CARD9 Mediates Pancreatic Islet Beta-Cell Dysfunction Under the Duress of Hyperglycemic Stress

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Abstract

Background/Aims: Published evidence implicates Caspase recruitment domain containing protein 9 (CARD9) in innate immunity. Given its recently suggested roles in obesity and insulin resistance, we investigated its regulatory role(s) in the onset of islet beta cell dysfunction under chronic hyperglycemic (metabolic stress) conditions.

Methods: Islets from mouse pancreas were isolated by the collagenase digestion method. Expression of CARD9 was suppressed in INS-1 832/13 cells by siRNA transfection using the DharmaFect1 reagent. The degree of activation of Rac1 was assessed by a pull-down assay kit. Interactions between CARD9, RhoGDIβ and Rac1 under metabolic stress conditions were determined by co-immunoprecipitation assay. The degree of phosphorylation of stress kinases was assessed using antibodies directed against phosphorylated forms of the respective kinases.

Results: CARD9 expression is significantly increased following exposure to high glucose, not to mannitol (both at 20 mM; 24 hrs.) in INS-1 832/13 cells. siRNA-mediated knockdown of CARD9 significantly attenuated high glucose-induced activation of Rac1 and phosphorylation of p38MAPK and p65 subunit of NF-κB (*RelA*), without significantly impacting high glucose-induced effects on JNK1/2 and ERK1/2 activities. CARD9 depletion also suppressed high

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glucose-induced CHOP expression (a marker for endoplasmic reticulum stress) in these cells. Co-immunoprecipitation studies revealed increased association between CARD9-RhoGDIβ and decreased association between RhoGDIβ-Rac1 in cells cultured under high glucose conditions.

Conclusion: Based on these data, we conclude that CARD9 regulates activation of Rac1-p38MAPK-NFκB signaling pathway leading to functional abnormalities in beta cells under metabolic stress conditions.

Keywords

Pancreatic islet; CARD9; Stress kinases; Metabolic stress; Diabetes

Introduction

Chronic exposure of pancreatic beta-cells to metabolic stress conditions, such as hyperglycemia, has been shown to increase the generation of intracellular reactive oxygen species (ROS; oxidative stress), endoplasmic reticulum (ER) stress, and mitochondrial dysregulation (mito stress) culminating in defective glucose-stimulated insulin secretion (GSIS) and apoptotic demise of the effete beta cell [1–10]. Published evidence implicates NADPH oxidases, specifically the phagocyte-like NADPH oxidase (Nox2), as contributors of oxidative stress in pancreatic beta cells following exposure to diabetogenic conditions [1, 11–16]. Regulatory roles of NADPH oxidases in islet beta cell dysfunction were confirmed in insulin-secreting beta cell lines, rodent islets and human islets under *in vitro* conditions, and in islets from animal models as well as humans with T2DM [1, 11, 17, 18]. Several previous studies have reported sustained (or constitutive) activation of Rac1, a small G protein, in pancreatic beta cells following exposure to chronic hyperglycemic and hyperlipidemic conditions as well as exposure to cell-permeable ceramides and pro-inflammatory cytokines [19–25]. Interestingly, hyperactivation of Rac1 under these pathological conditions has been shown to promote activation of Nox2 and stress kinases (e.g., p38MAPK) leading to dysregulation of beta cell function [17, 18, 26]. Several mechanisms have been put-forth to explain sustained activation of Rac1 under conditions of metabolic stress; these include increase in the dissociation of complexes of Rac1 and its GDP-dissociation inhibitors (RhoGDIs, e.g., RhoGDIβ) and accelerated activation of "free" Rac1 by its respective guanine nucleotide exchange factors (GEFS, e.g., Tiam1 and Vav2). The reader is referred to recent reviews on contributory roles of various GEFs, GDIs and other G protein regulatory factors in the activation-deactivation of candidate G proteins (e.g., Rac1) in pancreatic beta cells in normal health and under metabolic stress [21, 27–29].

Extant evidence from multiple laboratories implicates key roles for CARD9 in innate immunity [30–33]. Studies by Jia and coworkers have demonstrated that CARD9 promotes antifungal immunity *via* a mechanism involving activation of H-Ras, which, in turn, mediates Dectin-1 induced ERK activation [30]. Data accrued in studies by Wu et al. [31] have implicated CARD9 in microbe-mediated generation of ROS *via* promoting dissociation of Rac1-RhoGDIβ complex leading to activation of Nox2 and the onset of oxidative stress. Together, these studies suggest novel roles for CARD9 in G protein regulated cell function.

In the context of potential roles of CARD9 in islet beta cell function, we recently reported expression of CARD9 in a variety of insulin secreting cells, including human islets, rat islets, mouse islets and INS-1 832/13 cells. Interestingly, siRNA-mediated depletion of CARD9 markedly suppressed GSIS without significantly affecting glucose-induced activation of Rac1 under those conditions. We also noticed that CARD9 deletion markedly inhibited glucose-induced p38MAPK activation under conditions of GSIS inhibition [34]. These findings have led us to postulate that CARD9 might regulate GSIS *via* a Rac1-independent, but p38-dependent signaling module. Lastly, in an attempt to investigate potential regulatory roles of RhoGDIβ (also referred to as LyGDI, D4-GDI, RhoGDI2) in glucose-induced activation of Rac1 and insulin secretion, we recently demonstrated that siRNA-mediated knockdown of RhoGDIβ in INS-1 832/13 cells significantly attenuated glucose-induced Rac1 activation without affecting its translocation and membrane association. In addition, no significant effects of RhoGDIβ-depletion were observed on GSIS; these findings suggested differential regulatory roles of RhoGDIβ in Rac1 activation and GSIS [35].

Based on the findings highlighted above in other cell types and, albeit limited, in pancreatic beta cells, we undertook the current investigations to test the hypothesis that CARD9 plays key regulatory roles in the onset of beta cell dysfunction under the duress of chronic hyperglycemic conditions by regulating the RhoGDIβ-Rac1-p38MAPK-NF-κB signaling module. The first evidence in support of this hypothesis is presented below.

Materials and Methods

Materials

Antibodies directed against CARD9 (sc-374569), RhoGDIβ [Ly-GDI (D-7); sc-271108], BCL-10 (sc-5273) and agarose beads were from Santa Cruz Biotechnology (Dallas, TX, USA). Rac1 antibody (05-389-25UG) was from EMD Millipore (Burlington, MA, USA). Total and phospho antisera for p38MAPK [p38 MAPK; 9212S and P-p38 MAPK (Thr180/Tyr182); 9211S], total and phospho-JNK ½ antibodies (SAPK/JNK; 9258S and P-SAPK/JNK (Thr183/Tyr185); 9251S), total ERK ½ antibody [p44/42 MAPK (ERK1/2) (137F5); 4695S] and phospho- ERK ½ antibody [P-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) (D13.14.4E); 4370S], total and phospho antisera for p65 [NF-kappaBp65 (D14E12) XP(R); 8242S and P-NF-kappaB-p65 (Ser36) (93H1); 3033S], cleaved Caspase-3 antibody (Asp175; 9661S), CHOP antibody (2895S) and HRP-conjugated secondary antibodies were from Cell Signaling Technology, Inc (Danvers, MA, USA). β-actin antibody (A1978), sodium palmitate, BRD5529 inhibitor were from Sigma Aldrich (St. Louis, MO, USA). ON-TARGETplus Non-targeting siRNA (Control siRNA: Con-si; D001810-01-20), ON-TARGETplus Rat CARD9 siRNA-SMARTpool (CARD9-si; L-096926-02-20) and DharmaFect1 transfection reagent (T-2001–03) were from Horizon Discovery (Lafayette, CO, USA). Rac1 activation assay kits (pull-down) were from Cytoskeleton (Denver, CO, USA). Ceramide (C-2) was from Cayman Chemicals (Ann Arbor, MI, USA). Co-IP kit was from Thermo Scientific Inc. (Waltham, MA, USA).

Culture of INS-1 832/13 cells and mouse islets

INS-1 832/13 cells (passage #s 50–60) 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). As indicated in the text, cells were treated overnight with low serum/low glucose media prior to each experiment. Cells were incubated in the presence of low glucose (LG; 2.5mM) or high glucose (HG; 20mM) or HG and palmitic acid (PA; 500 µM; glucolipotoxic [GLT] condition) for 24 hrs unless specified otherwise.

All protocols for mouse islet isolation were reviewed and approved by Institutional Animal Care and Use Committees of Wayne State University and John D. Dingell Veterans Affairs Medical Center. Approximately 10 week-old (male and female) C57BL/6 mice (Charles River, Wilmington, MA, USA) were used for islet isolation using the Collagenase digestion [34]. Islets were hand-picked, and incubated overnight in islet media (RPMI-1640 medium containing 10% FBS supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, and 10 mM HEPES). These islets were then exposed to LG or HG treatment for 24 hrs. and cell lysates were prepared using the RIPA buffer and employed in studies described herein.

siRNA-mediated knockdown of expression of CARD9 in INS-1 832/13 cells

Endogenous expression of CARD9 was suppressed using CARD9-si as we recently reported in [34]. Briefly, cells were transfected with siRNA at a final concentration of 100 nM using the DharmaFect1 reagent. Specificity of RNA interference was accessed using cells transfected with non-targeting siRNA (i.e., control siRNA; Con-si). Following specific treatments, as indicated in the text, the cell lysates were prepared in RIPA lysis buffer containing protease and phosphatase inhibitors. Efficiency of the knockdown was determined by western blotting. Additional details on expression of CARD9 in various insulin-secreting cells, and characterization of the antibody directed against CARD9 can be found in one of our recent publications [34].

Western Blotting

Cell lysates (30–50 µg for INS-1 832/13 cells and 50 µg for mouse islets) were resolved by SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked in 3% BSA for one hour and probed overnight with appropriate primary antibodies. The membranes were then washed and then probed with the appropriate secondary antibody for 1 hr. The immune complexes were detected using ECL detection kit (ThermoScientific, Waltham, MA, USA). The band intensities were quantified using Image Studio Lite imaging software (Li- COR Biosciences, Lincoln, NE, USA).

Rac1 activation assay

Con-siRNA or CARD9-siRNA transfected INS-1 832/13 cells were exposed to either LG or HG for 24 hrs. and the magnitude of Rac1 activation was determined using a pull-down assay kit as we reported previously [18, 19, 34].

Co-immunoprecipitation assay

Immunoprecipitation was performed according to the manufacture's protocol. Briefly, 500 μg of lysates from LG- or HG-treated INS-1 832/13 cells were incubated overnight with respective primary antibody as indicated in the text. Pre-cleared lysates were incubated (continuous agitation) with agarose beads for 3 hrs. at 4°C. After multiple washes with lysis buffer, the resulting immunoprecipitates were subjected to SDS-PAGE and western blotting to detect and quantify the abundance of proteins of interest in the immunoblots [36].

Statistical analysis

Data are presented as mean ± SEM of three-six independent experiments. Statistical analysis was done using the student t-test. A *p*-value of < 0.05 was considered statistically significant.

Results

We previously reported that exposure of INS-1 832/13 cells to HG (20mM; 24 hrs.) results in dysregulation of mitochondrial function (Caspase-3 activation) and nuclear abnormalities (nuclear lamin-B degradation) leading to impaired GSIS and beta cell demise [26, 37, 38]. We employed this experimental model in the following investigations to assess the regulatory roles of CARD9 in the induction of metabolic dysfunction under conditions of chronic hyperglycemic conditions.

Diabetogenic stimuli increase the expression of CARD9 in insulin-secreting INS-1 832/13 cells and mouse islets

Overall objective of the studies described herein is to determine contributory roles of CARD9 in metabolic dysregulation of the islet beta cell exposed to metabolic stress conditions. To address this, at the outset, we determined the effects of a variety of diabetogenic stimuli on the expression of CARD9 in mouse islets and INS-1 832/13 cells. Data depicted in Fig. 1 (Panels A and B) indicate a significant increase (~2-fold) in the expression of CARD9 following exposure of mouse islets to chronic hyperglycemic conditions (20 mM; 24 hrs.). A significant increase in the expression of CARD9 (~1.7-fold) was also seen in INS-1 832/13 cells (Fig. 1; Panels C and D) following exposure to hyperglycemic conditions. Mannitol (20 mM; 24 hrs.), used as an osmotic control, elicited no effects on CARD9 expression suggesting that the effects of HG conditions are specific to glucose, not due to osmolality (Fig. 1; Panels C and D). In addition, exposure of INS-1 832/13 cells to palmitate (PA; 500 μM; 24 hrs.; lipotoxic conditions; LT), also increased (~2.5-fold) the expression of CARD9 (Fig. 1; Panels E and F). Lastly, exposure of these cells to a combination of glucose plus palmitate (to mimic gluco-lipotoxic conditions; GLT) exerted similar effects (~2.5-fold) on the expression of CARD9 in INS-1 832/13 cells (Fig. 1; Panels G and H). Interestingly however, we failed to see any significant effects on the expression of CARD9 in INS-1 832/13 cells following exposure to ceramide (50 μM; 24 hrs.) or IL-1β (25ng/ml; 24 hrs.; additional data not shown). Taken together, data represented in Fig. 1 suggest significant effects of HG, LT or GLT conditions on the expression of CARD9 in insulin-secreting cells.

CARD9 is involved in the sustained activation of Rac1 in INS-1 832/13 cells exposed to chronic hyperglycemic conditions

Several recent investigations in pancreatic beta cells and other cell types have shown that chronic metabolic stress promotes activation of Rac1, a small G protein belonging to Rho subfamily of G Proteins, leading to activation of phagocyte-like NADPH oxidases (Nox2) and stress kinases (e.g., p38 MAPK). Therefore, in the current set of studies, we determined putative roles of CARD9 in HG-induced activation of Rac1 in INS-1 832/13 cells. As shown in Fig. 2 (Panels A and C), siRNA-mediated knockdown of CARD9 abolished HG-induced Rac1 activation. Interestingly, knockdown of CARD9 promoted activation of Rac1 under LG conditions (i.e., inappropriate activation under basal conditions). Data in Fig. 2 (Panel B) indicate ~87% knockdown of CARD9 in cells exposed to LG conditions using our transfection protocol. Under these conditions, we noticed nearly 76% knockdown of CARD9 expression in INS-1 832/13 cells exposed to HG conditions. Together, data in Fig. 2 suggest key regulatory roles for CARD9 in the sustained activation of Rac1 in pancreatic beta cells under the duress of chronic metabolic stress conditions.

CARD9 promotes p38MAPK activation under chronic hyperglycemic conditions without significantly affecting glucose-induced regulatory effects on JNK1/2 and ERK1/2 in pancreatic beta cells

As indicated above, earlier studies have suggested that Rac1 activation represents an upstream signaling event for the activation of stress kinases, such as p38 MAPK, in pancreatic beta cells exposed to metabolic stress conditions [17, 18, 26]. Therefore, in the next set of studies, we investigated contributory roles of CARD9-Rac1 signaling axis in p38MAPK activation in INS-1 832/13 cells incubated under HG conditions. Data shown in Fig. 3 (Panel A) demonstrate significant inhibitory effects of HG-induced activation of p38MAPK in CARD9-depleted INS-1 832/13 cells. Pooled data from multiple studies are provided in Fig. 3 (Panel B). It is noteworthy that HG-induced activation of JNK1/2 appear to be resistant to CARD9 knockdown under our current experimental conditions (Fig. 3; Panels C, D and E). Lastly, no significant effects of CARD9 deletion on ERK1/2 phosphorylation were demonstrable under LG and HG exposure conditions (Fig. 3; Panels F, G and H). Taken together, data accrued from studies highlighted in Fig. 3 suggest key roles for CARD9-Rac1 signaling module in HG-induced activation of p38MAPK in pancreatic beta cells.

CARD9-TRIM62 module may not be involved in p38MAPK activation in INS-1 832/13 cells under chronic hyperglycemic conditions

Earlier studies by Leshchiner et al. have indicated BRD5529 as a selective inhibitor of CARD9 activation [39]. Mechanistically, BRD5529 binds to CARD9, thereby preventing cross-talk/interaction between CARD9 and TRIM62, a known E3 ubiquitin ligase for CARD9, resulting in functional inactivation of CARD9. Therefore, we examined the effects of BRD5529 (50 μM; 24 hrs.) on p38MAPK activation in INS-1 832/13 cells following exposure to high glucose conditions. Data accrued in these studies have indicated no clear effects of BRD5529 on high glucose-induced activation of p38MAPK. Ratios of phospho-p38MAPK to total-p38MAPK in high glucose exposed cells were 1.534 ± 0.16 and 1.53

± 0.27 in the absence and presence of BRD5529, respectively (n=8 experiments; p= not significant). Based on these data, we conclude that CARD9-TRIM62 signaling pathway may not be involved in high glucose-induced p38MAPK activation under conditions of the current study.

CARD9 is necessary for increased phosphorylation of the p65 NF-κB (RelA) subunit in INS-1 832/13 cells exposed to hyperglycemic conditions

Several lines of evidence have affirmed regulatory roles of CARD proteins in the induction of cellular dysfunction and apoptosis *via* modulation of NF-κB activity [40]. Furthermore, CARD9 has been implicated in promoting inflammation *via* activation of MAPK and NF-κB signaling modules in innate immune cells, which, in turn, promotes multiple metabolic diseases, including obesity and insulin resistance [41]. Therefore, we next asked if CARD9 contributes to increased phosphorylation of p65 subunit under conditions of exposure to high glucose conditions. Data shown in Fig. 4 (Panel A) demonstrate increased phosphorylation of p65 under high glucose conditions, which was markedly attenuated in INS-1 832/13 cells following siRNA-mediated knockdown of CARD9. Pooled data from multiple experiments are provided in Fig. 4 (Panel B). Together, data presented in Fig. 3 and 4 suggest that CARD9 plays important regulatory roles in high glucose-induced p38MAPK and NF-κB signaling modules. Furthermore, as in the case of p38MAPK (above), BRD5529, a known inhibitor of CARD9-TRIM62 signaling step, exerted no significant effects on high glucose-induced p65 phosphorylation (Fig. 4; Panels C and D). Taken together, our observations suggest minimal regulatory roles of CARD9-TRIM62 module in the activation of p38MAPK-NF-κB signaling pathways in INS-1 832/13 cells exposed to hyperglycemic conditions.

CARD9 promotes ER stress (i.e., CHOP expression) under chronic hyperglycemic conditions in INS-1 832/13 cells

Based on the existing evidence that HG exposure conditions induce endoplasmic reticulum stress (ER stress) resulting in mitochondrial and nuclear dysregulation in the pancreatic beta cell [37, 38, 42, 43], we next investigated if CARD9 contributes to ER stress response in INS-1 832/13 cells exposed to HG conditions. Western blot data shown in Fig. 5 (Panel A) indicate a significant increase in CHOP (a marker for ER stress) expression in these cells under the duress of HG conditions. siRNA-mediated knockdown of CARD9 significantly attenuated HG-induced CHOP expression. Pooled data from multiple experiments are included in Fig. 5 (Panel B). Data in Fig. 5 (Panel C) demonstrate ~40% inhibition of HG-induced CHOP expression following CARD9 knockdown in these cells under our current experimental conditions. Altogether, data depicted in Fig. 3 and 4 implicate key roles for CARD9 in HG-induced stress kinase (p38MAPK) and ER stress signaling pathways.

Lack of clear regulatory roles of CARD9 in high glucose-induced activation of Caspase-3 in INS-1 832/13 cells

Next set of experiments were aimed at determining if CARD9-mediated stress kinase activation and/or induction of ER stress contributes to Caspase-3 activation in pancreatic beta cells exposed to HG. Data highlighted in Fig. 6 (Panels A and B), as expected, suggest a significant increase in Caspase-3 activation in Control-siRNA transfected cells

incubated under HG conditions. Interestingly however, knockdown of CARD9 appears to have modest effects on HG-induced activation of Caspase-3. Even though there appears to be directional evidence for modest suppression of Caspase-3 activation in CARD9-siRNA treated cells under HG conditions, they did not achieve statistical significance compared to Control-siRNA transfected cells under those conditions. Based on these data we conclude that CARD9 contributes to HG-induced Rac1 activation and associated oxidative and ER stress in pancreatic beta cells. However, its role in HG-mediated Caspase-3 activation needs further examination. The following experiments were conducted to gain additional mechanistic insights in CARD9-mediated, HG-induced metabolic dysregulation of the islet beta cell.

Co-IP studies suggest alterations in the interaction between CARD9, RhoGDIβ and Rac1 in INS-1 832/13 cells exposed to chronic hyperglycemic conditions

It is well established that activation-deactivation cycles of small G proteins (e.g., Rac1) is mediated by concerted regulatory roles of GTP/GDP exchange factors (GEFs), GDP dissociation inhibitors (GDIs), and GTPase activating proteins (GAPs). Several of these regulatory factors have been identified and characterized in relation to their roles in physiological insulin secretion and metabolic dysfunction of the islet beta cell [21, 28]. In the context of CARD9-mediated regulation of Rac1 functions, a recent report by Wu and coworkers have identified RhoGDIβ-Rac1 complex as one of the target sites for CARD9-mediated effects in microbial killing in macrophages [31]. They provided compelling evidence to suggest that CARD9 promotes sustained activation of Rac1 *via* its binding (i.e., complexation) with RhoGDIβ thereby dissociating the RhoGDβI-Rac1 complex. Such a signaling step was shown to promote activation of Rac1 leading to activation of downstream signaling steps including generation of reactive oxygen species [31]. We recently reported expression of RhoGDIβ in human islets, rodent islets and INS-1 832/13 cells and demonstrated key regulatory roles for glucose-induced activation of Rac1, but not insulin secretion [35, 44]. Based on our evidence for RhoGDIβ's role in Rac1 functional regulation [44], and the data from studies by Wu et al. [31], we aimed at understanding potential interactions (alterations in these signaling steps, if any) between CARD9, RhoGDIβ and Rac1 in INS-1 832/13 cells incubated with LG and HG. We employed Co-IP approach to decipher those interactions.

Data shown in Fig. 7 (Panel A) demonstrate increased interaction between CARD9 and RhoGDIβ in INS-1 832/13 cells under HG conditions. Data from 4 independent experiments are shown in Fig. 7 (Panel B). Furthermore, under the same experimental conditions, we noticed a marked reduction in the interaction between RhoGDIβ and Rac1 in cells exposed to HG conditions. These data provide the first experimental evidence to indicate that HG conditions promote a significant increase in the interaction between CARD9 and RhoGDIβ, resulting in dissociation of RhoGDIβ-Rac1 complex to enable subsequent activation of Rac1 by candidate GEF proteins.

Evidence to implicate CARD9 in the increased expression of BCL 10 in pancreatic beta cells exposed to chronic hyperglycemic conditions

Several lines of evidence provide strong support for involvement of specific CARD9 associated proteins (CARD9 signalome) for innate antifungal immunity [32, 33, 45]. For example, studies have shown that signaling complexes, comprising of CARD9-BCL10-Malt 1, play critical roles in the activation of NF-κB signaling pathway in C-type lectin receptor (CLR) induced inflammatory responses [46]. Published evidence also suggests strong connection between over expression of CARD9 in the activation of p38 MAPK during inflammation [47, 48]. Lastly, studies by Wang and coworkers have demonstrated that obesity promotes activation of p38MAPK through the upregulation of CARD9-BCL10 complex [49]. As a logical extension to our current experimental findings supporting CARD9-mediated regulation of p38MAPK in pancreatic beta cells under HG conditions, we quantified BCL10 expression levels in INS-1 832/13 cells following exposure to HG or GLT conditions. Data depicted in Fig. 8, ~ 1.75-fold increase in the expression of BCL10 was seen under HG or GLT conditions (Panels A and B). Lastly, in two independent experiments, we observed inhibition (43–77%) of HG-induced expression of BCL10 following siRNA-mediated knockdown of CARD9. A representative blot of the two studies is provided in Panel C. Taken together, these findings support a close relationship between CARD9 and BCL10 in HG-mediated effects on islet beta cell dysregulation (see below).

Discussion

In addition to its contributory roles in innate immune response [30–33], CARD9 has been implicated in a variety of pathologies, including cardiovascular [50] and metabolic [41] diseases. One of the objectives of our current investigation was to assess the regulatory role of CARD9 in pancreatic islet beta-cell dysfunction under the duress of chronic hyperglycemic conditions. Our findings suggest that: [i] CARD9 expression is significantly increased in mouse islets and INS-1 832/13 cells under HG and GLT exposure conditions; [ii] siRNA-mediated knockdown of CARD9 significantly attenuated high glucose-induced activation of Rac1, p38MAPK and NF-κB signaling steps without significantly affecting JNK1/2 and ERK1/2 activities; [iii] depletion of CARD9 markedly suppressed high glucose-induced CHOP expression, but not Caspase-3 activation; and [iv] BCL10 expression is significantly increased under HG conditions, which appears to be downstream to CARD9.

As stated above, published evidence suggests key regulatory roles for CARD9 in innate immunity. Evidence from studies of Wu et al. [31] and Jia et al. [30] suggests that the regulatory effects of CARD9 may be mediated *via* activation of small G proteins, such as Rac1 and Ras. From a mechanistic standpoint, Jia and coworkers have demonstrated that Dectin-1 induces phosphorylation of Ras-GRF1, which then triggers association of phosphorylated Ras-GRF1 with CARD9 and subsequent association of Ras with GRF1-CARD9 (Ras-GRF1-CARD9 complex) to promote ERK-NF-κB signaling pathway leading to the production of pro-inflammatory cytokines (IL-6, IL-12, IL-1β, and TNFα) [30]. Indeed, such interactions highlight potential crosstalk between GRFs and CARD in the regulation of cell function. While these aspects have not been addressed in the context of CARD9-GRF interactions in the pancreatic beta cells, earlier studies have described

regulation of islet functions by GRFs [21]. Future studies will assess the roles of Ras-GRF-CARD9 signaling pathway in pancreatic islet beta cell function in health and metabolic stress. Studies by Wu et al. [31] demonstrate increased interaction between CARD9 and GDIs (e.g., RhoGDIβ) in the cascade of events leading to G protein (e.g., Rac1) activation. More importantly, data accrued in our current studies are compatible with those reported by Wu and coworkers in that, under hyperglycemic conditions, CARD9 appears to complex with RhoGDIβ, thereby promoting dissociation of RhoGDIβ-Rac1 complex, thus providing conditions conducive for the activation of Rac1 by its respective GEFs. However, whether CARD9 exerts direct regulatory effects of putative GEFs for Rac1 leading to its activation remains unclear at this time. Along these lines, Roth and coworkers have implicated Vav-CARD9 signaling axis in C-type lectin receptor (CLR)-mediated antifungal host defense [45]. In the context of the islet beta cell, it may be germane to point out that using specific inhibitors of GEFs for Rac1, namely NSC23766 (an inhibitor for Tiam1-Rac1 signaling axis), and Ehop-016 (an inhibitor for Vav2-Rac1 signaling module), we reported significant inhibition by these inhibitors of p38MAPK activation in pancreatic beta cell under the duress of chronic hyperglycemia [17]. Additional investigations are needed to further assess the roles of these GEFs in CARD9-Rac1 signaling in the islet beta cell.

Besides its aforestated roles in innate immunity, CARD9 has been implicated in diet-induced inflammation, obesity and metabolic pathologies [41]. For example, using a CARD9 knockout animal model, Zeng and associates have reported significantly higher insulin resistance and impairment of glucose tolerance in WT animals following high fat feeding compared to those in which CARD9 is deleted [47]. Interestingly, high fat-induced expression of p38MAPK, JNK and ERK were significantly lower in the liver from CARD9 depleted animals. Based on these findings, the authors have concluded that absence of CARD9 affords protection against diet-induced obesity and related pathologies *via* down-regulating CARD9-p38MAPK signaling module [47]. Using a high fat fed animal model, Wang and coworkers provided compelling evidence for key regulatory roles of BCL10-CARD9-p38MAPK signaling module in obesity-related cardiac hypertrophy [49]. Using a variety of complementary experimental approaches, these investigators have reported increase in the expression of BCL10, CARD9 and p38MAPK activation in the heart in high fat fed animals. Pharmacological inhibition of p38MAPK in high fat fed animals corrected p38MAPK activation with minimal effects on increased expression of BCL10 and CARD9, thus suggesting that BCL10-CARD9 are upstream to p38MAPK activation. siRNA mediated knockdown of BCL10 expression in cultured cardiomyocytes inhibited palmitate-induced p38MAPK. Lastly, supplementation of zinc rescued obesity-induced cardiac hypertrophy in these animals by suppressing the activation of BCL10-CARD9-p38 MAPK signaling module [49]. Published evidence also suggests that CARD9 knockdown prevents metabolic abnormalities and myocardial dysfunction related to high fat diet-induced obesity [51], and regional ischemia/reperfusion injury. The latter has been shown to occur *via* suppression of acute inflammatory response [52]. Our current findings on regulatory roles of CARD9 in the onset of metabolic dysregulation of the islet beta cell under chronic hyperglycemic conditions provide additional clues with regard to overall roles of this scaffolding protein in the pathology of metabolic disorders.

Based on existing data in other cell types and data accrued during the current investigation in pancreatic beta cells, we propose key roles for CARD9-RhoGDIβ-Rac1-p38MAPK-NF-κB signaling module in the cascade of events leading to beta cell dysfunction under the duress of chronic hyperglycemic conditions. We postulate that exposure of pancreatic beta cells to chronic hyperglycemic conditions leads to increased expression of CARD9 (Fig. 1), leading to CARD9-mediated dissociation RhoGDIβ-Rac1 complex *via* association of CARD9 with RhoGDIβ under these conditions (Fig. 7) resulting in increased activation of Rac1 (Fig. 2). This, in turn, promotes induction of oxidative stress (*via* activation of phagocyte-like NADPH oxidase; [11, 18]) and ER stress (*via* the CHOP pathway; Fig. 5). Increase in metabolic (oxidative and ER) stress accelerates p38MAPK and NF-κB signaling pathways (Fig. 3 and 4) leading to mitochondrial dysregulation and beta cell dysfunction [17, 26, 53]. Our findings also suggest increased expression of BCL10 (Fig. 8), which has been implicated in CARD9-mediated p38MAPK activation in other cell types (see above). Potential involvement of BCL10 in islet beta cell dysregulation under diabetogenic conditions needs further investigation. It may be germane to point out that our current findings also suggest alterations in association between CARD9-RhoGDIβ-Rac1 signalome in pancreatic beta cells following exposure to hyperglycemic stress conditions (Fig. 7). Potential consequences of these signaling steps, including alterations in targeting of Rac1 to the appropriate subcellular compartments, need to be investigated further.

Earlier investigations by Dib and coworkers in human neutrophils demonstrated beta2 integrin-induced, PI 3-kinase-mediated relocalization of Rac1/Rac2 to the plasma membrane following dissociation of these G proteins from RhoGDIβ [54]. Data from studies by Duan et al. in small cell lung cancer cells have shown that mir-34a-induced down regulation of RhoGDIβ results in the activation and membrane translocation of Rac1 [55]. Along these lines, observations of Zhang and coworkers in MDA-MB-231 cells have indicated constitutive activation and increased membrane association and activation of p38MAPK and JNK1/2 stress kinases following silencing of RhoGDIβ [56]. More recent investigations from our laboratory have demonstrated no significant effects of siRNA-mediated depletion of RhoGDIβ on glucose-induced membrane association of Rac1 under acute exposure conditions to stimulatory glucose [44]. Future studies will address the regulatory roles of various GDIs (i.e., RhoGDIα. RhoGDIβ, and RhoGDIγ), specifically RhoGDIβ, in the functional regulation of Rho G proteins (e.g., Rac1) under metabolic stress conditions [57].

Based on published evidence [30, 31], and findings from the current investigations it appears that specific scaffolding proteins, such as CARD9 are able to functionally regulate G protein functions at multiple levels. For example, CARD9 has been shown to mediate Ras activation *via* its complexation with Ras-GRF1 and H-Ras leading to activation of downstream signaling pathways [30]. In addition, CARD9 has been shown to interact with RhoGDIβ thereby dissociating RhoGDIβ-Rac1 complex leading to subsequent activation of Rac1 ([31] and current study). Along these lines, evidence from the Thurmond laboratory [58] suggested novel regulatory roles for Caveolin-1 (Cav-1), another scaffolding protein, as a GDI for Cdc42, a small G protein belonging to Rho subfamily of G proteins. Based on data from complementary approaches, these investigators proposed that Cav-1 functions as a GDI for Cdc42 in pancreatic beta cells, thereby maintaining Cdc42 in an inactive (GDP-bound) state, thus regulating basal secretion in the absence of appropriate stimulus

for insulin secretion. Earlier studies from our laboratory have implicated Cav-1 as one of the intermediary proteins in the cascade of events leading to cytokine-induced, H-Ras mediated inducible nitric oxide synthase activation and nitric oxide generation in pancreatic beta cells [59]. Lastly, we proposed earlier that scaffolding or adaptor proteins, such as IQGAPs could contribute to small G protein-mediated cytoskeletal remodeling and vesicular trafficking, signaling steps which are necessary for insulin secretion [60, 61]. Interestingly, genome-wide association analysis identified IQGAP2 as one of the candidate genes for insulin resistance and T2DM [62]. Potential regulatory roles of IQGAP1 in the activation of (non-prenylated) Rho G proteins (e.g., Rac1) and cytokine production in macrophages has been demonstrated recently [63]. While regulatory roles of BCL10 in islet beta cell function in health and metabolic stress remain unknown, clinical and immunological features of human BCL10 have been reviewed recently [64]. Taken together, these data provide fresh insights into potential regulation of small G proteins by various adaptor/scaffolding proteins in cell function in health and pathological states.

Lastly, using a dual systems genetic approach, Kaur and coworkers have reported CARD9 as one of the 9 genes identified in the "T1D-T2D islet expression quantitative trait locus interaction network" in human islets [65]. Furthermore, extended network analysis of these 9 genes identified several signaling pathways in which CARD9 might be involved in. These include CLR, CLEC7A (Dectin-1), NLR, immune system and NOD1/2 signaling pathways. As pointed out by these investigators, additional studies are warranted to precisely identify roles of these genes (e.g., CARD9) in islet beta cell function in health and diabetes [65]. At least at the outset, our current findings of novel regulatory roles of CARD9 in the pathogenesis of islet beta cell dysregulation under the duress of chronic hyperglycemic conditions gain further strength (and translational impact/relevance) and form basis for future investigations in the field of CARD9 biology in islet beta cell function.

Conclusion

In the current study, we presented the first evidence to implicate novel roles for CARD9 in islet beta cell dysfunction under diabetogenic conditions. It is noteworthy that CARD9 appears to exert dual regulatory roles in islet beta cell function. Our earlier findings suggested p38MAPK-dependent, and Rac1-TRIM62-ERK1/2-independent regulation by CARD9 of GSIS [34]. Interestingly, CARD9 appears to contribute to hyperglycemia-induced metabolic dysfunction of the islet beta cell *via* modulation of Rac1-p38MAPK-NFκB-CHOP-BCL10-dependent, and TRIM62-JNK1/2-ERK1/2-independent mechanisms. Much remains to be understood about this pathway, specifically on putative roles of this module in islet beta cell dysregulation in animal models of diet-induced obesity and T2DM. These aspects are being addressed in our laboratory currently.

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Abbreviations

BCL10	B-cell lymphoma/leukemia 10
BRD5529	a known inhibitor of CARD9-E3 ubiquitin ligase
CARD9	Caspase recruitment domain containing protein 9
Cav-1	caveolin-1
CHOP	C/EBP homologous protein
CLR	C-type lectin receptor
Ehop-016	N4-(9-ethyl-9H-carbazol-3-yl)-N2-(3-morpholinopropyl)pyrimidine-2,4-diamine
ERK	extracellular signal-regulated kinase
ER stress	endoplasmic reticulum stress
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide ex-change factor
GRF-1	guanine nucleotide releasing factor-1
GSIS	glucose-stimulated insulin secretion
LyGDI	Rho-GDP dissociation inhibitor-β
Nox2	phagocyte-like NADPH oxidase
NSC23766	6-N-[2-[5-(diethylamino)pentan-2-ylamino]-6-methylpyrimidin-4-yl]-2-methylquinoline-4,6-diamine
p38MAPK	p38 mitogen-activated protein kinase
Rac1	Ras-related C3 botulinum toxin substrate 1
ROS	reactive oxygen species
siRNA	small interfering RNA
T2DM	type 2 diabetes mellitus
Tiam1	T-lymphoma invasion and metastasis-inducing protein 1
TRIM62	tripartite motif containing 62

| **Vav2** | Vav guanine nucleotide exchange factor 2 |

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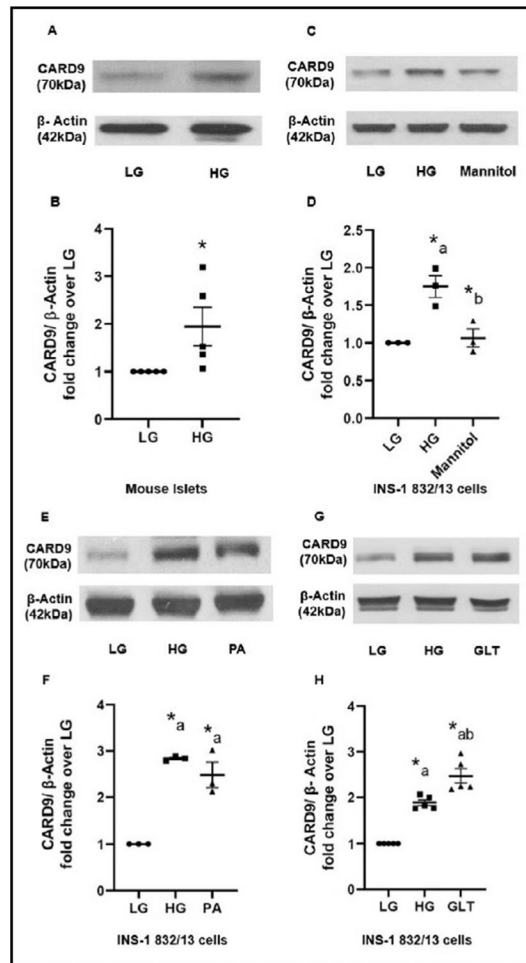
Fig. 1.

Diabetogenic stimuli increase the expression of CARD9 in mouse islets and INS-1 832/13 cells. Panel A: Mouse islets were treated with LG (2.5mM) or HG (20mM) for 24 hrs and expression of CARD9 was determined by western blotting. A representative blot from 5 independent experiments was shown in Panel A. β-Actin was used as the loading control. Panel B: Densitometric quantification of the pooled data from panel A is provided herein. Data are represented as mean ± SEM (n=5). (Comparisons-*: significant compared with LG; *p<0.05). Panel C: INS-1 832/13 cells were incubated with LG (2.5 mM), HG (20 mM) or mannitol (20mM) for 24 hrs. Relative abundance of CARD9 in lysates from these cells was determined by western blotting. β-Actin was used as the loading control. Panel D: Pooled data from studies in Panel C are shown here. The data are mean ± SEM (n=3). (Comparisons-a: significant compared with LG; b: significant compared with HG; *p<0.05). Panel E: INS-1 832/13 cells were treated with LG (2.5 mM), HG (20 mM) or Palmitic Acid (PA; 500 μM) for 24 hrs. Expression levels of CARD9 were determined by western blotting. β-Actin was used as the loading control. Panel F: Pooled data from studies in Panel E are shown here. The data are presented as mean ± SEM (n=3). (Comparisons-a: significant compared with LG; *p<0.05). Panel G: Lysates of INS-1 832/13 cells treated with LG (2.5 mM), HG (20 mM) or a combination of HG (20mM) and PA (500 μM) (GLT) for 24 hrs were used to determine the expression of CARD9 by western blotting. β-Actin was used as

loading control. Panel H: Pooled data from multiple studies shown in Panel G are shown here. Data are presented as mean ± SEM (n=5). (Comparisons-a: significant compared with LG; b: significant compared with HG; *p<0.05)
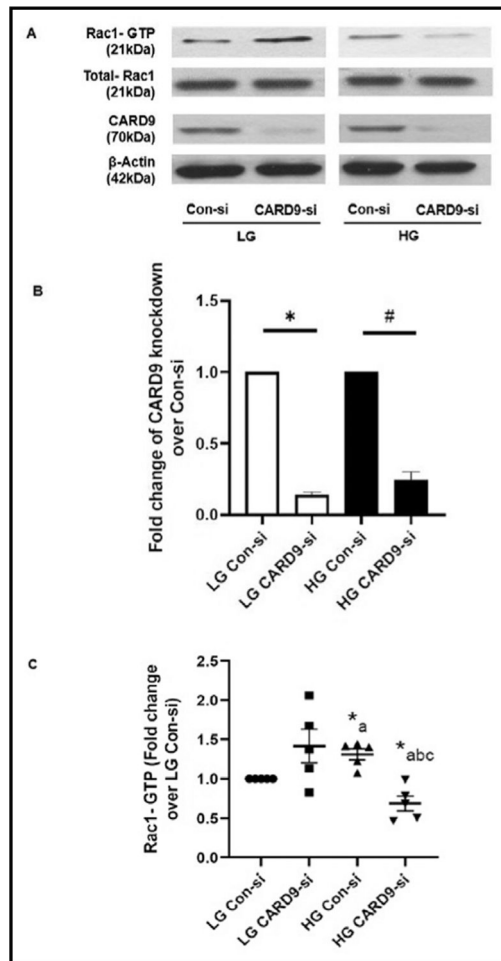
Fig. 2.
siRNA-mediated knockdown of CARD9 inhibits high glucose induced activation of Rac1 in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were transfected with Con-si or CARD9-si. 48 hrs post transfection the cells were treated with LG or HG for 24 hrs. Degree of Rac1 activation in these cells was determined by the pull-down assay (see Methods). The degree of CARD9 knockdown and total Rac1 expression in these cell lysates is also provided. β-Actin was used as loading control. A representative blot from five independent studies is shown here. Panel B: Efficiency of CARD9 knockdown under our current experimental conditions is indicated. *-significant compared with LG Con-si and #-significant compared with HG Con-si. Data are expressed as mean ± SEM (n=5 independent experiments). Panel C: Pooled Rac1 activation data from multiple experiments (shown in Panel A) are provided in Panel C. Data are expressed as fold change over LG Con-si. Data are mean ± SEM (n=5). (Comparisons: a: significant compared with LG Con-si; b: significant compared with LG CARD9-si; c: significant compared with HG Con-si; *p<0.05).
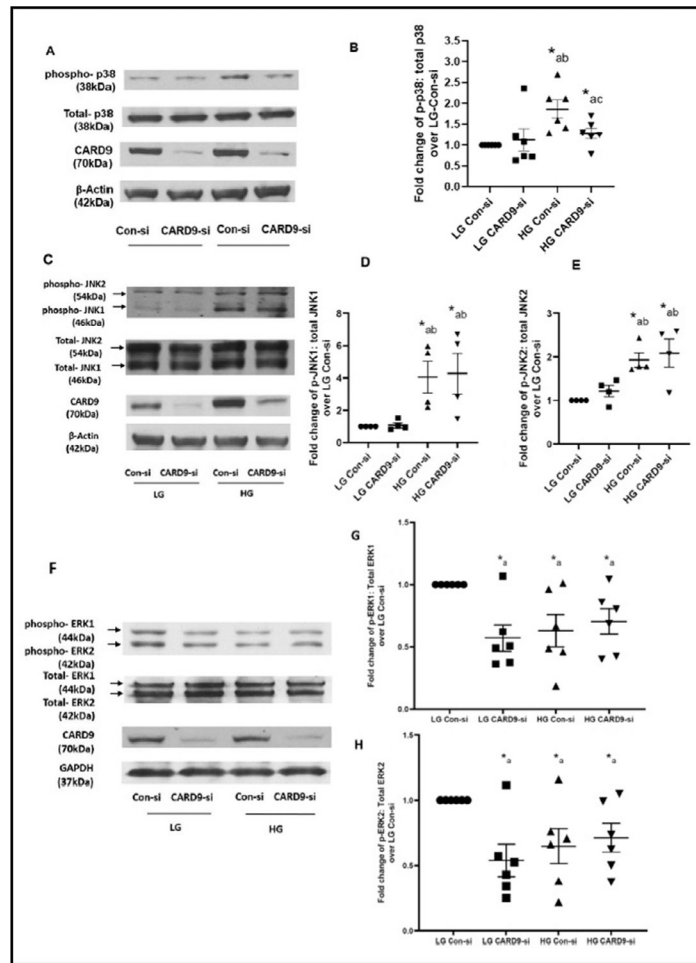
Fig. 3.
CARD9 knockdown attenuates high glucose-induced activation of p38MAPK but does not affect regulatory effects of high glucose on JNK1/2 and ERK1/2 in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were transfected with Con-si or CARD9-si. Next, 48 hrs post transfection the cells were treated with LG or HG for 24 hrs. Relative abundance of phosphop38 and total-p38 in lysates from these cells was assessed by western blotting. Western blot data for CARD9 expression in these cell lysates is shown to demonstrate knockdown of CARD9 under these experimental conditions. β-Actin was used as loading control for these conditions. A representative blot from six independent studies is shown here. Panel B: Band intensities were quantified by densitometry for phospho-p38MAPK and Total-p38MAPK and the ratios (phospho-p38MAPK: Total-p38MAPK) are provided herein. Data are represented as fold change from LG Con-si and are given as mean ± SEM (n=6). (Comparisons: a: significant compared with LG Con-si; b: significant compared with LG CARD9-si; c: significant compared with HG Con-si; *p<0.05). Panel C: INS-1 832/13 cells were transfected with Con-si or CARD9-si. 48 hrs. post transfection the cells were treated with LG or HG for 24 hrs. Relative abundance of phospho- and total-JNK1 and JNK2 in lysates from these cells was assessed by western blotting. Western blot data for CARD9 expression in these cell lysates is shown to demonstrate knockdown of CARD9 under these experimental conditions. β-Actin was used as loading control for

these conditions. A representative blot from four independent studies is shown here. Panels D and E: Densitometric analysis of pooled data from four independent experiments (in Panel C) is provided in Panel D (JNK1) and Panel E (JNK2). The data are shown as fold change from LG Con-si (mean ± SEM; n=4). (Comparisons: a: significant compared with LG Con-si; b: significant compared with LG CARD9-si; *p<0.05). Panel F: INS-1 832/13 cells were transfected with Con-si or CARD9-si. 48 hrs. post transfection the cells were treated with LG or HG for 24 hrs. Relative abundance of phospho- and total-ERK1 and ERK2 in lysates from these cells was assessed by western blotting. Western blot data for CARD9 expression in these cell lysates is shown to demonstrate knockdown of CARD9 under these experimental conditions. GADPH was used as loading control for these conditions. A representative blot from six independent studies is shown here. Panels G and H: Densitometric analysis of pooled data from six independent experiments (in Panel F) is provided in Panel G (ERK1) and Panel H (ERK2). The data are shown as fold change from LG Con-si (mean ± SEM; n=6). (Comparisons: a: significant compared with LG Con-si; *p<0.05).
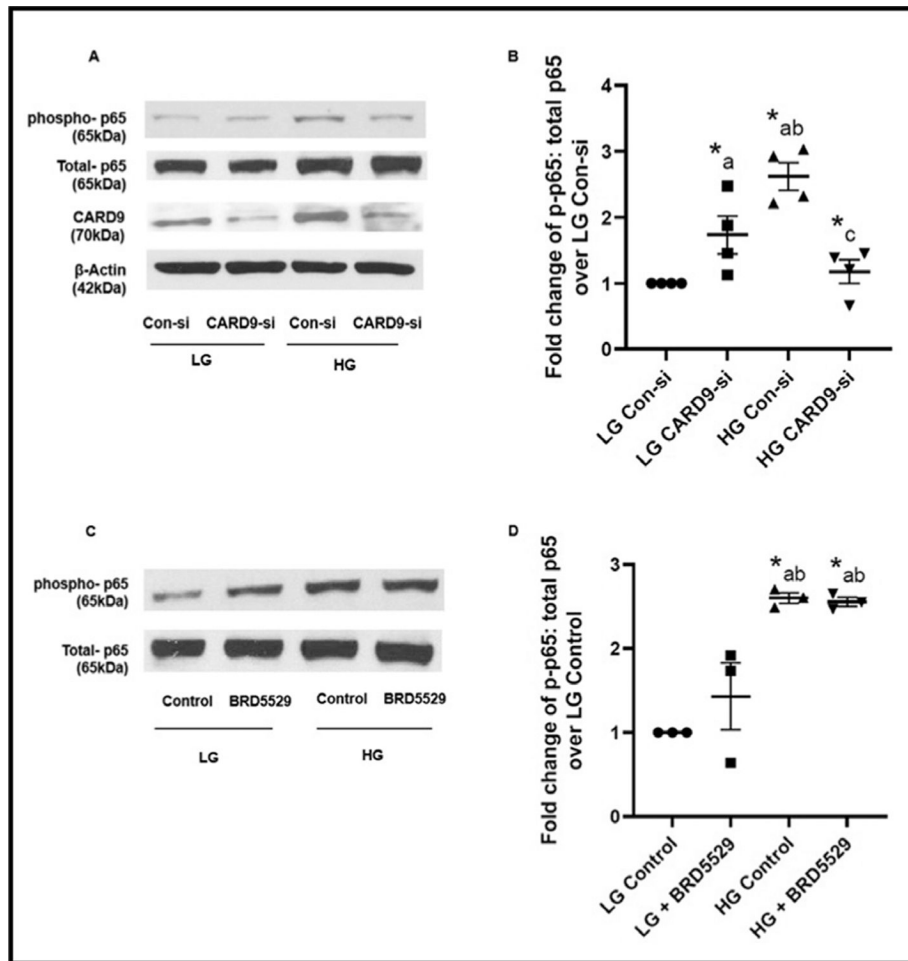
Fig. 4.

siRNA-mediated knockdown of CARD9 inhibits high glucose-induced phosphorylation of p65 and evidence for lack of regulation by the CARD9-TRIM62 module. Panel A: INS 832/13 cells were transfected with Con-si or CARD9-si. 48 hours post transfection the cells were treated with LG or HG for 24 hours. Cell lysates were resolved immediately using SDS-PAGE gel technique following lysate processing to access phosphorylation of p65. The same blots were re-probed with Total-p65 and used as the loading control. Western blotting was done on these lysates to determine the degree of CARD9 knockdown. (β-Actin was used as loading control). Four independent studies were done. The blots given are representative of those studies. Panel B: phospho-p65: Total-p65 ratios were obtained (by densitometry) for the four independent experiments and were plotted and expressed as fold change from LG Con-si. Data are given as mean ± SEM. (Comparisons: a: significant compared with LG Con-si; b: significant compared with LG CARD9-si; c: significant compared with HG Con-si; *$p<0.05$). Panel C: INS-1 832/13 cells were treated for 24 hours with LG or HG with BRD5529 (50μM) or without BRD5529/DMSO (control). Following treatment, cell lysates were prepared and subsequently western blotting was carried out and blots obtained were probed for phospho-p65. The same blots were re-probed for Total-p65. Representative blots from multiple experiments are shown. Panel D: Densitometry analysis was performed for phospho-p65 and Total-p65 bands. The ratios of phospho-p65: Total-p65

in multiple experiments were pooled and plotted as fold change from LG Control (mean ± SEM). (Comparisons: a: significant compared with LG Control; b: significant compared with LG + BRD5529; n=3; *p<0.05).
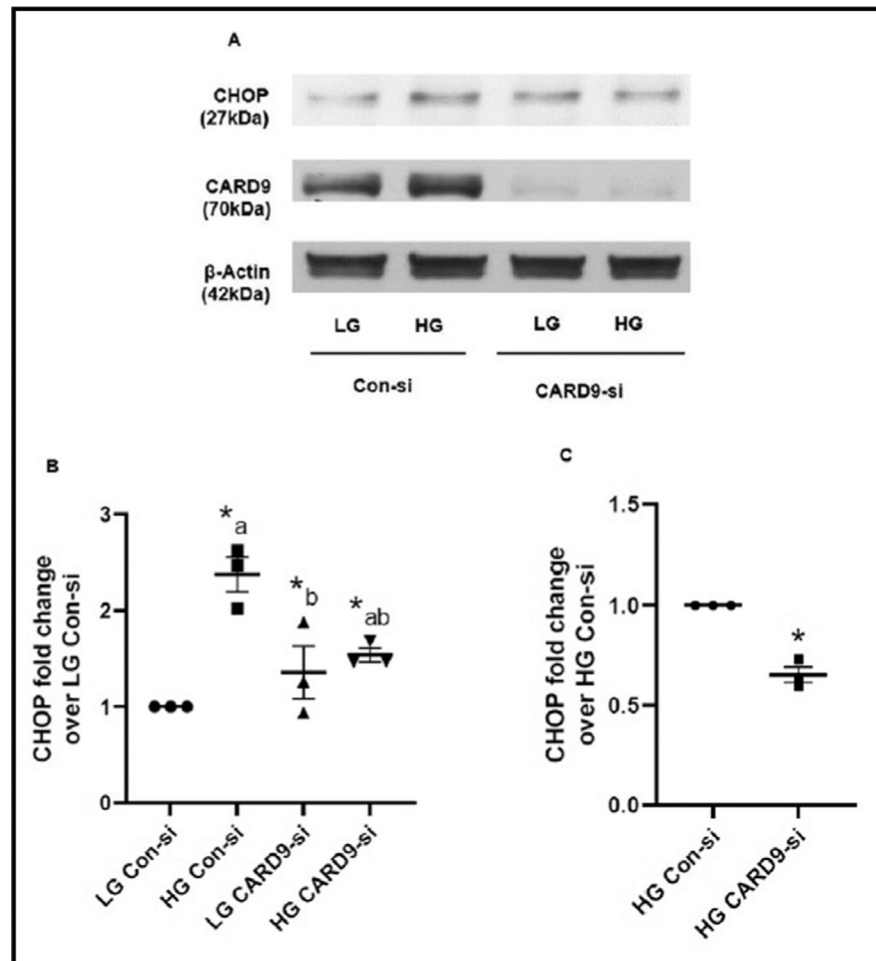
Fig. 5.
siRNA-mediated knockdown of CARD9 attenuates high glucose-induced CHOP expression in INS-1 832/13 cells. Panel A: Following transfection with Con-si or CARD9-si as described previously, INS-1 832/13 cells were treated with LG or HG for 24 hrs. Relative abundance of CHOP and CARD9 in the cell lysates were determined by western blotting. β-Actin was used as the control for protein loading. A representative blot from 3 independent experiments is shown here. Panel B: Densitometric quantification of CHOP expression in 3 independent experiments is shown here. Data are expressed as fold change relative to LG Con-si (* $p < 0.05$). (Comparisons: a: significant compared with LG Con-si; b: significant compared with HG Con-si; *$p < 0.05$). Panel C: Data depicting degree of inhibition of HG-induced CHOP expression in CARD9 depleted cells is shown here (* $p < 0.05$; n=3).
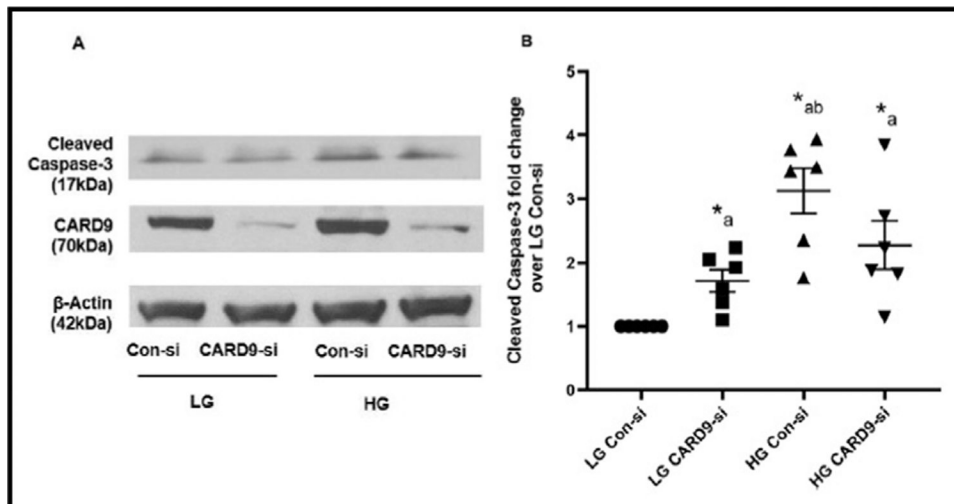
Fig. 6.
CARD9 knockdown does not appear to impact high glucose-induced Caspase-3 activation in INS-1 832/13 cells. Panel A: Following transfection with Con-si or CARD9-si, INS-1 832/13 cells were treated with LG or HG for 24 hrs. Relative abundance of cleaved Caspase-3 (an index for Caspase-3 activation) and CARD9 in the cell lysates was determined by western blotting. β-Actin was used as the control for protein loading. A representative blot from 6 independent experiments is shown here. Panel B: Densitometric quantitation of cleaved caspase-3 in Panel A is depicted herein. The pooled data are expressed as fold change relative to LG Con-si (mean ± SEM; n=6). (Comparisons: a: significant compared with LG Con-si; b: significant compared with LG CARD9-si; *p<0.05).
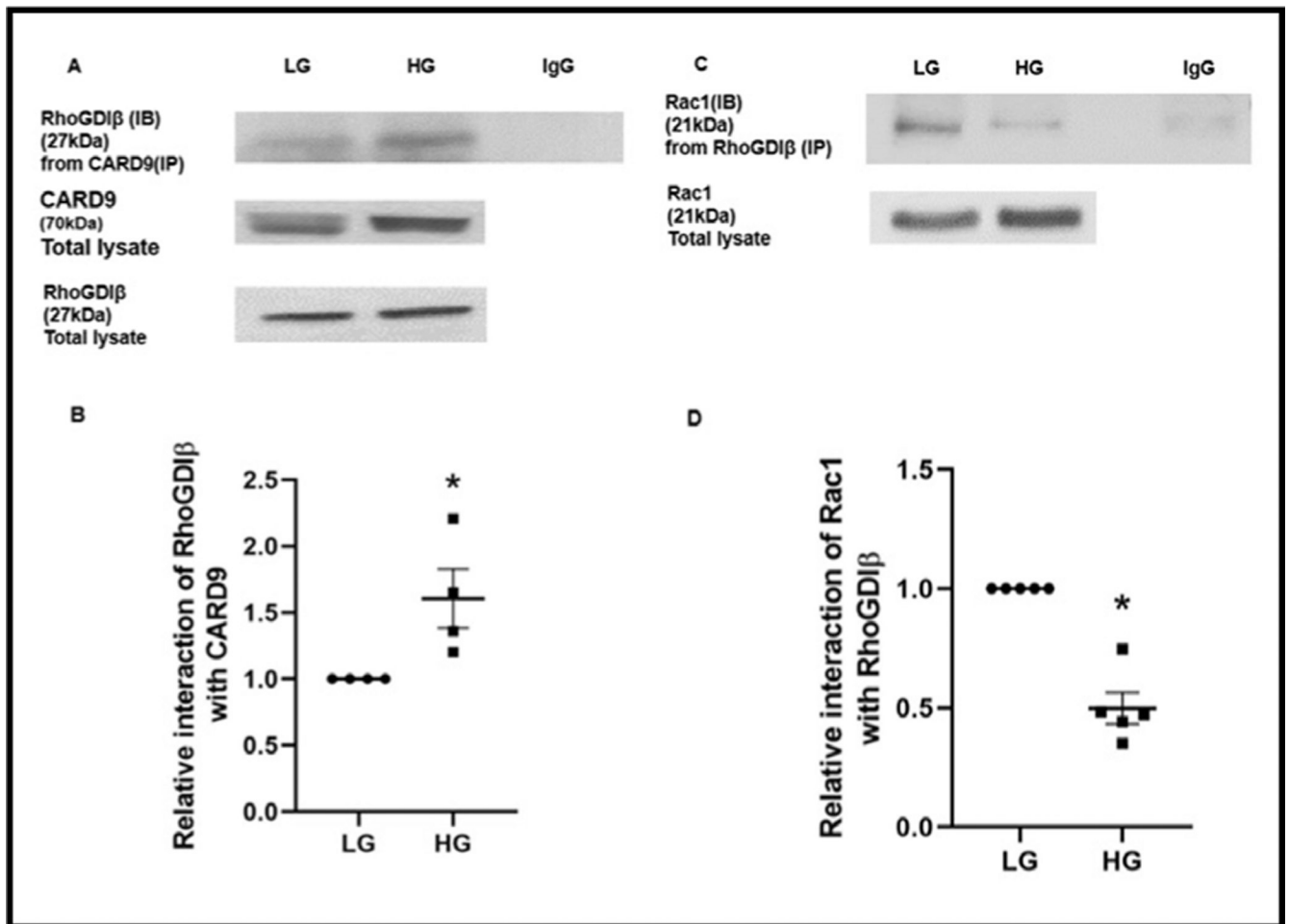
Fig. 7.
Co-immunoprecipitation studies reveal significant alterations in interaction between RhoGDIβ, CARD9 and Rac1 in INS-1 832/13 cells under chronic hyperglycemic conditions. CARD9 (Panel A) or RhoGDIβ (Panel C) was immunoprecipitated (IP) from pre-cleared lysates derived from LG or HG treated (24 hrs) INS-1 832/13 cells. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted (IB) for RhoGDIβ (Panel A) or Rac1 (Panel C). Immunoglobulin G (IgG) validated the specificity of the protein-protein interactions and total lysates were subjected to western blotting to demonstrate equal protein expression under both LG and HG conditions. Data in Panel B and Panel D represent densitometric quantification of protein bands to highlight interactions between RhoGDIβ-CARD9 (n=4) and RhoGDIβ-Rac1 (n=5). The pooled data are expressed as fold change relative to LG. Data are represented as mean ± SEM; $*p<0.05$).
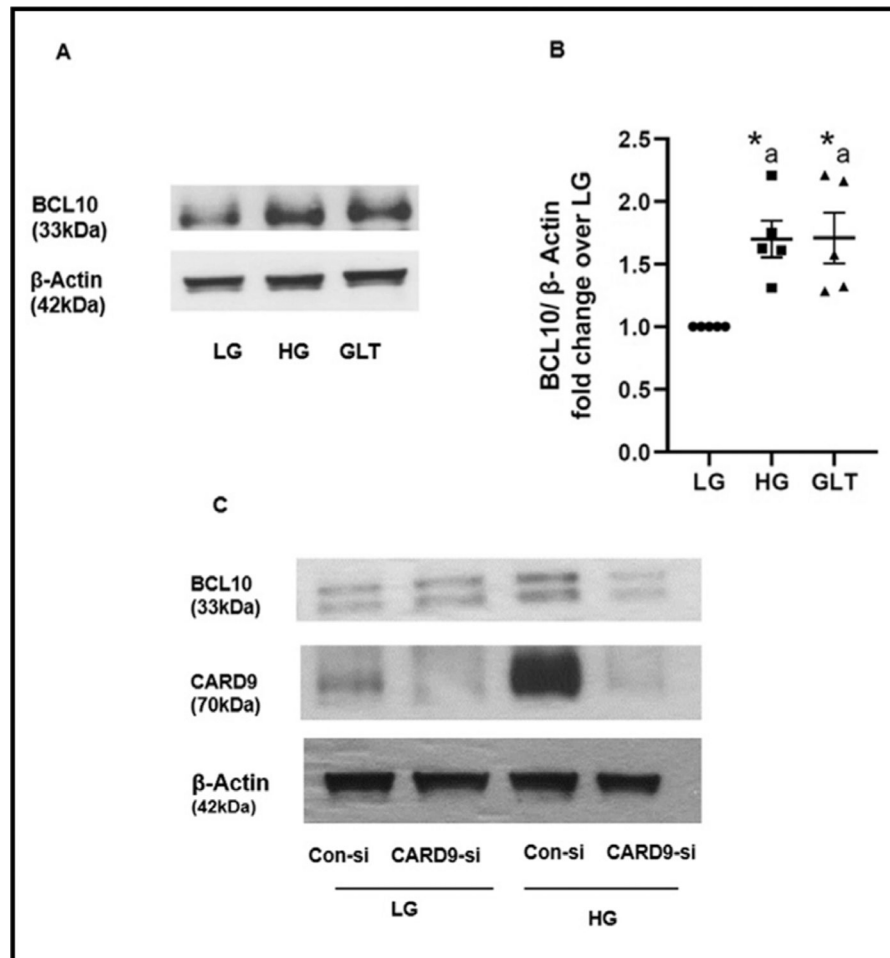
Fig. 8.
Gluco- and gluco-lipotoxic conditions increase the expression of BCL-10 in INS-1 832/13 cells: Evidence for CARD9 as a modulator of BCL10 expression under chronic hyperglycemic conditions. Panel A: INS-1 832/13 cells were cultured under LG, HG or GLT conditions as described in Methods. Relative abundance of BCL10 in the cell lysates was determined by western blotting. β-actin was used as a loading control. Data in Panel B represent fold change in the expression of BCL10 over LG conditions. Data are mean ± SEM from 5 independent experiments. Data in Panel C is a representative blot from two independent experiments demonstrating suppression in HG-induced expression of BCL10 in INS-1 832/13 cells following siRNA-mediated knockdown of CARD9. Expression levels of BCL10, CARD9 and β-actin (loading control) are indicated in this figure.