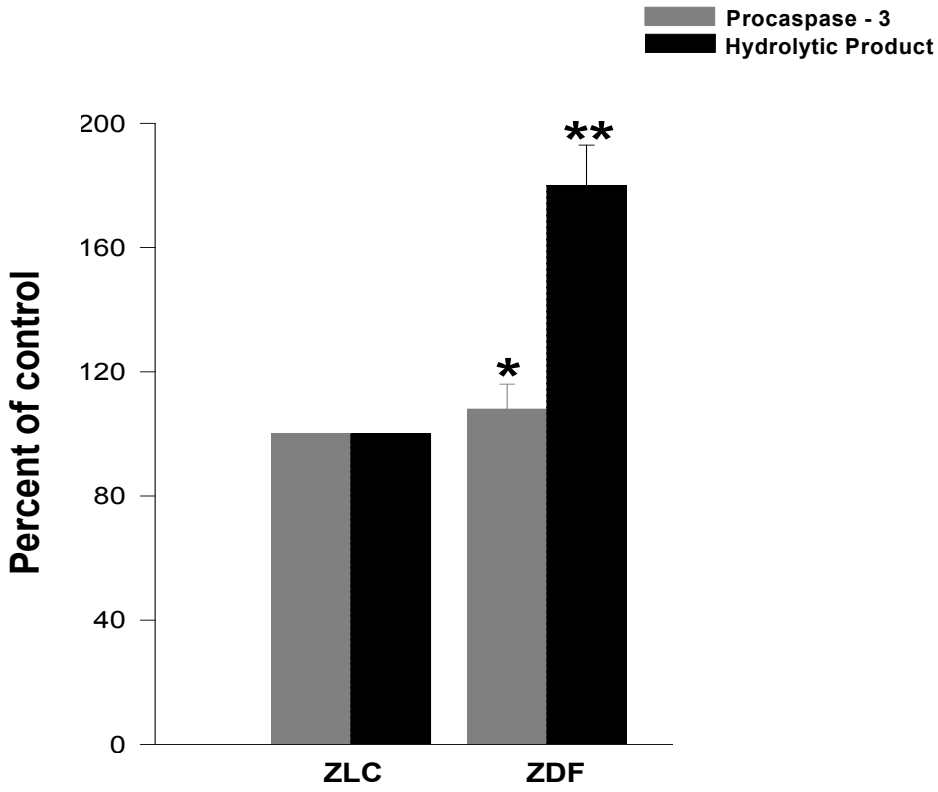
B]



C]



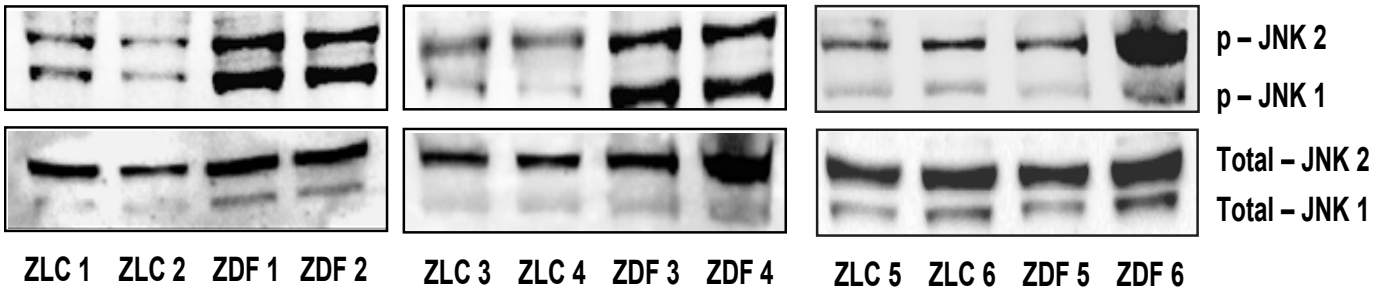
D]



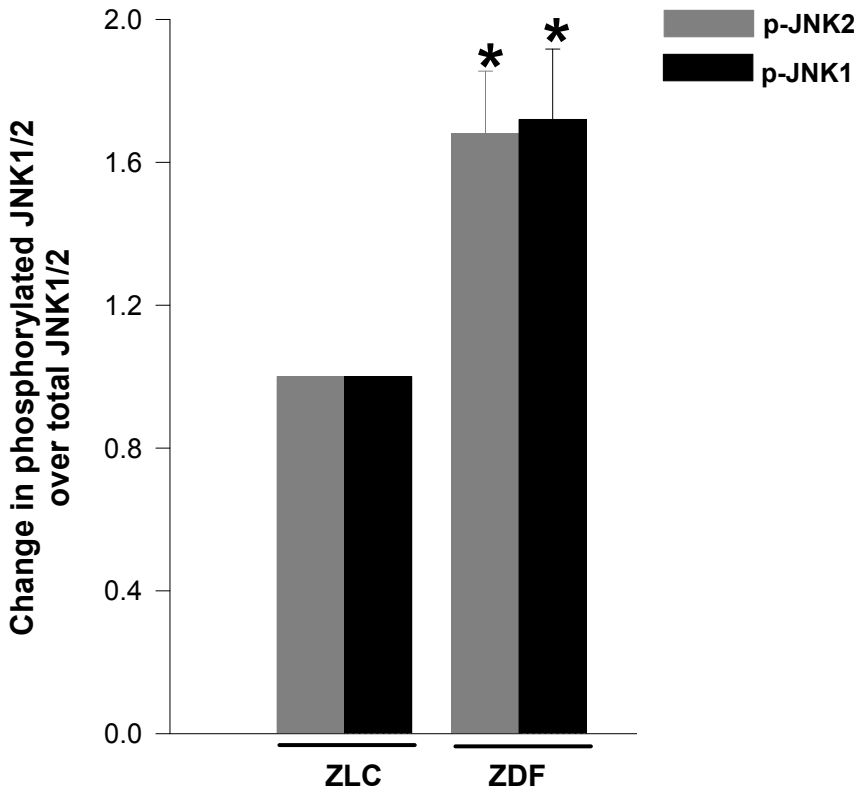
Lysates derived from ZLC and ZDF rats were used for the determination of expression of $gp91^{phox}$ by Western blotting [Panel A]. β-actin was used as loading control. The protein bands were analyzed densitometrically, expressed as percent increase over lean control [Panel B]. Data are mean ± SEM from islet preparations from five rats in each group. * $p< 0.05$ *vs*. ZLC islets. In a separate set of studies, islet lysates from ZLC and ZDF rats were resolved by SDS-PAGE and immunoprobed for caspase-3 activation. β-actin was used as loading control. A representative blot from three independent experiments yielding similar results is shown here [Panel C]. The density of the procaspase and its hydrolytic product-bands was quantitated and expressed as percent control [Panel D]. Data in Panel D are mean ± SEM from islet lysates from three rats in each group. * $p< 0.05$ *vs*. procaspase values of lean control. ** $p< 0.05$ *vs*. caspase cleavage product of ZLC islets.

Figure 35:

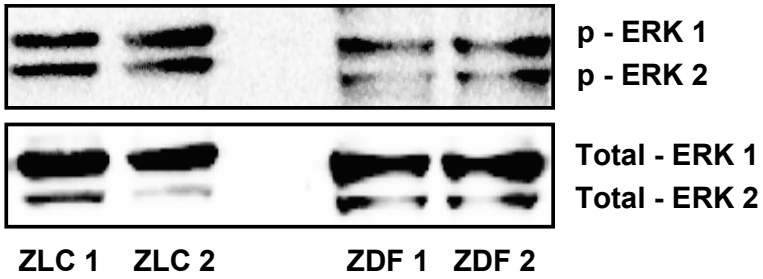
A]



B]



C]



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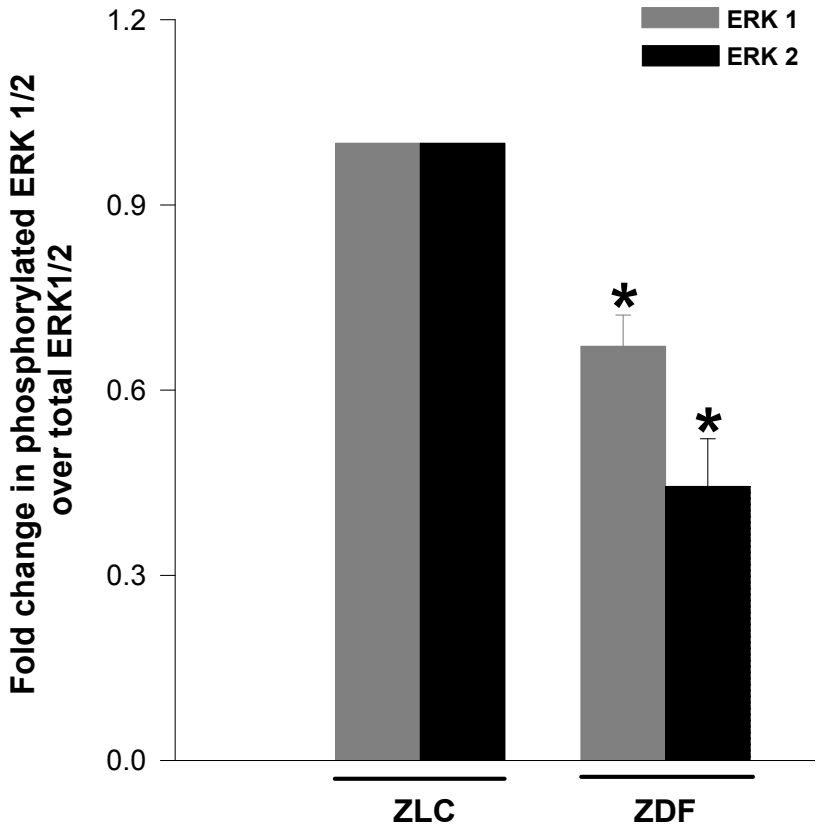
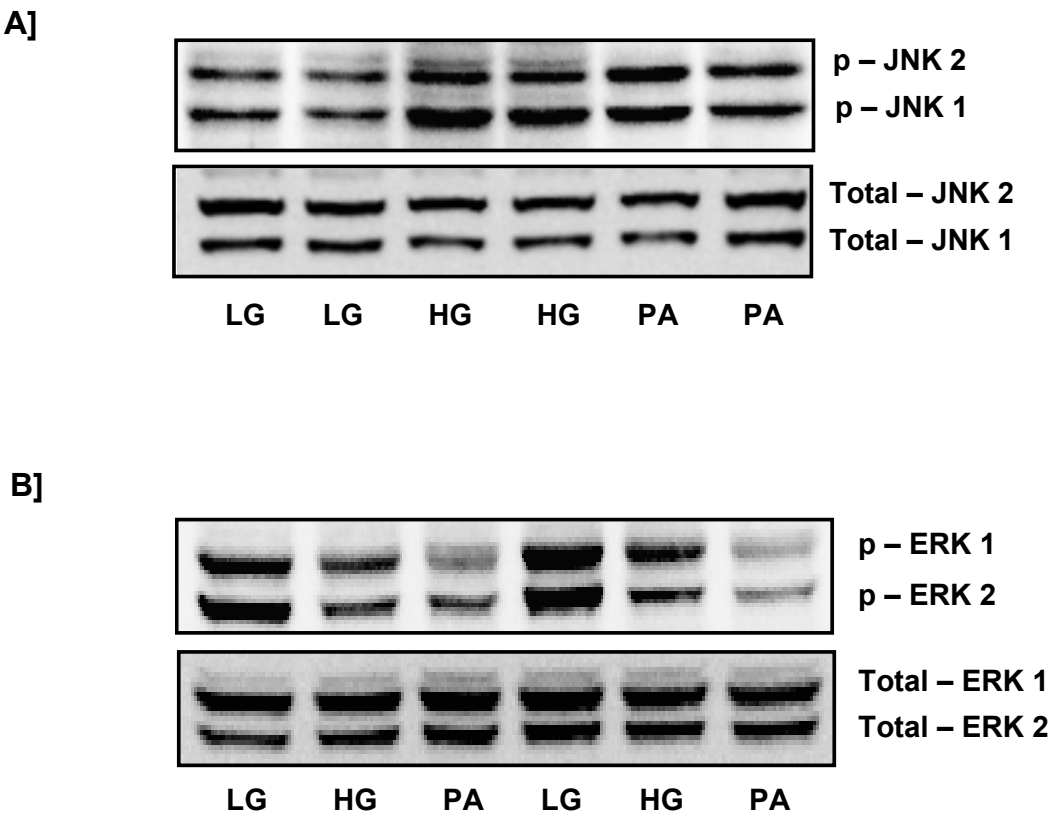


Islet lysates from ZLC and ZDF rats were prepared in RIPA buffer. Total and phospho-JNK1/2 were determined by Western blotting [Panel A] and analyzed densitometrically. Data are expressed as fold change in phosphorylation over total JNK 1/2 [Panel B]. Data are mean ± SEM from islet lysates derived from six rats in each group are shown herein. * $p < 0.05$ *vs*. ZLC islets. Lysates of islets from ZLC and ZDF rats were prepared in RIPA buffer. An equal amount of lysate protein was resolved by SDS-PAGE. Relative abundance of total and phospho-ERK1/2 were determined by Western blotting [Panel C] followed by densitometry [Panel D]. Data are expressed as fold change in phosphorylation over total ERK 1/2 and are mean ± SEM from islets from six rats in each group. * $p < 0.05$ *vs*. ZLC islets.

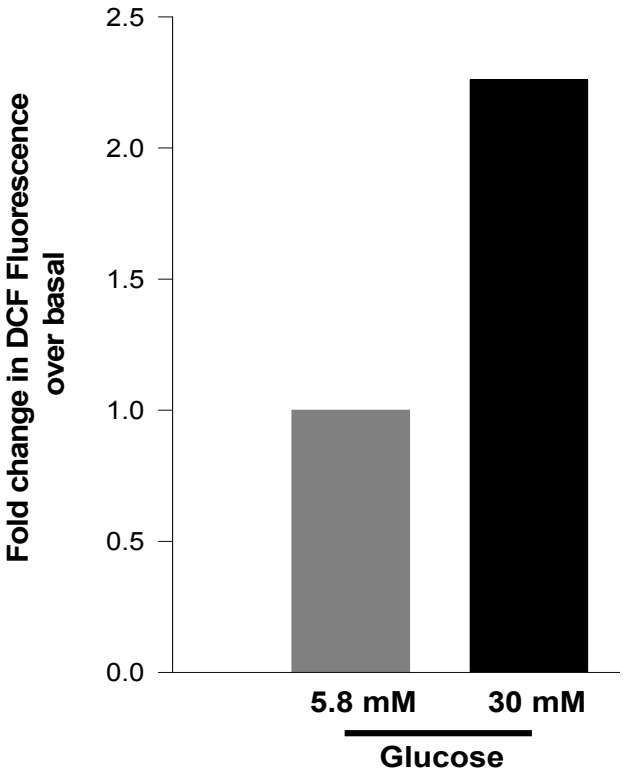
Figure 36:



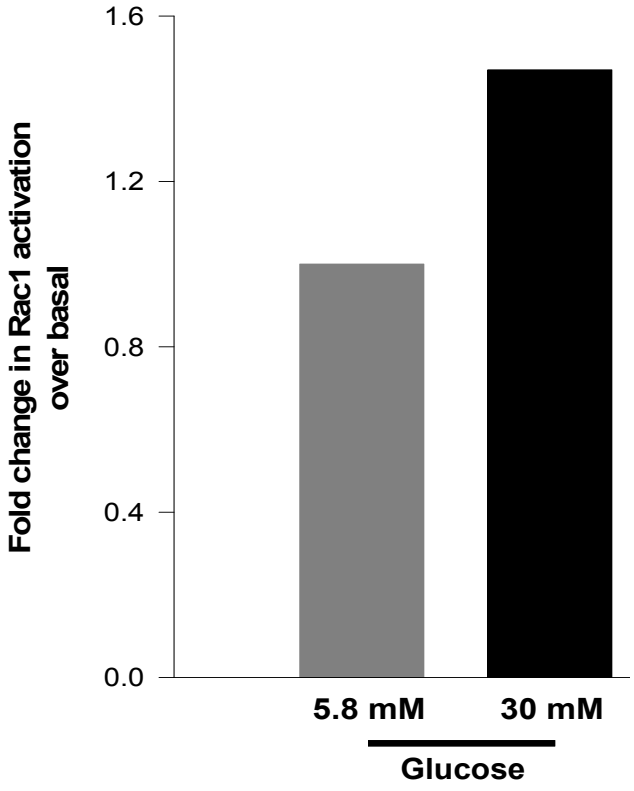
INS 832/ 13 cells were cultured in the presence of low glucose [LG; 2.5 mM], high glucose [HG; 20 mM] or palmitate [PA; 400 μM] for 48 h. At the end of incubation cells were lysed and the expression of total and phosphorylated JNK 1/2 [Panel A] and ERK 1/2 [Panel B] was determined by Western blotting.

Figure 37:

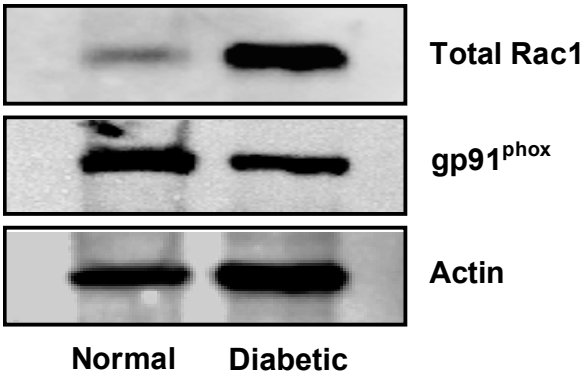
A]



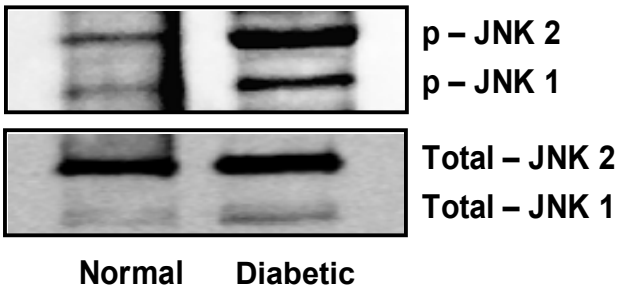
B]



C]



D]



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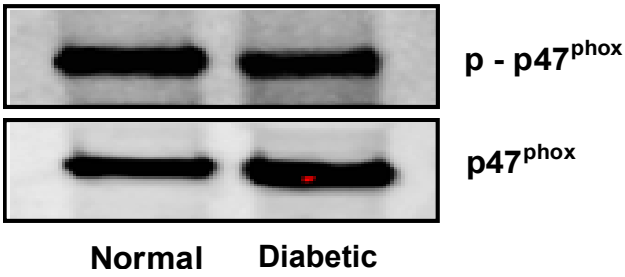
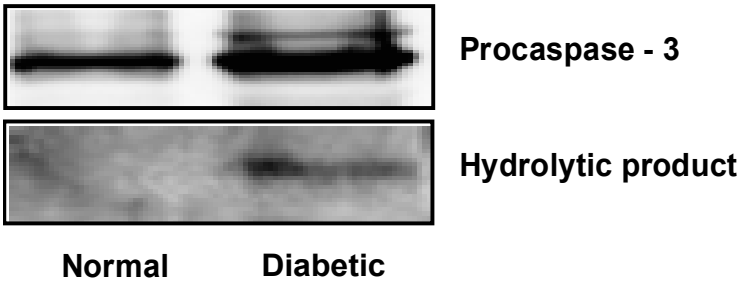
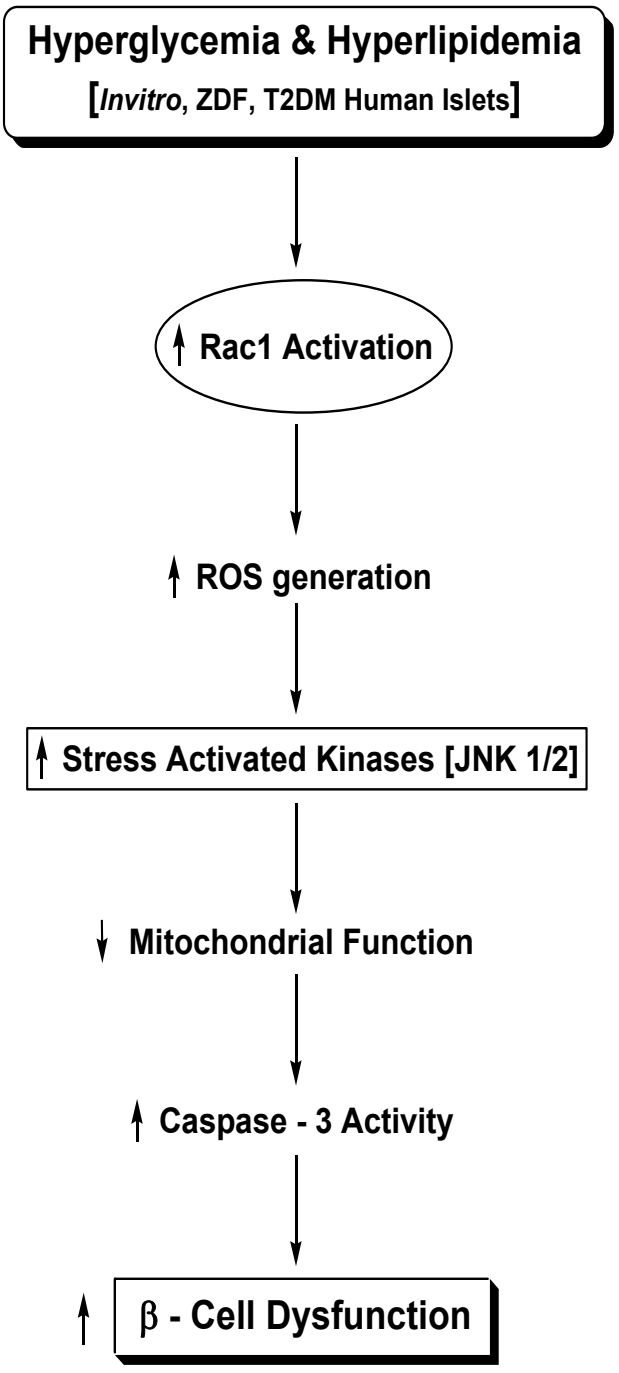


F]



Normal human islets were cultured in PMI medium in presence of 5.8 mM or 30 mM glucose for 48 h. Generation of ROS [Panel A; mean value from triplicate measurements] was quantitated by DCF fluorescence. Rac1 activation [Panel B; mean value from duplicate measurements] was quantitated by GLISA. In a separate set of studies, islets derived from control or diabetic human donors were lysed in RIPA buffer and lysate proteins were resolved by SDS-PAGE. The expression of total Rac1 and $gp91^{phox}$ [Panel C], phosphorylated and total JNK 1/2 [Panel D], phosphorylated and total $p47^{phox}$ [Panel E] and caspase-3 [Panel F] were determined by Western blotting. Corresponding house keeping genes were also measured in parallel to confirm equal loading.

Figure 38:



Based on the data accrued from the current studies [Chapter III], I propose a model for the Nox-ROS-JNK signaling in the metabolic dysfunction of pancreatic β-cell under the duress of hyperglycemia and hyperlipidemia. Glucolipotoxicity induces Nox activation by promoting the phosphorylation of $p47^{phox}$ and Rac1 activation. Nox activation and excessive ROS generation leads to the activation of stress-activated kinases [JNK 1/2] culminating in mitochondrial dysfunction and caspase-3 activation. I hypothesize that the collective effects of ROS generation, ERK1/2 inhibition and JNK1/2 activation may elicit maximal damaging effects on islet β-cell in diabetes.

DISCUSSION

Existing evidence in multiple cell types, including the pancreatic β-cell clearly implicates post-translational modification [e.g., phosphorylation and prenylation] of individual components as a requisite for the optimal activation of Nox **[219, 221]**. The main objective of the current study was to determine functional status of Nox in islets derived from ZDF rat, a well established model for obesity and type 2 diabetes, and to determine potential regulation of Nox components in human islets under the duress of glucolipotoxicity and diabetes. Salient findings of the current study include demonstration of increased expression and phosphorylation of $p47^{phox}$ subunit; Rac1 activation; $gp91^{phox}$ subunit expression and associated increase in ROS generation in the ZDF islet. These findings also suggested that differential regulation of JNK1/2 [i.e., activation] and ERK1/2 [i.e., inhibition] in the ZDF islet may, in part, be due to the gluco or lipotoxic effects, since *in vitro* exposure of INS 832/13 cells or normal human islets to high glucose or palmitate elicited similar effects. Lastly, data in diabetic human islets corroborated my findings in ZDF islets.

Several recent studies have demonstrated activation of Nox following exposure to physiological concentrations of glucose in a variety of insulin-secreting cells **[13, 14, 110, 112]**. Pharmacological and molecular biological inhibition of Nox revealed that a tonic increase in Nox-derived ROS is necessary for GSIS **[14, 118]**. In addition, recent findings demonstrated that prenylation of Rac1 is necessary for glucose-induced Nox activation and ROS generation in isolated β-cells **[219]**. Recent studies have also implicated Nox in metabolic dysfunction of the islet β-cell under conditions of

glucolipotoxicity and exposure to cytokines **[179, 180].** These studies demonstrated an increase in the expression and phosphorylation of Nox subunits [i.e., $p47^{phox}$], together with significant activation of Rac1. In addition, the activation status of Rac1 was shown to be under precise control of Tiam1, a known guanine nucleotide exchange factor for Rac1, but not Cdc42 and Rho G-proteins in isolated β-cells **[204]**. In further support of this, we observed a marked reduction in high glucose-, high palmitate- cytokine-induced Rac1 and Nox activation and ROS generation in isolated β-cells following exposure to NSC23766, a selective inhibitor of Tiam1/Rac1 signaling axis **[179, 180]**. Furthermore, using selective inhibitors of protein prenylation, Subasinghe et al demonstrated a critical requirement of prenylation of Rac1 for Nox-mediated β-cell dysfunction **[179]**. Taken together, previous *in vitro* findings clearly implicated participatory roles of Nox in exerting effects at the mitochondrial level including loss in membrane potential, cytochrome C release and activation of caspase-3 culminating in islet β-cell dysfunction **[179]**.

In addition to an increase in $p47^{phox}$ and $gp91^{phox}$ expression, Rac1 activation, and ROS generation, I observed a significant increase in the phosphorylation of JNK1/2 in the ZDF islets compared to the ZLC islets. I also observed a marked inhibition in ERK1/2 phosphorylation in cognate cellular preparations. It is noteworthy that similar changes in the phosphorylation status of JNK1/2 [activation] and ERK1/2 [inhibition] were demonstrable in INS 832/13 cells following incubation with either high glucose or palmitate. Together, these findings suggest that glucolipotoxicity may promote cellular dysfunction in the ZDF islet. However, whether JNK1/2 activation and ERK1/2 inhibition lie upstream to mitochondrial defects remains to be determined. Along these lines, a

recent study by Kim and associates **[222]** suggested roles for Nox-ROS-JNK1/2 signaling pathway in the onset of mitochondrial dysfunction induced by genipin in FaO rat hepatoma cells and hepatocarcinoma Hep3B cells. SP600125, a selective inhibitor of JNK1/2, suppressed genipin-induced apoptosis in these cells suggesting a role for JNK1/2 activation in genipin-induced cell demise. Further, DPI significantly attenuated genipin-induced ROS generation, JNK1/2 and caspase activation and cell death thereby establishing a role for ROS in genipin-induced, JNK1/2-mediated cell death. In this context, several recent studies have also demonstrated inhibition of caspase-3 activation following inhibition of JNK 1/2 activation in models of cellular apoptosis **[227 - 229]**.

The observed reduction of ERK1/2 activation under glucolipotoxic conditions in the ZDF rat islets *in vivo* and INS 832/13 cells *in vitro* are indicative of impaired metabolic function and β-cell proliferation. These current findings on reduction in ERK1/2 phosphorylation in INS 832/13 cells are in accord with studies of Costes and associates **[230]** who demonstrated a significant reduction in ERK1/2 phosphorylation in MIN6 cells following exposure to 25 mM glucose for 24 h. Based on further studies, these investigators concluded that glucotoxic conditions downregulate ERK1/2-CREB signaling pathway leading to the apoptotic demise of the β-cell.

Recent studies by Zhang and associates **[231]** demonstrated a significant increase in JNK 1/2 phosphorylation and reduction in ERK 1/2 phosphorylation during mevastatin-induced apoptosis of salivary adenoid carcinoma cells, suggesting a potential inverse relationship between JNK 1/2 and ERK 1/2 phosphorylation in the

induction of cellular apoptosis. Together, my observations in INS 832/13 cells, ZDF islets and diabetic human islets support involvement of Nox-ROS-stress activated signaling axis in the metabolic dysfunction. However, additional studies are needed to substantiate this formulation. Recent studies by Nakayama et al **[232]** demonstrated the functional activation of Nox in islets of *db/db* mice and Otsuka Long-Evans Tokushima Fatty rats. Treatment of these animals with angiotensin II type-1 receptor antagonists reduced Nox activation and oxidative stress. It may be germane to point out that Valle and coworkers **[233]** recently examined potential changes in Nox in islets derived from high fat-fed obese animals. In contrast to islets from *db/db* mice and Otsuka Long-Evans Tokushima Fatty rats **[232]** and ZDF rat [current study], islets from high fat-fed animals exhibited markedly lower expression levels of $p47^{phox}$ and $gp91^{phox}$ subunits and ROS production compared to control rat islets. These investigators attributed this toward increased glucose oxidation and GSIS seen in islets from high fat fed animals in response to glucose **[233]**.

Lastly, Fontes and coworkers **[226]** have reported a significant stimulation in ERK1/2 phosphorylation in MIN6 cells and normal rat islets when cultured in the presence of glucose and palmitate. Similar increase in the phosphorylation of Erk1/2 was seen in the presence of ceramide, a biologically active sphingolipid, which is biosynthesized from palmitate *via* the *de novo* pathway. Based on data accrued from additional studies these investigators concluded that ERK 1/2 activation represents one of the signaling steps involved in palmitate-induced inhibition of insulin gene expression. These findings are in contrast to my current findings of inhibition of ERK 1/2

phosphorylation by palmitate. Additional studies are needed to address potential differences between these findings.

Based on the existing information and current findings, I propose a model for Nox-mediated induction of β-cell dysfunction in diabetes [Figure 38]. Herein, I propose that exposure of isolated β-cells to glucolipotoxic conditions or islets derived from the diabetic condition in ZDF rats or humans, results in increased activation of Rac1 and Nox. Consequential generation of ROS and the associated oxidative stress, in turn, promote activation of JNK1/2 and mitochondrial dysregulation. Alternatively, activation of cytosolic Nox-ROS-JNK1/2 signaling pathway increases superoxide generation that impairs the functional efficiency of mitochondria; this proposal is supported by findings of Bindokas and associates **[133]** demonstrating excessive superoxide levels in islet mitochondria from the ZDF rat.

In summary, my current findings implicate Nox as one of the sources of oxidative stress in the diabetic islet. It will be interesting to determine if pharmacological intervention of Nox activation seen in islets under diabetic conditions can be restored to its normal function. Such intervention modalities include NSC23766, a selective inhibitor of Tiam1/Rac1, which I have utilized in *in vitro* experiments to restore mitochondrial function in β-cells exposed to elevated glucose, lipids and cytokines **[179, 180]**. In this context, recent investigations have successfully utilized NSC23766, a selective inhibitor of Tiam1-Rac1 signaling axis to correct Nox-mediated effects on cellular function *in vitro* and *in vivo* [**117** for a review]. Using streptozotocin diabetic mouse model, Shen et al **[120]** have demonstrated a regulatory role for Rac1 in hyperglycemia-induced apoptosis in cardiomyocytes. They demonstrated upregulation of Rac1, Nox activity,

increased ROS generation leading to apoptosis of cardiomyocytes under the duress of hyperglycemia. Treatment of diabetic *db*/*db* mice with NSC23766 significantly inhibited Nox activity and cell apoptosis **[231]**. Additional studies are needed to pin-point regulatory roles of Tiam1-Rac1-Nox-ROS signaling in the metabolic dysfunction in the diabetic islet.

WORKING MODEL

Based on the available evidence and my current findings, I propose that Nox-mediated ROS generation requires intermediacy of Tiam1/Rac1 signaling axis in isolated pancreatic β-cells following long-term exposure to glucose, palmitate or inflammatory cytokines. Under lipotoxic conditions, activation of Rac1 may, in part, be due to *de novo* synthesis of ceramide from palmitate, since Fumonisin B1, a selective inhibitor of ceramide biosynthesis from palmitate, markedly attenuated the increase in palmitate-induce ROS generation. As I observed, glucotoxic conditions also elicit Tiam1/Rac1 activation in Nox-mediated ROS generation. In this context, it is possible that such effects of glucose on Tiam1/Rac1 are direct, and not mediated *via* ceramide; however, this needs to be verified. Alternatively, activation of endogenous sphingomyelinases or endoplasmic stress may lead to accumulation of intracellular ceramide during glucotoxic conditions. This needs to be verified as well. Lastly, my data also identified similar involvement of Tiam1/Rac1 activation in inflammatory cytokine-induced Nox activation and ROS in pancreatic β-cells. A potential role for ceramide in this signaling needs to be examined further.

Under the conditions of oxidative stress in pancreatic β-cells, Nox-mediated ROS generation gets amplified, thus creating an environment in which mitochondrial membrane potential is reduced leading to the release of cytochrome c, activation of caspase-3 thereby leading to loss in cell viability and demise of the pancreatic β-cells. Coprovision of inhibitors for Nox [e.g., apocynin and DPI], Rac1 [e.g., GGTI-2147 and NSC23766] or siRNA mediated knockdown of $p47^{phox}$ leads to inhibition of Nox activation and partial restoration of mitochondrial dysfunction. Not included in this

current model are the other known mechanisms through which chronic hyperglycemia exerts damaging effects on β-cells, presumably not involving the Nox activation **[158]**. A recent review by Kowluru highlights the importance of Tiam1/Rac1 signaling axis in the generation of ROS under short-term conditions in the presence of stimulatory glucose and under conditions of long-term exposure to supra-physiological glucose concentrations leading to the metabolic regulation of the islet **[117]**. As recently suggested by Poitout and Robertson **[158]** other mechanisms such as oxidative phosphorylation, sorbitol metabolism, hexosamine metabolism, protein kinase C activation by DAG etc., may also underlie the generation of oxidative stress in the β-cells during the duress of glucolipotoxicity.

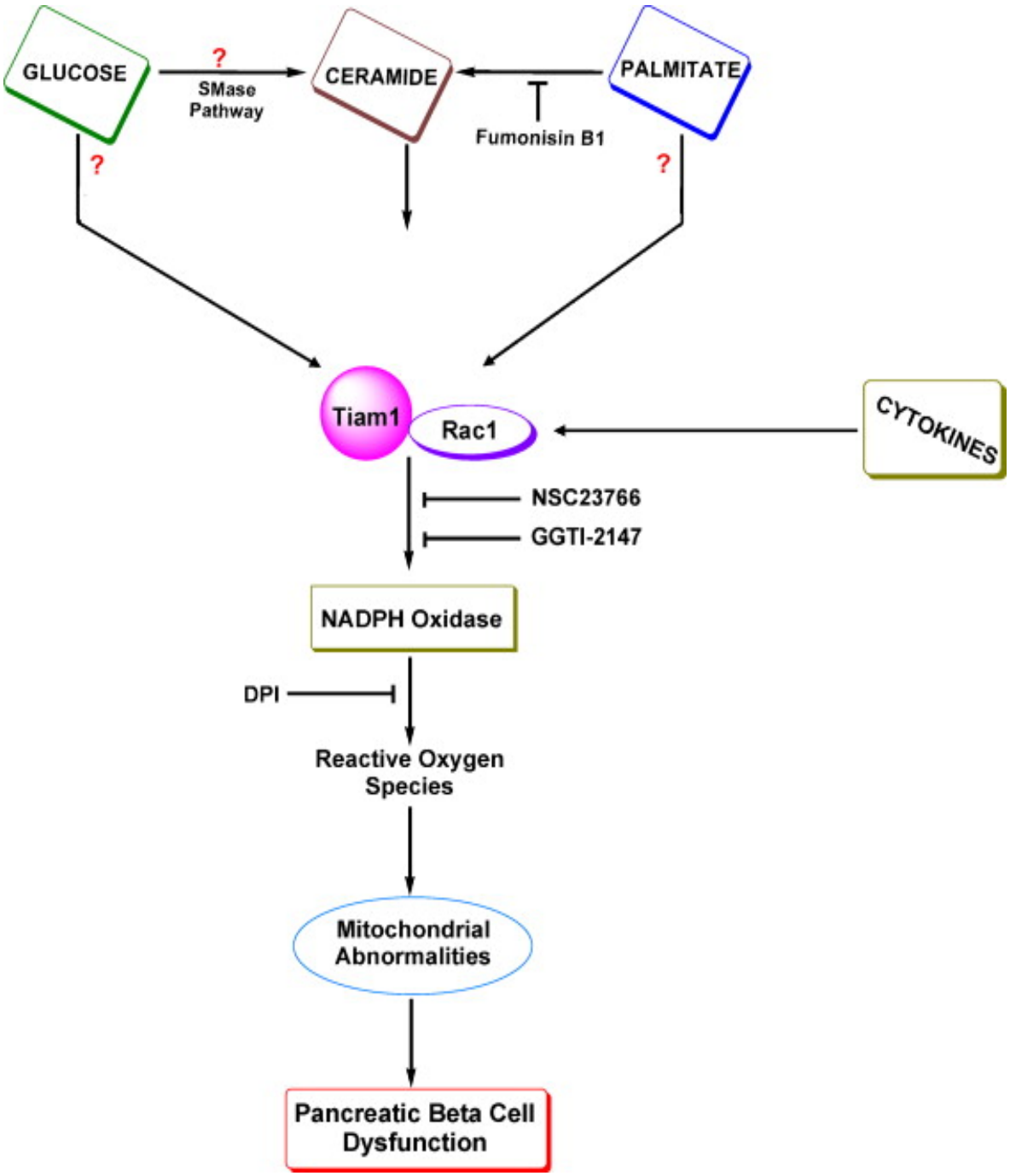


Figure 39: Proposed model illustrating potential involvement of Tiam1/Rac1 signaling axis in high glucose-, palmitate-, ceramide- or cytokines-induced metabolic dysfunction of the islet β-cell.

Nox-mediated oxidative stress, Potential Therapeutic Targets and Interventional Modalities

Inhibition of Nox holoenzyme assembly

Data accrued from my studies clearly suggested positive and negative modulatory roles for Nox-derived ROS in islet β-cell function. The holoenzyme assembly could represent a novel therapeutic drug target for either minimizing or halting excessive generation of Nox-mediated ROS and subsequent oxidative damage to the islet during hyperglycemia and/or hyperlipidemia. El-Benna and associates **[234]** recently proposed that $gp91^{phox}$, $p47^{phox}$ and $p67^{phox}$ might serve as potential drug targets due to their selective association in the Nox holoenzyme complex, but not other NADPH oxidases.

Along these lines Mizrahi et al developed $p47^{phox}$-$p67^{phox}$-Rac1 chimera as a quintessential single molecule activator of Nox **[235]** to study the effects of Nox activation, and suggested that a prenylation step is critical for Rac1 regulatory roles. These observations are in agreement with my findings, where I have demonstrated a decrease in glucose-mediated Nox-induced ROS generation in the presence of prenylation inhibitors. Developing inhibitors for such activators might provide a novel therapeutics to minimize Nox-mediated β-cell dysfunction.

Inhibition of Tiam1/Rac1/Nox signaling axis

It may be germane to point out that, many investigators have utilized NSC23766, an inhibitor for Tiam1-mediated Rac1 activation to inhibit Nox-induced ROS generation

both *in vitro* and *in vivo* **[179, 180, 207]**. Shen et al for the first time using streptozotocin-induced diabetic model demonstrated a regulatory role for Rac1 in hyperglycemia-induced apoptosis in cardiomyocytes **[120]**. Under these conditions, Rac1 and Nox activation were significantly augmented which were attenuated by pharmacological and microbiological approaches. Moreover, treatment of diabetic db/db mice with NSC23766 showed a decrease in Nox activity and cell death *via* apoptosis. Additional support for regulatory roles for Rac1 in the onset of myocardial remodeling in type 1 diabetes, came from Li et al who have demonstrated that Rac1 knockout or apocynin-treatment considerably attenuated Nox subunit[s] expression and activation, ROS production and cardiac collagen deposition. Moreover, Rac1 deficiency in myocardiocytes led to decreased hypertrophy and myocardial fibrosis and improved myocardial function **[236]**. Together, these studies provide compelling evidence in support of the hypothesis that Tiam1/Rac1 signaling axis plays a critical role in Nox-mediated cell dysfunction in diabetes. These facts also elevate the possible need for the development of more specific modalities to exclusively inhibit this signaling pathway. However, as discussed above, at least in the context of the islet β-cell, this strategy may not be ideal since the Tiam1/Rac1/Nox signaling pathway is also implicated in the signaling cascade leading to physiological insulin secretion, including actin remodeling, granule mobilization and tonic increase in ROS **[84, 219]**. On the other hand, it is expected that slight decrease in the Tiam1/Rac1 signaling pathway might be beneficial to the islet function. This needs experimental verification. Moreover, Bosco and associates **[237]** recently demonstrated that Rac1 regulates various cellular functions including microtubule stability, actin organization, transcription, superoxide generation

and nuclear signaling in normal physiological states. Together, the above mentioned positive modulatory roles of Rac1 in normal cell function implicate Rac1 as low priority target protein for therapeutic development.

Use of antioxidants

Proper maintenance of antioxidant defenses might be effective for slowing progression of diabetes itself by sustaining functional pancreatic β-cells. As islet β-cells hold a poor antioxidant defense mechanism as reviewed by Acharya and Ghaskadbi **[238]**, counterbalancing oxidative environment by antioxidant treatment or overexpressing antioxidant enzymes proves to be useful in regulating β-cell function. Such approaches have been successful in preserving the number of insulin-positive β-cells in presence of antioxidants. Quantitation of gene expression profiles of antioxidant enzymes in rodent islets yielded very low values compared to their respective counterparts in the liver. For an instance, relative abundance of CuZn superoxide dismutase, Mn-superoxide dismutase and glutathione peroxidase in islets corresponds only to 38%, 30% and 15% of the liver values, respectively. Catalase activity was undetectable in islets [**238** and references therein]. In addition, studies by Modak et al. **[239]** have demonstrated very poor DNA repair mechanism in β-cells due to oxidative stress compared to other cell types [e.g., liver cells].

Moreover, treatment with antioxidants like α-lipoic acid has been demonstrated to improve functional outcomes, like insulin sensitivity in type 2 diabetic subjects **[240]**. As an antioxidant, vitamin E improves outcomes related to pancreas physiology in diabetes **[241]**, which may improve functional outcomes of diabetes in animal models. Asayama

et al found that rats deficient in vitamin E, selenium, or both had decreased insulin secretory reserves, suggesting that vitamin E status can directly affect pancreatic islet function. In a mouse model of type II diabetes, treatment with vitamin E combined with vitamin C and n-acetyl cysteine resulted in large number of pancreatic islets than controls at 10 and 16 weeks **[242]**. Along these lines, studies from Robertson's laboratory have capitulated valuable insights into positive effects of overexpression of anti-oxidant enzymes like glutathione peroxidase against the damaging effects of oxidative stress **[243, 244]**. Studies from Xiao and group in humans **[159]** have also suggested beneficial effects of antioxidant therapy [e.g., taurine], which affectively restored lipid-induced reductions in plasma biomarkers of oxidative stress, insulin sensitivity and β-cell function. A recent review by Giacca et al. **[245]** provides additional advances in the area of lipid-induced pancreatic β-cell dysfunction, specifically focusing on *in vivo* studies. Moreover, it has been shown that NAC prevents impairment of GSIS *in vivo* in perfused pancreas of 96-h Intralipid-infused rats **[160]**. Together these studies further highlight antioxidant therapy as one of the feasible options in attenuating glucolipotoxicity-induced oxidative stress in the islets.

Use of polyphenolic extracts

Studies by Tao et al demonstrated an increase in Nox expression in the heart from adiponectin knockout mice **[246]**. In addition, studies by Dong et al reported significant increase in the expression Nox protein by leptin in murine cardiomyocytes **[247]**. In this milieu, beneficial effects of polyphenolic grape seed extract [GSE] against high-fat diet mediated obesity, adiponectin–leptin disparity and oxidative stress markers in hamsters have been demonstrated **[248]**. Following GSE therapy, these studies have

shown a marked reduction in high-fat-induced abdominal fat, plasma glucose, triglycerides and insulin resistance in these animal models; furthermore, plasma levels of adiponectin and leptin were normalized, in the conditions where there is increased cardiac production of superoxides and Nox expression. Together, these findings implicate regulatory roles of adiponectin and leptin in Nox activity. Further, they provide evidence for the therapeutic efficacy of grape phenolics in the prevention of Nox-mediated effects on cellular functions. Potential roles of Tiam1/Rac1 axis in this signaling cascade remains to be examined.

Use of angiotensin receptor antagonists

Studies from Nakayama et al have demonstrated a significant enhancement in islet function in diabetic OLETF rats and db/db mice following treatment with angiotensin1 receptor antagonist **[232]**. Following treatment with Valsartan, a known angiotensin1 receptor antagonist, they demonstrated attenuation in the expression of $gp91^{phox}$ and $p22^{phox}$ and associated oxidative stress. These conclusions are compatible with *in vitro* observations by Hirata et al demonstrating a significant activation of Nox-mediated and superoxide generation in rat pancreatic islets subsequent to exposure with angiotensin II **[221]**. Collectively, these data are indicative of novel cytoprotective effects of angiotensin receptor antagonists against cell damage induced by glucolipotoxicity and/or proinflammatory cytokines.

Inhibition of JNK signaling pathway

Under diabetic conditions, oxidative stress activates JNK cascade, which in turn suppresses insulin biosynthesis **[249]**. Thus, the hemin-dependent reduction of JNK

and subsequent increase of insulin are important mechanisms for the enduring antidiabetic effects. In agreement with this is a recent study to suggest that hemeoxygenase system abates JNK activity **[250]**. Lastly, the regulation of insulin release by the hemeoxygenase system has been well documented in the Goto-kakizaki rat, a nonobese model for type II diabetes **[251, 252]**.

CONCLUSION

GSIS involves a series of metabolic events involving interaction between a variety of signaling pathways to facilitate the transport of insulin-laden granules to the plasma membrane for fusion and release of insulin. Compelling evidence supports involvement of small G-proteins like Rac1 and Cdc42 in the cytoskeletal reorganization, which is necessary for GSIS to occur. Findings from our laboratory further validate that Tiam1 represents one of the GEFs for Rac1 and that Tiam1/ Rac1 signaling axis is requisite for GSIS. Nox appears to be an effector protein for Tiam1/Rac1 signaling and that its activation leads to a tonic increase in the generation of ROS under the stimulatory conditions of glucose and fatty acids leading to insulin release. In addition to this, Tiam1/Rac1 signaling axis appears to play an important role in the generation of Nox-mediated ROS generation under the duress of excessive glucose, palmitate, ceramide and cytokines culminating in oxidative stress and metabolic dysfunction of pancreatic β-cells. Together, my findings suggest positive and negative modulatory roles for Tiam1-Rac1-Nox signaling pathway in islet function. Therefore, it may be difficult to pin-point as to how much of ROS generation is good for the normal function of the islet as opposed to and how much is bad to elicit damaging effects on the pancreatic β-cell. The Figure 40 depicted below is indicative of potential effects of ROS on islet at different stages. It also indicates known metabolic alterations at each stage. It is likely that there may be a “window of opportunity” or “point of return” for the islet β-cell to recover from the Noxious effects of excessive ROS due to accelerated Tiam1-Rac1-Nox signaling pathway in the presence of elevated glucose, FFA, ceramide or cytokines.

Despite the fact that, Nox is identified as a cause for development of oxidative stress, its possible loci for the action of ROS and mechanistic insights into the cytosolic subunits within the β-cells require a thorough study. However, published evidence from our laboratory **[96]** demonstrated roles for biologically active phospholipids in the dissociation of Rac1/GDI complexes, which is ought to be essential for activation and translocation of Rac1 to the membrane; and this step involves intermediacy of Tiam1. Moreover, post-translational geranylgeranylation of Rac1 is essential for activation of Nox complex as manifested by inhibition of Nox-mediated ROS generation in presence of GGTI2147. It should also be noted that Rap1, one of the membranic components of Nox holoenzyme, also gets geranylgeranylated and is inhibited by GGTI2147. A recent review by Kowluru **[117]** suggests that activation of Rac1 is necessary for Nox activation, and that very little is known about potential regulatory roles of Rap1 in this signaling cascade. Further, Rap1 could potentially be under the control of specific GEFs [e.g., *Epac2*] and related mechanisms.

In summary, I believe that my findings provide fresh insights into potential therapeutic targets and interventional modalities to prevent these metabolic defects. The *in vitro* observations are supported by my finding in islets derived from the diabetic rodents [the ZDF rat] and diabetic human islets. I truly hope that these findings form basis for the development of small molecule inhibitors in halting the metabolic defects, thereby retaining normal β-cell mass.

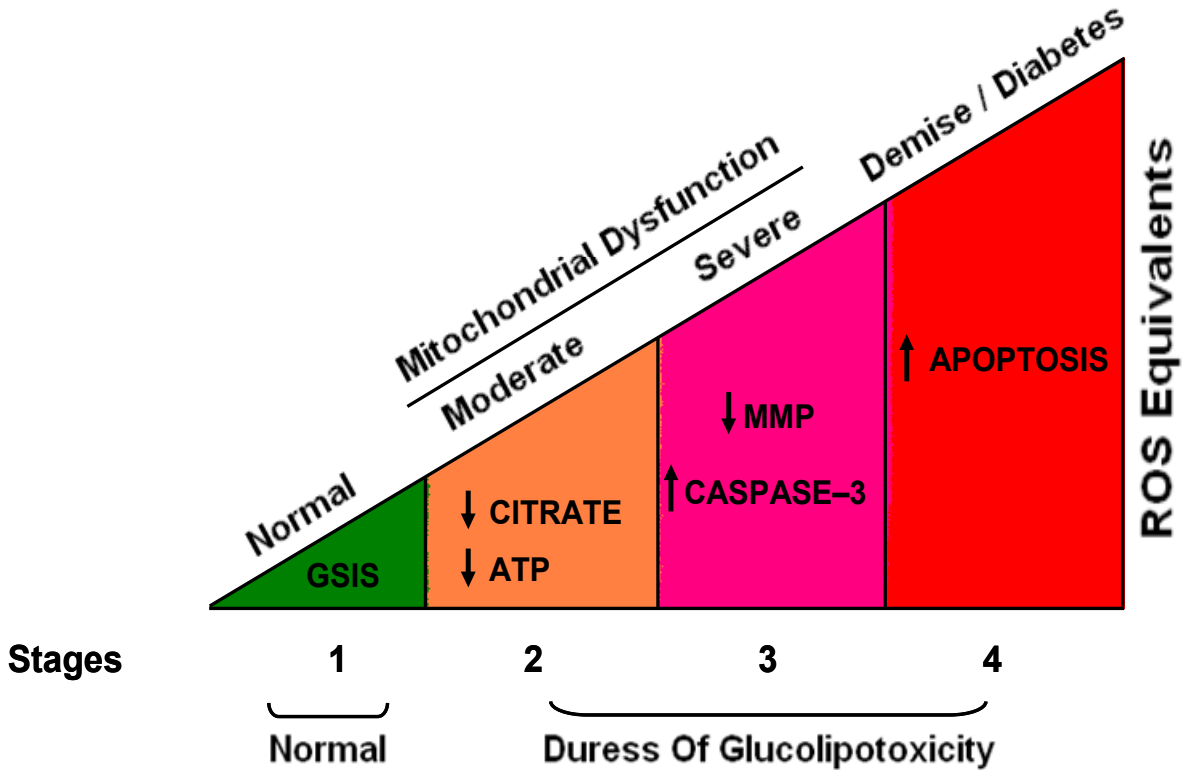


Figure 40: Hypothetical model for ROS generation in identifying the effects on pancreatic β-cells.

Appendix A

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Phagocyte-like NADPH oxidase generates ROS in INS 832/13 cells and rat islets: role of protein prenylation

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Syed I, Kyathanahalli CN, Kowluru A. Phagocyte-like NADPH oxidase generates ROS in INS 832/13 cells and rat islets: role of protein prenylation. *Am J Physiol Regul Integr Comp Physiol* 300: R756–R762, 2011. First published January 12, 2011; doi:10.1152/ajpregu.00786.2010.—Recent evidence suggests that an acute increase in the generation of phagocyte-like NADPH-oxidase (Nox)-mediated reactive oxygen species (ROS) may be necessary for glucose-stimulated insulin secretion. Using rat islets and INS 832/13 cells, we tested the hypothesis that activation of specific G proteins is necessary for nutrient-mediated intracellular generation of ROS. Stimulation of β-cells with glucose or a mixture of mitochondrial fuels (mono-methylsuccinate plus α-ketoisocaproic acid) markedly elevated intracellular accumulation of ROS, which was attenuated by selective inhibitors of Nox (e.g., apocynin or diphenyleneiodonium chloride) or short interfering RNA-mediated knockdown of $p47^{phox}$, one of the subunits of Nox. Selective inhibitors of protein prenylation (FTI-277 or GGTI-2147) markedly inhibited nutrient-induced ROS generation, suggesting that activation of one (or more) prenylated small G proteins and/or γ-subunits of trimeric G proteins is involved in this signaling axis. Depletion of endogenous GTP levels with mycophenolic acid significantly reduced glucose-induced activation of Rac1 and ROS generation in these cells. Other immunosuppressants, like cyclosporine A or rapamycin, which do not deplete endogenous GTP levels, failed to affect glucose-induced ROS generation, suggesting that endogenous GTP is necessary for glucose-induced Nox activation and ROS generation. Treatment of INS 832/13 cells or rat islets with pertussis toxin (Ptx), which ADP ribosylates and inhibits inhibitory class of trimeric G proteins (i.e., G_i or G_o), significantly attenuated glucose-induced ROS generation in these cells, implicating activation of a Ptx-sensitive G protein in these signaling cascade. Together, our findings suggest a prenylated Ptx-sensitive signaling step couples Rac1 activation in the signaling steps necessary for glucose-mediated generation of ROS in the pancreatic β-cells.

G protein; pancreatic islets; Rac1 activation; pertussis toxin; inosine monophosphate dehydrogenase

GLUCOSE-INDUCED INSULIN SECRETION (GSIS) involves a series of metabolic and cationic events, leading to translocation of insulin-laden secretory granules from a distal site toward the plasma membrane for fusion and release of insulin into circulation. It is widely accepted that vesicular transport and fusion involves interplay between signaling proteins, including vesicle-associated membrane proteins on the secretory granule and docking proteins on the plasma membrane (23, 28, 33). Furthermore, interaction between these proteins is widely felt to require cytoskeletal remodeling, which is under the fine control of small molecular mass G proteins belonging to the Rho subfamily (e.g., Cdc42 and Rac1; see Ref. 17 for a recent review). Several effector proteins for these small G proteins have been identified in the islet β-cell, including phospholipases, p21-activated kinase-1 kinase, and ERK1/2 kinases (17, 40, 42).

In the context of G proteins, it is well established that they undergo posttranslational modifications for optimal activation, membrane trafficking, and effector interactions. The majority of small G proteins undergoes a series of modifications at their COOH-terminal cysteine residues, which include prenylation (i.e., farnesylation and geranylgeranylation), carboxylmethylation (CML), and palmitoylation. In addition to small G proteins, the γ-subunits of trimeric G proteins undergo prenylation and CML (2, 13, 16, 17, 40). Indeed, using pharmacological and molecular biological approaches, several recent studies have confirmed the requisite nature of these modifications in GSIS in a variety of insulin-secreting cells, including clonal β-cells, normal rodent islets, and human islets (see Ref. 17 for a recent review).

A growing body of recent evidence implicated roles for reactive oxygen species (ROS) in metabolic dysfunction of the islet β-cell under the duress of glucolipotoxicity, cytokines, and ceramide (26, 38, 39). It has been shown that increased ROS generation seen under the above experimental conditions is derived from the activation of phagocyte-like NADPH oxidase (Nox), since inhibition of this enzyme by selective inhibitors [e.g., diphenyleneiodonium chloride (DPI) or apocynin] or transfection of short interfering RNA (siRNA) against individual subunits of Nox (e.g., $p47^{phox}$) significantly attenuated deleterious effects of aforementioned noxious stimuli (38, 39).

Despite the negative modulatory role(s) of ROS in cell function, recent evidence appears to indicate that a tonic increase in the ROS generation may be necessary for GSIS and fatty acid-induced insulin secretion (5, 29–32). ROS have also been shown to modulate many physiological processes, including ion transport and protein phosphorylation (1, 4, 9, 21). As reviewed recently by Pi and Collins (32), ROS plays "second messenger" role in modulating islet β-cell function. Along these lines, studies by Pi and coworkers (31) have demonstrated that glucose-mediated generation of H_2O_2 alters intracellular redox status, leading to augmented GSIS; such effects were attenuated by coprovision of antioxidants. These findings were further strengthened by Leloup and colleagues (20), suggesting that generation of mitochondrial ROS is a requisite stimulus for GSIS to occur. Together, these data implicate an essential role for Nox-derived ROS as a signaling molecule involved in the regulation of β-cell function, specifically at the level of insulin secretion. The present studies are undertaken to determine potential mechanisms underlying nutrient-induced elevation of ROS levels in INS 832/13 cells and normal rat

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islets. Specifically, we have determined the roles of G proteins in this signaling cascade; this was accomplished by selective inhibitors of protein prenylation (e.g., GGTI-2147 and FTI-277), which have been used to verify the roles for G proteins in GSIS (17). In addition, we have examined permissive roles for endogenous GTP in nutrient-induced ROS generation. Our findings implicate that prenylation-sensitive signaling steps are necessary for glucose- and mitochondrial fuel-induced intracellular generation of ROS in INS 832/13 cells and normal rat islets.

MATERIALS AND METHODS

Materials. DPI, apocynin, pertussis toxin (Ptx), mycophenolic acid (MPA), cyclosporine A, rapamycin, mono-methylsuccinate, α-keto-isocaproic acid, and 2′,7′-dichlorofluorescein diacetate (DCHFDA) were from Sigma (St. Louis, MO). $p47^{phox}$ siRNA and $p47^{phox}$ antiserum were from Santa Cruz Biotechnology (Santa Cruz, CA). FTI-277 and GGTI-2147 were from Calbiochem (San Diego, CA). Rac1 activation kit was from Cytoskeleton (Denver, CO).

Insulin-secreting cells. INS 832/13 cells were provided by Dr. Chris Newgard (Duke University Medical Center, Durham, NC) and 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 μM 2-mercaptoethanol, and 10 mM HEPES (pH 7.4). The medium was changed twice, and cells were subcloned weekly. Islets from normal Sprague-Dawley rats were isolated by collagenase digestion method described previously (41). All experiments, including isolation of pancreatic islets from normal Sprague-Dawley rats, were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee.

Quantitation of ROS. This was carried out as our laboratory described recently in Ref. 39. In brief, INS 832/13 cells were seeded in six-well plate and treated with various insulin secretagogues and inhibitors (or their respective diluents), as indicated in the text. Following incubation, the medium was removed, and cells were further incubated with DCHFDA (10 μM) at 37°C for 30 min in RPMI. DCHFDA, being a nonpolar compound, diffuses rapidly into the cells and hydrolyzes readily by cellular esterases into polar 2′,7′-dichlorofluorescein. In the presence of ROS, 2′,7′-dichlorofluorescein readily oxidizes to fluorescent dichlorofluorescein. The cells were washed with ice-cold phosphate-buffered saline and harvested, and equal amounts of protein were taken for fluorescence measurements (emission wavelength: 485 nm and excitation wavelength: 535 nm) using luminescence spectrophotometer (PerkinElmer, Waltham, MA).

Inhibition of Nox activity via molecular biological or pharmacological approaches. INS 832/13 cells were seeded in a 24-well plate and at 50–60% confluence either mock transfected or transfected with antisense $p47^{phox}$ siRNA at a final concentration of 150 nM and cultured for 24 h. Following this, cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 1 h. At the end of stimulation, culture medium was removed; cells were incubated further with DCHFDA (10 μM) at 37°C for 30 min in RPMI, washed with ice cold PBS, and harvested; equal amount of proteins were taken; and fluorescence was measured (excitation wavelength: 485 nm, and emission wavelength: 535 nm) using luminescence spectrophotometer as described above. Alternatively, Nox activity was inhibited via a pharmacological approach by incubating INS 832/13 cells either with apocynin (100 μM; 12 h) or DPI (5 μM; 2 h) in low-serum, low-glucose-containing medium. Following incubation, cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 1 h in the continuous absence or presence of inhibitors, and NADPH activity was measured by DCHFDA assay, as described above. The amount of fluorescence recorded is directly correlated with the amount of superoxide radicals generated due to Nox activity.

Rac1 activation assay. This was accomplished using a pull-down assay that our laboratory described recently (18). Briefly, INS 832/13 cells were starved overnight in low-serum, low-glucose-containing medium in either the presence or absence of MPA (10 μM). At the end of incubation, cells were stimulated with low glucose (2.5 mM) or high glucose (20 mM) for 30 min in the continuous presence or absence of MPA. Lysates (~500 μg protein) were clarified by centrifugation for 5 min at 4,800 *g*, and p21-activated kinase-binding domain beads (20 μl) were added to the supernatant. The mixture was then rotated for 1 h at 4°C and pelleted by centrifugation at 4,000 *g* for 3 min. The pellet was washed once with lysis buffer followed by a rinse (3×) in wash buffer (25 mM Tris, pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl, and 150 mM EDTA). Proteins in the pellet were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and Western blotting method determined the relative abundance of activated Rac1.

Other assays and statistical analysis of data. Protein concentrations were determined by Bradford's dye-binding method using bovine serum albumin as the standard. Statistical significance of differences between diluent and experimental groups was determined by Student's *t*-test and ANOVA analysis. $P < 0.05$ was considered significant.

RESULTS

Pharmacological inhibitors or siRNA-$p47^{phox}$ markedly attenuate glucose-induced ROS generation in insulin-secreting cells. At the outset, we determined whether stimulatory glucose promotes the generation of ROS, and whether selective inhibition of Nox attenuates such an effect in this model system. Data in Fig. 1*A* demonstrated a significant increase (~1.7-fold) in glucose-induced ROS generation in INS 832/13 cells, which was markedly attenuated by inhibitors of Nox holoenzyme (e.g., apocynin and DPI). The above observations were further validated by knockdown of $p47^{phox}$, a cytosolic subunit of Nox. Data in Fig. 1*B* indicated ~50% inhibition in the expression of $p47^{phox}$ subunit after siRNA transfection, and under these conditions we noticed a marked attenuation of glucose-induced ROS generation (Fig. 1*C*).

Selective inhibitors of protein prenylation markedly attenuate glucose-induced ROS generation in INS 832/13 cells and normal rat islets. Several earlier studies have demonstrated that posttranslational farnesylation and geranylgeranylation of specific G proteins are necessary for GSIS (17, 42). With this in mind, using a pharmacological approach, we examined whether glucose-induced ROS generation in isolated β-cells is sensitive to inhibition of protein prenylation. Data in Fig. 2 demonstrated a significant reduction in glucose-induced ROS generation by selective inhibitors of farnesylation (e.g., FTI-277) or geranylgeranylation (e.g., GGTI-2147) in INS 832/13 cells (*A*) or rat islets (*B*). Together, these findings suggested involvement of farnesylated and geranylgeranylated proteins in the signaling events, leading to glucose-induced ROS generation.

Protein prenylation is also necessary for mitochondrial fuel-, but not KCl-induced ROS generation. We next examined if a mixture of mitochondrial fuels (e.g., α-keto-isocaproic acid and mono-methylsuccinate), which elicits insulin secretion from INS 832/13 cells (6), also promotes Nox-mediated generation of ROS in these cells. Data in Fig. 3 demonstrated that mitochondrial fuels increased ROS generation in a manner akin

to glucose. Furthermore, we observed that such a signaling step was inhibited by FTI-277 and GGTI-2147, albeit to a lesser degree (Fig. 3) compared with glucose-induced ROS generation (Fig. 2). Together, data in Figs. 2 and 3 implicate protein farnesylation and geranylgeranylation in the cascade of events, leading to nutrient-induced generation of ROS in INS 832/13 cells. It should be noted that ROS generation appears to be specific for nutrient secretagogues, since a depolarizing concentration of KCl (40 mM), which facilitates insulin release via membrane depolarization and associated increase in cytosolic calcium, failed to promote ROS generation. (i.e., 109 ± 1.2% of control values; mean ± SE; $n = 3$; additional data not shown).

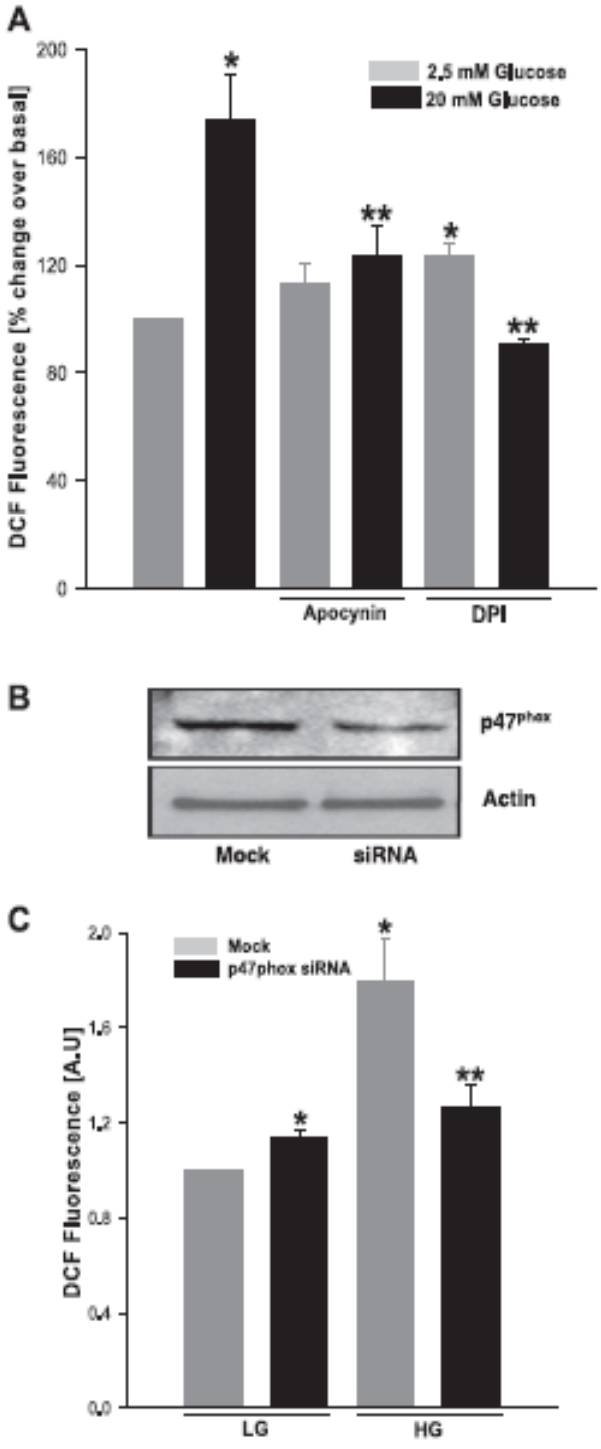

Fig. 1. Selective inhibitors of NADPH oxidase or short interfering RNA (siRNA)-p47^{phox} inhibits glucose-stimulated reactive oxygen species (ROS) generation in insulin-secreting cells. INS 832/13 cells were incubated with either diluent or apocynin (100 μM, 12 h; *A*) or diphenyleneiodonium chloride (DPI; 5 μM, 2 h; *A*) or transfected with p47^{phox} siRNA (*B* and *C*), following which they were stimulated with either low (2.5 mM; LG) or high glucose (20 mM; HG) for 1 h. ROS generated was quantified as dichlorofluorescein (DCF) fluorescence and expressed as arbitrary units (AU). *B*: transfection efficiency of p47^{phox} siRNA was determined by immunoblotting. Values are means ± SE from three independent experiments done in triplicates in each case. *$P < 0.05$ vs. LG alone or mock transfected LG. **$P < 0.05$ vs. HG alone or mock transfected HG.

Depletion of intracellular GTP inhibits glucose-induced Rac1 activation and ROS generation in INS 832/13 cells. Several previous studies have demonstrated a critical requirement for endogenous GTP in physiological insulin secretion by selectively inhibiting inosine monophosphate dehydrogenase (IMPDH) with MPA (24, 25). Herein, using MPA, we examined if endogenous GTP is required for glucose-induced Nox activation and associated ROS generation in INS 832/13 cells. Cyclosporine A and rapamycin were included as negative controls, which, like MPA, are endowed with immunosuppressive actions, but not GTP-lowering properties. Data in Table 1 suggested a marked attenuation in glucose-induced ROS generation by MPA, but not cyclosporine A or rapamycin. These data indicate a critical requirement for endogenous GTP for glucose to promote ROS generation in these cells. Together, data in Figs. 2 and 3 and Table 1 indicate potential involvement of prenylated G protein, requiring newly synthesized GTP due to the catalytic activation of IMPDH in the signaling events leading to ROS generation.

We next examined if GTP depletion impedes glucose-induced activation of specific G proteins involved in GSIS. To test this, we quantitated glucose-induced activation of Rac1 in MPA-treated (i.e., GTP-depleted) INS 832/13 cells. The premise underlying the selection of Rac1 in these studies is based on the evidence that *1*) it has been shown to be activated by glucose and involved in GSIS; *2*) it undergoes geranylgeranylation, and GGTI-2147 (above) inhibits glucose-induced Rac1 activation and GSIS; and *3*) it is a member of the Nox holoenzyme. Data shown in Fig. 4 demonstrated that stimulatory concentration of glucose failed to activate Rac1 in INS 832/13 cells following depletion of endogenous GTP using MPA.

A Ptx-sensitive G protein mediates glucose-induced ROS generation in INS 832/13 cells. In the last series of studies, we determined the nature of the prenylated protein that might be involved in glucose-induced ROS generation shown in Figs. 2 and 3. In this context, our laboratory recently reported that coprovision of FTI-277 or FTI-2628 or siRNA-mediated knockdown of farnesyltransferase β-subunit resulted in a significant inhibition of glucose-stimulated activation of ERK1/2, Rac1, and insulin secretion, further ruling out the potential involvement of Ras in these signaling steps (18). Based on

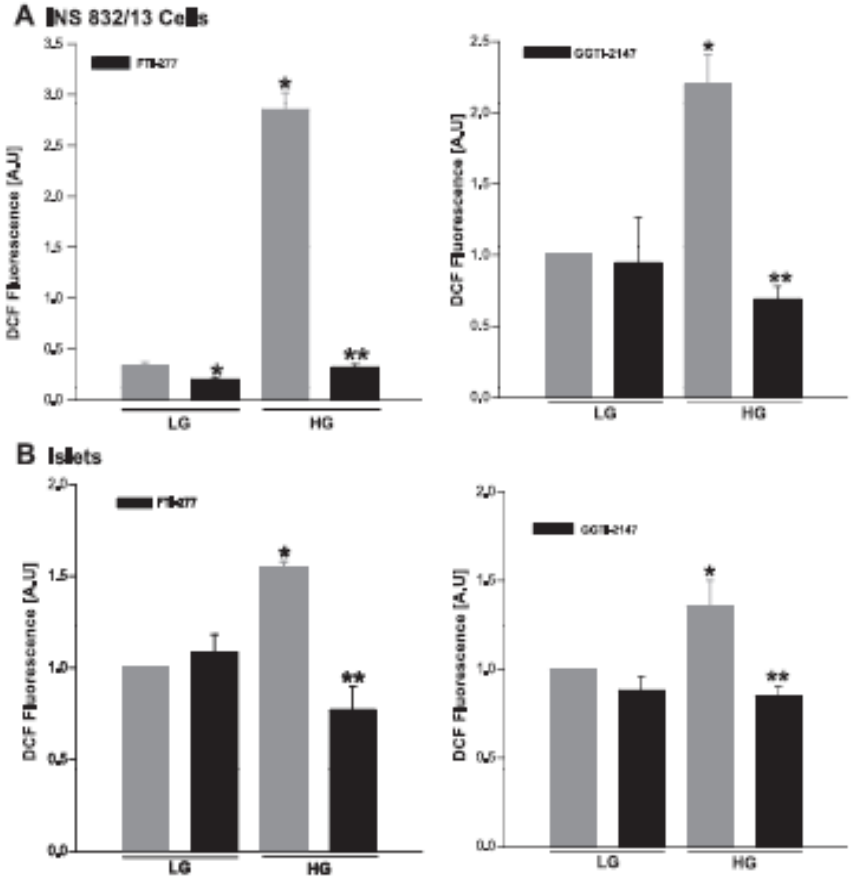

Fig. 2. Selective inhibitors of protein farnesylation or geranylgeranylation markedly attenuate glucose-induced ROS generation in INS 832/13 cells and normal rodent islets. INS 832/13 cells (*A*) or normal rat islets (*B*) were incubated overnight in the absence or presence of FTI-277 (5 μM; *left*) or GGTI-2147 (10 μM; *right*), followed by stimulation with either LG (2.5 mM) or HG (20 mM) for 1 h. ROS generated was quantified as DCF fluorescence and expressed as AU. Values are means ± SE from three independent experiments done in triplicates (in INS 832/13 cells) and in duplicates (in islets) in each case. $^{*}P < 0.05$ vs. LG alone. $^{**}P < 0.05$ vs. HG alone.

these findings, we speculated a prenylated protein, most likely the γ-subunit(s) of trimeric G proteins, in the regulation of the above signaling cascade. Herein, we examined if a Ptx-sensitive trimeric G protein is involved in glucose-induced ROS generation. Data shown in Fig. 5 demonstrated marked attenuation of glucose-induced ROS generation in INS 832/13 cells (*A*) and normal rat islets (*B*) treated with Ptx.

DISCUSSION

The overall objective of the present study was to determine potential mechanisms underlying nutrient-induced generation of ROS in isolated β-cells. Salient features of our studies are as follows: *1*) glucose and mitochondrial fuels, but not membrane-depolarizing KCl, increase ROS generation significantly; *2*) an increase in ROS seen under these conditions is derived from Nox, since pharmacological or molecular biological inhibition of Nox inhibited ROS generation; *3*) such a regulatory effect of glucose requires the activation of farnesylated as well as geranylgeranylated proteins; *4*) MPA, but not rapamycin or cyclosporine A, completely inhibits glucose-induced ROS generation, implying that endogenous GTP is necessary for such an effect; and *5*) inactivation of Ptx-mediated ADP ribosylation of an inhibitory G protein(s) markedly attenuates glucose-induced ROS generation. Taken together, our findings provide insights into potential G protein-mediated regulation of ROS in the islet β-cells under conditions in which they regulate physiological insulin secretion.

Nox is a highly regulated membrane-associated protein complex that facilitates the one electron reduction of oxygen to superoxide anion involving oxidation of cytosolic NADPH. The Nox holoenzyme is composed of membrane as well as cytosolic components. The membrane-associated catalytic core consists of $gp91^{phox}$, $p22^{phox}$, and the small G protein Rap1. The cytosolic regulatory components include $p47^{phox}$, $p67^{phox}$, $p40^{phox}$, and the small G protein Rac. Following 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-sensitive phosphorylation of $p47^{phox}$ leads to its translocation to the membrane fraction (3). It has also been shown that functional activation of Rac1 (Rac1.GTP) is vital for the holoenzyme assembly and activation of Nox in insulin-secreting cells (38, 39).

Along these lines, Oliveira et al. (30) provided a detailed description of localization, expression, and functional regulation of Nox within the islet. More recent pharmacological and molecular biological observations by Morgan and coworkers (27) have provided compelling evidence for a regulatory role for Nox in glucose-stimulated insulin secretion in rat islets under static incubation and perifusion

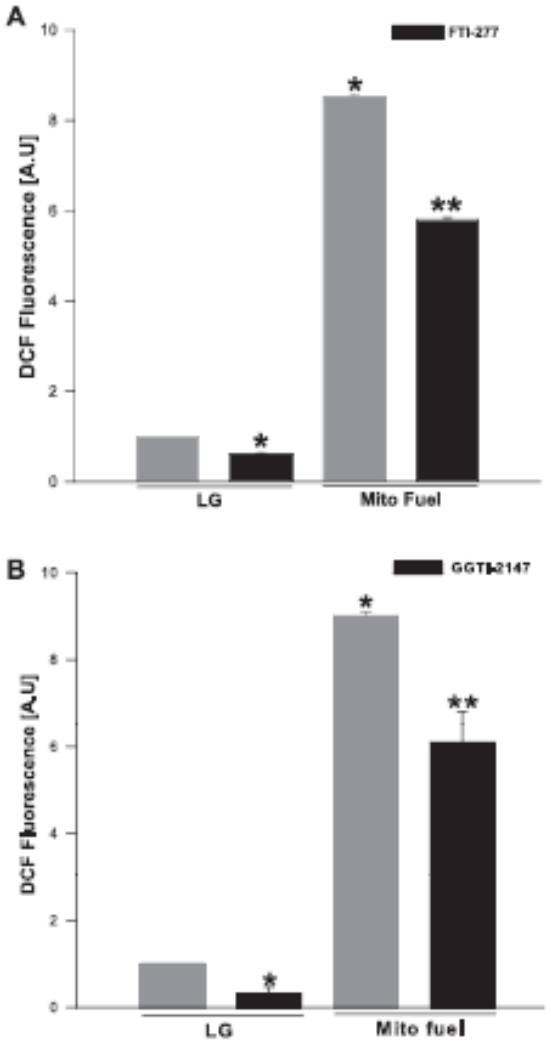

Fig. 3. Selective inhibitors of protein prenylation inhibit ROS generation induced by a mixture of mitochondrial (mito) fuels in INS 832/13 cells. INS 832/13 cells were incubated overnight in the presence or absence of FTI-277 (5 μM; *A*) and GGTI-2147 (10 μM; *B*), followed by stimulation with LG (2.5 mM) or a mixture of mito fuels [monomethyl succinate (MMS) = 20 mM and α-ketoisocaproic acid (KIC) = 5 mM] for 1 h in continuous presence or absence of inhibitors. ROS generated was quantified as DCF fluorescence and expressed as AU. Values are means ± SE from three independent experiments done in triplicates in each case. $*P < 0.05$ vs. glucose alone. $**P < 0.05$ vs. mito fuels alone.

conditions. Follow-up studies from this group have demonstrated key roles for Nox-derived ROS in palmitate-induced insulin secretion in the presence of submaximal concentration of glucose in islets (5). Under the above conditions, palmitate not only promoted translocation of $p47^{phox}$ to the membrane fraction, but also upregulated the protein content of $p47^{phox}$ and the mRNA levels of $p22^{phox}$, $gp91^{phox}$, $p47^{phox}$, proinsulin, and the G protein-coupled receptor 40. Essential role for Nox in palmitate-induced effects on β-cells was further strengthened by their observations to indicate a marked inhibition of fatty acid stimulation of insulin secretion in the presence of high-glucose concentration by inhibition of Nox activity. Based on these findings, it is evident that Nox plays key roles in islet function, including gene regulation and insulin secretion.

Table 1. *Depletion of endogenous GTP markedly attenuates glucose-induced ROS generation in INS 832/13 cells*

Condition	Degree of ROS Generation, fold over basal glucose
Low glucose	1.00
High glucose	1.58 ± 0.06†
Low glucose + mycophenolic acid	1.08 ± 0.02*
High glucose + mycophenolic acid	1.16 ± 0.04‡
Low glucose +cyclosporine A	1.06 ± 0.09*
High glucose + cyclosporine A	1.46 ± 0.14†
Low glucose + rapamycin	1.05 ± 0.08*
High glucose + rapamycin	1.42 ± 0.15†

Values are means ± SE from three independent experiments in each case. INS 832/13 cells were incubated with low glucose (2.5 mM) and low serum in the presence or absence of mycophenolic acid (10 μM), cyclosporine-A (5 μM), and rapamycin (100 nM) for 24 h. Following this, cells were stimulated either with low (2.5 mM) or high glucose (20 mM) for 1 h in the continuous presence and or absence of the inhibitors, as indicated. At the end of stimulation, cells were incubated with 2′,7′-dichlorofluorescein diacetate (10 μM) for 30 min and harvested for dichlorofluorescein fluorescence. ROS generated was quantified as dichlorofluorescein fluorescence and expressed as arbitrary units. *No significant difference vs. low glucose alone. †$P < 0.05$ vs. low glucose alone. ‡$P < 0.05$ vs. high glucose alone.

Our present observations also implicate roles for farnesylated and geranylgeranylated proteins in nutrient-induced Nox activation and associated ROS generation; the geranylated protein involved in nutrient-mediated activation of Nox might be Rac1, since it is one of the components of the Nox holoenzyme (39). Pharmacological (i.e., generic as well as more selective inhibitors of geranylgeranylation of Rac1), as well as molecular biological (i.e., dominant-negative mutants of prenyltransferases; Ref. 22) studies from our laboratory have clearly implicated Rac1 in islet function, including insulin secretion (17, 15, 41). The identity of the farnesylated protein, which is required for nutrient-induced ROS generation, remains to be determined. It is likely that it might represent the γ-subunit of a Ptx-sensitive G protein, since our laboratory has demonstrated earlier regulation of Ptx-sensitive G proteins by glucose in clonal β-cells, normal rat islets, and human islets (10–12, 14). Several earlier studies by Seaquist et al. (35), Robertson et

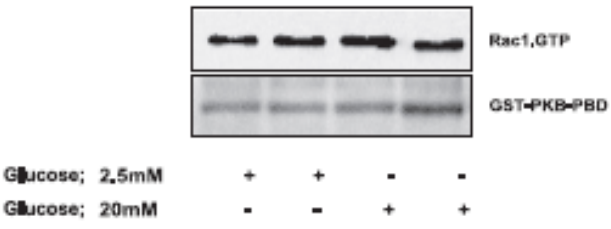

Fig. 4. Endogenous GTP levels are required for glucose-induced Rac1 activation and subsequent ROS generation in pancreatic β-cells. INS 832/13 cells were incubated overnight with either diluent or mycophenolic acid (MPA; 10 μM), followed by stimulation with either LG (5 mM) or HG (20 mM) for 30 min. The degree of Rac1 activation was determined by p21-activated kinase-binding domain (PAK-PBD) pull-down assay, as described in MATERIALS AND METHODS. A representative blot from two pull-down assays yielding similar data is depicted here.

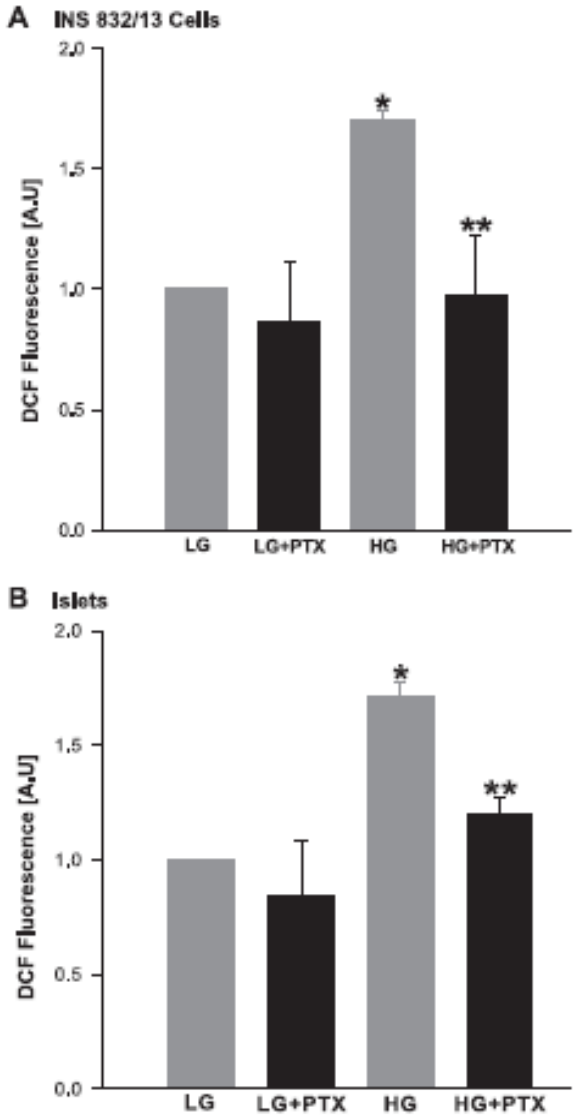

Fig. 5. Pertussis toxin (Ptx) pretreatment attenuates glucose-induced ROS generation in INS 832/13 cells or normal rat islets. Untreated or Ptx-treated (100 ng/ml) INS 832/13 cells (*A*) or normal rat islets (*B*) were stimulated with either LG (2.5 mM) or HG (20 mM) for 1 h. ROS generated was quantified as DCF fluorescence and expressed as AU. Values are means ± SE from three independent experiments done in triplicates (in INS 832/13 cell) and in duplicates (in islets) in each case. *$P < 0.05$ vs. LG alone. **$P < 0.05$ vs. HG alone.

al. (34), and Sharp (36) have provided evidence for the expression of inhibitory (e.g., G_i or G_o) class of Ptx-sensitive heterotrimeric proteins in the islet β-cell. Furthermore, studies from our laboratory (10) and those of Konrad and coworkers (7) have demonstrated functionally active heterotrimeric G proteins on the insulin granules in isolated β-cells. Lastly, using clonal β-cells, normal rat islets, and human islets, we have been able to demonstrate activation of the CML of γ-subunits by glucose; such effects of glucose were shown to be sensitive to Ptx, GTP, and extracellular calcium (14). Existing experimental evidence also implicates role(s) for trimeric G proteins, specifically the inhibitory G_i class of proteins in the regulation of NADPH-oxidase activity. For example, using human fat cells, Kreuzer and coworker's (19) demonstrate insulin-induced activation of NADPH-dependent H_2O_2 generation in human adipocyte plasma membranes is mediated by $G\alpha_{i2}$, which is regulated via ADP-ribosylation by Ptx. Additional studies are needed to conclusively determine the identity of this protein. However, based on our laboratory's recently published evidence (18), it is unlikely that the farnesylated protein is Ras, since inhibition of Ras (a farnesylated protein) had no effects on glucose-induced ERK1/2 phosphorylation, Rac1 activation, and insulin secretion.

Our findings also suggested that depletion of endogenous GTP by MPA results in a decreased activation of glucose-induced Rac1 and ROS generation. In this context, original studies by Metz and coworkers (24, 25) have documented permissive roles for endogenous GTP in physiological insulin secretion. MPA, which selectively inhibits GTP biosynthesis by inhibiting IMPDH, has been shown to inhibit GSIS and mastoparan-induced insulin secretion (24, 37). Even though inhibition of G protein activation was speculated to be one of the underlying mechanisms in the inhibition of insulin secretion following GTP depletion by MPA, very little information is available to substantiate that speculation. In this context, our laboratory has described earlier the inability of glucose to increase the CML (and activation) of small G proteins in GTP-depleted cells (13). The present studies identify Rac1 as one of the target proteins for glucose-mediated, endogenous GTP-dependent effects in β-cells. Our present findings are also in agreement with observations of Krotz et al. (19a), demonstrating inhibition of endothelial Nox by MPA via a Rac1-dependent mechanism.

Perspectives and Significance

Our studies provide the first evidence to suggest that prenylation-dependent, Nox-mediated generation of ROS is necessary for nutrient-induced insulin secretion in the pancreatic β-cell. Our findings also suggested that IMPDH-derived generation of GTP is necessary for glucose-induced ROS generation and subsequent activation of Rac1 in insulin-secreting cells. Data accrued in studies involving Ptx suggested that glucose-induced Nox activation and ROS generation are under the fine control of a Ptx-sensitive G protein. Potential identity of the prenylated protein whose activation appears to be required for glucose-induced ROS generation (present study) and ERK1/2 phosphorylation, Rac1 activation, and insulin secretion (18) remains to be elucidated.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Appendix B

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Tiam1/Rac1 signaling pathway mediates palmitate-induced, ceramide-sensitive generation of superoxides and lipid peroxides and the loss of mitochondrial membrane potential in pancreatic β-cells☆

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ABSTRACT

The phagocytic NADPH oxidase [NOX] has been implicated in the generation of superoxides in the pancreatic β-cell. Herein, using normal rat islets and clonal INS 832/13 cells, we tested the hypothesis that activation of the small G-protein Rac1, which is a member of the NOX holoenzyme, is necessary for palmitate [PA]-induced generation of superoxides in pancreatic β-cells. Incubation of isolated β-cells with PA potently increased the NOX activity culminating in a significant increase in the generation of superoxides and lipid peroxides in these cells; such effects of PA were attenuated by diphenyleneiodonium [DPI], a known inhibitor of NOX. In addition, PA caused a transient, but significant activation [i.e., GTP-bound form] of Rac1 in these cells. NSC23766, a selective inhibitor of Rac1, but not Cdc42 or Rho activation, inhibited Rac1 activation and the generation of superoxides and lipid peroxides induced by PA. Fumonisin B-1 [FB-1], which inhibits *de novo* synthesis of ceramide [CER] from PA, also attenuated PA-induced superoxide and lipid peroxide generation and NOX activity implicating intracellularly generated CER in the metabolic effects of PA; such effects were also demonstrable in the presence of the cell-permeable C2-CER. Further, NSC23766 prevented C2-CER-induced Rac1 activation and production of superoxides and lipid peroxides. Lastly, C2-CER, but not its inactive analogue, significantly reduced the mitochondrial membrane potential, which was prevented to a large degree by NSC23766. Together, our findings suggest that Tiam1/Rac1 signaling pathway regulates PA-induced, CER-dependent superoxide generation and mitochondrial dysfunction in pancreatic β-cells.

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1. Introduction

Several lines of evidence from multiple laboratories suggests that chronic exposure of isolated β-cells to elevated saturated fatty acids [e.g., palmitic acid; PA] leads to a significant metabolic dysregulation and eventual demise of the β-cell [1–3]. Multiple mechanisms have been put forth to explain PA-induced metabolic defects; one of these include generation of intracellular oxidative stress [e.g., reactive oxygen species; ROS; 4–6], albeit recent studies by Moore et al. [7] appear to argue against fatty acid-induced oxidative stress in the pancreatic β-cell. A signaling step involved in the increased generation of ROS and associated induction of intracellular oxidative stress in the pancreatic β-cell is the activation of the phagocytic NOX system, which 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 consisting of $gp91^{phox}$, $p22^{phox}$ and the small G-protein Rap1. The cytosolic regulatory components include $p47^{phox}$, $p67^{phox}$ and the small G-protein Rac1 [8–12]. Following stimulation, the cytosolic components of NADPH oxidase translocate to the membrane for association with the catalytic core for holoenzyme assembly. Available evidence also suggests that a protein kinase Cζ-sensitive phosphorylation of $p47^{phox}$ leads to its translocation to the

☆ Portions of this work were presented in the 69th Annual Meetings of the American Diabetes Association Meetings held in New Orleans, USA, in 2009 and the 2nd International Brussels Pancreatic islet Symposium held in Brussels, Belgium, in 2009.

Abbreviations: C2-CER, C2-Ceramide; DCHFDA, 2′,7′-dichlorodihydrofluorescein diacetate; DHC, Dihydroceramide; DPI, diphenyleneiodonium; FB-1, Fumonisin B-1; GEF, guanine nucleotide exchange factor; MDA, malondialdehyde; MMP, mitochondrial membrane potential; NBT, nitroblue tetrazolium; PA, palmitic acid; Rac1, Ras-related C3 botulinum toxin substrate 1; ROS, reactive oxygen species; Tiam1, T-lymphoma invasion and metastasis 1.

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membrane fraction [13]. It has also been shown that functional activation of Rac [i.e., GTP-Rac] is vital for the holoenzyme assembly and activation of NOX [14–16].

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 including elevated levels of glucose, saturated fatty acids and proinflammatory cytokines [6,17–19]. It has also been demonstrated that pharmacological inhibition of NOX by diphenyleneiodonium chloride [DPI] or anti-sense oligonucleotides for $p47^{phox}$ markedly attenuated glucose-induced ROS production and oxidative stress, suggesting a critical involvement of NOX in the metabolic dysfunction induced by glucose [20]. These data implicate a significant contributory role for NOX in the metabolic dysfunction of the β-cell under conditions of oxidative stress [21–23].

Despite the aforementioned compelling lines of evidence, very little has been studied with regards to the potential contributory roles of small G-proteins [e.g., Rac1] in the cascade of events leading to PA-induced NOX-mediated superoxides generation in β-cells. Based on this reasoning, we undertook the current investigation to test our overall hypothesis that PA induces generation of superoxides and lipid peroxides in INS 832/13 cells and rodent islets by increasing Rac1 activation, which represents one of the signaling events necessary for the functional regulation of the endogenous NOX holoenzyme assembly and its catalytic activity. Herein, we describe evidence to implicate NOX signaling pathway in the generation of superoxides and lipid peroxides in PA-mediated effects on isolated β-cells. We also present the first evidence to suggest a critical modulatory role for Tiam1, a guanine nucleotide exchange factor [GEF] for Rac1 [28], in this signaling pathway leading to the onset of mitochondrial dysfunction.

2. Materials and methods

2.1. Materials

C2-Ceramide, Dihydroceramide and NSC23766 were from Calbiochem [San Diego, CA]. Nitroblue tetrazolinium salt, malondialdehyde, thiobarbituric acid, diphenyleneiodonium chloride, butylated hydroxytoulene, oleic acid and palmitic acid were from Sigma [St. Louis, MO]. Antibodies directed against $p47^{phox}$ and actin were from Santa Cruz Biotechnology [Santa Cruz, CA]. Rac1 activation kit was purchased from Cytoskeleton Inc. [Denver, CO]. JC-1 assay kit was from Cell Technology Inc. [Mountain View, CA]. Palmitate stock solutions were prepared as we described in Ref. [24].

2.2. Insulin-secreting cells

INS 832/13 cells were provided by Dr. Chris Newgard [Duke University Medical Center, Durham, NC] and 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 μM 2-mercaptoethanol, and 10 mM HEPES [pH 7.4]. The medium was changed twice and cells were subcloned weekly. Islets were isolated from normal Sprague–Dawley rats using the collagenases digestion method described previously [24].

2.3. Quantitation of superoxide generation by nitroblue tetrazolium [NBT] assay

INS 832/13 cells were plated in six-well plates and grown to subconfluency and then treated with PA [100 μM], C2-CER [30 μM], FB-1 [10 μM], DPI [5 μM] or NSC23766 [20 μM] in different combinations as described in the text. The medium was then removed and the cells were washed once with PBS and further incubated with 0.25% NBT for 30 min at 37 °C. Cells were then harvested and pelleted by low-speed centrifugation. The resulting pellet was resuspended in 50% acetic acid. The reduced NBT formazan product was quantified by measuring the absorbance at 510 nm using Beckman DU640 spectrophotometer.

2.4. Quantitation of superoxide generation by malondialdehyde [MDA] assay

INS 832/13 cell lysates derived from control or treated cells [100 μg protein] were treated with 10% trichloro acetic acid, 2% butylated hydroxytoulene, and freshly prepared 0.67% thiobarbituric acid. Following this, the samples were boiled for 15–20 min and then allowed to cool down at 4–8 °C for 15–20 min. The samples were then gently vortexed and centrifuged at 3500 rpm for 15 min. The resulting supernatant was used to measure the absorbance at 532 nm. A standard concentration curve was used to extrapolate MDA generated from various samples.

2.5. NOX assay

INS 832/13 cells were plated in six-well plates, grown to subconfluency and then treated with either diluent or PA [100 μM] or C2-CER [30 μM] for 6 h. After treatment the medium was removed and the cells were washed once with PBS and further incubated with 5 μM of 2′,7′-dichlorodihydrofluorescein diacetate [DCHFDA] for 30 min at 37 °C. Cells were then harvested and pelleted by low-speed centrifugation and the protein content of the pellet was determined using Bradford's assay. Following to this, equal amount of proteins were taken and fluorescence in each condition was recorded [excitation – 485 nm and emission – 530 nm]. The amount of fluorescence recorded is directly correlated to the amount of superoxide radicals generated due to NOX activity.

2.6. Rac1 activation assay

INS 832/13 cells were treated with either diluent or NSC23766 [20 μM] or C2-CER or PA or oleate. Before treatment, cells were incubated overnight with NSC23766 in a low serum–low glucose containing medium. Cells were further incubated with PA or C2-CER as indicated in the text in the continuous presence of either NSC23766 or diluent. Lysates [~500 μg protein] were clarified by centrifugation for 5 min at 4800 × g, and PAK-PBD [p21-activated kinase-binding domain] beads [20 μl] were added to the supernatant. The mixture was then rotated for 1 h at 4 °C and pelleted by centrifugation at 4000 × g for 3 min. The resulting pellet was washed once with lysis buffer followed by a rinse [3 ×] in wash buffer [25 mM Tris, pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl, and 150 mM EDTA]. Proteins in the pellet were resolved by SDS-PAGE and transferred onto a nitrocellulose membrane, and Western blotting method determined the relative abundance of activated Rac1.

2.7. Assessment of mitochondrial dysfunction by JC-1 assay

Loss of mitochondrial membrane potential in cells has been estimated using JC-1 assay kit. Briefly, INS 832/13 cells were grown at 80% confluency on the cover slips and were incubated with and without NSC23766 [20 μM] overnight in low serum–low glucose media. Cells were then treated with C2-CER [30 μM] or DHC [30 μM] for 6 h with or without NSC23766. At the end of incubation, cells were washed twice with assay buffer (provided

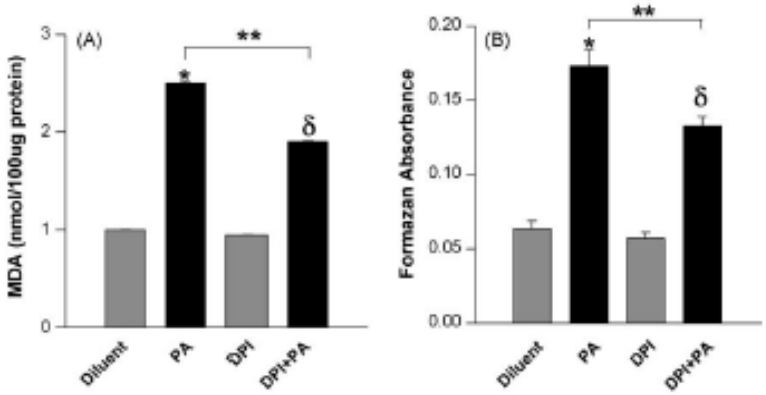

Fig. 1. PA induces generation of lipid peroxides and superoxides in INS 832/13 cells: protection by DPI. INS 832/13 cells were incubated [6 h] with either diluent or PA [100 μM] and/or DPI [5 μM] as indicated in the figure. Lipid hydroperoxide levels were measured as MDA equivalents [Panel A] and superoxide levels [Panel B] were quantitated as formazan equivalents. Data are mean ± SEM from three independent determinations. Values were considered significant at $p < 0.05$. *Significant effect of PA to diluent. $^{\delta}$Significance between DPI and DPI + PA. **Significance between PA and DPI + PA.

with the kit) and were further incubated for 15 min with JC-1 dye [1×]. Cells were then washed twice with assay buffer and the cover slips were mounted onto a glass slide and images were taken at 40× magnification using Olympus IX71 microscope [Olympus America Inc., Center Valley, PA].

2.8. Other assays

Protein concentrations were determined by Bradford's dye-binding method using bovine serum albumin as the standard. Statistical significance of differences between diluent and experi-

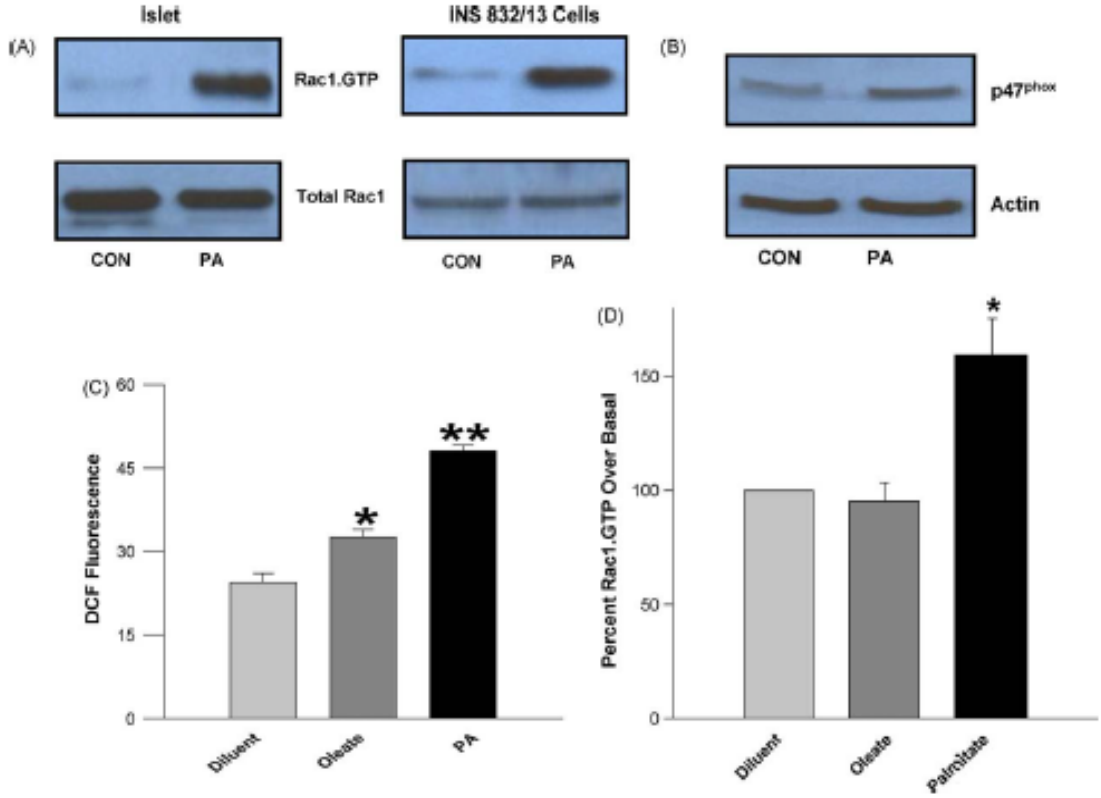

Fig. 2. PA, but not oleate, induces Rac1 activation and NOX activation in β-cells. Normal rat islets and INS 832/13 cells were treated with diluent or PA [100 μM; Panel A]. The relative amounts of activated Rac1 [i.e., Rac1-GTP] were determined from these lysates by PAK-PBD pull down assay. Data are representative of two independent experiments. Panel B: lysates derived from INS 832/13 cells treated in the absence or presence of PA [100 μM] were separated by SDS-page, and probed for $p47^{phox}$ and actin expression. A representative blot from two independent experiments is shown here. Panel C: lysates derived from INS 832/13 cells treated in the absence or presence of PA or oleate [100 μM each] were processed for NOX activity and were quantitated by the DCHFDA assay and are expressed as DCF fluorescence units. Data are mean ± SEM from two individual measurements for DCF fluorescence. *,**$p < 0.05$ vs. diluent. Panel D: INS 832/13 cells were treated with diluent and/or oleate [100 μM] or PA [100 μM] and the relative amounts of activated Rac1 were determined by PAK-PBD pull down assay. Data presented in here are densitometric analysis of the blots and are mean ± SEM from four independent experiments. *$p < 0.05$ vs. diluent.

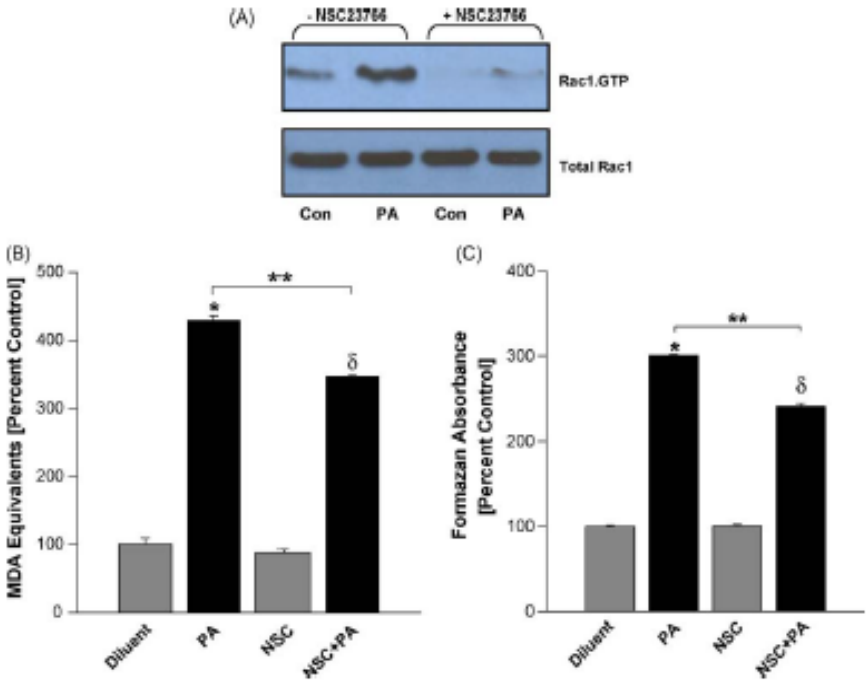

Fig. 3. NSC23766, a specific inhibitor of Tiam1-mediated activation of Rac1, markedly attenuates PA-induced Rac1 activation in INS 832/13 cells. INS 832/13 cells were incubated overnight with either diluent or NSC23766 [20 μM]. The cells were further incubated [3 h] in the presence of either low glucose [5 mM] or PA [100 μM] in the continuous presence of NSC23766 or diluent. The degree of Rac1 activation was determined by PAK-PBD pulldown assay. Panel A: data are representative of two independent experiments. Levels of lipid hydroperoxides [Panel B] or ROS [Panel C] generated in PA or diluent-treated INS 832/13 cells in the absence or presence were measured as MDA equivalents or formazan equivalents, respectively. Data are mean ± SEM from three determinations. Values were considered significant at $p < 0.05$. *Significant effect of PA to diluent. $^{\delta}$Significance between NSC and NSC + PA. **Significance between PA and NSC + PA.

mental groups was determined by Student's *t*-test and ANOVA analysis. $p < 0.05$ was considered significant.

3. Results

3.1. PA induces generation of lipid peroxides and ROS in insulin-secreting cells

At the outset, we determined if exposure of isolated β-cells to PA results in the generation of superoxides and lipid peroxides. Data shown in Fig. 1 suggest that incubation of INS 832/13 cells with PA [100 μM; 6 h] significantly increased lipid peroxide levels [~2.5-fold; expressed as MDA equivalents; Panel A] and ROS levels [~2.7-fold; Panel B]. Furthermore, coprovision of DPI, a known inhibitor of NOX attenuated the PA-induced lipid peroxide levels [~37%] and ROS generation [~31%]. Together, these data suggest that PA-mediated generation of lipid peroxides and ROS in isolated β-cells may, in part, be due to activation of NOX.

3.2. PA induces activation of NOX in pancreatic β-cells

Data described above prompted us to further investigate potential regulation of NOX activity by PA in insulin-secreting cells. As stated above, $p47^{phox}$ represents one of the subunits of the NOX holoenzyme which is subjected to regulation in cells under the duress of oxidative stress. It has been shown that small G-protein Rac1, also a member of the NOX assembly, is also activated under conditions of oxidative stress leading to activation of NOX activity. Data described in Fig. 2 suggested that incubation of normal rat islets [Panel A; left] or INS 832/13 cells [Panel A; right] with PA significantly increased the activation [i.e., GTP-bound form] of Rac1 as determined by the PAK-pulldown assay [see Section 2 for additional details]. In addition, we observed a marked increase in the expression of $p47^{phox}$ in these cells following exposure to PA [Fig. 2; Panel B]. Together, data in Panels A and B suggest upregulation of expression and function of key components of NOX holoenzyme in cells exposed to PA. We next

Table 1
Time-dependent effects of PA on NOX activity in INS 832/13 cells: potential roles for ceramide.

Treatment	Time (h)				
	0	3	6	12	24
PA	100 ± 3.11$^{\delta}$	157 ± 5.73$^{*\delta}$	145 ± 2.99*	72 ± 1.41$^{*\delta}$	62 ± 5.06$^{*\delta}$
PA + FB1	109 ± 2.27$^{\delta}$	156 ± 3.70$^{\delta}$	113 ± 1.84†	65 ± 4.64$^{\delta}$	60 ± 9.02$^{\delta}$

INS 832/13 cells were treated with PA [100 μM] in the absence or presence of FB-1 [10 μM] for different time intervals as indicated. NOX activity was quantitated by the DCHFDA assay. Data are expressed as DCF fluorescence units and are mean ± SEM from three determinations.

$^{\delta}$ No significant difference between the two treatment groups.

* $p < 0.05$ between PA induced ROS *vs.* control (0 h).

† $p < 0.05$ between PA induced ROS in the presence of FB1 *vs.* PA alone.

quantitated the NOX activity to determine if PA-induced activation of Rac1 [Panel A] and $p47^{phox}$ expression [Panel B] culminates in the functional activation of the enzyme. Indeed, findings described in Fig. 2 [Panel C] suggested a significant increase [~97%] in the catalytic activation of NOX in cells treated with PA. It should be noted that under these conditions, oleate exerted a modest effect on the NOX activity [Fig. 2; Panel C] without significantly affecting Rac1 activation in INS 832/13 cells [Fig. 2; Panel D]. Together, our findings suggest that PA, but not oleate, elicits stimulatory effects on Rac1 activation and NOX activity.

3.3. Tiam1, a GEF for Rac1, is involved in PA-induced Rac1 activation and generation of superoxides and lipid peroxides in pancreatic β-cells

It has been demonstrated in many cells types, and more recently in pancreatic β-cells, that Rac1 activation is mediated by GEFs, such as Tiam1 [25,26]. Recent studies from our laboratory have provided immunological evidence for Tiam1 in insulin-secreting cells, and further indicated that NSC23766, a specific inhibitor of Tiam1, specifically inhibits GTP-loading onto Rac1, but not Cdc42 and Rho [25]. Therefore, we investigated if pre-treatment of isolated β-cells to NSC23766 prevents PA-induced Rac1 activation and associated increase in the generation of superoxides and lipid peroxides. Data shown in Fig. 3 [Panel A] demonstrated a near complete inhibition of PA-induced Rac1 activation by NSC23766 suggesting potential requirement for Tiam1 in PA-induced Rac1 activation. Furthermore, we observed that PA-induced generation of lipid peroxides [Panel B] and reactive oxygen species [Panel C] in INS 832/13 cells was also reduced [~20–30%] following inhibition of Tiam1-mediated activation of Rac1. Together, these data implicate a novel regulatory role(s) for Tiam1/Rac1 signaling step(s) in PA-mediated generation of superoxides and lipid peroxides in isolated β-cells.

3.4. PA-induced generation of lipid peroxides and superoxides may, in part, be due to intracellular generation of CER via the de novo pathway

Since PA is the precursor for the *de novo* biosynthesis of CER, in the next series of studies we investigated potential roles of intracellularly generated CER in aforementioned PA-induced

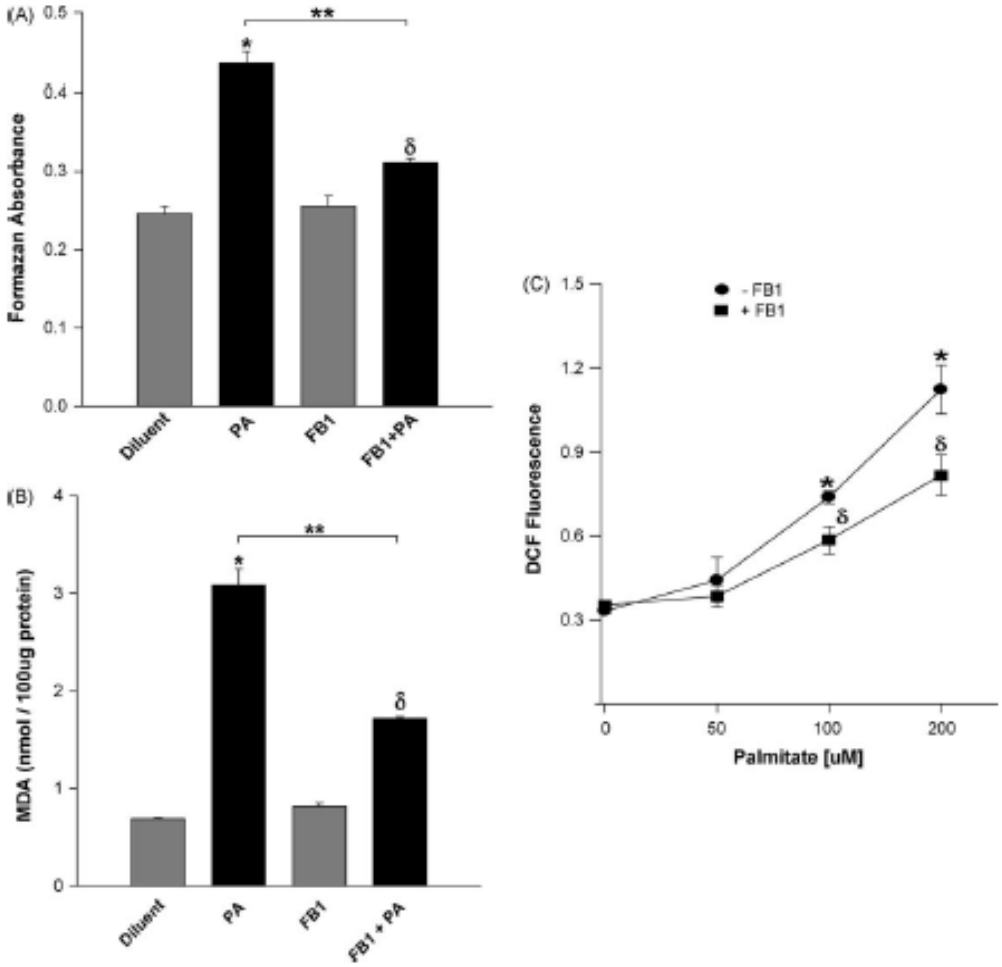

Fig. 4. Fumonisin B-1, an inhibitor of *de novo* biosynthesis of CER from PA, markedly reduces PA-induced generation of lipid peroxides and superoxides in INS 832/13 cells. INS 832/13 cells were pre-treated in the presence or absence of FB-1 [10 μM] prior to the addition of PA [100 μM] and lysates derived from these cells were assessed for generation of superoxides and lipid peroxides. Superoxide generation was quantitated by NBT method and expressed as formazan equivalents [Panel A]. Lipid peroxide levels were quantitated by the MDA assay, and expressed as nanomoles of MDA formed/100 μg protein [Panel B]. Data are mean ± SEM from three determinations. Values were considered significant at $p < 0.05$. *Significant effect of PA to diluent. $^{\delta}$Significance between FB1 and FB1 + PA. **Significance between PA and FB1 + PA. Furthermore, cells were pre-treated in the presence or absence of FB1 [10 μM] prior to the addition of PA at different concentrations [0–200 μM]. Lysates derived were processed for NOX activity and were quantitated by the DCHFDA assay [Panel C] and are expressed as DCF fluorescence. Data are mean ± SEM from three determinations. Graph with different symbols is statistically significant at $p < 0.001$. *PA-induced ROS *vs.* diluent. $^{\delta}$PA + FB1 induced ROS *vs.* FB1.

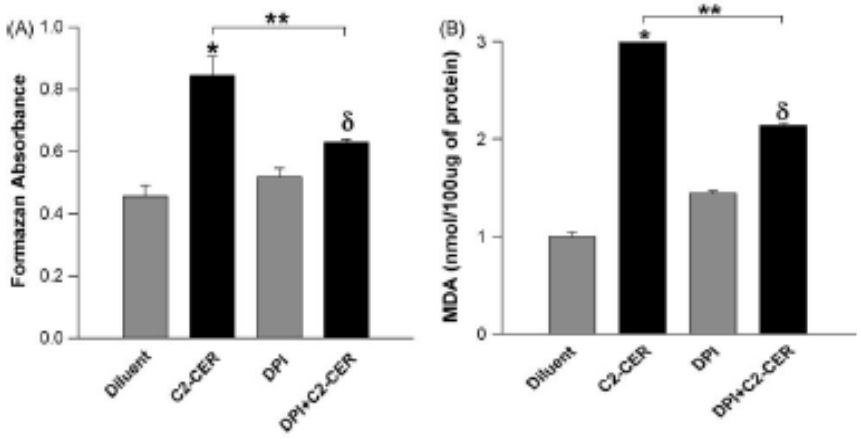

Fig. 5. C2-CER promotes generation of lipid peroxides and ROS in INS 832/13 cells by activating endogenous NADPH oxidase activity. INS 832/13 cells were treated with either diluent or C2-CER [30 μM] and/or DPI [5 μM] in various combinations as indicated in the figure. The degree of ROS generation was quantitated by the NBT method and is expressed as formazan equivalents [Panel A]. The amount of lipid hydroperoxide generation was quantitated by the MDA assay and is expressed as MDA equivalents [Panel B]. Data are mean ± SEM from three determinations in each case. Values were considered significant at $p < 0.05$. *Significant effect of C2-CER *vs.* diluent. $^{\delta}$Significance between DPI and DPI + C2-CER. **Significance between C2-CER and DPI + C2-CER.

effects on isolated β-cells. To address this, we quantitated PA-induced generation of reactive oxygen species and lipid peroxides in cells pre-treated in the absence or presence of FB-1, a known inhibitor of *de novo* biosynthesis of CER from PA [28], incubation of isolated β-cells with 100 μM PA in presence of FB-1 significantly reduced PA-induced generation of ROS [~72%; Panel A] and lipid peroxides [~62%; Panel B] without significantly affecting these parameters in cells incubated with the diluent.

We next quantitated PA-induced effects on NOX activity as a function of period of incubation and the concentration of PA. Data in Table 1 indicated that PA elicited significant stimulatory effects on NOX activity. Maximal effects were seen between 3 and 6 h of incubation. Interestingly, PA effects were not seen beyond 6 h time point as the NOX activity fell even below the control values. In addition, pre-incubation of these cells with FB-1 resulted in a significant inhibition in NOX activity at 6 h time point suggesting potential regulation of NOX activity by intracellularly generated CER [Table 1]. We next quantitated NOX activity in these cells as a function of PA concentration [0–200 μM] in the absence or presence of FB-1. Data in Fig. 4 [Panel C] suggested a concentration-dependent activation of NOX by PA. Further, we noticed a significant inhibition of PA-induced NOX activity by FB-1. Together, these data suggest that PA-induced effects on lipid and superoxide levels and NOX activity may, in part, be due to the intracellularly generated CER.

3.5. A cell-permeable analog of CER mimics PA effects in isolated β-cells

We next investigated if coprovision of a cell-permeable CER [e.g., C2-CER] mimics PA-induced oxidative stress in INS 832/13 cells, and if such an increase is mediated *via* activation of endogenous NOX. To address this, INS 832/13 cells were incubated with diluent or C2-CER, which has been effectively used to determine CER-induced metabolic dysfunction in isolated β-cells [27,28] in the absence or presence of DPI to inhibit endogenous NOX. Data described in Fig. 5 showed a marked reduction in C2-CER-induced ROS levels [~71%; Panel A] or lipid peroxides [~69%; Panel B] in cells exposed to DPI. It should be noted that DPI exerted a modest increase in the generation of lipid peroxides in the absence of C2-CER without significantly affecting the basal superoxide generation [Panels A and B; lanes 1 *vs.* 3]. Taken together, these findings implicate NOX activity in C2-CER-induced generation of ROS and lipid peroxides in pancreatic β-cells.

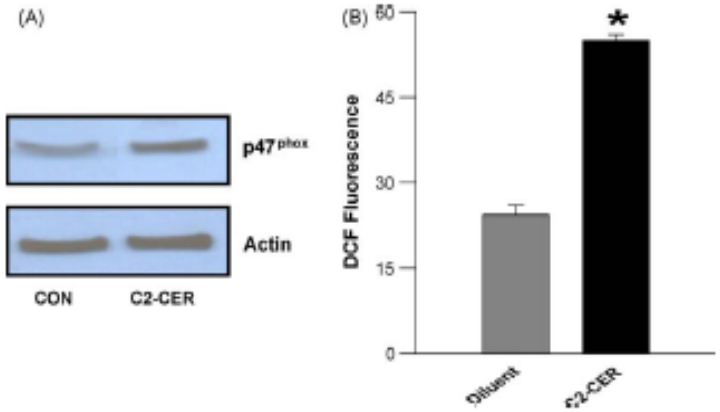

Fig. 6. C2-CER increases the expression of $p47^{phox}$ and NOX activity in INS 832/13 cells. INS 832/13 cells were treated with either diluent or C2-CER [30 μM] and examined for relative increases in $p47^{phox}$ expression and NADPH oxidase activity. Panel A: lysate proteins derived from diluent or C2-CER-treated cells were separated by SDS-page and probed for $p47^{phox}$ and actin expression. A representative blot from two independent experiments is shown here. Panel B: NOX activity in diluent or C2-CER-treated cells was quantitated by the DCHFDA fluorescence assay and is expressed as DCF fluorescence. Data are mean ± SEM from two independent determinations. *$p < 0.05$ *vs.* diluent.

3.6. C2-CER mimics PA effects in inducing $p47^{phox}$ expression and NOX activity in isolated β-cells

As a logical extension to the studies described in Fig. 5, we examined if C2-CER induces $p47^{phox}$ expression and NOX activity in pancreatic β-cells. Data in Fig. 6 [Panel A] show that incubation of INS 832/13 cells with C2-CER significantly increased $p47^{phox}$ expression. Moreover, in a manner akin to PA, C2-CER increased [more than 2-fold] the NOX activity in INS 832/13 cells [Fig. 6; Panel B]. Together, these data in Figs. 5 and 6 demonstrate that a cell-permeable analog of C2-CER mimics the effects of PA on isolated β-cells by increasing the NOX activity.

3.7. C2-CER-induced generation of superoxides and lipid peroxides is mediated by the Tiam1/Rac1 signaling pathway

Herein, we examined the possible involvement of Tiam1/Rac1 signaling cascade in C2-CER-induced oxidative stress in β-cells. Data shown in Fig. 7 suggested a significant activation of Rac1 by C2-CER in INS 832/13 cells [Panel A; left] and normal rat islets [Panel A; right]. Moreover, coprovision of NSC23766 substantially inhibited C2-CER-induced Rac1 activation in both cell types. These data clearly suggest that C2-CER-induced effects on isolated β-cells may, in part, be due to activation of a Rac1-dependent signaling mechanism. Furthermore, we noticed that C2-CER-induced generation of lipid peroxides [Fig. 7; Panel B] or superoxides [Fig. 7; Panel C] was reduced [~27–60%] by NSC23766, thus suggesting novel regulation of CER-mediated effects by a Tiam1/Rac1-dependent signaling mechanism [see below].

3.8. C2-CER, but not its inactive analogue, promotes mitochondrial dysfunction in INS 832/13 cells in a Tiam1/Rac1 signaling pathway

We have recently reported that exposure of isolated β-cells to C2-CER results in significant abnormalities in mitochondrial function including loss in membrane potential and leakage of cytochrome-C into the cytosolic compartment [28]. Therefore, in the last set of experiments we verified if Tiam1/Rac1 signaling step might underlie the CER-induced mitochondrial dysfunction in INS 832/13 cells. To address this, mitochondrial membrane potential [MMP] was quantitated by the JC-1 staining method in cells

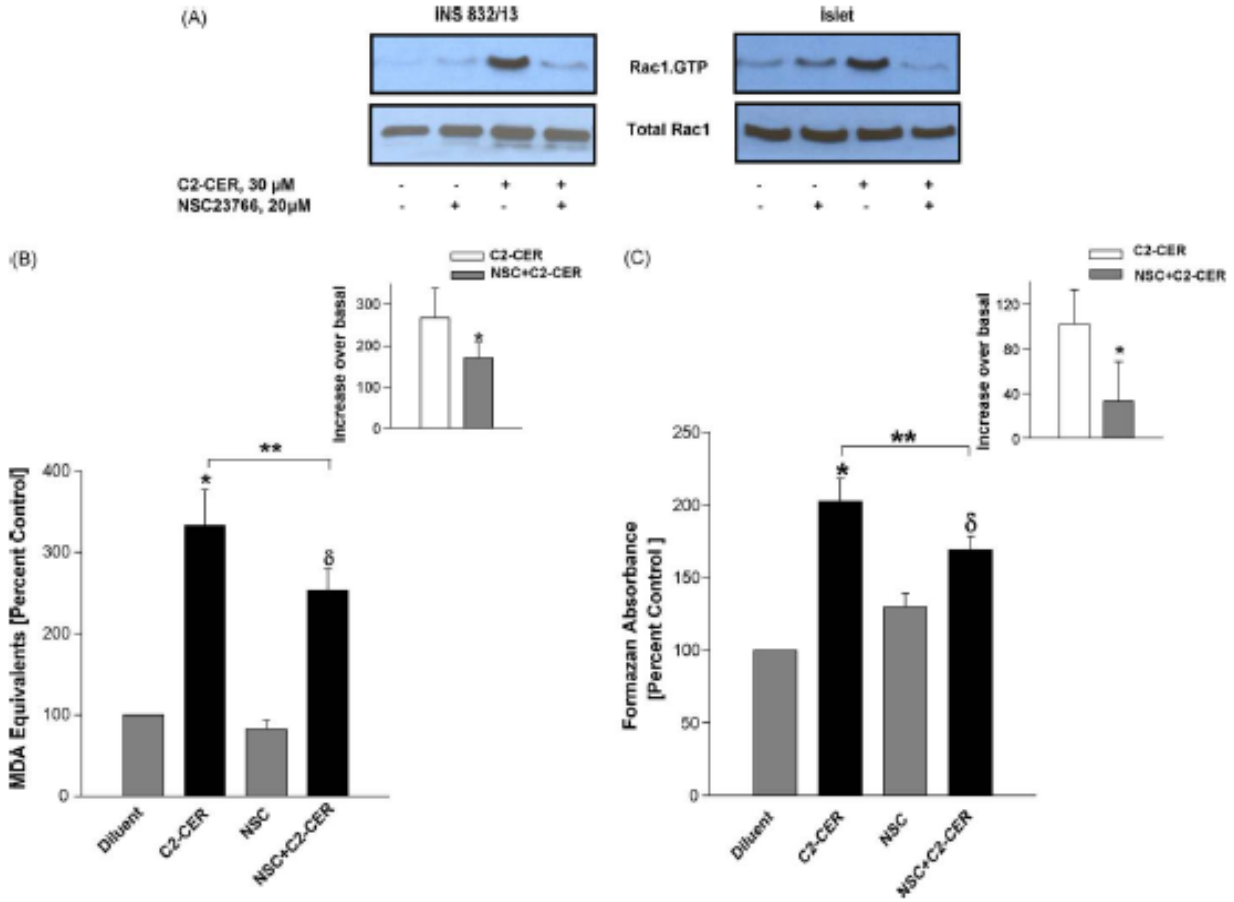

Fig. 7. NSC23766 inhibits C2-CER-induced Rac1 activation and generation of lipid peroxides and superoxides in pancreatic β-cells. INS 832/13 cells and rat islet were treated with either diluent or NSC23766 [20 μM] and cultured overnight in low glucose/low serum media. Cells were further incubated in the presence of C2-CER [30 μM] for 30 min in INS 832/13 cells and 3 h in islet in the continuous presence of NSC23766 or diluent. The relative amounts of activated Rac1 [i.e., Rac1-GTP] were determined by PAK-PBD pull down assay. Data are representative of two independent experiments [Panel A]. Panel B: INS 832/13 cells were incubated [6 h] with either diluent or with C2-CER [30 μM] or NSC23766 [20 μM; alone or in combination]. Lipid hydroperoxides were measured as MDA equivalents and plotted as increase over basal. Panel C: INS 832/13 cells were incubated [6 h] with either diluent or with C2-CER [30 μM] or NSC23766 [20 μM; alone or in combination as indicated in the figure]. Superoxide generation was measured as formazan equivalents and plotted as increase over basal. Data in the insets represent incremental response to C2-CER in the absence or presence of NSC23766. Data are mean ± SEM from three determinations in each case. Values were considered significant at $p < 0.05$. *Significant effect of C2-CER vs. diluent. $^{\delta}$Significance between NSC and NSC + C2-CER. **Significance between C2-CER and NSC + C2-CER.

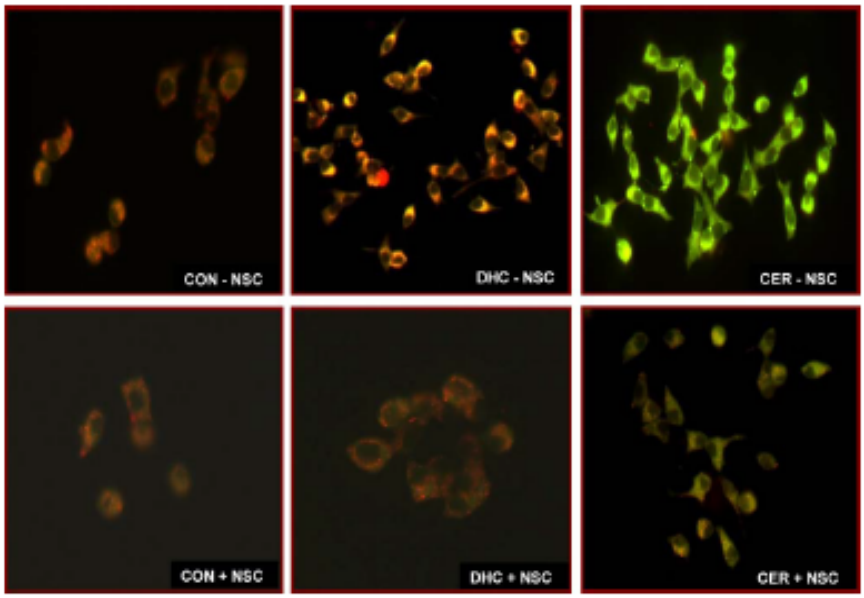

Fig. 8. NSC23766 inhibits C2-CER-induced mitochondrial dysfunction in pancreatic β-cells: INS 832/13 cells were treated with either diluent or NSC23766 [20 μM] and cultured overnight in low glucose and low serum media. Cells were further incubated in the presence of C2-CER [30 μM] and/or DHC [30 μM] for 6 h in the continuous presence of NSC23766 or diluent. Mitochondrial dysfunction was determined by JC-1 assay. Data are representative of two independent experiments.

exposed to diluent or C2-CER in the absence or presence of NSC23766. To determine the specificity of CER effects, we also utilized Dihydroceramide [DHC], an inactive analogue of CER, on MMP in INS 832/13 cells. Data in Fig. 8 indicated that exposure of these cells to C2-CER [lower left panel], but not DHC [middle left panel] significantly lowered the MMP as evidenced by staining of the majority of cells in green due to reduced MMP. Furthermore, NSC23766 prevented C2-CER-induced loss in membrane potential [as evidenced by a strong J-aggregation; red color] in these cells, further supporting the hypothesis that Tiam1/Rac1 signaling pathway contributes to CER-induced metabolic dysfunction in the pancreatic β-cell.

4. Discussion

One of the main objectives of this study was to test the hypothesis that activation of the Tiam1/Rac1 signaling pathway is necessary for PA-mediated generation of superoxides and lipid peroxides in isolated β-cells. The salient findings of our study are: [i] exposure of isolated β-cells to PA leads to the generation of reactive oxygen species and lipid peroxides, which may, in part, be due to increased NOX activity; [ii] PA-induced effects on NOX activity are largely due to intracellularly generated CER from the *de novo* biosynthetic pathway; [iii] PA-induced, CER-mediated activation of NOX and the resultant increase in intracellular oxidative stress require activation of Rac1; [iv] PA-induced, CER-sensitive activation of Rac1 requires the intermediacy of Tiam1, a GEF for Rac1; [v] inhibition of Tiam1/Rac1 signaling pathway leads to restoration of mitochondrial membrane potential. Together, our data provide the first evidence for Tiam1/Rac1 signaling pathway in PA-induced, CER-mediated increase in the oxidative environment in INS 832/13 cells and normal rodent islets.

As stated above, our current findings implicate the involvement of Tiam1 in PA- or C2-CER-induced activation of Rac1. In the context of potential regulation of Rac1, multiple GEFs have been identified in other cell types. These constitute the diffuse B cell lymphoma [Dbl] family of GEFs, including Trio and Tiam1. Recently, Zheng and co-workers have developed NSC23766, which is a soluble first generation small molecule inhibitor of Tiam1-mediated activation of Rac1 [29]. These investigators have reported significant inhibition of Rac1-GTP-loading by NSC23766 without significantly affecting the GTP-loading onto other small G-proteins including Cdc42 and Rho A. Under these conditions, NSC23766 also attenuated cell proliferation induced by Tiam1, which is a Rac1-specific GEF. Based on these data, Zheng and co-workers concluded that NSC23766 represents a specific inhibitor of Tiam1-mediated activation of Rac1. Several other laboratories have utilized NSC23766 since then to decipher the potential contributory roles for Tiam1/Rac1 signaling pathway in cellular functions [25,30 and references therein]. Recently, we have confirmed the selectivity of NSC23766-mediated inhibition of Rac1 activation in insulin-secreting cells [25]. In the present study, we demonstrated that NSC23766 not only attenuated PA or C2-CER-induced Rac1 activation, but also markedly reduced PA or C2-CER-induced generation of superoxides and lipid peroxides, implicating novel regulatory roles for Tiam1/Rac1 signaling pathway in the activation of phagocytic-like NOX in β-cells. Using molecular biological approaches Yi et al. [31] have recently demonstrated roles of Vav2, another GEF for Rac1, in homocysteine-induced Rac1/NOX activation in mesangial cells.

Several recent studies have demonstrated regulatory roles of Rac1 in high glucose-induced metabolic dysregulation and cell death. For example, Shen et al. [30] have recently reported a significant increase in cardiomyocyte apoptosis under hyperglycemic conditions. Using cultured myocytes, these investigators demonstrated a significant upregulation of Rac1 and NOX activity which was attenuated in cells overexpressing a dominant negative mutant of Rac1. Moreover, treatment of diabetic animals with NSC23766 significantly reduced NOX activity and cell demise followed by restoration of myocardial function [30]. These findings further support the involvement of Tiam1/Rac1 signaling pathway in hyperglycemia-induced metabolic dysfunction and demise of myocytes. It may be germane to point out that unpublished observations from our laboratory have suggested similar regulatory roles of Rac1 in high glucose-induced activation of NOX activation and the associated increase in oxidative stress in INS

832/13 cells and normal rat islets [Syed and Kowluru, unpublished].

Along these lines, studies by Cacicedo et al. in cultured retinal pericytes have demonstrated a role for NOX in PA-induced apoptosis [32]. A significant increase in NOX activity, oxidative stress and caspase-3 activity was demonstrable in cells exposed to PA. Overexpression of dominant negative mutants of $p67^{phox}$ and Rac1 [N17Rac1] markedly inhibited the increase in caspase-3 activation. Furthermore, overexpression of an active mutant of Rac1 [V12Rac1] increased caspase-3 activity suggesting that constitutive activation of Rac1 results in NOX activation culminating in the generation of oxidative stress and metabolic dysfunction in these cells.

Using FB-1, a specific inhibitor of *de novo* synthesis of CER from PA, we have demonstrated that PA-induced effects may, in part, be due to intracellularly generated CER. Data accrued in studies using C2-CER further support this postulation. Published evidence along these lines suggests that CER-mediated effects are indeed mediated *via* activation of Rac1 in many cell types. For example, using C2-CER, Kim and Kim have reported activation of c-fos serum response element *via* the Rac1 signaling pathway in Rat-2 fibroblasts [33]. Interestingly, using NIH 3T3 cells, Embade et al. have demonstrated novel relationships between FasL generation and CER production in Rac1-induced apoptosis [34]. In another study, Deshpande et al. [35] have demonstrated intermediacy of intracellularly generated CER in Rac1-induced mitochondrial oxidative stress and premature senescence in human umbellical vein endothelial cells. Together, these data appear to implicate CER/Rac1 signaling pathways in oxidative stress and metabolic dysfunction in multiple cells types. Therefore, based on these and other supporting evidence we presented in this study, we believe that PA effects on lipid peroxides, superoxides and NOX activity are specific and that they require the intermediacy of Tiam1/Rac1 signaling pathway. It should be noted that we also observed modest effects of oleate on NOX activity without significantly affecting the Rac1 activation [Fig. 2] suggesting a clear distinction between the modes of action of these two fatty acids.

It is important to note that several recent studies have implicated physiological roles for a tonic increase in NOX activation and subsequent generation of reactive oxygen species in the stimulus-secretion coupling of glucose-stimulated insulin secretion [36]. Besides this, existing evidence in the literature clearly demonstrates increase in the insulin secretion by fatty acids under acute incubation conditions [17]. Therefore, one might ask the question if increase in Tiam1/Rac1 activation and NOX activation could contribute towards the physiological insulin secretion rather than inducing metabolic abnormalities in the isolated β-cell. While it appears likely, under specific experimental conditions, chronic activation of NOX by specific stimuli [e.g., high levels of glucose, fatty acids, CER or cytokines] leads to metabolic dysfunction and demise of the β-cell. For example, our recent observations [28] suggested significant abnormalities in mitochondrial function [i.e., loss in MMP] in cells exposed to C2-CER under acute conditions. Further, we have reported significant leakage of mitochondrial cytochrome-C into the cytosolic compartment in C2-CER-treated cells [28]. Furthermore, it should be noted that our current observations [Fig. 8] indicate that mitochondrial dysfunction, which is demonstrable in cells incubated with C2-CER, but not DHC, is prevented, to a large degree, by inhibition of Tiam1/Rac1 signaling pathway, further implicating the Tiam1-Rac1-NOX signaling pathway in the onset of metabolic dysfunction in the presence of C2-CER. Therefore, we speculate that early biochemical and cellular changes that we reported herein might be paving way to metabolic dysfunction and demise of the islet β-cell. It may be germane to point out that recent studies by Moore et al. [7] have provided compelling evidence to argue against potential involvement of oxidative stress in fatty acid-induced metabolic dysfunction of the islet β-cell. It is, therefore, likely that additional regulatory mechanisms might underlie β-cell demise seen under the duress of lipotoxic conditions including those involving progressive alterations in the mitochondrial membrane permeability transition pore as suggested by recent studies of Koshkin et al. [37] in MIN6 and INS-1 cells. Furthermore, PA-induced CER-mediated effects might also include regulation of key target proteins such as the CER-activated protein phosphatase 2A that we have characterized in isolated β-cells [27,38], leading to the inactivation of key cellular events including inhibition of extracellular-regulated kinase and inhibition of proinsulin gene expression [39].

In conclusion, we present the first evidence for a novel role of Tiam1/Rac1 signaling pathway in PA-induced, CER-sensitive metabolic activation of NOX and associated production of superoxides

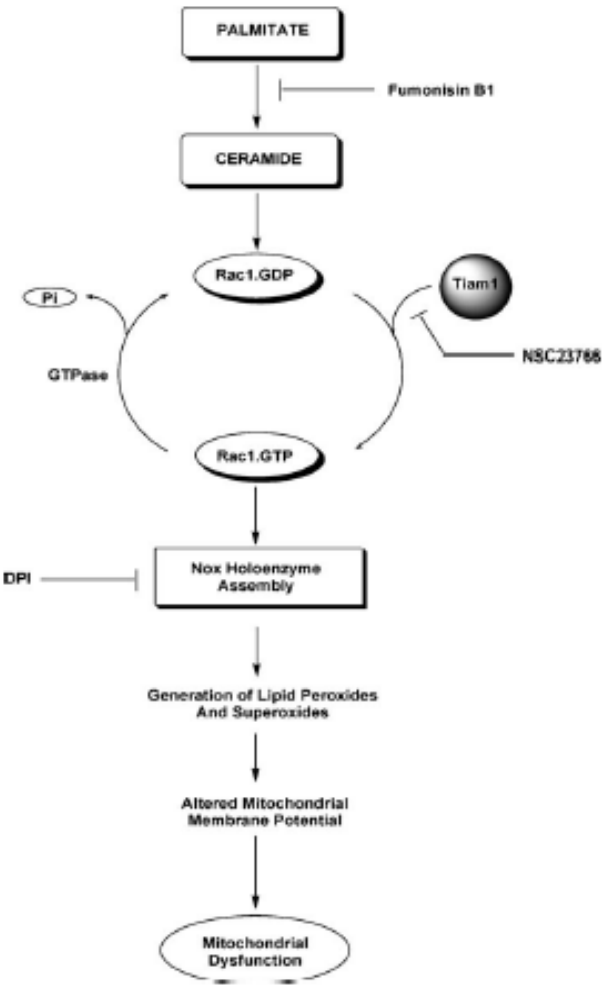

Fig. 9. A model to implicate Tiam1/Rac1 signaling pathway in PA-induced, CER-mediated effects on isolated β-cells. Based on the data accrued in the current studies we propose that incubation of isolated β-cells to either PA [but not oleate] or C2-CER [but not DHC] leads to Tiam1-mediated activation of Rac1, which is a key member of the NOX assembly. In addition, PA or C2-CER promotes the expression of $p47^{phox}$ in these cells. Together, these signaling steps promote the assembly and activation of NOX holoenzyme leading to the production of superoxides and lipid peroxides. These, in turn, exert damaging effects on the mitochondria including reduction in MMP leading to the onset of mitochondrial dysfunction. Appropriate sites of inhibition of these signaling steps by FB-1, NSC23766 and DPI are also indicated in the figure.

and lipid peroxides in pancreatic β-cells. It is likely that Tiam1 could serve as a novel drug target for inhibition of generation of superoxides and lipid peroxides in isolated β-cells under lipotoxic conditions. Based on these data we propose a working model [Fig. 9] to suggest that PA/CER increase the Rac1 activation [GTP-bound active form] and deactivation [GDP-bound inactive form] to generate signals that may be necessary for triggering cellular events leading to NOX activation, increased oxidative milieu, mitochondrial dysregulation in the pancreatic β-cell. It should be noted that while the proposed model principally addressed the roles of Tiam1–Rac1–NOX connection in PA/CER-mediated effects, relative contributory roles of other sources of reactive oxygen species, including the glutathione peroxidase, manganese-sensitive superoxide dismutase, catalase signaling cascades must also be recognized as key contributors to the mitochondrial dysfunction in isolated β-cells under the duress of lipotoxic conditions [1–3,40]. However, additional studies are needed to further understand these signaling steps in the islet β-cell.

Acknowledgements

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Appendix C

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CALL FOR PAPERS | *Mitochondrial Function/Dysfunction in Health and Disease*

Phagocyte-like NADPH oxidase promotes cytokine-induced mitochondrial dysfunction in pancreatic β-cells: evidence for regulation by Rac1

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Subasinghe W, Syed I, Kowluru A. Phagocyte-like NADPH oxidase promotes cytokine-induced mitochondrial dysfunction in pancreatic β-cells: evidence for regulation by Rac1. *Am J Physiol Regul Integr Comp Physiol* 300: R12–R20, 2011. First published October 13, 2010; doi:10.1152/ajpregu.00421.2010.—Reactive oxygen species (ROS) are important mediators of cellular signal transduction cascades such as proliferation, migration, and apoptosis. Chronic exposure of isolated β-cells to proinflammatory cytokines elevates intracellular oxidative stress leading to the demise of pancreatic β-cells culminating in the onset of diabetes. Although the mitochondrial electron transport chain is felt to be the primary source of ROS, several lines of recent evidence suggest that phagocyte-like NADPH oxidase plays a central role in cytokine-mediated ROS generation and apoptosis of β-cells. However, the precise mechanisms underlying the regulation of NADPH oxidase remain unknown. To address this, insulin-secreting INS 832/13 cells were treated with cytomix (IL-1β, IFN-γ, and TNF-α; 10 ng/ml each) for different time intervals (0–24 h). A significant, time-dependent increase in NADPH oxidase activation/intracellular ROS production, $p47^{phox}$ subunit, but not $p67^{phox}$ subunit, expression of the phagocyte-like NADPH oxidase were demonstrable under these conditions. Furthermore, siRNA-$p47^{phox}$ transfection or exposure of INS 832/13 cells to apocynin, a selective inhibitor of NADPH oxidase, markedly attenuated cytomix-induced ROS generation in these cells. Cytomix-mediated mitochondrial dysfunction in INS 832/13 cells was evident by a significant loss of mitochondrial membrane potential (MMP) and upregulated caspase 3 activity. Cytomix treatment also caused a transient (within 15 min) activation of Rac1, a component of the NADPH oxidase holoenzyme. Furthermore, GGTI-2147 and NSC23766, known Rac1 inhibitors, not only attenuated the cytomix-induced Rac1 activation but also significantly prevented loss of MMP (NSC23766 > GGTI-2147). However, NSC23766 had no effect on cytomix-induced NO generation or caspase 3 activation, suggesting additional regulatory mechanisms might underlie these signaling steps. Together, these findings suggested that Rac1-mediated regulation of phagocyte-like NADPH oxidase contributes to cytokine-mediated mitochondrial dysfunction in the β-cell.

T-lymphoma invasion; metastasis 1; geranylgeranylation; mitochondrial membrane potential; pancreatic β-cell

TYPE-1 DIABETES IS CHARACTERIZED by an absolute insulin deficiency arising from progressive autoimmune destruction of pancreatic β-cells (2–3, 8, 22). During the progression of the disease, proinflammatory cytokines, particularly IL-1β, TNF-α, and IFN-γ, are released into islets of Langerhans by infiltrated, activated T cells and macrophages (8, 25, 27). However, the exact cellular mechanisms by which cytokines induce β-cell demise is only partially understood (3). Though cytokines modulate the activity of several destructive signaling cascades, apoptosis is considered as the primary mode of cell death in human and mouse models (2, 21–22). Apoptosis is a highly regulated, genetically encoded, and energy-dependent cell death process activated by extracellular signals (11, 13, 24). Caspases, a family of cysteine proteases, play a critical role in apoptosis. In the presence of apoptotic stimuli, caspase signaling axis is activated, in which activation of initiator caspases (i.e., caspases 8 and 9) leads to the downstream activation of executioner caspases (e.g., caspase 3). It is well established that once activated, caspase 3 cleaves ~40 different cellular substrates (3, 6, 11, 24).

There are three possible mechanisms by which cells undergo death via apoptosis. Recent studies indicate cytokines may signal apoptosis via an intrinsic apoptotic pathway, which involves damage to the mitochondrial membrane and subsequent release of cytochrome *c* from the intermembranous space into cytosol, leading to the activation of caspase cascade (6, 27). A growing body of recent evidence suggests upregulated oxidative stress from reactive oxygen species (ROS), and nitric oxide (NO) contributes to the damage in mitochondrial membrane, eventually causing defects in the membrane potential. In contrast with most other mammalian cell types, β-cells comprise relatively lower levels of redox-regulating enzymes, making them more vulnerable to oxidative damage (7). Recently, members of the NADPH oxidase family have emerged as one of the sources of redox signaling and pathological oxidative stress. Under basal conditions, this multicomponent enzyme system is inactive, and its respective subunits are dispersed between the soluble and membranous compartments. The membrane-bound catalytic core consists of flavocytochrome b_{558} components $p22^{phox}$ and $gp91^{phox}$ and small G-protein Rap1. The regulatory core consisting of $p47^{phox}$, $p67^{phox}$, and $p40^{phox}$ subunits and the small G-protein Rac1 reside in the cytosol. Upon stimulation, the cytosolic components are translocated to the membrane for holoenzyme assembly and activation of the enzyme (7). It has also been suggested that functional activation of Rac1 (i.e., GTP-Rac) is vital for the NADPH holoenzyme assembly (9).

Several recent studies have demonstrated localization and functional activation of the phagocyte-like NADPH in clonal β-cells, normal rat islets, and human islets under the duress of various stimuli, including elevated levels of glucose, saturated

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fatty acids, and proinflammatory cytokines (14). It has also been documented that pharmacological inhibition of NADPH oxidase by diphenyleneiodonium chloride (DPI) or anti-sense oligonucleotides for $p47^{phox}$ markedly attenuate glucose-induced ROS production and oxidative stress, suggesting critical involvement of NADPH oxidase in the metabolic dysfunction induced by long-term exposure to elevated glucose (14, 15). These data implicated a significant contributory role for NADPH oxidase in the onset of metabolic dysfunction of the β-cell under a condition of oxidative stress (17, 19–20, 26). Despite the aforementioned compelling lines of evidence, very little has been studied with regard to the potential contributory roles of Rac1 in the cascade of events leading to cytokine-induced NADPH oxidase-mediated superoxide generation and mitochondrial dysfunction in pancreatic β-cells. On the basis of this reasoning, we undertook the current study to test the hypothesis that cytokines induce ROS generation and oxidative stress in pancreatic β-cells by promoting Rac1 activation, which represents one of the signaling events necessary for the functional regulation of the endogenous phagocyte-like NADPH oxidase holoenzyme assembly and its catalytic activity. Herein, we describe the first evidence to suggest a critical modulatory role for Tiam1, a guanine nucleotide exchange factor (GEF) for Rac1 (30), in this signaling pathway leading to the onset of mitochondrial dysfunction. We also report that posttranslational prenylation of Rac1 is also necessary for the optimal activation of NADPH oxidase elicited by cytomix in insulin-secreting cells.

MATERIALS AND METHODS

Materials. Interleukin-1β, IFN-γ, and TNF-α were obtained from R&D Systems (Minneapolis, MN). Rac1 activation assay kit was obtained from Cytoskeleton (Denver, CO). JC-1 mitochondrial membrane potential detection kit was obtained from Cell Technology (Mountain View, CA). Caspase 3 antiserum was obtained from Cell Signaling Technology (Danvers, MA). $p47^{phox}$ siRNA, $p47^{phox}$, and $p67^{phox}$ antisera were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). NSC23766 and GGTI-2147 were obtained from Calbiochem (San Diego, CA). Apocynin was obtained from Sigma-Aldrich (St. Louis, MO).

Cell lines and culture conditions. INS 832/13 cells (kindly provided by Dr. Chris Newgard, Duke University Medical Center, Durham, NC) were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, and 10 mM HEPES (pH 7.4). The cultured cells were subcloned twice weekly following trypsinization and passages 53–61 were used for the study. For the inhibitor studies, INS 832/13 cells were cultured up to 70–80% confluence in RPMI medium supplemented with 10% heat-inactivated FBS prior to inhibitor exposure. Cells were then incubated overnight with low serum-low glucose (LS-LG) media in the presence or absence of NSC23766 and GGTI-2147 at 20 μM and 10 μM, respectively.

Quantitation of cytokine-induced NO release. INS 832/13 cells were incubated with cytomix (IL-1β, IFN-γ, TNF-α; 10 ng/ml; each) or IL-1β (25 ng/ml), for 12 and 24 h in the presence and absence of inhibitors, as indicated in the text. At the end of incubation period, the medium was collected and centrifuged at 1,000 *g* for 5 min. Equal volumes of media and Griess reagent were mixed, and absorbance (540 nm) was measured using microplate reader (Molecular Devices, Sunnyvale, CA).

Quantitation of ROS. INS 832/13 cells were seeded in a 6-well plate and treated with either diluent or cytomix in the presence and absence of inhibitors (i.e., NSC23766, GGTI-2147) for a 12- and 24-h period, as indicated in the text. Following that, media were removed, and cells were incubated further in 2′,7′-dichlorofluorescein diacetate (DCHF-DA) (10 μM) at 37°C for 30 min. DCHF-DA is a nonpolar compound that diffuses rapidly into the cells and hydrolyzes readily by cellular esterases into polar DCFH. In the presence of ROS, DCFH is readily oxidized to fluorescent DCF (1, 6). The cells were washed twice with ice-cold PBS and harvested; equal amounts of proteins (50 μg) were taken, and fluorescence was measured (Em: 485 nm and Ex: 535 nm) using a luminescence spectrophotometer (PerkinElmer, Waltham, MA).

Molecular biological or pharmacological inhibition of NADPH oxidase activity. INS 832/13 cells were seeded in a 24-well plate up to 50% confluence and transfected with mock or antisense siRNA-$p47^{phox}$ (150 nM) and allowed to grow up to 80% or higher confluence. Then the cells were treated either with diluent or cytomix for a 12-h period. Following this, culture medium was removed, and cells were incubated further in DCHF-DA (10 μM) at 37°C for 30 min, washed twice with ice-cold PBS, and harvested; equal amounts of proteins (50 μg) were taken, and fluorescence was measured (Ex: 485 nm and Em: 535 nm) using luminescence spectrophotometer (PerkinElmer, Waltham, MA). Alternatively, NADPH oxidase was inhibited via a pharmacological approach by treating INS 832/13 cells with either diluent or cytomix for 12 or 24 h in the absence or presence of apocynin (75 μM), and NADPH activity was measured with DCFH-DA assay, as described above.

Rac1 activation assay. The relative degree of Rac1 activation (GTP-bound form) was determined using Rac1 pull-down assay, as described by Syed et al. (23). In brief, INS 832/13 cells were pretreated with either the diluent or pharmacological inhibitors followed by treatments with either diluent or Cytomix for 15 min in the absence and presence of NSC23766 (20 μM) or GGTI-2147 (10 μM). Cell lysates (~250–300 μg) were clarified by centrifugation. Then PAK-PBD (p21-activated kinase-p21-binding domain) beads (20 μl) 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.

Determination of mitochondrial membrane potential. INS 832/13 cells were plated on sterile glass cover slips placed in 6-well plates and pretreated with cytomix for 12 and 24 h in the presence and absence of NSC23766 (20 μM) or GGTI-2147 (10 μM). At the end of treatment, cells were incubated with JC-1 (1: 200) dye for 15 min at 37°C in a 5% CO_2 incubator. Cells were washed with assay buffer, mounted onto glass slides, and observed under IX71 inverted fluorescence microscope (X100, Olympus America, Center Valley, Pennsylvania), as we described previously (23). The ratios of red-to-green fluorescence emissions were quantitated to further estimate the extent of mitochondrial membrane damage.

Western blot analysis. Treated INS 832/13 cells were harvested and homogenized in mannitol-protease inhibitor cocktail buffer (250 mM mannitol, 70 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM DTT, and protease inhibitor cocktail). Protein samples (~20–30 μg) were resolved by SDS-PAGE and transferred onto nitrocellulose membrane. The blots, after blocking with 5% BSA in 20 mM Tris-HCl, pH 7.5, 137 mM NaCl, and 0.1% Tween 20, were immunoprobed with corresponding primary antibody followed by secondary polyclonal rabbit/mouse antibody conjugated to horseradish peroxidase (1:1,000). The protein signal band was detected with enhanced chemiluminescence system (ECL, Amersham Biosciences, Little Chalfont, UK) and developed using Kodak Pro Image 400 R (New Haven, CT). The blots were stripped and reprobed for β-actin to ensure equal loading and transfer of proteins.

Statistical analyses. Data are presented as means ± SE. Statistically significant differences between values were evaluated by Student's *t*-test or ANOVA where appropriate. $P < 0.05$ was considered to be statistically significant.

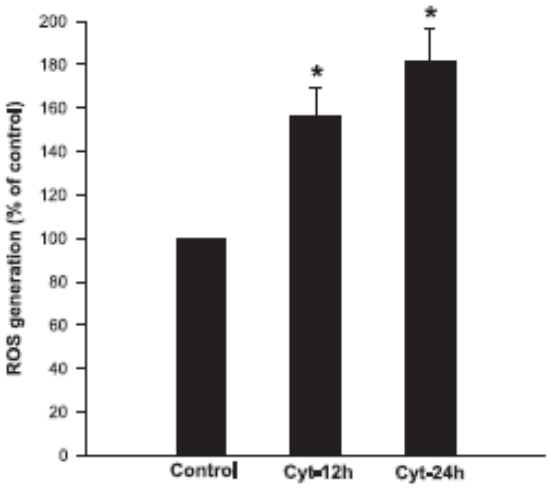

Fig. 1. Incubation of INS 832/13 cells with cytomix leads to a time-dependent increase in reactive oxygen species (ROS). INS 832/13 cells were incubated with either diluent or cytomix for 12 or 24 h, as indicated in the figure, and ROS generation was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA) assay. Intracellular levels of ROS in treated cells were expressed as a percent of control cells. Data represent means ± SE from four independent experiments. *Significantly different ($P < 0.05$) from control.

RESULTS

Cytomix induces phagocyte-like NADPH oxidase activation in INS 832/13 cells. At the outset, we quantitated NADPH oxidase activity in INS 832/13 cells exposed to cytomix (i.e., IL-1β, IFN-γ, TNF-α; 10 ng/ml each). The amount of ROS generation and the degree of expression of NADPH subunits ($p47^{phox}$ and $p67^{phox}$) were determined following a 12-h or 24-h incubation of these cells with cytomix. Data in Fig. 1 showed a significant increase in ROS generation at these time points (~60 and 85% above the control at 12 h and 24 h, respectively). Compatible with these findings are data presented in Fig. 2, *A* and *B*, indicating a significant increase in the expression of $p47^{phox}$ in these cells following exposure to cytomix. However, no effect of cytomix on the expression of $p67^{phox}$ was demonstrable under these conditions (Fig. 2, *C* and *D*).

To further assess whether the cytomix-induced ROS are derived from NADPH oxidase, we quantitated cytomix-induced ROS generation following inhibition of NADPH oxidase via two independent approaches. In the first approach, we used apocynin, a selective inhibitor of NADPH oxidase. Data from these studies indicated a marked inhibition in cytomix-induced ROS generation by apocynin. The values for cytomix-mediated ROS generation represented 154.0 ± 3.9% and 167.8 ± 6.5% at 12 h and 24 h, respectively. The corresponding values in the presence of apocynin reached basal levels (i.e., 98.1 ± 5.2% and 106.6 ± 9.1% at 12 and 24 h, respectively; $n = 3$ experiments in each case; $P < 0.05$). In the second approach, endogenous expression of the $p47^{phox}$ was knocked down by transfecting cells with siRNA-$p47^{phox}$. Under the current experimental conditions employed in the study (see MATERIALS AND METHODS), we were able to reduce $p47^{phox}$ expression by ~60–70% in siRNA-$p47^{phox}$-transfected cells. Furthermore, the ability of cytomix to induce ROS generation (following 12 h of incubation) was completely abolished in siRNA-$p47^{phox}$-transfected cells (i.e., 102.0 ± 5.5% of control; $n = 3$ experiments), suggesting that NADPH oxidase might be the principal contributor in cytomix-induced generation of ROS.

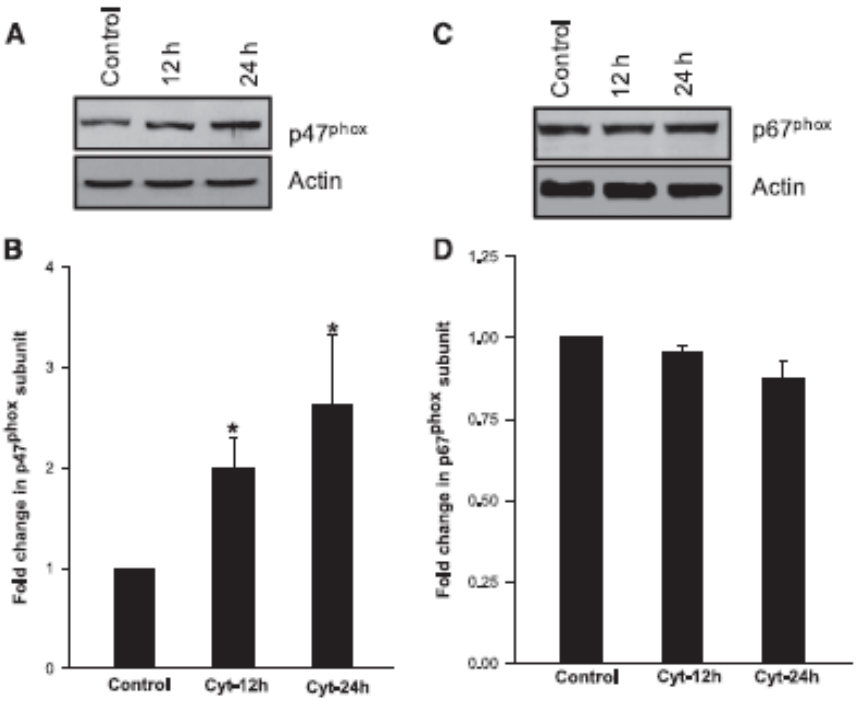

Fig. 2. Incubation of INS 832/13 cells with cytomix increases expression of $p47^{phox}$, but not $p67^{phox}$ subunits of NADPH-oxidase. *A*: INS 832/13 cells were exposed to cytomix for 12 or 24 h as indicated in the figure. Relative degree of expression of $p47^{phox}$ was determined by Western blot analysis. $p47^{phox}$ expression was normalized to actin content in individual lanes. Pooled data from three independent experiments are provided in *B*. *Significantly different ($P < 0.05$) from control. *C*: INS 832/13 cells were exposed to cytomix for 12 or 24 h as indicated in the figure. Relative degree of expression of $p67^{phox}$ was measured by Western blot analysis. $p67^{phox}$ expression was normalized to actin content in individual lanes. Pooled data from three independent experiments are provided in *D*.

Cytomix transiently increases Rac1 activation in INS 832/13 cells: potential requirement for Tiam1 as a guanine nucleotide exchange factor for Rac1. As stated above, Rac1, a small G protein, is one of the components of the NADPH oxidase holoenzyme assembly. Therefore, we next examined whether cytomix-induced activation of NADPH oxidase is mediated via activation of Rac1. This was accomplished by quantitating the GTP-bound Rac1 (active configuration) by a pull-down assay (see MATERIALS AND METHODS for additional details). Data depicted in Fig. 3 suggested a significant (~1.7-fold), but transient, activation of Rac1 (within 15 min) in INS 832/13 cells following exposure to cytomix. Rac1.GTP levels reached basal levels at 30 min of exposure. These data implicate Rac1 activation as one of the signaling steps involved in cytomix-mediated effects on isolated β-cells.

Recently, we reported the expression and functional activation of GEFs for small G proteins in pancreatic β-cells (29). The primary function of these proteins is to facilitate GTP/GDP exchange. Our findings in INS 832/13 cells and primary rat islets have indicated that Tiam1 serves as a GEF for Rac1 (29). In the current study, we investigated whether Tiam1 is required for cytomix-induced activation of Rac1 in INS 832/13 cells. This was accomplished using pharmacological inhibitor, NSC23766, which selectively inhibits Tiam1-mediated activation of Rac1, but not Cdc42 or Rho in insulin-secreting β-cells (5). Data in Fig. 3*C* suggested a significant reduction in cytomix-induced activation of Rac1 by NSC23766 in INS 832/13 cells. These findings support the viewpoint that Tiam1 plays a key regulatory role in Rac1 activation elicited by cytomix in insulin-secreting cells.

It is well established that posttranslational geranylgeranylation is necessary for optimal activation of Rac1 in pancreatic β-cells (see Ref. 12, for a review). Therefore, we examined whether geranylgeranylation of Rac1 is necessary for cytomix-induced activation of Rac1. This was accomplished via a pharmacological approach, which involved quantitation of cytomix-induced activation of Rac1 in cells exposed to diluent or in the presence of GGTI-2147, a known inhibitor Rac1 geranylgeranylation (12, 28). Data in Fig. 3*D* showed a marked reduction in cytomix-induced Rac1 activation in cells exposed to GGTI-2147. Together, data depicted in Fig. 3, *A–D* suggested that cytomix induces Rac1 activation in INS 832/13 cells, which is sensitive to inhibition of Tiam1 activation and posttranslational geranylgeranylation.

NSC23766 and GGTI-2147 markedly reduce cytomix-induced ROS generation in INS 832/13 cells. As a logical extension to the above studies, we asked whether inhibitors of Rac1 attenuate cytomix-induced ROS generation. Data shown in Fig. 4 indicate a marked reduction in cytomix-induced ROS generation at both 12- and 24-h time points by NSC23766 and GGTI-2147. It is noteworthy that GGTI-2147, but not NSC23766, also reduced the ROS generated under basal conditions (Fig. 4, *A* and *B*). Taken together, our findings establish a direct role for Tiam1-dependent, prenylation-sensitive Rac1

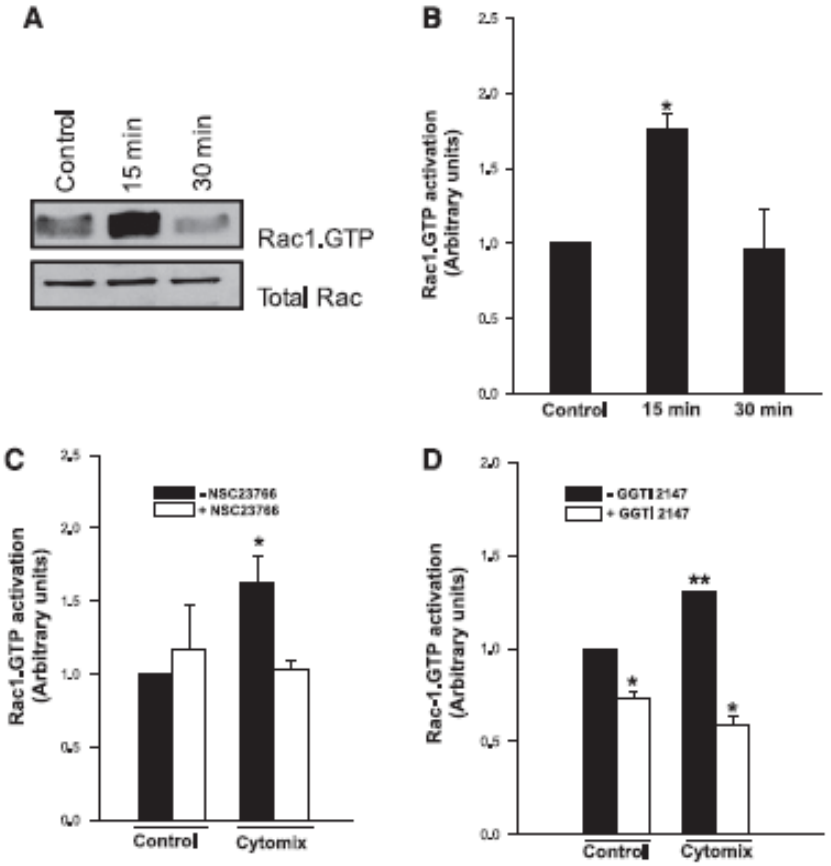

Fig. 3. Cytomix induces transient activation of Rac1 in INS 832/13 cells: inhibition of this signaling step by NSC23766 and GGTI-2147. *A*: cytomix causes transient activation of small G-protein Rac1 in INS 832/13 cells, as determined by the pull-down assay followed by Western blot analysis (see MATERIALS AND METHODS). Total Rac1 in the lysates is also provided as a loading control. A representative blot of three independent experiments is shown here. *B*: pooled activation data from three independent experiments are shown here. *C*: NSC23766 inhibition of cytomix-induced activation of Rac1. Pooled data from three independent studies are depicted in the figure. *D*: GGTI-2147 inhibits cytomix-induced Rac1 activation in INS 832/13 cells. Pooled data from three independent studies are depicted in the figure. *Significantly different ($P < 0.05$) from control. *, **Different symbols represent the values that are significantly different at $P < 0.05$.

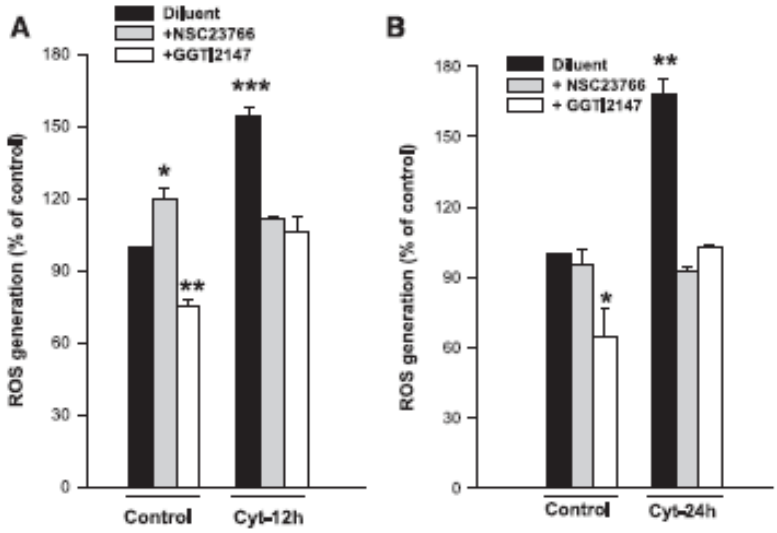

Fig. 4. Cytomix-induced ROS generation is inhibited by NSC23766 and GGTI-2147 in INS 832/13 cells. INS 832/13 cells were treated with either diluent or cytomix in the presence and absence of NSC23766 (20 μM) or GGTI-2147 (10 μM) for 12 h (*A*) and 24 h (*B*), as indicated in the figure and intracellular levels of ROS was measured using DCHF-DA assay. Data are representative of three independent experiments, expressed as a percentage of control cells and represent means ± SE. Bars with different symbols (*, **, ***) are significantly different at $P < 0.05$.

activation in the signaling cascade leading to cytomix-induced NADPH oxidase and ROS generation in INS 832/13 cells.

Inhibitors of Rac1 activation reduce cytomix-induced mitochondrial defects in INS 832/13 cells. It is widely felt that cytokine-mediated effects on pancreatic β-cells may, in part, be mediated via alterations in mitochondrial membrane properties, including loss of MMP leading to cytochrome-*c* release and caspase 3 activation (2, 16, 30). Therefore, we examined whether inhibitors of Rac1 activation exert protective effects on cytomix-induced loss in MMP. This was accomplished using JC-1 (5,5′,6,6′-tetrachloro-1,1′3,3′-tetraethylbenzimidazolyl-carbocyanine iodide) assay. JC-1 is a lipophilic dye, which fluoresces red when aggregated above the critical concentration within mitochondria. In cells in which mitochondrial membrane is damaged, JC-1 remains in the cytoplasm, as a green fluorescence monomer. Data from these studies, which are depicted in Fig. 5, suggested a significant loss of MMP in INS 832/13 cells treated with cytomix following a 12- or 24-h exposure. Coprovision of NSC23766, a Tiam1 inhibitor, and GGTI-2147, a prenylation inhibitor, modestly, but significantly protected these cells against cytomix-induced damage to the mitochondrial potential (Fig. 5). Quantitation of fluorescence intensity ratios of red to green further confirmed these conclusions (Fig. 6). Further, these data also suggested that the protective effects were more prominent in the case of NSC23766 compared with GGTI-2147 (Fig. 5, *A–D*, Fig. 6, *A* and *B*). It should be mentioned that NSC23766 exerted inhibitory effects on MMP in control (i.e., diluent-treated cells). Regardless of this inhibitory effect, it markedly prevented cytomix-induced loss in MMP at both time points. Together, these data indicate that Rac1 activation might be requisite for cytomix-induced mitochondrial defects in pancreatic β-cells.

Tiam1/Rac1 signaling axis is not necessary for cytomix-induced caspase 3 activation in INS 832/13 cells. The observed protective effects of NSC23766 against cytomix-induced loss in MMP (Figs. 5 and 6) prompted us to investigate whether caspase 3 activation, which is a hallmark of cellular apoptosis, is inhibited by Tiam1-mediated activation of Rac1. To accomplish this, INS 832/13 cells were treated with cytomix (as above) or IL-1β alone (25 ng/ml) for 12 or 24 h. Activated caspase 3 in the lysates was determined by Western blot analysis using an antiserum that identifies both the native procaspase and degradative product of caspase 3. Under these conditions we noticed no significant effects of NSC23766 on either cytomix-induced or IL-1β-mediated caspase 3 activation at either time points. Cytomix-induced caspase 3 activation represented 1.55 ± 0.11 units and 1.83 ± 0.24 units at 12 and 24 h, respectively. The corresponding values in the presence of NSC23766 were 1.40 ± 0.14 units and 2.06 ± 0.32 units, respectively ($n = 3$ determinations in each case, not significantly different from each other). Likewise, IL-1β-induced caspase 3 activation represented 1.27 ± 0.10 units and 1.65 ± 0.23 units at 12 and 24 h, respectively. The corresponding values in the presence of NSC23766 were 1.23 ± 0.09 units and 1.71 ± 0.22 units, respectively ($n = 3$ determinations in each case, not significantly different from each other). Together, these data indicate that additional mechanisms might underlie caspase 3 activation in these cells elicited by cytokines.

Evidence to further suggest that the Tiam1/Rac1 signaling step may not be required for cytokine-induced NO release from INS 832/13 cells. It is well established that cytokine-mediated effects on isolated β-cells are mediated via inducible nitric oxide synthase (iNOS) expression and associated NO release. It has also been suggested that NO exerts damaging effects on mitochondria leading to caspase 3 activation. Therefore, in the last set of studies, we investigated whether Tiam1/Rac1 activation is necessary for cytokine-induced NO release in INS 832/13 cells. Data in Fig. 7 demonstrated no significant effect of NSC23766 on either IL-1β or cytomix-induced NO release in INS 832/13 cells either at 12 or 24 h. Together, the above data suggest that Tiam1/Rac1 signaling step is not involved in cytokine-induced NO release and caspase 3 activation and that additional regulatory steps might be necessary for these to occur (see the proposed model below).

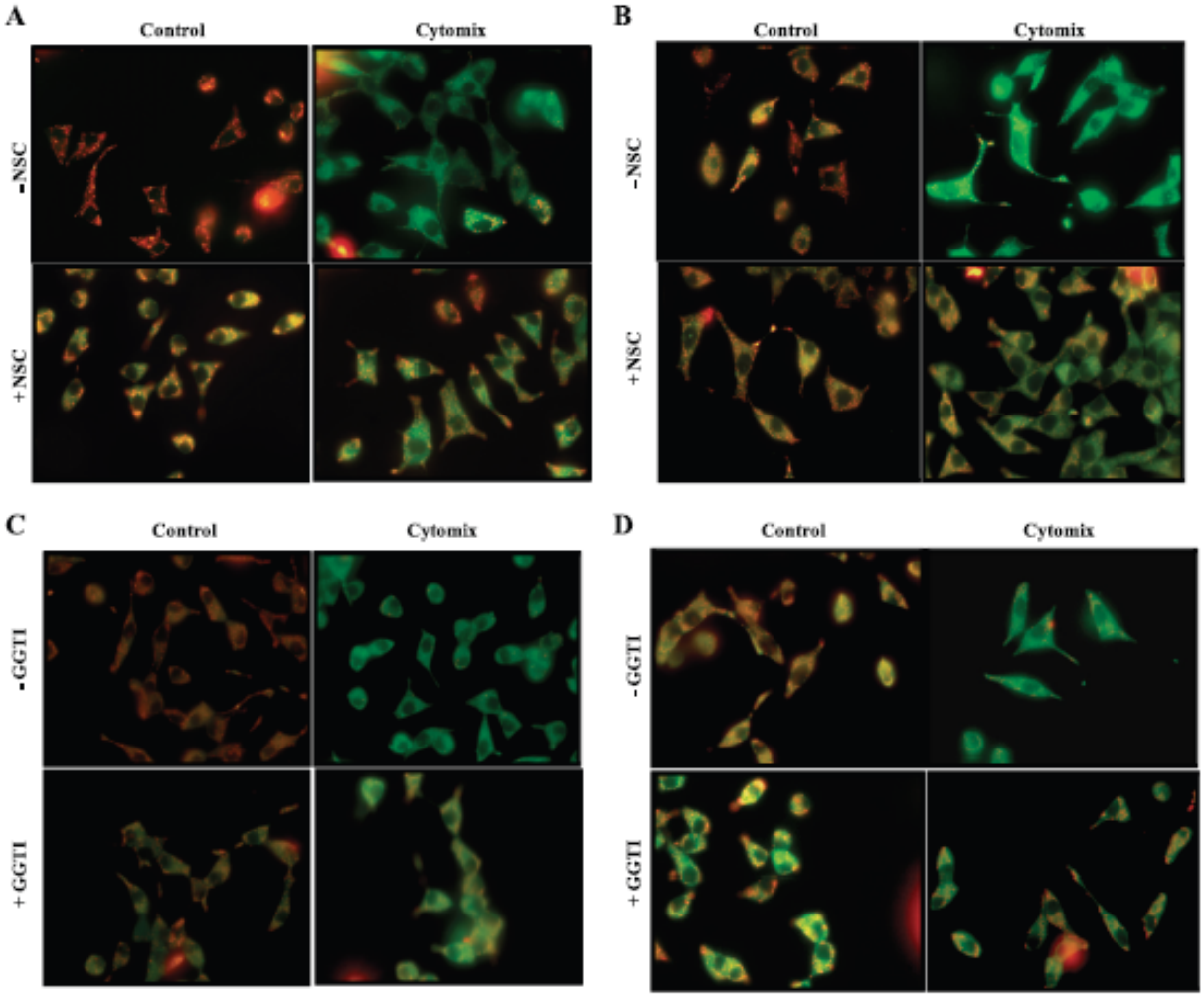

Fig. 5. Cytomix-induced loss in mitochondrial membrane potential is partially prevented by NSC23766 and GGTI-2147. INS 832/13 cells were treated with either diluent alone or cytomix for 12 (*A* and *C*) and 24 h (*B* and *D*) in the presence and absence of NSC23766 (20 μM) or GGTI-2147 (10 μM), as indicated in the figure. The mitochondrial membrane potential was measured with JC-1 assay kit. Data are representative of three independent experiments with comparable results.

DISCUSSION

A growing body of evidence supports the hypothesis that damaging effects of elevated glucose, saturated fatty acids (e.g., palmitate), or proinflammatory cytokines on isolated β-cells may, in part, be due to their ability to increase the generation of superoxides and lipid peroxides leading to increased intracellular oxidative stress culminating in mitochondrial dysfunction and the demise of the effete β-cell (10, 14, 20). More recent evidence (14, 18) suggests that intracellular oxidative stress is largely due to the activation of phagocyte-like NADPH oxidase in these cells. Such a postulation was further supported by pharmacological (e.g., DPI) and molecular biological (e.g., antisense $p47^{phox}$) approaches. Using apocynin and siRNA-$p47^{phox}$, we have demonstrated herein that the majority of ROS generated in INS 832/13 cells in the presence of cytomix is derived via the activation of NADPH oxidase. Furthermore, as described in the following sections, our current findings provide additional novel insights into regulatory mechanisms underlying the regulation of NADPH oxidase by cytokines in the islet β-cell.

Our findings implicate a requirement for Tiam1 in the cascade of events leading to cytokine-induced activation of Rac1 in insulin-secreting cells. Using a selective inhibitor of Tiam1-mediated activation of Rac1 (i.e., NSC23766), we have been able to demonstrate that Tiam1 serves as a GEF for Rac1 activation induced by cytomix. These data were further substantiated by our observations to demonstrate a significant reduction in cytomix-induced ROS generation/NADPH oxidase activity by NSC23766. It should be noted in this context that Rac1 activation is transient in nature and that it might be adequate to "initiate" signaling cascade leading to activation of NADPH oxidase. It appears that activation of Rac1 by cytomix seen in the current studies may be attributable primarily to the effects of IL-1β in the cytomix, since we observed a significant

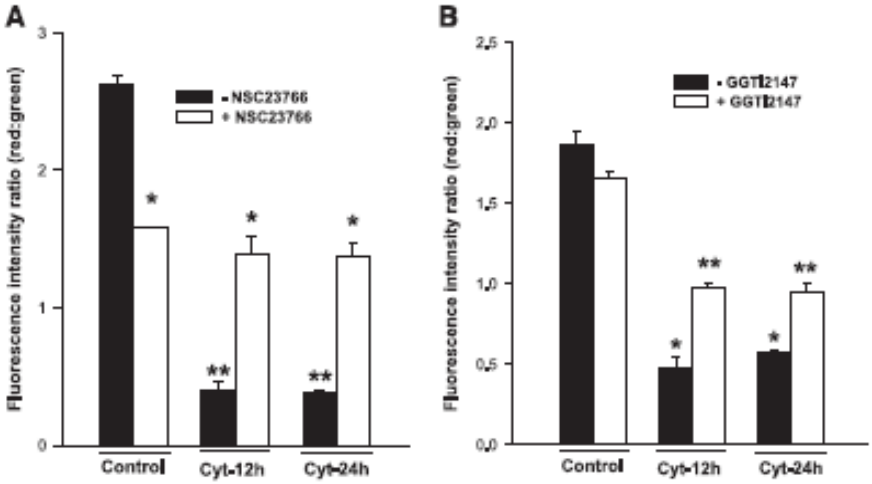

Fig. 6. Cytomix-induced loss in mitochondrial membrane potential is partially prevented by NSC23766 and GGTI-2147. Cytomix induced changes in mitochondrial membrane potential was measured with JC-1 assay kit, as described in Fig. 5 and red: green fluorescence ratio was calculated by sampling ($n = 10$ data points per image) for three independent experiments with comparable results. *, **Bars with different symbols are significantly different $P < 0.05$. *A*: data from cells treated with NSC23766 (20 μM). *B*: data from cells treated with GGTI-2147 (10 μM).

(and transient) activation of Rac1 (2 ± 0.4-fold stimulation; $n = 3$ determinations) in INS 832/13 cells exposed to IL-1β (25 ng/ml). No significant effects were observed with either TNF-α (10 ng/ml) or IFN-γ (10 ng/ml) under these conditions (additional data not shown). Our findings also suggest that IL-1β-mediated Tiam1/Rac1 signaling pathway may not be necessary for iNOS expression and NO release since NSC23766 failed to exert any significant effects on IL-1β (or cytomix)-induced NO release in INS 832/13 cells. Lastly, in the context of transient activation of Rac1 by cytokines, it must be noted that earlier studies from our laboratory and others have demonstrated a significant translocation and membrane association of Rac1 following its activation (see Ref. 12, for a recent review). Therefore, it is likely that the activated Rac1 translocates to the membrane fraction for the NADPH oxidase holoenzyme assembly and activation. However, this remains to be verified.

Our findings provide the first evidence to suggest that prenylation of Rac1 is necessary for cytokine-mediated activation of Rac1 and subsequently the NADPH oxidase. We found that GGTI-2147, a selective inhibitor of protein geranylgeranylation, but not farnesylation, markedly attenuated Rac1 and NADPH oxidase activation mediated by cytokines. In this context, using molecular biological (e.g., dominant-negative Rac1 mutant or siRNA-Rac1) and pharmacological (e.g., GGTI-2147 and 3-allyl or vinyl geranyl geraniols), we have shown recently that geranylgeranylation of Rac1 is necessary for its optimal activation and membrane association in clonal β-cells and normal rats islets (28). It must be pointed out that the NADPH oxidase membrane core component is comprised of Rap1, which also undergoes geranylgeranylation like Rac1. Therefore, the inhibitory effects of GGTI-2147 on cytokine-induced NADPH oxidase may, in part, be due to inhibition of geranylgeranylation of Rac1 and Rap1. Our findings accrued from NSC23766 studies directly support the involvement for Taim1/Rac1 in this signaling cascade since Tiam1 serves as a GEF for Rac1, but not other small G proteins. Taken together, on the basis of the current data accrued from NSC23766 and GGTI-2147 studies, we propose that Tiam1-mediated and geranylgeranylation-sensitive activation of Rac1 is necessary

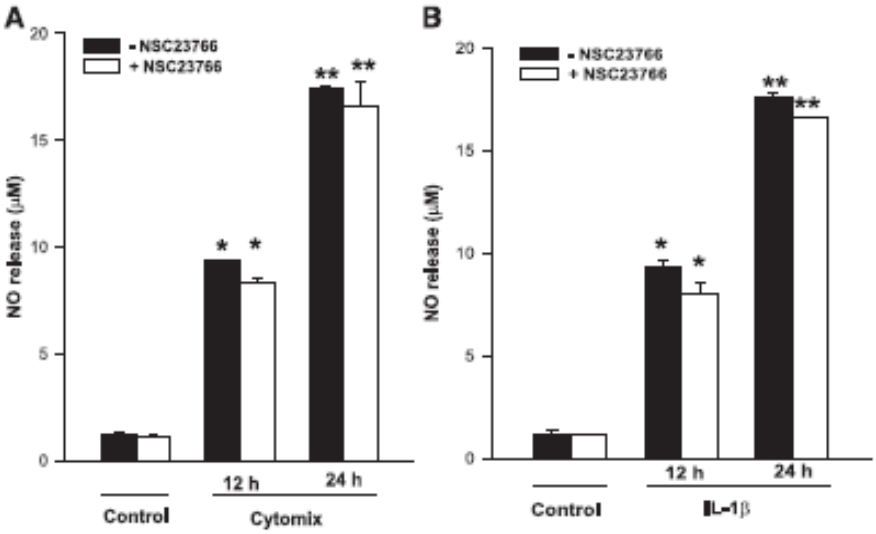

Fig. 7. NSC23766 fails to inhibit cytomix-induced NO release in INS 832/13 cells. INS 832/13 cells were treated with diluent, cytomix (*A*) or IL-1β (*B*) for 12 or 24 h. NO released into the medium was measured using Griess assay. Data are expressed as means ± SE from 3 independent experiments. *, **Bars with different symbols represent the values that are significantly different.

for cytokine-mediated effects of NADPH oxidase and generation of oxidative stress in the islet β-cell.

The currently described evidence (Figs. 5 and 6) is also suggestive of protective effects of the Rac1 inhibitors against cytokine-induced loss in MMP. Our data suggested that NSC23766 affords a better protection compared with GGTI-2147. Therefore, it appears that additional signaling mechanisms might be controlling mitochondrial membrane potential, which are distinct from NADPH oxidase-derived ROS. Compatible with these observations are our findings that demonstrated relative lack of effects of NSC23766 on caspase 3 activation. As in the context of Rac1 activation mentioned above, such steps may be related to direct metabolic effects of IL-1β, but not TNF-α or IFN-γ (also present in the cytomix), since IL-1β-mediated caspase 3 activation and NO release were not affected by Tiam1 inhibition.

On the basis of these observations, we propose a model for cytokine-mediated effects on islet β-cell as they relate to NADPH oxidase and ROS generation (Fig. 8). Cytomix induces NADPH oxidase activation by promoting the expression of $p47^{phox}$ and activation of Rac1. The Rac1 activation step not only requires the intermediacy of Tiam1, but also prenylation as evidenced by inhibition of the signaling step by GGTI-2147. NADPH oxidase activation leads to an increase in the oxidative stress, resulting in alterations in mitochondrial membrane properties. IL-1β-mediated effects also include an increase in the expression of iNOS and subsequent release of NO, which has been shown to affect mitochondrial function directly leading to further damage and release of cytochrome *c* followed by activation of caspase 3. Please note that iNOS expression and NO release were found to be independent of Tiam1/Rac1 signaling pathway. It is likely that combined effects of intracellularly generated NO (via activation of iNOS) and ROS (via activation of NADPH oxidase) contribute to maximal damage of the mitochondrial membrane properties leading to caspase 3 activation and metabolic dysfunction of the β-cell.

It may be germane to point out that additional regulatory mechanisms might underlie cytokine-mediated stimulatory effects on NADPH oxidase. These include phosphorylation of the cytosolic $p47^{phox}$, which appears to be necessary for its translocation to the membrane. Published evidence implicates PKC in the phosphorylation of this protein (4). Indeed, studies by Morgan et al. (14) have demonstrated partial restoration of IL-1β-induced ROS to normal levels following exposure to GF109203X, a known inhibitor of PKC. Other regulatory mechanisms might also include regulation of Tiam1/Rac1/NADPH oxidase signaling cascade by sphingolipids, such as ceramide. In this context, we have recently reported palmitic acid-mediated activation of Tiam1/Rac1 and associated increase in NADPH oxidase activation in insulin-secreting β-cells (23). Palmitate effects were inhibited by fumonisin-B1, a known inhibitor of de novo biosynthesis of ceramide from palmitate. In addition, we observed that C2-ceramide, a cell-permeable analog of ceramide exerted similar stimulatory effects on NADPH oxidase in a NSC23766-sensitive manner (23). Additional studies are needed to determine whether intracellular generation of ceramide represents a regulatory mechanism in cytokine-challenged β-cells. Together, these data accrued in the current studies suggest that Rac1-mediated regulation of NADPH oxidase function contributes to cytokine-mediated mitochondrial dysfunction in the β-cell.

Perspectives and Significance

A growing body of evidence supports the hypothesis that damaging effects of elevated proinflammatory factors on isolated β-cells involves increased oxidative stress leading to demise of the β-cell. This may, in part, be due to activation of phagocyte-like NADPH oxidase endogenous to the islet β-cell. On the basis of the extant data and our current findings, it is reasonable to speculate that NADPH oxidase-derived oxidative stress exerts cytotoxic effects on the islet β-cell under the

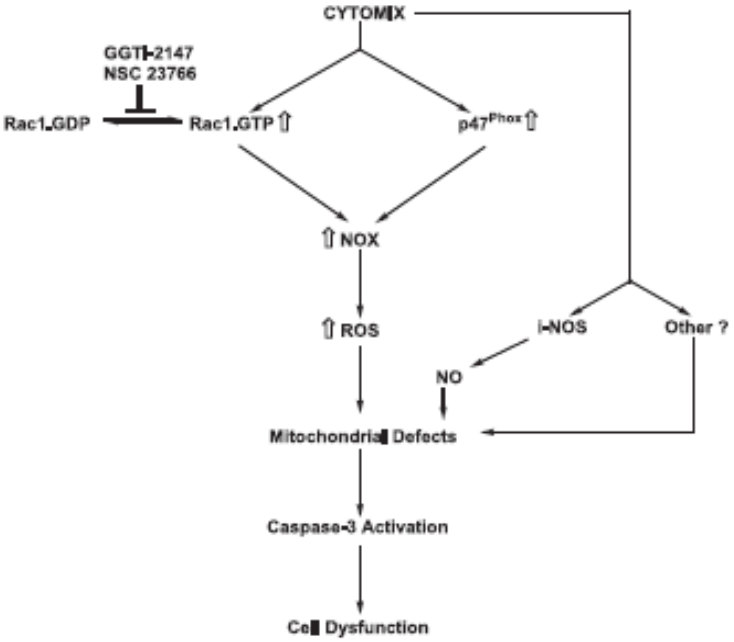

Fig. 8. A model for Rac1-dependent NADPH-oxidase-mediated cytomix-induced mitochondrial dysfunction in pancreatic β-cells. On the basis of the data accrued from the current studies, we propose a model for the Rac1-mediated regulation of NADPH oxidase activity under the duress of cytokines. Cytomix induces NADPH oxidase activation by promoting the expression of $p47^{phox}$ and activation of Rac1. The Rac1 activation step not only requires the intermediacy of Tiam1 (i.e., inhibition by NSC23766), but also prenylation, as evidenced by inhibition of the signaling step by GGTI-2147. NADPH oxidase activation leads to an increase in the oxidative stress culminating in loss of mitochondrial membrane potential. IL-1β-mediated effects also include an increase in the expression of iNOS and subsequent release of NO, which has been shown to affect mitochondrial function directly leading to further damage and release of cytochrome *c* followed by activation of caspase 3. Our findings suggested that iNOS expression and NO release are independent of Tiam1/Rac1 signaling pathway. It is likely that combined effects of intracellularly generated NO (via activation of iNOS) and ROS (via activation of NADPH oxidase) contribute to maximal damage of the mitochondrial membrane properties leading to caspase 3 activation and metabolic dysfunction of the β-cell. NOX, phagocyte-like NADPH oxidase; iNOS, inducible nitric oxide synthase; NO, nitric oxide.

duress of noxious stimuli, including chronically elevated glucose, saturated fatty acids, ceramides, and cytokines. From these studies, it is becoming increasingly evident that Rac1 signaling axis plays a critical role in the functional regulation of NADPH oxidase. Further, it appears that inhibition of Tiam1-mediated activation (using NSC23766) or inhibition of posttranslational geranylgeranylation of Rac1 (using GGTI-2147) restores some of these toxic effects. Unfortunately, however, these signaling steps/enzymes cannot be used as drug targets since they have been shown to play key roles in the normal functioning of the islet β-cells, including glucose-stimulated insulin secretion. Therefore, additional studies are needed to develop novel tools/probes to prevent the constitutive/chronic activation of NADPH oxidase and generation of oxidative stress following exposure of the islet β-cell to aforestated stimuli and prevent the associated metabolic dysfunction, loss of β-cell mass, and the onset of diabetes.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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ABSTRACT

MECHANISMS OF REGULATION OF ISLET FUNCTION BY NADPH OXIDASE

by

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Glucose stimulated insulin secretion (GSIS) involves a series of metabolic and cationic events, leading to translocation of insulin-laden secretory granules from a distal site toward the plasma membrane for fusion and release of insulin into circulation. Vesicular transport and fusion events are tightly regulated by signals which coordinate between vesicle- and membrane-associated docking proteins. It is now being accepted that reactive oxygen species [ROS] plays a second messenger role in islet β-cell function. Further, evidence from multiple laboratories suggests a tonic increase in ROS generation is necessary for GSIS and fatty acid-induced insulin secretion. On the other hand, excessive ROS generated during glucolipotoxic / exposures to cytokines and ceramide have proved to be detrimental for islet β-cells. Recent studies have shown activation of phagocyte-like NADPH oxidase [Nox] to be underlying cause for increased ROS generation observed under the above pathological conditions.

The overall objective of the present study is to i) determine potential mechanism[s] underlying nutrient-induced generation of ROS; ii) contributory roles of Tiam1-Rac1-

Nox signaling in free fatty acid (e.g., palmitate) and cytokines- induced β-cell dysfunction. Findings from current study suggest that posttranslational prenylation is a requisite for signaling G-proteins involved in the activation of Nox and generation of ROS for nutrient-induced insulin secretion from islet β-cells. Studies with pertussis toxin [Ptx] suggested that glucose-induced Nox-mediated ROS generation is regulated by inhibitory class of G-proteins [Go/Gi]. Our next set of studies, directed towards understanding the mechanism of Nox activation under chronic exposure to high palmitate, cytokines and C2-ceramide implicate increased expression of Nox subunits to precede the functional activation of the holoenzyme and excessive ROS generation resulting in mitochondrial dysfunction. This study also provide first evidence for a critical modulatory role of Tiam1, a guanine nucleotide exchange factor [GEF] in Rac1-Nox signaling axis.

The next set of studies validated the above observations in Zucker Diabetic Fatty [ZDF] rat model, which mimics type2 diabetes in humans, characterized by obesity, hyperinsulinemia, hyperglycemia and gradual decline in β-cell function. The results obtained were comparable with clonal β-cells. Islets derived from ZDF-rats presented high levels of Nox subunit expression [$p47^{phox}$, $gp91^{phox}$, Rac1] which constitutively activated Nox-holoenzyme and augmented ROS levels. The increased oxidative stress under conditions of diabetes activated Jun-N-terminal kinases [JNK 1/2, stress-activated kinases] leading to mitochondrial abnormalities and eventual demise of islet β cells. A similar pattern of induction in Nox subunit expression/activation, ROS generation and JNK 1/2 were also observed in type 2 diabetes human islets. Taken together, herein I propose that high levels of oxidative stress, activation of stress-activated kinases

[JNK1/2] and mitochondrial abnormalities underlies pancreatic β-cell dysfunction[s] during diabetes. Additional studies are needed to understand the precise regulatory roles for Tiam1-Rac1-Nox-ROS-JNK1/2 signaling to develop therapeutic strategies in the treatment of metabolic disorder.

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Publications:

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