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Original Paper

RhoG-Rac1 Signaling Pathway Mediates Metabolic Dysfunction of the Pancreatic Beta-Cells Under Chronic Hyperglycemic Conditions

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Key Words
Islet beta-cell • Metabolic stress • Diabetes • Beta-cell dysfunction • Mitochondrial dysregulation • Rac1 • RhoG

Abstract
Background/Aims: Published evidence suggests regulatory roles for small G proteins (Cdc42 and Rac1) in glucose-stimulated insulin secretion (GSIS) from pancreatic beta-cells. More recent evidence suggests novel roles for these G proteins, specifically Rac1, in the induction of metabolic dysfunction of the islet beta-cell under the duress of a variety of stress conditions. However, potential upstream regulators of sustained activation of Rac1 have not been identified in the beta-cell. Recent studies in other cell types have identified RhoG, a small G protein, as an upstream regulator of Rac1 under specific experimental conditions. Herein, we examined putative roles for RhoG in islet beta-cell dysregulation induced by glucotoxic conditions. ***Methods:*** Expression of RhoG or GDIγ was suppressed by siRNA transfection using the DharmaFect1 reagent. Subcellular fractions were isolated using NE-PER Nuclear and Cytoplasmic Extraction Reagent kit. The degree of activation of Rac1 was assessed using a pull-down assay kit. Extent of cell death was quantified using a Cell Death Detection ELISA^{plus} kit. ***Results:*** RhoG is expressed in human islets, rat islets, and clonal INS-1 832/13 cells. siRNA-RhoG markedly attenuated sustained activation of Rac1 and caspase-3 in INS-1 832/13 cells exposed to hyperglycemic conditions (20 mM; 24 hours). In a manner akin to Rac1, which has been shown to translocate to the nuclear fraction to induce beta-cell dysfunction under metabolic stress, a significant increase in the association of RhoG with the nuclear fraction was observed in beta-cells under the duress of metabolic stress. Interestingly, GDIγ, a known regulator of RhoG, remained associated with non-nuclear fraction under conditions RhoG and Rac1 translocated to the membrane. Lastly, siRNA-RhoG modestly attenuated

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pancreatic beta-cell demise induced by high glucose exposure conditions, but such an effect was not statistically significant. ***Conclusion:*** Based on these data we conclude that RhoG-Rac1 signaling module plays critical regulatory roles in promoting mitochondrial dysfunction (caspase-3 activation) of the islet beta cell under metabolic stress.

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Introduction

It is well established that chronic exposure of pancreatic beta-cells to diabetogenic conditions (high glucose and/or saturated fatty acids, sphingolipids, and pro-inflammatory cytokines) leads to the induction of metabolic (oxidative, nitrosative, and endoplasmic reticulum) stress, which, in turn, results in stress kinase activation, mitochondrial dysregulation, and nuclear collapse culminating in the demise of the effete beta-cell. Several recent reviews have highlighted observations from *in vitro* and *in vivo* studies from multiple laboratories to affirm this model [1-8]. In further support of such an experiment formulation, multiple lines of evidence indicated novel regulatory roles of Rac1, a small G protein, in metabolic stress-induced beta-cell dysfunction and demise [8-10]. It has been shown that metabolic stress promotes activation of phagocyte-like NADPH-oxidase (Nox2), which, in turn, promotes the generation of intracellular reactive oxygen species (ROS) and increased intracellular oxidative stress culminating the activation of p38, JNK1/2, p53, and mitochondrial dysfunction [9-13]. It has also been demonstrated that Rac1, which is a member of the cytosolic core of Nox2, is activated under metabolic stress, leading to its translocation to the membrane for association with the membranous core of Nox2 to enable holoenzyme assembly [11, 12]. These findings were replicated in *in vitro* and *in vivo* models of glucotoxicity and in islets derived from human subjects with type 2 diabetes [11]. In further support of this model, studies have demonstrated that pharmacological inhibition of Rac1 (NSC23766, Ehop-016) or Nox2 (apocynin, *gs-tat*-peptides) markedly attenuated beta-cell dysfunction under metabolic stress conditions [9, 11-14]. Despite this evidence, the identity of putative regulators of Rac1 activation in beta-cell models of metabolic stress has not been examined thus far.

RhoG, a small G protein, with significant homology to other G proteins, namely Cdc42 (62% homology) and Rac1 (72% homology), has been shown as a modulator of Rac1 function [15]. Based on the evidence for its roles in the regulation of Rac1-sensitive signaling pathways in other cell types [16-21] , we undertook the current investigation to determine putative regulatory roles of RhoG in Rac1-mediated metabolic dysregulation of pancreatic beta-cells exposed to chronic hyperglycemic (glucotoxicity) conditions. We present evidence in support of the hypothesis that the RhoG-Rac1 signaling axis mediates metabolic dysfunction in INS-1 832/13 cells exposed to glucotoxic conditions.

Materials and Methods

Materials

Antibodies for RhoG, GDIγ, GAPDH, and HRP-conjugated secondary antibodies were from Santa Cruz Biotechnology (Dallas, TX, USA and Cell Signaling, Danvers, MA, USA). Antisera directed against cleaved caspase-3 (active form) and lamin B were obtained from Cell Signaling (Danvers, MA, USA). Rac1 antibody was from EMD Millipore (St. Louis, MO, USA). The on-target RhoG-siRNA SMARTpool, GDIγ-siRNA, non-targeting control-siRNA, and DharmaFect1 reagent were from Dharmacon (Lafayette, CO, USA). The antibody for β-actin was obtained from Sigma Aldrich (St. Louis, MO, USA). The NE-PER® Nuclear and Cytoplasmic Extraction Kit was purchased from Thermo Fisher Scientific [Carlsbad, CA]. The pull-down assay kit for the quantification of Rac1 activation was from Cytoskeleton (Denver, CO, USA). The cell permeable C2-ceramide was from Cayman Chemicals (Ann Arbor, MI, USA).

INS-1 832/13 cells, rat islets, and human Islets

INS-1 832/13 cells were kindly provided by Prof. Chris Newgard. Islets were isolated from normal 10-week old male Sprague-Dawley rats by the collagenase digestion method [22, 23]. All protocols were reviewed and approved by the Wayne State University and John D. Dingell VA Medical Center Institutional Animal Care and Use Committees. Human islets were from Prodo Laboratories (Aliso Viejo, CA). Studies involving human islets were approved by the Biosafety Committee at the John D. Dingell VA Medical Center.

Cell culture and experimental conditions

INS-1 832/13 cells were cultured in RPMI-1640 medium containing 10% FBS supplemented with 100 IU/ ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μM 2-mercapto-ethanol and 10 mM HEPES (pH 7.4). The cultured cells were sub-cloned twice weekly following trypsinization [11, 13]. Cells were starved overnight in a low glucose /low serum growth medium (2.5 mM glucose and 2.5% FBS) prior to the treatment with different concentrations of glucose (2.5 or 20 mM) for various time points, as indicated in the text. In select experiments, in addition to high glucose, putative effect of a cell-permeable C2-ceramide (50 μM) was tested on the expression of RhoG as described in the following sections.

Western blotting

Following incubation under specific experimental conditions, the cells were homogenized and lysed in RIPA buffer containing protease and phosphatase inhibitors. Lysates (30-50 μg proteins) were separated by SDS-PAGE and electro-transferred onto the nitrocellulose membrane. The membranes were blocked with 3% BSA in PBS-T at room temperature for 1 hour and were then incubated with corresponding primary antibodies (1:3000 dilution for RhoG and lamin B; 1:10,000 dilution for β-actin and GAPDH and 1:1000 dilution for cleaved caspase-3; all dilutions in 1.5% BSA in PBS-T) overnight at 4°C. The membranes were then washed (3 times for 5 minutes each) with PBS-T and probed with appropriate HRP-conjugated secondary antibody in 1.5% BSA in PBS-T at room temperature for 1 hour. After washing (3 times for 10 minutes each), the target protein signal band was detected by chemiluminescence system. Band intensities were then quantified by densitometry [9, 11, 13].

siRNA-mediated knockdown of endogenous expression of RhoG in INS-1 832/13 cells

The endogenous expression of RhoG was suppressed by siRNA transfection as per the manufacturer's protocol. Cells were transfected with siRNA-RhoG or siRNA-GDIγ (at a final concentration of 100 nM) using the DharmaFect1 reagent. Non-targeting RNA (i.e., scrambled siRNA) duplexes (specific for rat genome) were used as a control. Transfected cells were maintained in complete growth medium for 48 and 96 hours for RhoG and GDIγ, respectively. The degrees of RhoG or GDIγ knockdown were determined by Western blot analysis.

Isolation of non-nuclear and nuclear fractions from INS-1 832/13 cells

INS-1 832/13 cells were incubated under basal (2.5 mM) or high (20 mM) glucose exposure conditions for 24 hours. Cell fractionation was carried out using NE-PER Nuclear and Cytoplasmic Extraction Reagents kit as we reported in [24]. The purity of non-nuclear and nuclear fractions was determined using specific protein markers, namely GAPDH and lamin B, respectfully.

Rac1 activation assay

The degree of activation of Rac1 was quantified using a pull-down assay kit [11, 25]. In brief, INS-1 832/13 cells were grown to ~70% confluency in RPMI complete growth medium. Cells were then cultured in low glucose-low serum medium (2.5 mM glucose and 2.5% FBS) starvation medium overnight followed by incubation with basal (2.5 mM) or high (20 mM) glucose for 24 hours. Lysates were clarified by centrifugation and p21-binding domain of p21-activated kinase beads were added to the supernatant. The mixture was rotated for 1 h at 4°C and pelleted by centrifuging at 4,000*g* for 3 min. The resulting pellets were washed once with wash buffer and the relative abundance of Rac1 was determined by Western blotting.

Cell death detection assay

INS-1 832/13 cells were incubated with low (2.5 mM) or and high (20 mM) glucose for 24 hours and analyzed using a Cell Death Detection ELISA^{plus} kit according to the manufacturer's instructions. Briefly,

cells were washed with PBS and lysed using the lysis buffer provided with the kit. The cell lysates were further centrifuged at 200g for 10 min, supernatants were collected and incubated in a streptavidin-coated microplate along with the immuno-reagent containing anti-histone-biotin and anti-DNA-peroxidase for 2 hours. The complexes were further detected photometrically using ABTS substrate. Absorbance was measured at 405nm.

Statistical analysis

Data are represented as mean ± SEM from three or more independent experiments. Statistical analysis for differences between groups was done using the Student *t*-test. A p value of < 0.05 was considered statistically significant.

Results

Earlier investigations from our laboratory have demonstrated that exposure of INS-1 832/13 cells to high glucose (20 mM for 24 hours) results in mitochondrial dysfunction (caspase-3 activation) and nuclear collapse (nuclear lamin-B degradation) leading to impaired GSIS and accelerated β-cell apoptosis [26, 27]. We employed this experimental model in our current studies to determine the regulatory roles of RhoG in the activation of Rac1, caspase-3, and cell demise in INS-1 832/13 beta-cells under the duress of glucotoxic conditions.

Expression of RhoG is significantly increased in pancreatic beta-cells exposed to glucotoxic conditions

As depicted in Fig. 1 (Panel a), RhoG is expressed in a variety of insulin-secreting cells, including INS-1 832/13 cells, normal rat islets and human islets. Furthermore, the expression of RhoG was significantly increased in INS-1 832/13 cells exposed to high glucose conditions (Fig. 1; Panel b). Data pooled from multiple experiments indicated greater than 2.5-fold increase in RhoG expression under these conditions (Fig. 1; Panel c).

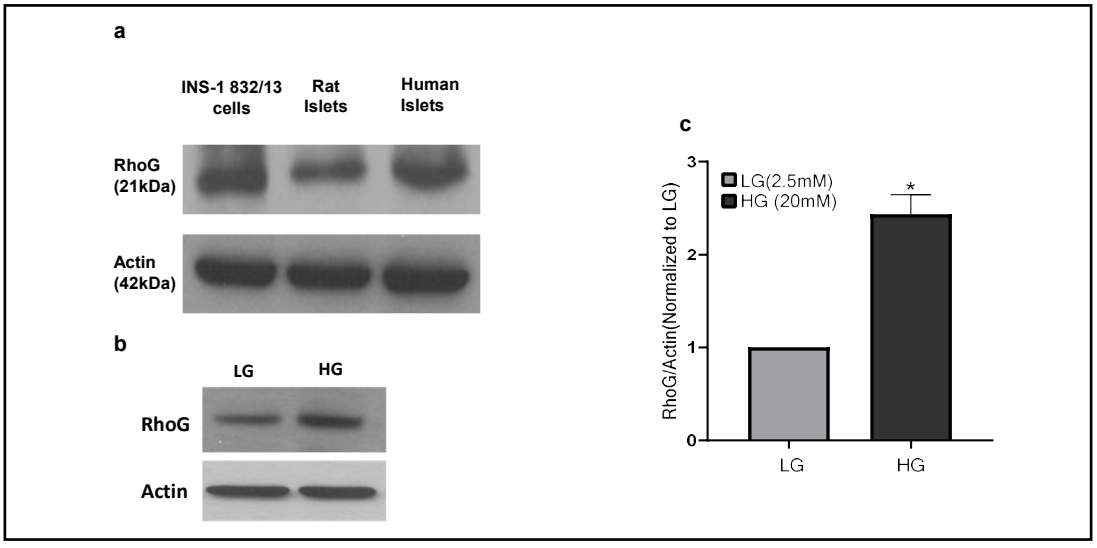


Fig. 1. Panel a: Expression of RhoG in INS-1 832/13 cells, rat islets and human islets. Lysates from INS-1 832/13 cells, rat and human islets were analyzed for RhoG protein expression by Western blot analysis. Actin was used as loading control. Panel b: Expression of RhoG is increased in INS-1 832/13 cells following chronic exposure to high glucose conditions. INS-1 832/13 cells were incubated with basal glucose (2.5 mM) or high glucose (20 mM) for 24 hours and lysates were analyzed by western blotting for the expression of RhoG. Actin was used as loading control. Panel c: Densitometric quantitation of RhoG band intensities depicted in Panel b is shown here. The results are presented as means ±SEM. The data are expressed as fold change relative to LG (n=3;*p< 0.05).

siRNA-mediated knockdown of RhoG inhibits sustained activation of Rac1 in INS-1 832/13 cells exposed to glucotoxic conditions

We reported earlier sustained activation of Rac1 in a variety of insulin-secreting cells under diabetogenic conditions, including exposure to high glucose, palmitate, ceramide, and pro-inflammatory cytokines [9, 11, 26, 28, 29]. These findings were also confirmed in islets derived from animal models of obesity and diabetes (e.g., ZDF rat) and islets from humans with type 2 diabetes [11]. Based on published evidence in other cell types indicating upstream regulatory roles of RhoG to Rac1 [16-21], we asked if Rac1 activation seen in pancreatic beta-cells exposed to high glucose conditions is mediated *via* RhoG. This was addressed by quantifying high glucose-induced activation of Rac1 in INS-1 832/13 cells in which RhoG expression was compromised *via* the siRNA approach. Data in Fig. 2 (Panel a) indicated significant knock-down of the expression of RhoG in INS-1 832/13 cells following transfection with siRNA-RhoG (RhoG-si), but not control siRNA (Cont-si). Pooled data from multiple experiments indicated greater than 50% reduction in the expression of RhoG under these conditions (Fig. 2; Panel b). We then quantified high glucose-induced activation of Rac1 in RhoG depleted INS-1 832/13 cells. Data in Fig. 2 (Panel c) represent Western blots for total Rac1 and Rac1-GTP (active form). Pooled data from multiple studies are included in Fig. 2 (Panel d). Together, these data indicated a significant degree of activation of Rac1 under glucotoxic conditions in mock or Cont-si transfected cells. However, high glucose-induced activation of Rac1 was abolished in INS-1 832/13 cells transfected with RhoGsi. Based on these data we conclude that RhoG plays a regulatory role in promoting sustained activation of Rac1 under hyperglycemic conditions.

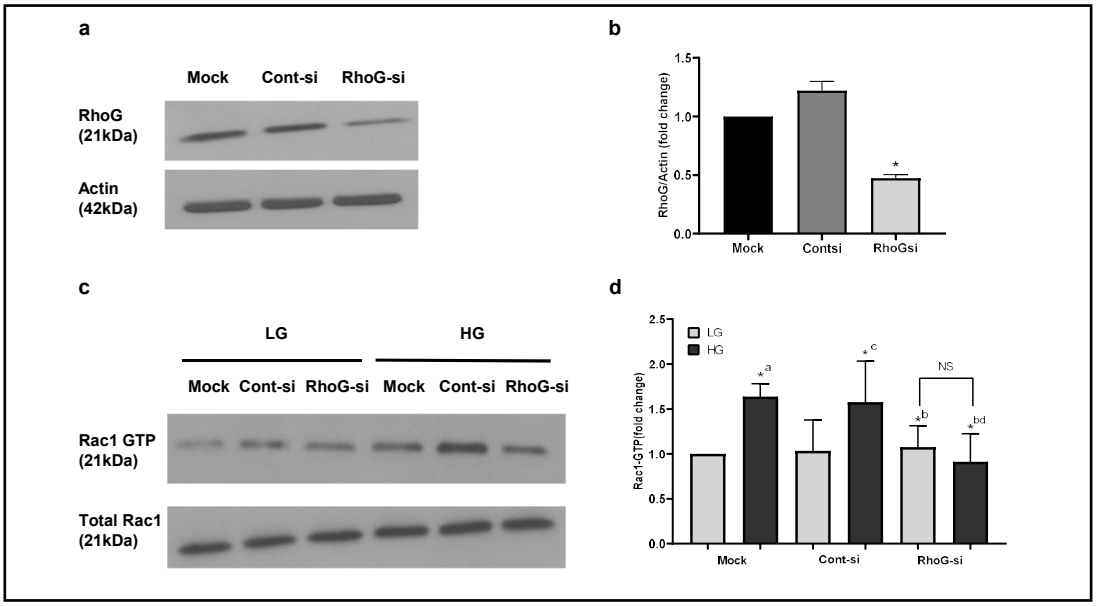


Fig. 2. siRNA-mediated depletion of endogenous RhoG markedly attenuated sustained activation of Rac1 in INS-1 832/13 cells. Panel a: INS-1 832/13 cells were transfected with control-siRNA or RhoG-siRNA. Cell lysates were analyzed by Western blotting for the expression of RhoG. Actin was used as loading control. A representative blot from n=3 independent studies is shown here. Panel b: Pooled data from three independent experiments was shown. Data are expressed as mean ± SEM from three experiments. The data are expressed as fold change relative to Mock. (n=3; * $p< 0.05$). Panel c: INS-1 832/13 cells were transfected with control-siRNA or RhoG-siRNA. After 48 hours of transfection, cells were subjected to overnight starvation and then were treated with LG (2.5 mM) or HG (20 mM) for 24 hours. Rac1 activation was quantified by Rac1 pull down assay. Representative blots from three independent studies are provided. Panel d: Densitometric quantitation of activated Rac1 in Panel a is shown here. The results from three independent experiments are presented as means ± SEM. The data are expressed as fold change relative to LG-mock (n=3; * $p< 0.05$). Comparisons shown: a - significant compared with LG-treated mock; b - significant compared with HG treated Mock; c - significant compared with LG treated Cont-si; d - significant compared with HG treated Cont-si.

RhoG translocates to nuclear fraction in INS-1 832/13 cells under the duress of glucotoxicity

In the next set of studies, to further gain mechanistic insights, we determined potential alterations, if any, in the subcellular distribution of RhoG in INS-1 832/13 cells exposed to high glucose exposure conditions. The premise underlying these studies is based on our recent observations demonstrating significant translocation of Rac1 to the nuclear fraction in INS-1 832/13 cells, normal rat islets and human islets exposed to glucotoxic conditions [24]. Based on these observations, we proposed that nuclear translocation (i.e., inappropriate or mislocalization) of Rac1 might promote activation of downstream signaling proteins in the nuclear fraction (e.g., p53 and ATM kinase) leading to transcriptional regulation of key apoptotic signaling pathways culminating in beta-cell dysfunction [24]. Therefore, at the outset, we examined the relative abundance of RhoG in non-nuclear and nuclear compartments isolated from INS-1 832/13 cells exposed to hyperglycemic conditions. To address this, non-nuclear and nuclear fractions were isolated from these cells as we reported in [24]. The purity of these fractions was assessed by determination of the enrichment of marker proteins, such as GAPDH (cytosolic marker) and lamin-B (a marker for nuclear compartment). It is noteworthy that we detected modest levels of GAPDH in the nuclear fraction, which is not surprising since it has been shown to be associated with nuclear components, such as cytoskeletal proteins [30]. Data in Fig. 3 (Panel a) indicated increased association of RhoG with the nuclear fraction derived from INS-1 832/13 cells exposed to high glucose conditions. Pooled data from multiple studies suggested an increase in the abundance of RhoG in the nuclear fraction under these conditions. Based on these data we conclude that, in a manner akin to Rac1, RhoG translocates to the nuclear fraction under metabolic stress conditions; such a signaling step might promote/accelerate signaling pathways leading to cellular dysregulation. Additional investigations are needed to substantiate this formulation.

Subcellular localization of GDIγ, a known regulator of RhoG function, is not altered in INS-1 832/13 cells exposed to glucotoxic conditions

The GDI class of signaling proteins play critical modulatory roles in G protein function at three levels. First, they inhibit dissociation of GDP from G proteins, thus maintaining the GDP-bound G proteins in their inactive conformation and preventing their activation by

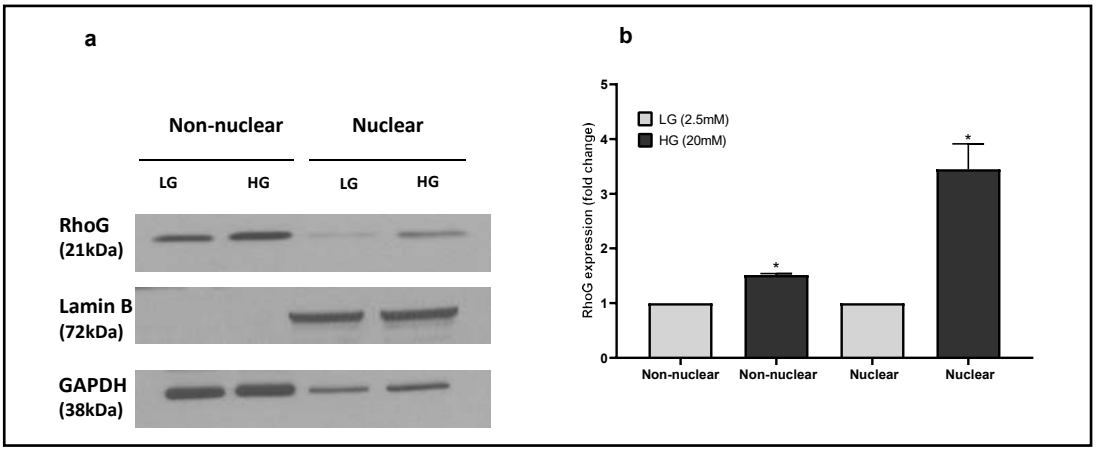


Fig. 3. Chronic exposure to high glucose promotes nuclear association of RhoG in INS-1 832/13 cells. Panel a: INS-1 832/13 cells were exposed to either basal glucose (LG, 2.5 mM) or high glucose (HG, 20 mM) for 24 hours. Following incubation, total nuclear and cytosolic fractions were isolated, and relative abundance of RhoG was determined in these fractions by Western blotting. GAPDH and Lamin B were used as marker proteins for non-nuclear and nuclear fractions, respectively. A representative blot from four independent studies is shown here. Panel b: Densitometric quantitation of relative abundance of RhoG in cytosolic and nuclear fractions isolated form basal and high glucose treated INS-1 832/13 cells is shown here. The data are expressed as fold change relative to respective LG (mean ± SEM; n=4 ; * p< 0.05).

guanine nucleotide exchange factors. Second, they interact with GTP-bound G proteins, thus preventing their inactivation by GTPase activating proteins. Finally, they participate in the cycling of G proteins from cytosol and membrane [8, 31]. In this context, we recently reported the expression of GDIγ in INS-1 832/13 cells, rat islets and human islets [30]. Therefore, in the next set of experiments, we studied the impact of hyperglycemic exposure conditions on the levels of expression and distribution (non-nuclear *vs.* nuclear) of GDIγ, which is a known regulator of RhoG functions [32-34], in INS-1 832/13 cells incubated under low and high glucose conditions. Data indicated in Fig. 4 suggested no appreciable changes in the expression of GDIγ in lysates derived from INS-1 832/13 cells incubated with high glucose for 24 (Panel a) or 48 (Panel b) hours. Data depicted in Fig. 4 (Panel c) indicated that Rho GDIγ is primarily localized in the non-nuclear fraction. Furthermore, we did not observe any changes, either in the expression or distribution of GDIγ under the duress of glucotoxicity. Taken together, data presented in Fig. 3 and 4 indicate distinct effects of hyperglycemic conditions on the distribution of RhoG and GDIγ in that they appear to promote association of RhoG with the nuclear fraction without significantly affecting the distribution of its GDI. Additional studies are needed to explain potential impact of these observations in the overall activation of RhoG-Rac1 signaling axis in the islet beta cell exposed chronically to hyperglycemic conditions.

siRNA-mediated knockdown of RhoG inhibits caspase 3 activation in INS-1 832/13 cells exposed to glucotoxic conditions

Published evidence suggests that exposure of pancreatic beta cells to hyperglycemic conditions leads to mitochondrial dysfunction as evidenced by cytochrome-C release from mitochondria into the cytosolic compartment and subsequent activation of caspase-3 [11, 24, 26, 27]. Therefore, in the next series of studies, we determined the potential roles

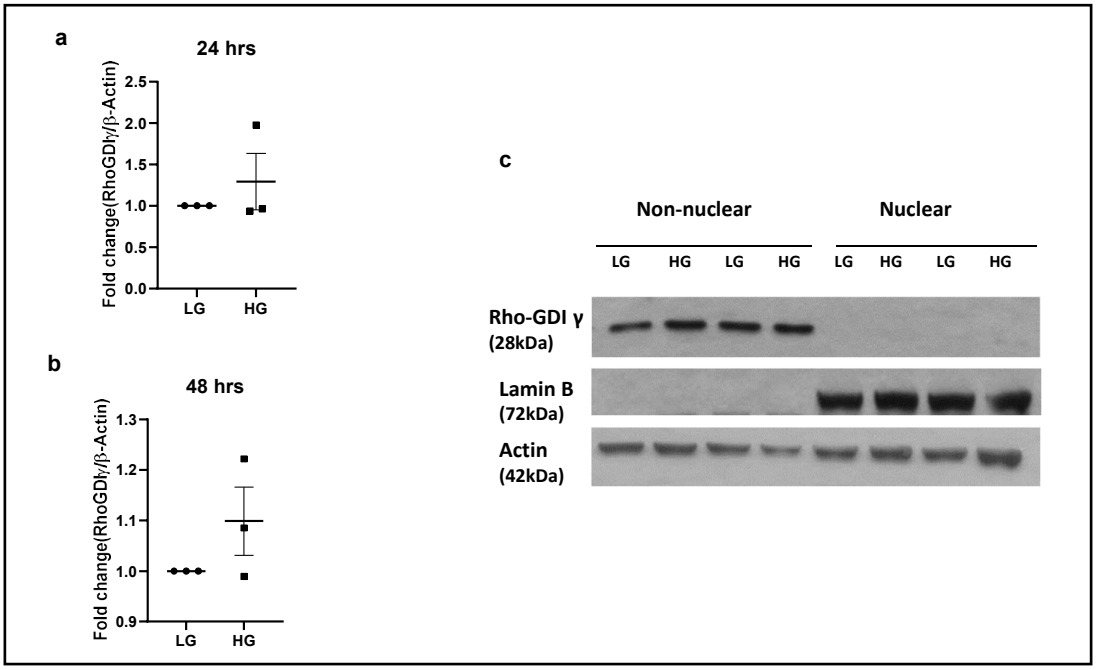


Fig. 4. Unlike RhoG, expression and subcellular distribution of GDIγ are not affected by hyperglycemic conditions. INS-1 832/13 cells were exposed to either basal glucose (LG, 2.5 mM) or high glucose (HG, 20 mM) for 24 (Panel a) or 48 (Panel b) hours. Relative abundance of RhoG in lysates was determined by Western blotting and band intensities were quantified by densitometry. Data are from three determinations in each condition. Panel c: INS-1 832/13 cells were exposed to either basal glucose (LG, 2.5 mM) or high glucose (HG, 20 mM) for 24 hours. Following incubation, nuclear and non-nuclear fractions were isolated, and relative abundance of Rho-GDIγ was determined in these fractions by Western blotting. Lamin B was used as marker protein for the nuclear fraction. Data from two individual experiments are shown here.

of RhoG in caspase-3 activation in INS-1 832/13 cells exposed to glucotoxic conditions. Data shown in Fig. 5 (Panel a) demonstrated a significant increase in caspase-3 activation (evidenced by increased abundance of the cleaved product of pro caspase-3) in cells incubated under hyperglycemic conditions in mock or Cont-si-transfected cells. However, the degree of activation of caspase-3 was markedly attenuated in INS-1 832/13 cells transfected with RhoGsi. Based on data accrued from multiple investigations (Fig. 5; Panel b), we conclude that RhoG plays key regulatory roles in the induction of caspase-3 activation in pancreatic beta-cells under glucotoxic conditions.

siRNA-mediated knockdown of RhoG fails to prevent cell death in INS-1 832/13 cells exposed to glucotoxic conditions

In the next set of studies, we determined if depletion of endogenous RhoG expression protects high glucose-induced cell death in INS-1 832/13 cells. Data depicted in Fig. 6 suggested a significant increase in cell death under conditions of chronic exposure to high glucose conditions in mock and Cont-si transfected INS-1 832/13 cells. It is noteworthy that siRNA-mediated knockdown of RhoG exerted partial protection (26%) against high glucose-induced cell death, but such effects did not achieve statistical significance. Based on these findings we conclude that RhoG might play regulatory roles in high glucose-induced activation of Rac1 and caspase-3. However, knockdown of RhoG appears not be sufficient to prevent high glucose-induced cell dysregulation in pancreatic beta-cells (see below).

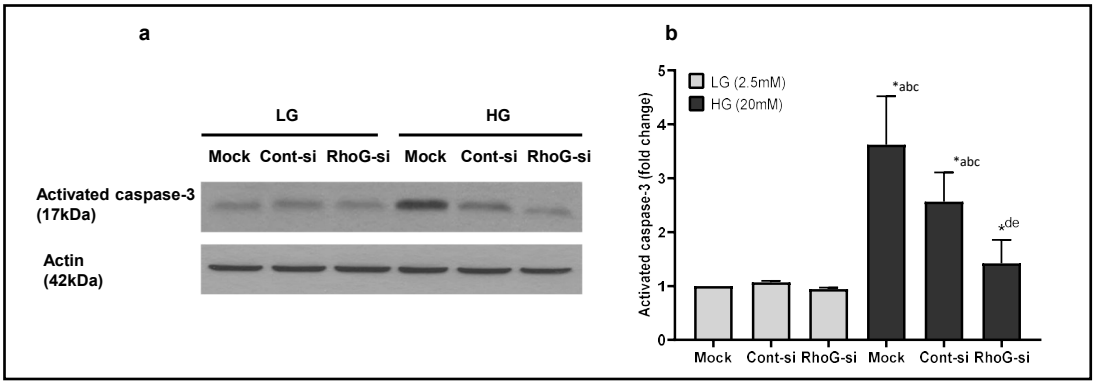
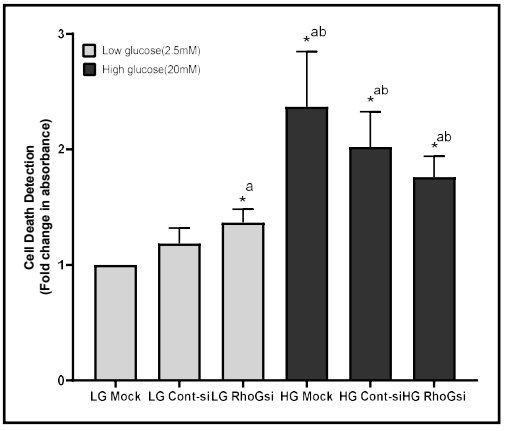


Fig. 5. siRNA mediated knockdown of RhoG leads to inhibition of caspase-3 activity in INS-1 832/13 cells. Panel a: INS-1 832/13 cells were cultured in RPMI media containing basal glucose (2.5 mM) or high glucose (20 mM) for 24 hrs. Lysate proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane and then probed for cleaved caspase-3. Uniform protein loading was ensured by re-probing the blots with antibody against β-actin. Representative blots from four independent studies are provided. Panel b: Densitometric quantitation of activated caspase-3 in Panel a is shown here. The results are shown as means ± SEM. The data are expressed as fold change relative to LG-mock (n=4; * p< 0.05). Comparisons shown: a - significant compared with HG-treated mock; b - significant compared with HG treated Cont-si.

Fig. 6. Lack of significant effects of siRNA-mediated knockdown of RhoG on high glucose-induced cell death in INS-1 832/13 cells. Following incubation with Low glucose (2.5 mM) and High glucose (20 mM) for 24 hours, cells were analyzed using Cell Death detection ELISA^{plus} kit. Absorbance was measured at 405nm and data are expressed as fold change relative to LG Mock (mean ± SEM; n=4; *p<0.05). Comparisons shown: a - significant compared with LG-treated mock; b - significant compared with LG-treated Cont-si.



Discussion

A growing body of experimental evidence suggests that exposure of pancreatic beta-cells to diabetogenic conditions (high glucose, saturated fatty acids, ceramide, and pro-inflammatory cytokines) leads to significant metabolic defects including increased oxidative stress, stress kinase activation, mitochondrial dysregulation, and nuclear collapse culminating in the loss of GSIS and eventual demise of the beta-cell [1, 3, 4, 8, 10, 12, 35]. Along these lines, original contributions from our laboratory have identified sustained activation and mislocalization of Rac1 as one of the triggers of metabolic dysregulation of the islet beta-cell under the aforestated pathological conditions [10, 12, 31]. However, precise molecular and cellular mechanisms, including the identity of upstream regulators, that mediate sustained activation of Rac1 remain less understood. Therefore, we undertook these studies to explore potential signaling pathways that might underlie Rac1 activation in pancreatic beta-cells exposed to high glucose conditions. Specifically, we aimed at understanding the regulatory roles of RhoG in the cascade of events leading to Rac1 activation and downstream mitochondrial dysregulation. Our findings indicated that: [i] RhoG is expressed in a variety of insulin-secreting cells, including INS-1 832/13 cells, normal rat islets, and human islets, and is upregulated under high glucose exposure conditions; [ii] siRNA-mediated knockdown of RhoG results in significant attenuation of sustained activation of Rac1 and the downstream activation of caspase-3 in pancreatic beta-cells exposed to hyperglycemic conditions, leading to prevention of cell demise under these conditions; and [iii] exposure to chronic hyperglycemic conditions promotes increased association of RhoG, but not its putative GDI (GDIγ) with the nuclear fraction. Altogether, the findings from our study provide the first evidence in support of the hypothesis that RhoG plays a key upstream regulatory role in the induction of Rac1 and Caspase-3 activation in pancreatic beta-cells under the duress of chronic hyperglycemic conditions.

Several lines of evidence in multiple cells implicates RhoG as an upstream modulator of small G protein (Cdc42 and Rac1) activation under a variety of experimental conditions. For example, Katoh and Negishi reported essential roles of RhoG in the activation of Rac1 in the signaling pathway involving Elmo-Dock180 in the control of neurite growth and morphology [16]. They also provided additional evidence to implicate RhoG in the regulatory control of Rac1 activation in the formation of lamellipodia in migrating cells in response to wound healing [17]. Gong et al. have reported the involvement of RhoG-ELMO1-Rac1 signaling axis in the suppression of phagocytosis in TM4 cells by mono-butyl phthalate [18]. Specifically, these studies demonstrated significant down-regulation of key signaling proteins involved in phagocytosis (RhoG, ELMO1 and Rac1) in cells exposed to mono-butyl phthalate [18]. Kwlatkowska and coworkers have reported key roles for RhoG in the activation of Rac1 mediated by growth factors, including HGF and EGF during glioblastoma cell invasion. They also reported regulatory roles for RhoG in Rac1-dependent formation of lamellipodia and invadopodia, thus identifying upstream regulation of Rac1 functions by RhoG [19]. El Atat and associates also demonstrated novel regulatory roles of a novel phosphatidyl inositol-3-kinase-mediated activation of RhoG-Cdc42-Rac1 module in tube formation in vascular endothelial cells [20]. Lastly, investigations by Xu and coworkers have implicated RhoG-Rac1 signaling pathway in migration and invasion of salivary adenoid cystic carcinoma cells [36]. Altogether, the available evidence provides compelling evidence for regulatory roles of RhoG in Rac1-mediated signaling events in multiple cells. Our current observations provide a novel RhoG-mediated regulatory mechanism for activation of Rac1 and caspase-3 in pancreatic beta-cells under conditions of chronic hyperglycemia.

Data accrued in the current investigation suggest that RhoG is upstream to Rac1 activation. What then are potential signaling steps that could couple RhoG to Rac1 activation in the pancreatic beta-cell under conditions of hyperglycemia? Earlier studies from our laboratory have identified Nox2 activation as one of the potential mechanisms whereby Rac1-mediates metabolic dysfunction of the pancreatic beta-cell under diabetogenic conditions [11, 12]. In this context, investigations by Condliffe et al. have demonstrated RhoG as a critical

component of G protein-coupled receptor-mediated regulation of NADPH oxidase in murine neutrophils. Albeit an indirect evidence, these studies have demonstrated modest inhibition of agonist-induced Rac1 activation (~25%) in RhoG-null cells [37]. Based on these and data from other complementary investigations, the authors concluded that RhoG could contribute directly to Rac1-mediated events and/or other signaling steps leading to NADPH oxidase activation. Studies of Peotter et al. have proposed a novel link between Tiam1 and RhoG-ILK/ELMO2 signaling modules in the activation of Rac1-induced phagocytosis in human trabecular meshwork cells [38]. Given our earlier findings demonstrating regulatory roles for Tiam1 in the activation of Rac1-mediated Nox2, it will be interesting to further investigate potential cross-talk between Tiam1 and RhoG in promoting Rac1-mediated signaling pathways leading to metabolic dysfunction of the beta-cell under conditions described above. Lastly, Bagci and coworkers have recently identified PLEKHG3, a known promoter of Rac1-mediated membrane ruffling downstream of RhoG [39]. It is likely that RhoG-mediated effects on Rac1 in beta-cells might involve such regulatory proteins, but recent proteomics investigations have failed to identify PLEKHG3, as one of the interacting proteins for Rac1 in pancreatic beta-cells exposed to hyperglycemic conditions [40]. Additional studies are needed to validate this postulation.

We also presented evidence to indicate that siRNA-mediated depletion of RhoG markedly attenuated high glucose-induced caspase-3 activation in INS-1 832/13 cells. Even though we have not provided a direct role for Rac1 in caspase-3 activation, our earlier findings suggested that inhibition of stress kinase activation, which is mediated *via* Rac1 activation, markedly attenuated caspase-3 activation under diabetogenic conditions [11]. Along these lines, Jin and associates tested the hypothesis that Rac1 mediates TNFα-induced apoptosis in IEC-6 cells. They reported significant activation of Rac1 during TNFα-induced apoptosis, and that NSC-23766, a Tiam1-Rac1 inhibitor or expression of dominant-negative Rac1 significantly protected cells from TNF-α induced apoptosis by inhibiting caspase-3, -8, and -9 activities, thus affirming roles for Tiam1-Rac1 module in TNFα-induced apoptosis [41]. Interestingly, our findings suggested that high glucose-induced cell demise since siRNA-mediated depletion of endogenous RhoG partially prevented high glucose-induced cell death. These data implicate additional control mechanisms that might underlie regulatory roles of RhoG in the induction of metabolic dysregulation of the islet β-cell.

We observed a significant association of RhoG, but not GDIγ, with the nuclear fraction in INS-1 832/13 cells exposed to high glucose conditions. This observation may be significant in the context of Rac1-mediated beta-cell dysfunction under these conditions in that we have recently demonstrated a significant degree of association of Rac1 with the nuclear fraction (mislocalization or mistargeting) in INS-1 832/13 cells, normal rat islets and human islets exposed to glucotoxic conditions [24]. Based on these observations, we have surmised that nuclear Rac1 promotes activation of apoptotic factors such as p53, which we have demonstrated to translocate to the nuclear fraction under metabolic stress conditions, leading to accelerated apoptotic signaling pathways [9, 24, 42]. Although not proven herein, it may be likely that nuclear RhoG-Rac1-p53 signaling module might play key roles in the onset of cell dysfunction and demise under metabolic stress conditions. Furthermore, from a translational relevance standpoint, we have reported that metformin, a biguanide anti-diabetic drug, at a clinically relevant concentration, prevents β-cell defects (Rac1 activation, nuclear association, stress kinase and caspase-3 activation, and loss in metabolic viability) under the duress of glucotoxicity [24]. Studies are underway to assess potential beneficial effects of metformin therapy in halting islet beta-cell dysfunction under metabolic stress by targeting RhoG-Rac1-Caspase-3 signaling axis. It may be germane to point out that Lu and associates have reported contributory roles for GDIγ in the differentiation of neural stem cells. Interestingly, they reported significant reduction of GDIγ regulated genes (e.g., RhoA, Cdc42, LimK2 and N-WASP) in these cells following siRNA-mediated depletion of GDIγ [34]. A slight increase in the expression of Rac1 was reported under these conditions. Along these lines, preliminary studies in INS-1 832/13 cells have suggested no significant effects of GDIγ knockdown on the expression of Rac1 and RhoG (additional data not shown).

Conclusion

Despite the recently reported evidence for lack of regulatory effects of RhoG in glucose-stimulated insulin secretion [43], data from our current investigations identify RhoG as an upstream mediator of sustained activation of Rac1 and caspase-3 in pancreatic beta-cells under the duress of chronic hyperglycemia. It is noteworthy that, in addition to hyperglycemic conditions, expression of RhoG appears to be regulated by other metabolic stress conditions including exposure to a cell permeable ceramide (1.64 fold increase in the presence of 50 μM C2-ceramide vs. 1.67 fold increase in the presence of 20 mM glucose incubated for 24 hours; n=2 experiments). Earlier observations from our laboratory have suggested that ceramide-induced metabolic defects on pancreatic beta-cells might underlie activation of Rac1 and caspase-3 [44]. Additional studies are needed to further assess the potential interaction between these two small G proteins (RhoG and Rac1) and their regulatory molecules (GEFs, GDIs, and GAPs) in the propagation of signals leading to mitochondrial dysregulation under a variety of pathological conditions conditions [8].

Abbreviations

Arf6 (ADP-ribosylation factor 6); Cdc42 (cell division control protein 42); ELMO (Engulfment and cell motility); EGF (Epidermal growth factor); GDIγ (Guanosine dissociation inhibitor γ); GSIS (Glucose-stimulated insulin secretion); HGF (Hepatocyte growth factor); ILK (Integrin linked kinase); IL1β (Interleukin 1β); IFNγ (Interferon γ); JNK 1/2 (Jun NH (2)- terminal kinase); NOX2 (Phagocyte-like NADPH oxidase 2); PLEKHG3 (Pleckstrin homology and RhoGEF containing G3); p38MAPK (p38 mitogen activated protein kinase); Rac1 (Ras related C3 botulinum toxin substrate 1); ROS (Reactive oxygen species); RhoG (Ras homology Growth-related protein); siRNA (Small (or short) interfering RNA); Tiam1 (T-lymphoma invasion and metastasis-inducing protein 1); TNFα (Tumor necrosis factor alpha); ZDF (Zucker diabetic fatty rat).

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Author Contributions

SAC, AH, MH, NG, SG conducted experiments, analyzed experimental data and edited the manuscript. AK participated in design of the experiments, overall execution of the studies, and wrote the manuscript.

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Statement of Ethics

All protocols were reviewed and approved by the Wayne State University and John D. Dingell VA Medical Center Institutional Animal Care and Use Committees. Human islets were from Prodo Laboratories (Aliso Viejo, CA). Studies involving human islets were approved by the Biosafety Committee at the John D. Dingell VA Medical Center.

Disclosure Statement

The authors have no conflicts of interest to declare.

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