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Phagocyte-like NADPH oxidase (Nox2) promotes activation of p38MAPK in pancreatic β -cells under glucotoxic conditions: Evidence for a requisite role of Ras-related C3 botulinum toxin substrate 1 (Rac1)

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Abstract

It is well established that glucotoxicity (caused by high glucose concentrations; HG) underlies pathogenesis of islet dysfunction in diabetes. We have recently demonstrated that Nox2 plays a requisite role in the generation of reactive oxygen species (ROS) under HG conditions, resulting in mitochondrial dysregulation and loss of islet β -cell function. Herein, we investigated roles of Nox2 in the regulation of downstream stress kinase (p38MAPK) activation under HG conditions (20 mM; 24 h) in normal rodent islets and INS-1 832/13 cells. We observed that gp91-ds-tat, a specific inhibitor of Nox2, but not its inactive analog, significantly attenuated HG-induced Nox2 activation, ROS generation and p38MAPK activation, thus suggesting that Nox2 activation couples with p38MAPK activation. Since Rac1, is an integral member of the Nox2 holoenzyme, we also assessed the effects of Rac1 inhibitors (EHT 1864, NSC23766 and Ehop-016) on HG-induced p38MAPK activation in isolated β -cells. We report a significant inhibition of p38MAPK phosphorylation by Rac1 inhibitors, implying a regulatory role for Rac1 in promoting the Nox2-p38MAPK signaling axis in the β -cell under the duress of HG. 2-Bromopalmitate, a known inhibitor of protein (Rac1) palmitoylation, significantly reduced HG-induced p38MAPK phosphorylation. However, GGTI-2147, a specific inhibitor of geranylgeranylation of Rac1, failed to exert any significant effects on HG-induced p38MAPK activation. In conclusion, we present the first evidence that the Rac1-Nox2 signaling module plays novel regulatory roles in HG-induced p38MAPK activation and loss in glucose-stimulated insulin secretion (GSIS) culminating in metabolic dysfunction and the onset of diabetes.

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Authors' contribution

Vaibhav Sidarala, Rajakrishnan Veluthakal and Khadija Syeda performed the experimental work, analyzed the data and edited the manuscript prior to publication. Cornelis Vlaar and Philip Newsholme provided scientific input and edited the manuscript prior to submission. Anjan Kowluru designed the studies, wrote the manuscript and edited prior to submission.

Keywords

Rac1; p38MAPK; Glucotoxicity; Pancreatic islet β -cell; Diabetes

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1. Introduction

Glucose-stimulated insulin secretion (GSIS) is initiated by the entry of glucose into the pancreatic β -cell and subsequent glucose metabolism which results in the formation of metabolic stimulus-secretion coupling factors, thereby, promoting several intracellular events which facilitate the movement of insulin granules toward the membrane for fusion and release [1]. Small G proteins such as Rac1 and Cdc42 have been implicated to play a critical role in cytoskeletal remodeling and GSIS, as shown in several *in vitro* and *in vivo* studies [2–7]. A growing body of evidence suggests that alterations in the function of these G proteins could represent plausible mechanisms underlying impaired insulin secretion, commonly associated with type 2 diabetes (T2D) [7,8].

In the context of physiological function of the islet β -cell, evidence from several laboratories, has shown that physiological and relatively low levels of intracellular reactive oxygen species (ROS) are requisite for GSIS [9]. Studies by Leloup et al. have demonstrated that suppression of ROS generation with antioxidants resulted in altered calcium mobilization and decreased GSIS, in rodent pancreatic islets [10]. Although several intracellular processes including the mitochondrial electron-transport chain play a role in generating ROS, recent investigations have focused on the phagocyte-like NADPH oxidase (Nox2), which is a major source of extra-mitochondrial superoxide in the pancreatic β -cell. Nox2 is a trans-membrane protein complex consisting of several membrane-associated and cytosolic components. This holoenzyme complex catalyzes one electron reduction of oxygen, accompanied by oxidation of cytosolic NADPH, resulting in the generation of intracellular superoxide. The superoxide molecule is rapidly converted to the more stable hydrogen peroxide by superoxide dismutase. While the Nox2 membrane associated components include gp91^{phox} and p22^{phox}, the cytosolic components include p47^{phox}, p67^{phox}, p40^{phox} and a small G-protein, Rac1. Recent evidence from our own laboratory has demonstrated activation of Nox2 and the involvement of Rac1 in the generation of ROS, facilitating GSIS [11,12].

Insulin resistance and decreased glucose utilization, in T2D, results in chronic exposure of pancreatic β -cells to elevated levels of glucose and free-fatty acids (termed as glucolipotoxicity). Glucolipotoxicity has been demonstrated to be the central cause for several T2D complications, including β -cell dysfunction and cell death [13]. In this context, several studies have implicated hyperactivity of Rac1 and Nox2, leading to excess ROS generation and oxidative stress, to play a mediatory role in β -cell dysfunction and apoptosis [14]. Studies from our own laboratory have demonstrated increased Nox2 activity in the ZDF rat, a model for T2D and human islets exposed to glucotoxic conditions [15]. However, the signaling mechanisms that mediate the deleterious effects of glucotoxic conditions and consequent abnormal Rac1-Nox2 activity need to be further elucidated.

To further investigate the downstream effects of Nox2-derived ROS generation under glucotoxic conditions, we investigated the involvement of p38 mitogen-activated protein kinase (p38MAPK) in the metabolic dysfunction of the pancreatic β -cell. As demonstrated in various cell types, p38MAPK undergoes activation by phosphorylation at Thr180/Tyr182 residues, and mediates cellular responses to stress stimuli, which include cell proliferation, senescence and apoptosis [16]. It has been suggested that activation of p38MAPK upon prolonged exposure of β -cells to stress stimuli results in apoptosis, possibly mediated by down-stream pro-apoptotic signaling targets including p53 transcription factor and caspases [17,18]. Additionally, *in vivo* studies with mice lacking p38 δ , an isoform of p38MAPK, have shown that genetic depletion of this stress kinase resulted in prevention of high-fat diet-induced insulin resistance and β -cell dysfunction [19]. These observations indicate a mediatory role of p38MAPK in oxidative-stress induced β -cell dysfunction and cell death. Based on this evidence, in this study, we primarily focused on demonstrating the role of Rac1-Nox2 driven ROS generation in metabolic dysfunction and apoptosis of the pancreatic β -cell under glucotoxic conditions, mediated by the p38 family of stress kinases. We have utilized several pharmacological inhibitors with distinct mechanisms of action (listed in Table 1), targeting various signaling steps involved in activation of the Rac1-Nox2 enzyme complex, to study their effects on p38MAPK activation.

2. Materials and methods

2.1. Materials

Rabbit polyclonal antibody for phospho-p38MAPK (Thr 180/Tyr 182) and total-p38MAPK were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved caspase-3 and lamin B antibodies were purchased from Cell Signaling (Danvers, MA). Actin antibody was from Sigma–Aldrich (St. Louis, MO). Anti-rabbit IgG-horseradish peroxidase conjugate and enhanced chemiluminescence (ECL) kits were from Amersham Biosciences (Piscataway, NJ). IRDye® 800CW anti-rabbit was obtained from LICOR (Lincoln, NE). NSC23766 and GGTI-2147 were purchased from Calbiochem (San Diego, CA). Ehop-016 was synthesized as we described in [20]. EHT1864 was from R&D systems (Minneapolis, MN). Scrambled gp91-ds-tat (inactive) and active gp91-ds-tat were from Anaspec, Inc. (Fremont, CA). 2-Bromo-palmitate (2-BP), 2',7'-dichlorofluorescein diacetate (DCFDA), N,N'-dimethyl-9,-9'-bisacridiniumdinitrate (lucigenin), were from Sigma–Aldrich (St. Louis, MO). The rat insulin ELISA kit was purchased from American Laboratory Products Co (Wind-ham, NH). All other reagents used in the studies were obtained from Sigma–Aldrich (St. Louis, MO).

2.2. Insulin-secreting cells: isolation, culture and incubations

Pancreatic islets were isolated from 6 to 8 weeks old Sprague-Dawley male rats (Harlan laboratories, Oxford) by collagenase digestion method as previously described [15]. Rat islets and INS-1 832/13 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS supplemented with 100 IU penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol (not added in medium for rat islets) and 10 mM HEPES (pH 7.4) at 37 °C and 5% CO₂ in a humidified incubator. INS-1 832/123 cells were sub cloned twice weekly following trypsinization and passages 53–61 were used for the

studies. Following overnight incubation in 2.5 mM glucose and 2.5% serum RPMI media, the cells were treated with glucose (2.5 mM, low glucose; LG or 20 mM, high glucose; HG) in absence or presence of gp91-ds-tat peptide (2.5 μ M) or scrambled peptide (2.5 μ M), EHT 1864 (10 μ M), NSC23766 (20 μ M), Ehop-016 (5 μ M), GGTI-2147 (10 μ M) and 2-bromopalmitate (2-BP; 100 μ M) for the indicated time. After harvesting, samples were separated by SDS-PAGE and processed by Western Blotting.

2.3. Quantification of glucose-stimulated insulin release

Following overnight starving, INS-1 832/13 cells were incubated with glucose (2.5 mM, LG and 20 mM, HG) for 24 h. Cells were then pre-incubated in Krebs-Ringer Bicarbonate buffer (KRB, pH 7.4) and further stimulated with either 2.5 mM LG or 20 mM HG for 45 min at 37 °C. The supernatants were then collected and insulin released into the medium was quantified using ELISA kit, as we described previously [4].

2.4. Western Blotting

After incubation with glucose (2.5 mM, LG and 20 mM, HG) in the absence or presence of various inhibitors as indicated, cells were lysed using RIPA buffer containing protease inhibitor cocktail, 1 mM NaF, 1 mM PMSF and 1 mM Na_3VO_4 . Cell lysates (~40 μ g for INS-1 832/13 cells and ~25 μ g for rat islets) were then resolved by SDS-PAGE, and transferred onto nitrocellulose membranes. Membranes were blocked in 5% non-fat dry milk in TBS-T buffer (10 mM Tris-HCl pH-7.6, 1.5 M NaCl and 0.1% Tween 20) or 0.1% Casein in PBS and then incubated with appropriate primary antibody (cleaved Caspase-3, Lamin B, phospho-p38MAPK and total-p38MAPK) diluted with 5% non-fat dry milk in TBS-T or 0.1% Casein in PBS-T, for 1 h at room temperature. The membranes were then washed 5 $\times$ for 5 min with TBS-T or PBS-T, and then probed with the appropriate secondary antibody (Anti-rabbit IgG-horseradish peroxidase conjugate or IRDye® 800CW anti-rabbit). The immune complexes were then detected using ECL detection kit (CareStream® Imaging system or HyBlot CL® Autoradiography Film) or Odyssey® Imaging Systems. The band intensities were quantified using CareStream® Molecular Imaging Software.

2.5. Quantification of ROS generation

Total ROS levels were quantified fluorometrically using 2',-7'-dichlorofluorescein diacetate (DCFDA; Sigma-Aldrich, St. Louis, MO, USA). Briefly, 20–30 μ g of protein derived from INS-1 832/13 cells treated with glucose (LG and HG) alone or in the presence of gp91-ds-tat peptide (2.5 μ M) or scrambled peptide (2.5 μ M), was incubated with 2 μ M of DCFDA for 10 min and the resulting fluorescence was measured at 485 nm and 530 nm as excitation and emission wavelengths, respectively.

2.6. Determination of Nox2 activity

Nox2 activity was measured in lysates derived from INS-1 832/13 cells treated with glucose (LG and HG) in absence or presence of gp91-ds-tat peptide (2.5 μ M) or scrambled peptide (2.5 μ M) by luminescence assay using 20 μ M lucigenin as electron acceptor and 100 μ M NADPH. The Nox2 activity was expressed as nmoles of NADPH oxidized/min/ μ g of protein.

2.7. Statistical analysis of experimental data

Results are expressed as means with their standard errors as indicated. The statistical significance of differences between control and experimental groups was evaluated by ANOVA followed by SNK *Post Hoc* test where appropriate. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Our in vitro experimental model for glucotoxicity of the islet β -cell

The overall objective of the current study was to assess the contributory roles of Nox2 in promotion of stress kinase (p38MAPK) activation in pancreatic β -cells exposed to glucotoxic conditions. Unless stated otherwise, as a model for glucotoxicity, pancreatic β -cells were incubated in the presence of either 2.5 mM or 20 mM glucose for 24 h. We have chosen these experimental conditions since we observed a significant increase in mitochondrial dysfunction which promotes caspase 3 activation (Fig. 1; Panel A). Furthermore, as we reported recently [21], a significant increase in caspase 3-mediated lamin degradation was also seen under these conditions (Fig. 1; Panel A), culminating in metabolic dysfunction and loss in GSIS (Fig. 1; Panel B). To accomplish our objectives, we utilized gp91-ds-tat, a specific inhibitor of Nox2, and its scrambled peptide (as a negative control) to delineate the regulatory roles of Nox2 in HG-induced p38MAPK activation. Furthermore, we tested specific inhibitors of Rac1, which is a component of the Nox2 holoenzyme to further assess the contributory roles of the Rac1-Nox2 cascade in the induction of islet dysfunction under conditions of glucotoxicity. For brevity, specific details of pharmacological inhibitors and their modes of action are described in Table 1.

3.2. gp91-ds-tat peptide, a specific inhibitor of Nox2, but not its inactive congener, markedly attenuates HG-induced Nox2 activation and ROS generation in INS-1 832/13 cells

At the outset, we determined whether HG conditions induce Nox2 activation in INS-1 832/13 cells. To accomplish this, we quantified Nox2 activity in these cells exposed to LG (2.5 mM) or HG (20 mM) conditions for 24 h. Data in Fig. 2 (Panel A) demonstrate a significant increase in Nox2 activity under these experimental conditions. Further, gp91-ds-tat peptide, a specific inhibitor of Nox2 activation, markedly attenuated HG-induced Nox2 activation. These inhibitory effects of gp91-ds-tat peptide were specific, since its inactive analog failed to inhibit HG-induced Nox2 activity in these cells. Compatible with these findings are our observations, which suggested a significant increase in ROS generation in INS-1 832/13 cells incubated under HG conditions (Fig. 2; Panel B). In addition, HG-induced ROS generation was significantly suppressed by gp91-ds-tat peptide (bar 4 vs. bar 6), but not by its inactive analog. Taken together, our findings (Fig. 2) indicate a significant increase in Nox2 activity and associated ROS generation in INS-1 832/13 cells following exposure to HG conditions.

3.3. Nox2 activation is upstream to p38MAPK activation in pancreatic β -cells exposed to HG conditions

We next asked whether HG-induced activation of Nox2 and associated generation of ROS promote activation of p38MAPK, which has been implicated in cellular dysfunction under the duress of HG conditions in multiple cell types, including the islet β -cell. To address this, we quantified p38MAPK phosphorylation under basal or HG conditions in the absence or presence of gp91-ds-tat peptide. Data depicted in Fig. 3 demonstrate significant increase in p38MAPK phosphorylation under HG conditions, which was suppressed significantly by gp91-ds-tat peptide (Panel A). In line with our observations in Figs. 1 and 2, the inactive analog of gp91-ds-tat peptide exerted no inhibitory effects on HG-induced p38MAPK activation (Panel A). Pooled data from multiple experiments are provided in Panel B. Together, these data suggest Nox2 activation is upstream to p38MAPK activation in the cascade of events leading to loss of β -cell function, including GSIS under the duress of HG conditions.

3.4. Inhibition of guanine nucleotide association with Rac1 results in inhibition of HG-induced p38MAPK activation

The Nox2 holoenzyme complex is comprised of membranous (gp91^{phox}, p22^{phox}) and cytosolic (p47^{phox}, p67^{phox}) and the small molecular weight G-protein Rac1 [7,22]. Recent evidence from our laboratory has suggested that pharmacological or molecular biological inhibition of Rac1 prevents the activation of Nox2 under the duress of HG and high lipid exposure conditions [22,23]. Therefore, in the next series of experiments, we employed specific inhibitors of Rac1 function (Table 1) to further assess the regulatory roles of this G-protein in HG-induced p38MAPK activation in INS-1 832/13 cells. The first inhibitor we tested was EHT 1864, a small molecule inhibitor, which inhibits Rac1 activation *via* inhibition of guanine nucleotide association. Consequently, EHT 1864 retains Rac1 in an inactive/inert conformation [24,25]. Data in Fig. 4 (Panel A) demonstrate a marked reduction of HG-induced p38MAPK activation by EHT 1864, thus suggesting a regulatory role for Rac1 in the cascade of events leading to HG-induced p38MAPK activation. Data from multiple experiments confirming the inhibitory effects of EHT 1864 are provided in Fig. 4; Panel B.

3.5. Inhibition of GDP/GTP exchange on Rac1 suppresses HG-induced p38MAPK in pancreatic β -cells

It is well established that biological activation-inactivation of small G-proteins (*e.g.*, Rac1) is mediated by a variety of highly specific regulatory proteins/factors [7]. One such regulatory class of proteins are the guanine nucleotide exchange factors (GEFs), which promote activation of G-proteins *via* GDP/GTP exchange. Recent studies from our laboratory have identified two GEFs for Rac1 in pancreatic β -cells. They are Tiam1 [4] and Vav2 [20]. Using siRNA and pharmacological approaches (*e.g.*, NSC23766 for Tiam1 and Ehop-016 for Vav2), we defined novel roles for Tiam1-Rac1 and Vav2-Rac1 signaling pathways in islet function, including GSIS [4, unpublished data]. More recent investigations from our laboratory have implicated the Tiam1-Rac1 signaling pathway in the metabolic dysregulation of pancreatic β -cells under the duress of glucolipotoxic, ceramide and pro-

inflammatory cytokine exposure conditions [22,23,26]. We also reported regulatory roles of the Tiam1-Rac1 axis at the level of Nox2 activation [23,26]. Therefore, in the next set of studies, we examined the contributory roles of Tiam1-Rac1 and Vav2-Rac1 axes on HG-induced p38MAPK activation.

Data depicted in Fig. 5; Panel A demonstrates significant inhibition of HG-induced p38MAPK activation by NSC23766 in INS-1 832/13 cells. NSC23766 exerted no effects on basal p38MAPK. Data from multiple studies are pooled and presented in Fig. 5; Panel B. It is noteworthy that NSC23766 completely abolished HG-induced p38MAPK activation in normal rodent islets without significantly affecting basal activation (Fig. 5; Panel C). Pooled data from multiple studies are provided in Fig. 5; Panel D. Taken together, our findings in INS-1 832/13 cells and normal rodent islets indicate that functional inactivation of Tiam1-Rac1 signaling pathway, presumably, upstream to Nox2 activation [22,23,26], relieves HG-induced stress kinase (p38MAPK) activation.

In the next series of studies, we asked whether the Vav2-Rac1 signaling module mediates HG-induced p38MAPK activation in pancreatic β -cells. To accomplish this, we utilized Ehop-016, a specific inhibitor of Vav2-mediated activation of Rac1 [20]. Data in Fig. 6; Panel A demonstrate a significant inhibition of p38MAPK activation by Ehop-016 under glucotoxic conditions. However, unlike other Rac1 inhibitors tested above, we observed a significant stimulation of p38MAPK by Ehop-016 under basal glucose conditions (Fig. 6; Panel B; bar 1 vs. bar 2). Data accrued in multiple studies are included in Panel B. We observed similar inhibitory effects of Ehop-016 on p38MAPK phosphorylation in normal rodent islets exposed to glucotoxic conditions (Fig. 6; Panels C and D). Furthermore, akin to our findings in INS-1 832/13 cells (Fig. 6; Panels A and B) Ehop-016 significantly increased p38MAPK phosphorylation in rat islets under basal glucose conditions (Fig. 6; Panels C and D), raising a potential possibility that it might be regulating additional signaling mechanisms (see Section 4). Taken together, our findings shown in Figs. 5 and 6 suggest novel regulatory roles of GEFs (Tiam1 and Vav2) in Rac1-mediated activation of p38MAPK in pancreatic β -cells exposed to glucotoxic conditions. To the best of our knowledge this is the first evidence in support of involvement of more than one GEF in modulation of G-protein-mediated metabolic dysfunction of the islet β -cell. We speculate that these two GEFs might mediate HG-induced activation of Rac1-sensitive Nox2 (see Section 4).

3.6. Geranylgeranylation of Rac1 is not essential for HG-induced p38MAPK activation in INS-1 832/13 cells

It is well established in multiple cell types, including in our own observations in the islet β -cell, that small molecular weight G-proteins (*e.g.*, Rac1) undergo a series of post-translational modifications at their C-terminal cysteine residues (prenylation, carboxymethylation and palmitoylation). It has been shown that these modifications increase the hydrophobicity of modified G-proteins, thereby promoting their membrane targeting and effector activation [7,27–29]. Recent pharmacological and molecular biological evidence from our laboratory in human islets, rat islets and INS-1 832/13 cells implicate novel regulatory roles for these modifications in islet function, including GSIS [28–30]. Herein, we first investigated whether geranylgeranylation of Rac1 is critical for

promoting p38MAPK activation in INS-1 832/13 cells exposed to HG conditions. To address this, we quantified HG-induced p38MAPK phosphorylation in INS-1 832/13 cells incubated in the absence or presence of GGTI-2147, a known inhibitor of geranylgeranylation of Rac1. Data in Fig. 7 demonstrate no significant effects of this inhibitor on glucose-induced p38MAPK (Panel A). Pooled data from multiple experiments are included in Fig. 7; Panel B. Taken together, these findings indicate that geranylgeranylation of Rac1 may not be necessary for HG-induced p38MAPK activation.

3.7. Protein palmitoylation is requisite for HG-induced p38MAPK in INS-1 832/13 cells

In the last set of experiments, we determined whether protein (e.g., Rac1) palmitoylation underlies HG-induced p38MAPK activation. To address this, we employed 2-bromopalmitate (2-BP), a selective inhibitor of palmitoyl transferase, which incorporates palmitate into the cysteine residues, which are upstream to the prenylated cysteine [7]. Data in Fig. 8; Panel A (representative Western blot) and Panel B (pooled data from multiple studies) demonstrate significant attenuation of HG-induced p38MAPK activation by 2-BP (Fig. 8; Panel B) suggesting that protein palmitoylation represents one of the signaling events leading to HG-mediated stress kinase activation and metabolic dysregulation of the islet β -cell.

4. Discussion

It is well established that chronic exposure of pancreatic β -cells to elevated glucose concentrations (glucotoxicity) results in severe metabolic dysregulation including inhibition of GSIS [13,14]. The current study was based on the hypothesis that HG induces Nox2 activation leading to subsequent activation of the downstream stress kinase signaling pathway culminating in the loss of GSIS in pancreatic β -cells. We have tested this hypothesis using small molecule inhibitors of Nox2 and Rac1 activation (Table 1) in insulin-secreting pancreatic β -cells and normal rodent islets. Salient findings are discussed below.

Our current findings suggest that high glucose-induced p38MAPK activation is a consequence of increased Nox2 activation and associated ROS generation, since gp91-ds-tat peptide, but not its inactive (sgp91-ds-tat, scrambled) analog, markedly reduced HG-induced Nox2 activation (Fig. 2A), ROS generation (Fig. 2B) and p38MAPK activation (Fig. 3A and B). Data accrued from previously published studies and our current investigations also suggest that p38MAPK activation by HG results in increased β -cell apoptosis. For example, recent studies have provided evidence to suggest that glucotoxic conditions increase stress kinase activation, including p38MAPK [18,31] resulting in increased apoptosis. In support of this, our recent findings have also suggested that glucotoxic conditions (identical to those employed in the current studies) significantly attenuated HG-stimulated insulin secretion and loss in metabolic cell viability [32,33]. Compatible with these findings are our current data (Fig. 1) indicating significant activation of caspase-3 and lamin B degradation in INS-1 832/13 cells under conditions of glucotoxicity.

Several lines of evidence in multiple cell types, specifically in the islet β -cell, suggests that Nox2 activation results in the generation of ROS under conditions of glucolipotoxicity and exposure to pro-inflammatory cytokines (IL-1 β , TNF α and IFN γ) and biologically active

sphingolipids (ceramide) [22,23,26]. The majority of the studies employed relatively selective inhibitors of Nox2, such as apocynin and DPI, to deduce its roles in the onset of cellular dysfunction [34,35]. In the current studies, we have utilized gp91-ds-tat peptide, a specific inhibitor of Nox2; this peptide has been shown to interfere with binding of the cytosolic core of Nox2 with the membranous gp91^{phox} [36]. In addition, we have employed a scrambled peptide analog, as a negative control, which exerted no effect on HG-induced Nox2 activation. Thus, our findings are the first to document the regulatory role of Nox2 in the generation of ROS under HG exposure conditions. We also have demonstrated that gp91-ds-tat peptide, but not its inactive analog, attenuated HG-induced p38MAPK, thereby providing the first evidence that links Nox2 activation to p38MAPK activation in insulin-secreting cells under glucotoxic conditions.

What then are potential mechanisms underlying HG-induced activation of Nox2 in pancreatic β -cells? It is well established that Nox2 activation results from translocation of cytosolic core of Nox2 subunits to the membrane for association with the membrane core of Nox2 proteins resulting in holoenzyme assembly and enzyme activation. Evidence from our laboratory indicates that activation of cytosolic Rac1, a member of Nox2 holoenzyme, represents a critical regulatory step involved in Nox2 activation induced by glucolipotoxic and cytokine exposure conditions [7,14]. Several recent studies have demonstrated that Rac1 activation is regulated by GEFs (Tiam1, Vav2 and Trio; [7]). Indeed, several recent *in vitro* and *in vivo* investigations have demonstrated that inhibition of the Tiam1/Rac1 signaling axis with NSC23766 prevents cellular dysfunction in islet β -cells, retinal endothelial cells and cardio-myocytes under conditions of glucolipotoxicity and diabetes [14,37,38]. Herein, we have provided further evidence to suggest that the Tiam1-Rac1 signaling pathway controls HG-induced p38MAPK activation, thus supporting the hypothesis that Tiam1 controls stress kinase activation in glucotoxic conditions. In addition, our current findings suggest a signaling role for Vav2, a known GEF for Rac1, in HG-induced p38MAPK activation. Recent observations from our laboratory have indicated that Vav2 is expressed in human islets, rodent islets and INS-1 832/13 cells, and that inhibition of Vav2 function, *via* pharmacological (Ehop-016) and siRNA approaches, attenuates glucose-induced Rac1 activation and insulin secretion [unpublished data]. In the current studies, we demonstrated that Ehop-016 blocks HG-induced p38MAPK activation, thus raising a possibility that HG-mediated effects on Rac1 activation and downstream p38MAPK activation are regulated by both Tiam1 and Vav2. Our findings gain further support from recent reports of dual regulation of Rac1 by Tiam1 and Vav2 in eliciting activation of Nox2. Liu et al. have recently discovered a novel polarity complex that directs localized Rac1 activation required for downstream ROS production. They reported that Vav2 is essential for the attainment of the active conformation of Rac1 (Rac1-GTP), whereas, Tiam1 functions as an adaptor in a VE-cadherin-p67^{phox}-Par3 polarity complex that directs localized activation of Rac1 [39]. Further, they reported that compromised Tiam1 function culminates in the disruption of redox signaling both *in vitro* and *in vivo*. Based on these findings, these authors concluded that both Vav2 and Tiam1 mediate regulation of Rac1 by linking components of the polarity complex to NADPH oxidase. Together, using pharmacological inhibitors of Rac1 activation (EHT 1864, NSC23766 and Ehop-016), we demonstrate a critical role for this small G-protein in the induction of stress kinase activation under HG exposure conditions in insulin-

secreting cells. It is noteworthy that Ehop-016 exerted stimulatory effects on p38MAPK in normal rat islets and INS-1 832/13 cells under basal glucose (2.5 mM) exposure conditions while inhibiting HG-induced p38MAPK (Fig. 6; Panels C and D). The reasons for such effects of this Rac1 inhibitor on basal p38MAPK are unknown at this time. We speculate that such effects might include activation of other Rho subfamily of G-proteins (*e.g.*, Rho) as demonstrated by Montalvo-Ortiz and associates recently [20]. Additional studies are needed to further address these findings.

The next question we answered from the current studies was if post-translational modifications of Rac1 (geranylgeranylation and palmitoylation) are necessary for HG-induced activation of p38MAPK. It is widely felt that small G-proteins undergo a series of post-translational modifications at their C-terminal residues; these include prenylation, carboxylmethylation and palmitoylation [7,27–29]. Using a variety of experimental approaches, including specific inhibitors, dominant negative mutants or siRNAs, we have demonstrated the requisite nature for these signaling steps in islet β -cell function, including GSIS [28,29]. We report herein that GGTI-2147, a known inhibitor of Rac1 function including membrane targeting [29], fails to exert significant effects on HG-induced p38MAPK activation in INS-1 832/13 cells. These findings suggest that Rac1 can mediate activation of p38MAPK without the requisite geranylgeranylation step under the duress of glucotoxic conditions. These findings are somewhat paradoxical since geranylgeranylation step has been implicated in optimal functioning of Rac1. However, recent data from the laboratory of Khan and associates support the notion that small GTPases (Rac1, Cdc42 and RhoA) can undergo activation without being geranyl-geranylated. These investigators demonstrated high levels of active Rac1, Cdc42 and RhoA in macrophages from GGTase-1 deficient mice. In addition, GGTase-I deficiency significantly accelerated p38MAPK and NF- κ B activation resulting in increased production of pro-inflammatory cytokines [40]. Therefore, additional studies are needed to determine potential alterations in the structure and function of protein prenyl transferases in islet β -cells exposed to glucolipotoxic conditions and associated consequences in the activation status of candidate G-proteins. Studies are underway to further understand these cellular events in β -cells exposed to various pathological stimuli. Our findings suggest that inhibition of protein palmitoylation (2-BP) results in inhibition of HG-induced p38MAPK activation. While our studies do not directly address whether or not Rac1 palmitoylation is increased under HG conditions, they, however, demonstrate that palmitoylation of specific proteins is necessary for regulation of stress kinase activation. There is paucity of published information supporting that Rac1 is palmitoylated *in vivo*. Using novel experimental methodology, Navarro-Lérida et al. have recently reported that Rac1 undergoes palmitoylation at cysteine 178, which results in Rac1 movement for stabilization at actin cytoskeleton-linked ordered membrane regions. They also demonstrated that Rac1-null cells or a palmitoylation-deficient mutant of Rac1 exhibited substantially higher content of disordered membrane domains, and defects in cell spreading and migration [41]. Future investigations will determine if HG conditions do indeed promote palmitoylation of Rac1 to promote activation of Nox2 and p38MAPK.

What are potential mechanisms of β -cell dysfunction induced by increase in cytosolic and mitochondrial ROS? We propose that increased cytosolic ROS in consequence to high glucose-induced activation of Nox2 promotes mitochondrial dysregulation including

activation of specific signaling cascades leading to increase in mitochondrial ROS generation. This, in turn, results in alterations in mitochondrial membrane properties (permeability pore transition) leading to release of proapoptotic factors (cytochrome C) into the cytosolic compartment resulting in activation of caspase-mediated degradation of cellular proteins, including nuclear lamins (Fig. 1). In this context, we have recently demonstrated temporal relationships between cytosolic and mitochondrial ROS generation in retinal endothelial cells exposed to glucotoxic conditions. We reported that activation of cytosolic Nox2 represents an early signaling event, which, in turn, promotes the generation of mitochondrial ROS. These findings were also reproduced in retina from diabetic animal models [37]. Additional studies are needed in the islet β -cell to confirm these postulations.

In conclusion, we have provided the first pharmacological evidence to implicate key regulatory roles for Rac1-Nox2 signaling pathway in HG-induced p38MAPK activation and attenuation of GSIS from pancreatic β -cells. Our findings also suggest that Rac1 activation, observed under these conditions, requires the intermediacy of GEFs, such as Tiam1 and Vav2. While it remains to be further examined, a signaling step involving post-translational palmitoylation of key signaling proteins (Rac1) appears to play a contributory role in HG-induced p38MAPK activation. Lastly, our findings also suggest that geranylgeranylation of Rac1 may not be necessary for activation of this signaling cascade. Based on these observations, we propose a working model (Fig. 9) for potential involvement of Nox2-mediated, Rac1 dependent activation of p38MAPK in the islet β -cell under conditions of glucotoxicity. More importantly, our model identifies potential therapeutic targets in this signaling pathway. It is hoped that future studies will be directed toward the development of optimized small molecule compounds that can be tested for halting metabolic defects of the islet β -cell in animal models of obesity and diabetes.

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Abbreviations:

2-BP	2-bromopalmitate
Cdc42	cell division control protein 42
DCFDA	2',-7'-dichlorofluorescein diacetate
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GGTI	geranylgeranyl transferase inhibitor

GSIS	glucose stimulated insulin secretion
HG	high glucose
LG	low glucose
Nox2	NADPH oxidase 2
p38MAPK	p38 mitogen activated protein kinase
Rac1	Ras-related C3 botulinum toxin substrate 1
ROS	reactive oxygen species
RhoA	Ras homolog gene family, member A

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Fig. 1.

Glucotoxic conditions promote caspase 3 activation, lamin B degradation and loss in GSIS in INS-1 832/13 cells.

Panel A: INS-1 832/13 cells were incubated in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 24 h and protein lysates (~40 mg) were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was probed for cleaved (active) caspase 3 and lamin B, and immune complexes were identified using ECL detection kit. To check equal protein loading, the membranes were stripped and reprobed for actin. A representative blot is shown here. These findings confirm our original observations in [21].

Panel B: INS-1 832/13 cells were cultured in the presence of low (2.5 mM; LG) or high (20 mM; HG) glucose for 24 h following which they were stimulated with low (2.5 mM) or high (20 mM) glucose for 45 min. Insulin released into the medium was quantified by ELISA (as described in Section 2). The data are expressed as insulin release (ng/ml) and are mean $\pm$ SEM from two independent experiments. * $P < 0.05$ vs. LG under 24 h low glucose treatment; ** $P < 0.05$ vs. HG under 24 h low glucose treatment.

Fig. 2.

gp91-ds-tat peptide a specific inhibitor of Nox2, but not its inactive analog, inhibits HG-induced Nox2 activation and ROS generation in INS-1 832/13 cells.

Panel A: INS-1 832/13 cells were pre-incubated with scrambled peptide (2.5 μM) or gp91-ds-tat peptide (2.5 μM) for 1 h and then treated with LG and HG in the continuous presence of scrambled peptide (2.5 μM) or gp91-ds-tat peptide (2.5 μM) for 24 h. Nox2 activity was quantified as described under Methods, and the activity was expressed as nmoles of NADPH oxidized/min/μg of protein. * $P < 0.05$ vs. low glucose. ** $P < 0.05$ vs. high glucose alone or in the presence of inactive peptide (mean $\pm$ SEM; $n = 6$).

Panel B: INS-1 832/13 cells were cultured and treatments were performed as described under Panel A. Intracellular levels of ROS were then measured using DCF-DA assay as described under Methods. ROS generation was expressed as fold change over 2.5 mM glucose. * $P < 0.05$ vs. low glucose. ** $P < 0.05$ vs. high glucose alone or in the presence of inactive peptide (mean $\pm$ SEM; $n = 6$).

Fig. 3.

Glucose induced p38MAPK activation is inhibited by gp91-ds-tat.

Panel A: INS-1 832/13 cells were pre incubated with scrambled peptide (2.5 μ M) or gp91-ds-tat peptide (2.5 μ M) for 1 h and then further treated with glucose (LG and HG) in the presence of scrambled peptide (2.5 μ M) or gp91-ds-tat peptide (2.5 μ M) for 24 h. Lysate proteins were then separated by SDS-PAGE, and levels of phosphorylated p38MAPK were determined by Western Blotting. After separation, proteins were transferred onto nitrocellulose membranes. The membranes were then blocked and probed with antibody raised against phosphorylated p38MAPK followed by incubation with anti-rabbit secondary antibody. The immune complexes were then detected using ECL detection kit. To ensure equal protein loading, phospho-p38MAPK was normalized with total p38MAPK, as loading control.

Panel B: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean $\pm$ SEM from three independent experiments and expressed as fold change of the ratios between phospho-p38MAPK and total p38MAPK. * P < 0.05 vs. low glucose, ** P < 0.05 vs. high glucose alone or in presence of inactive peptide.

Fig. 4.

Glucose-induced p38MAPK activation is inhibited by EHT 1864.

Panel A: Following treatment with glucose (LG and HG) in the absence or presence of EHT 1864 (10 μM) for 24 h, INS-1 832/13 cells were lysed and proteins were separated by SDS-PAGE. Lysate proteins were then transferred onto nitrocellulose membranes. The membranes were then blocked and incubated with antibody against phosphorylated p38MAPK followed by incubation with anti-rabbit secondary antibody. The same blots were stripped and reprobed with antibody raised against total p38MAPK.

Panel B: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean ± SEM from three independent experiments and expressed as fold change of the ratios between phospho-p38MAPK and total-p38MAPK. * $P < 0.05$ vs. low glucose, # $P < 0.05$ vs. high glucose.

Fig. 5.

NSC23766, a known inhibitor of Tiam1-Rac1 signaling pathway suppresses HG-induced p38MAPK activation in INS-1 832/13 cells and normal rodent islets.

Panel A: INS-1 832/13 cells, pre-incubated overnight with NSC23766 (20 μM), were further treated with glucose (LG and HG) in the absence or presence of NSC23766 (20 μM) for 24 h. Cells were then lysed and proteins were resolved by SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes. The membranes were then blocked and probed with antibody for detecting phosphorylated p38MAPK. The same blots were stripped and reprobed with antibody against total p38MAPK.

Panel B: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean ± SEM from three independent experiments and expressed as fold change of the ratios between phospho-p38MAPK and total-p38MAPK. * $P < 0.05$ vs. low glucose, # $P < 0.05$ vs. high glucose.

Panel C: After isolation, rat pancreatic islets were pre incubated overnight with NSC23766 (20 μM) and further treated with glucose (LG and HG) in the absence or presence of NSC23766 (20 μM) for 24 h. Lysate proteins (~25 μg) were then resolved by SDS-PAGE, and analyzed by Western Blotting, as described above, for detecting phosphorylated and total p38MAPK.

Panel D: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean ± SEM from three independent experiments and expressed as fold change of the ratios between phospho-p38MAPK and total p38MAPK. * $P < 0.05$ vs. low glucose, # $P < 0.05$ vs. high glucose.

Fig. 6.

Ehop-016, a specific inhibitor of Vav2-mediated activation of Rac1 attenuates HG-induced p38MAPK activation in INS-1 832/13 cells and normal rodent islets.

Panel A: INS-1 832/13 cells, pre incubated overnight with Ehop-016 (5 μM) were further treated with LG and HG in the absence or presence of Ehop-016 (5 μM) for 24 h. Cell lysate proteins were then separated and analyzed by Western Blotting as described above. After separation by SDS-PAGE, proteins were transferred onto nitrocellulose membranes. The membranes were blocked and probed with anti-phosphorylated p38MAPK followed by incubation with anti-rabbit secondary antibody. The same blots were stripped and reprobed with anti-total p38MAPK.

Panel B: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean ± SEM from five independent experiments and expressed as fold change of the ratios between phospho-p38MAPK and total-p38MAPK. * $P < 0.05$ vs. low glucose, # $P < 0.05$ vs. high glucose.

Panel C: Pancreatic islets isolated from normal rodents, were pre-incubated with Ehop-016 (5 μM) overnight and further incubated with glucose (LG and HG) in the continuous absence or presence of Ehop-016, for 24 h. Islets were then lysed and lysate proteins were separated by SDS-PAGE. Phosphorylated and total p38MAPK were then detected by Western Blot analysis.

Panel D: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean ± SEM from three independent studies and expressed as fold change in the ratios between phosphorylated and total p38MAPK. * $P < 0.05$ vs. low glucose, # $P < 0.05$ vs. high glucose.

Fig. 7.

Inhibition of geranylgeranylation by GGTI-2147 fails to exert significant effects on HG-induced p38MAPK activation.

Panel A: INS-1 832/13 were treated with LG and HG in the absence or presence of GGTI-2147 (10 μ M) for 24 h. Cells were then lysed and proteins were separated by SDS-PAGE, and then transferred onto a nitrocellulose membrane. The membranes were then blocked and probed with anti-phosphorylated p38MAPK followed by incubation with anti-rabbit secondary antibody. The same blots were stripped and reprobed with anti-total p38MAPK.

Panel B: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean $\pm$ SEM from three independent experiments and expressed as fold change of the ratios between phospho-p38MAPK and total-p38MAPK. * P < 0.05 vs. low glucose.

Fig. 8.

Inhibition of protein palmitoylation by 2-bromopalmitate results in inhibition of HG-induced p38MAPK activation.

Panel A: INS-1 832/13 were treated with LG and HG in the absence or presence of 2-BP (100 μM) for 24 h. Cells were then lysed and proteins were separated by SDS-PAGE, and then transferred onto a nitrocellulose membrane. The membranes were then blocked and probed with anti-phosphorylated p38MAPK followed by incubation with anti-rabbit secondary antibody. The same blots were stripped and reprobed with anti-total p38MAPK.

Panel B: Band intensities of phospho-p38MAPK and total-p38MAPK were quantified by densitometric analysis. Results are shown as mean $\pm$ SEM from three independent experiments and expressed as fold change of the ratios between phospho-p38MAPK and total-p38MAPK. * P < 0.05 vs. low glucose, # P < 0.05 vs. high glucose.

Fig. 9.

Our proposed model for Rac1-mediated, Nox2-dependent activation of p38MAPK in pancreatic β -cells under glucotoxic conditions. Our current findings implicate Rac1 in Nox2-mediated activation of p38MAPK in pancreatic β -cells under the duress of exposure to HG conditions. We demonstrated that inhibition of Rac1 activation or function at the level of guanine nucleotide exchange (EHT 1864, NSC23766 and Ehop-016) suppresses HG-induced activation of p38MAPK. We also provided evidence that post-translational acylation (2-BP), but not geranylgeranylation (GGTI-2147) is necessary for HG-induced Rac1-mediated activation of p38MAPK. Lastly, gp91-ds-tat peptide, a specific inhibitor of Nox2, markedly attenuated HG-induced Nox2 activation, ROS generation and p38MAPK activation. Together, our findings provide the first evidence to support our hypothesis that HG exposure conditions promote activation of Rac1, a key member of Nox2 holoenzyme, which, in turn, promote the activation of Nox2 culminating in the generation of excessive ROS. An increase ROS leads to the onset of mitochondrial dysfunction as evidenced by caspase-3 activation, which has been shown to mediate proteolytic degradation of lamin-B [21], to result in loss in metabolic function of the islet β -cell, including loss in GSIS.

Table 1

Small molecule inhibitors used in the current studies.

Inhibitor used	Modes of action
gp91-ds-tat peptide	Inhibits Nox2 activation by inhibiting holoenzyme assembly [33].
EHT 1864	Inhibits guanine nucleotide binding and activation of Rac1 [24,25]
NSC23766	Inhibits Tiam1-mediated activation of Rac1 [4]
Ehop-016	Attenuates Vav2-mediated activation of Rac1 [20]
GGTI-2147	Inhibits geranylgeranylation and membrane targeting of Rac1 [29]
2-Bromopalmitate	Inhibits palmitoylation and membrane anchoring of Rac1 [27]