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CARD9 mediates glucose-stimulated insulin secretion in pancreatic beta cells

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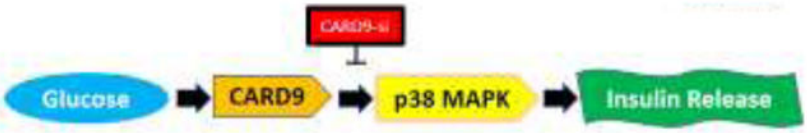
Abstract

Caspase recruitment domain containing protein 9 (CARD9) plays key regulatory role(s) in innate and adaptive immune responses. Recent evidence implicates CARD9 in the onset of metabolic diseases including insulin resistance. However, potential contributory roles of CARD9 in glucose-stimulated insulin secretion (GSIS) remain unknown. Herein, we report that CARD9 is expressed in human islets, rat islets, mouse islets and clonal INS-1 832/13 cells. Subcellularly, CARD9 is predominantly cytosolic (~75%) in INS 1– 832/13 cells. siRNA-mediated depletion of CARD9 expression significantly (~50%) suppressed GSIS in INS-1 832/13 cells. Interestingly, glucose-induced activation of Rac1, a small G-protein, which is a requisite for GSIS to occur, is unaffected in CARD9-si transfected cells, suggesting that CARD9-mediates GSIS in a Rac1-independent fashion. Furthermore, insulin secretion promoted by KCl or mastoparan, a global G protein activator, remained resistant to CARD9 depletion in INS-1 832/13 cells. In addition, pharmacological inhibition (BRD5529) of interaction between CARD9 and TRIM62, its ubiquitin ligase, exerted no significant effects on GSIS. Lastly, depletion of CARD9 prevented glucose-induced p38, not ERK1/2 phosphorylation in beta cells. Based on these observations, we propose that CARD9 might regulate GSIS *via* a Rac1-independent and p38-dependent signaling module.

Graphical Abstract



CARD9 mediates physiological insulin secretion

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Credit Author Statement

SG and MH performed the studies, carried out statistical analyses of the experimental data and edited the manuscript. AK participated in the design the overall execution of the studies. He wrote and edited the manuscript.

Conflict of Interests

The authors declare no conflict of interests

Keywords

CARD9; pancreatic beta cell; insulin secretion; Rac1

1. Introduction

Glucose-stimulated insulin secretion (GSIS) from the pancreatic beta cell involves a precise interplay between metabolic (e.g., generation of second messenger molecules) and cationic (e.g., increased intracellular calcium) events in a glucose-stimulated islet [1–5]. In addition, considerable evidence suggests key regulatory roles for a variety of GTP-binding proteins (G proteins; e.g., Arf6, Cdc42, Rac1 and Rab GTPases) as key regulators of GSIS, primarily at the levels of cytoskeletal remodeling, movement of insulin-containing secretory granules to the plasma membrane for fusion and release of insulin into the circulation [6–15]. In the context of G protein-mediated GSIS, several regulatory proteins of G protein function, namely guanine nucleotide exchange factors (GEFs; e.g., Tiam1, Vav2, p-REX1, and β-PIX), guanine nucleotide dissociation inhibitors (GDIs; e.g., Rho GDIα) and GTPase activating proteins (GAPs; e.g., RacGAP1) have also been identified and studied in the pancreatic beta cell [9, 16–18].

CARD9 is a scaffolding protein, which is expressed in many cell types, including macrophages, dendritic cells, monocytes and neutrophils [19–21]. A growing body of experimental evidence implicates CARD9 in promoting innate immunity; its primary role is ascribed to be a transducer of signals from the pattern recognition receptors (PPRs) localized on the cell membrane to various intracellular signaling pathways [19, 22]. It is noteworthy that, CARD9 has been shown to contribute to the onset of metabolic diseases, including insulin resistance and obesity [22]. Recent evidence in animal models with CARD9 deletion demonstrated roles for CARD9 in diet-induced inflammation, obesity and metabolic pathologies [23].

Despite the evidence highlighted above, virtually no information is available on the expression of, and regulation by CARD9, of islet beta cell function, including GSIS. Therefore, we attempted to answer some of these questions in insulin-secreting pancreatic beta cells. Our current findings suggest that CARD9 is expressed in human islets, normal rat islets, mouse islets and clonal INS-1 832/13 cells, and that it plays a modulatory role in GSIS. We also provide potential mechanistic details for CARD9's role in islet beta cell function.

2. Materials and Methods

2.1 Reagents

CARD9 antibody and THP-1 (human monocyte) cell lysate were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies against ERK1/2 (total and phospho), p38 MAPK (total and phospho), E-Cadherin, GAPDH and HRP-conjugated secondary antibodies were from Cell Signaling Technology (Danvers, MA, USA). Rac1 antibody was from EMD Millipore (Burlington, MA, USA). β-actin antibody was from Sigma Aldrich

(St. Louis, MO, USA). Mem-PER Plus Membrane Extraction kit was from Thermo Fisher Scientific (Waltham, MA, USA). ON-TARGETplus Non-targeting siRNA (Con-siRNA), ON-TARGETplus Rat CARD9 siRNA-SMARTpool and DharmaFect1 transfection reagent were from Horizon Discovery (Lafayette, CO, USA). Rat specific Insulin ELISA kit was from ALPCO (Salem, NH, USA). Rac1 activation kit (pull-down assay) was obtained from Cytoskeleton (Denver, CO, USA). Mastoparan and BRD2599 (CARD9 inhibitor) were purchased from Enzo Life Sciences (Ann Arbor, MI, USA) and Sigma-Aldrich (St. Louis, MO, USA), respectively.

2.2 Cell culture, rat islets, mouse islets and human islets

INS-1 832/13 cells were propagated in RPMI-1640 medium consisting of 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. Prior to a given study, cells were treated overnight with low serum/ low glucose media. Low glucose (LG) and high glucose (HG) treatments were done with 2.5 mM and 20 mM glucose, respectively. Rat (Sprague-Dawley) and mouse (C57BL/6) islets were isolated using the collagenase digestion method. All protocols received IACUC (Wayne State University and the JDD VA Medical Center, Detroit) approvals. Human islets were obtained from Prodo Labs (Aliso Viejo, CA, USA).

2.3 Depletion of endogenous expression of CARD9 via siRNA methodology

INS1 832/13 cells were transfected with CARD9-si (100 nM) using the DharmaFect1 reagent. Non-targeting control siRNA (Con-si) was employed to assess potential non-specific effects of siRNA delivery and for providing a baseline to compare to siRNA-treated samples. INS-1 832/13 cells transfected with Con-si or CARD9-si were propagated further (for 48 hours) in complete growth medium. The efficiency of CARD9 knockdown in Con-si and CARD9-si transfected cells was assessed by western blotting.

2.4 Western blotting

Following incubation under specific experimental conditions described under the Results section, the cells were washed with ice cold PBS and lysed in RIPA buffer containing protease and phosphatase inhibitors. Cell lysates (20–50 μg protein) were separated by 10% SDS-PAGE gels and transferred onto the nitrocellulose membranes at 110 V for 1 hour at 4°C using BIORAD PowerPac HC 250V equipped with Mini PROTEAN Tetra Cell bucket. The membranes were then blocked with 3% BSA in PBS-T at room temperature for 1 hour. Blots were then immunoprobed with corresponding primary antibody in 1.5% BSA in PBS-T (CARD9, total and phospho ERK1/2, total and phospho-p38, and Rac1– 1:1000, β- Actin- 1:5000) overnight at 4°C. The membranes were then washed three times for 15 minutes with PBS-T and probed with appropriate HRP-conjugated secondary antibody [Anti- mouse for CARD9, Rac1 and β- Actin and anti-rabbit for total and phospho ERK1/2 and p38] in 1.5% BSA in PBS-T at room temperature for 1 hour. After repeated washing (3 times; 10 minutes each), the target protein bands were detected by chemiluminescence and autoradiography. The intensities of candidate protein bands were then quantified by densitometry.

2.5 Isolation of total membrane and cytosolic fractions from INS-1 832/13 cells

Following overnight starvation, INS1 832/13 cells were treated with LG and HG for 45 minutes and total membrane and cytosolic fractions were isolated using Mem-PER Plus Membrane Extraction kit as we described recently in [24]. Western blotting was used to quantify protein expression as described above.

2.6 Insulin secretion assays

INS-1 832/13 cells, transfected with either Con-si or CARD9-si, were starved (overnight) followed by exposure to LG or HG for 45 minutes. KCl-induced insulin secretion studies were conducted in Con-si or CARD9-si transfected cells with LG or KCl (60 mM; osmolality balanced by adjusting NaCl) for 60 minutes [8]. The regulatory effects of Mastoparan (Mas) on insulin secretion were assessed following incubation in the absence (LG) or presence of 30 μM Mas for 45 minutes. Insulin released into the medium was quantified using an ELISA kit [24, 25]. We also investigated putative effects of BRD5529, a CARD9 inhibitor [26], on GSIS following exposure of INS-1 832/13 cells with BRD5529 (50 μM) for either 1 hour or overnight followed by incubation with LG or HG for 45 minutes, and then quantifying insulin released into the medium as described above. For quantification of rapid and slow phases of GSIS, INS-1 832/13 cells, transfected with either Con-si or CARD9-si, were stimulated with either LG or HG for 10 minutes. The culture medium was removed for quantification of insulin representing the rapid secretion event. Following this, the cells were cultured in fresh medium containing the same stimuli for an additional 30 minutes for assessment of slow phase secretion as we reported earlier [13, 27] .

2.7 Rac1 activation assay

Con-si or CARD9-si transfected INS-1 832/13 cells were exposed to either LG or HG for 15 minutes, and the magnitude of Rac1 activation (Rac1-GTP conformation) was determined using a pull-down assay kit as we reported previously [12, 24, 25, 28].

2.8 p38 MAPK and ERK1/2 phosphorylation assay

INS-1 832/13 cells were transfected with either Con-si or CARD9-si. Forty-eight hours after transfection, the cells were incubated in LG or HG for 30 minutes at 37°C. Immediately following the incubation, cells were lysed using RIPA buffer containing protease and phosphatase inhibitors. Western blotting was done to determine relative abundance of phospho and total forms of p38 and ERK 1/2. Band intensities were quantified by densitometry.

2.9 Statistical analysis

Data are presented as mean ± SEM of at least three independent experiments. Statistical analysis for differences between groups was done using the Student's *t*-test. p value of < 0.05 was considered statistically significant.

3. Results

3.1 Expression and subcellular distribution of CARD9 in insulin-secreting cells

As stated above, expression of CARD9 in pancreatic beta cells has not been investigated before. Therefore, at the outset, we determined by western blotting the expression of CARD9 in lysates derived from INS-1 832/13 cells, normal rat islets, mouse islets, and human islets. To affirm the specificity of the antibody for CARD9 that was employed in the current studies, we used THP-1 (human monocyte) cell lysate as the positive control for CARD9. Figure 1 (Panel A) is a representative western blot demonstrating the specificity of the antibody as evidenced by a band with an expected apparent molecular weight of 70 kDa (with increasing intensity as a function of increase in INS-1 832/13 cell lysate protein concentration; lanes B-D). Interestingly, in addition to CARD9, this antibody reacted with another protein (smaller in size) in THP-1 cell lysates (lane A). Together, data in Figure 1 (Panel B) indicated that CARD9 is expressed in INS-1 832/13 cells, rat islets, mouse islets and human islets. We next assessed the subcellular distribution (membrane *vs*. cytosolic) of CARD9 in INS-1 832/13 cells. Data depicted in Figure 1 (Panel C) suggested that CARD9 is predominantly cytosolic in distribution in these cells (~76% in cytosol vs. ~24% in the membrane).

3.2 Knockdown of CARD9 results in inhibition of GSIS in INS-1 832/13 cells

Next set of studies were aimed at examining putative regulatory roles of CARD9, if any, in GSIS. To address this question, we utilized CARD9-si to deplete endogenous expression of CARD9 in INS-1 832/13 cells. Data in Figure 2 (Panels A and B) demonstrated ~ 70% knockdown of CARD9 in these cells under our experimental conditions. Data shown in Figure 2 (Panel C) indicated a robust degree of GSIS in INS-1 832/13 cells transfected with Con-si. A modest, but significant inhibition of basal insulin secretion was seen in Con-si transfected cells. It is noteworthy that CARD9-si transfection in INS-1 832/13 cells resulted in a marked attenuation of GSIS (Figure 2; Panel C). Altogether, these observations suggested a novel regulatory role for CARD9 in GSIS in islet beta cells.

3.3 Suppression of CARD9 expression elicited no significant effects on insulin secretion promoted by a membrane depolarizing concentration of KCl in INS-1 832/13 cells

Next, we determined regulatory roles of CARD9 in insulin secretion promoted by a membrane depolarizing concentration of KCl. Insulin secretion under these conditions represents the exocytosis of readily releasable pool of insulin-laden secretory granules. As in the case of GSIS (Figure 2), we addressed this question by quantifying KCl-induced insulin secretion in INS-1 832/13 cells transfected with either Con-si or CARD9-si. Data depicted in Figure 3 (Panel A) demonstrated significant knockdown of CARD9 in these cells following transfection with CARD9-si. However, no significant effects on insulin secretion promoted by KCl were demonstrable under these experimental conditions (Figure 3; Panel B). It is noteworthy that, as in the case of studies of GSIS, a slight, but significant inhibition of insulin secretion was noted in these cells under LG exposure conditions following CARD9-knockdown (Figure 3; panel B). Together, these data suggest minimal regulatory roles for CARD9 in KCl-induced insulin secretion. Next series of studies were undertaken to gain potential mechanistic insights underlying CARD9 mediated regulation of GSIS.

3.4 Knock-down of CARD9 inhibits both rapid and slow phases of GSIS in INS-1 832/13 cells

We next examined the potential possibly of distinct regulatory roles of CARD9 in time-dependent changes in GSIS (i.e., biphasic secretion). To address this, we quantified GSIS from Cont-si or CARD9-si transfected beta cells during two consecutive static incubations, namely the initial 10 minute incubation corresponding to the rapid phase secretory event, followed by a 30-minute exposure to stimulatory glucose corresponding to the slow phase of insulin secretion [13, 27]. Data represented in Figure 4 (Panel A) demonstrated no significant effect of CARD9 knockdown on basal insulin secretion during the rapid or slow phase study conditions. Interestingly, however, significantly lower levels of GSIS were noted in CARD9 depleted INS-1 832/13 cells during both the rapid and sustained phases of GSIS. These data raise an interesting possibility for regulatory roles of CARD9 in both phases of GSIS.

3.5 Mastoparan, a known regulator of G protein activation, induces insulin secretion in INS-1 832/13 cells in a CARD9-independent fashion

Earlier studies from multiple laboratories have demonstrated that Mas, a tetra decapeptide (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH2) isolated from wasp venom, promotes insulin secretion from pancreatic β-cells *via* activation of G proteins [29–33]. Therefore, we investigated if Mas-induced insulin secretion is regulated by CARD9-derived signaling pathways. Data provided in Figure 5 (Panel A) indicated significant knockdown of CARD9 under conditions we employed in this set of investigations. As expected, Mas significantly stimulated insulin secretion in INS-1 832/13 cells transfected with Con-si. Interestingly, however, Mas-induced insulin secretion remained resistant to CARD9 knockdown (Panel B). Together, these data provide evidence that insulin secretion elicited by nutrient secretagogues (e.g., glucose), but not other regulators (e.g., KCl and Mas) is regulated by CARD9 (see Discussion).

3.6 Subcellular distribution of CARD9 is not altered under conditions of GSIS in INS-1 832/13 cells

We next asked if the subcellular (cytosolic *vs.* membrane) distribution is altered in INS-1 832/13 cells under conditions that promoted GSIS. To address this question, total membrane and cytosolic fractions were isolated (see Methods) from INS-1 832/13 cells exposed to basal (LG) or stimulatory (HG) glucose concentrations for 45 minutes. Relative abundance of CARD9 in these fractions was determined by western blotting. Data depicted in Figure 6 (Panel A) suggested that CARD9 is predominantly cytosolic in distribution in INS-1 832/13 cells, and that no significant alterations in the subcellular distribution of CARD9 were demonstrable in these cells exposed to HG. High abundance of E-cadherin (~ 135 kDa) and GAPDH (~37 kDa) in membrane and cytosolic fractions affirmed the purity of these fractions isolated under these experimental conditions [24]. These data suggest that CARD9 does not undergo changes in the subcellular distribution under conditions of GSIS.

3.7 Rac1 activation may not be requisite for CARD9-mediated GSIS in INS-1 832/13 cells

Extant data from numerous laboratories have indicated critical regulatory roles for small G proteins (e.g., Rac1) in GSIS [6, 15, 27, 34, 35]. Furthermore, these investigations have also revealed that glucose-, Mas, but not KCl-induced insulin secretion, requires activation of Rac1 [6, 17, 29]. In order to determine potential roles of glucose-induced Rac1 activation as one of the intermediary signaling steps in CARD9-mediated insulin secretion, we quantified glucose-induced activation of Rac1 in INS-1 832/13 cells transfected with Con-si or CARD9-si. As expected, data shown in Figure 6 (Panel B) indicated significant increase in Rac1 activation (i.e., Rac1-GTP) by glucose in cells transfected with Con-si. However, no significant changes in glucose-induced activation of Rac1 were seen in these cells following transfection with CARD9-si. Interestingly, a significant increase in Rac1 activation was also seen in CARD9-depleted cells under basal (LG) conditions. No changes in total Rac1 were noted under these experimental conditions. As indicated in Figure 6 (Panel B), CARD9-si significantly downregulated expression of CARD9 in these cells. Based on these data we surmise that CARD9 might exert its effect on insulin secretion *via* Rac1-independent mechanisms (see Discussion). Our findings also suggested that subcellular distribution of CARD9 is not altered under conditions that favor GSIS in these cells.

3.8 BRD5529, a known inhibitor of CARD9, fails to exert any inhibitory effects on GSIS in INS-1 832/13 cells

Recent investigations by Leshchiner and coworkers have reported BRD5529 (Figure 7; Panel A) as a selective inhibitor of CARD9. Mechanistic studies have demonstrated direct binding of BRD5529 to CARD9, thus preventing the interaction between CARD9 and TRIM62, a known E3 ubiquitin ligase for CARD9 culminating in the functional inactivation of CARD9 [26]. Therefore, in the next series of experiments we used this pharmacological approach to determine roles of TRIM62-CARD9 signaling axis, if any, in the sequence of events leading to GSIS. We have examined its effects under acute (60 min; Panel B) or longer (overnight; Panel C) exposure conditions prior to quantification of GSIS. Data from these investigations indicated no discernable effects of BRD5529 on GSIS at either exposure conditions. No significant effects of BRD5529 on basal insulin secretion were noted under these conditions. Altogether, these data imply that CARD9-TRIM62 axis might not underlie GSIS. Additional investigations are needed to further understand putative regulatory mechanisms that govern CARD9-mediated signaling steps involved in GSIS (see below).

3.9 Depletion of CARD9 suppresses glucose-induced phosphorylation of p38 MAPK, but not ERK1/2, in INS-1 832/13 cells

Several lines of evidence suggest that signaling steps that underlie GSIS might underlie MAPK activation [36–39]. Therefore, we asked if CARD9-mediated insulin secretion involves activation of MAPK, such as p38 and ERK1/2. To address this question glucose-induced phosphorylation of p38 and ERK1/2 was quantified in INS-1 832/13 cell transfected with Con-si or CARD9-si. Data in Figure 8 (Panel A) demonstrated significant knockdown of CARD9 under the conditions employed in the MAPK activation assays. Under these experimental conditions, we observed a significant stimulation of ERK1/2 phosphorylation by stimulatory glucose in cells transfected with Con-si. CARD9 knockdown did not

elicit any significant effects on glucose-induced ERK1/2 phosphorylation. No alterations in the expression of total ERK1/2 were seen under these conditions (Figure 8; Panel A). Our findings also suggested a significant increase in the phosphorylation of p38 in Con-si transfected INS-1 832/13 cells following exposure to stimulatory glucose (Figure 8; Panel A). Interestingly however, glucose-induced phosphorylation of p38 was significantly reduced in INS-1 832/13 cells following suppression of CARD9 expression following transfection of CARD9-si. Total p38 levels remained unchanged under these experimental conditions. Pooled data from multiple experiments on glucose-induced ERK1/2 and p38 phosphorylation in Con-si and CARD9-si transfected INS-1 832/13 cells were provided in Figure 8 (Panels B-D). Based on our current findings, we propose that glucose-induced p38 MAPK, but not ERK1/2 activation, might underlie cascade of events leading to CARD9-mediated GSIS.

4. Discussion

One of the goals of the current study was to determine the modulatory roles of CARD9 in GSIS. Salient observations of our studies are: [i] CARD9 is expressed in human islets, rat islets and mouse islets, and clonal INS-1 832/13 cells; [ii] CARD9 is predominantly cytosolic in distribution; [iii] siRNA-mediated knockdown of CARD9 results in glucose-, but not KCl- and Mas-induced insulin secretion; [iv] knockdown of CARD9 significantly inhibits both rapid and sustained phases of GSIS; [v] siRNA-mediated depletion exerts no significant effects on glucose-induced Rac1 activation; [vi] BRD5529, which inhibits interaction between CARD9 and TRIM62 resulting in inactivation of CARD9, fails to inhibit GSIS; and [vii] depletion of CARD9 expression elicits inhibition of glucose-induced p38, but not ERK1/2 phosphorylation. Potential significance of these findings in the context of CARD9-mediated GSIS are discussed below.

Our findings suggested that CARD9-mediates GSIS (during both phases) without significantly affecting KCl- and Mas-induced insulin secretion. We also observed that CARD9-mediated regulation of GSIS might not require activation of Rac1, which has been shown to be one of the key signaling steps in GSIS [6, 15, 27, 28, 34]. Furthermore, published evidence suggests that Rac1 activation may be necessary for Mas-induced, but not KCl-induced insulin secretion [6, 15, 28, 29, 40]. In addition, previous studies have also reported that Rac1 activation may be necessary for the sustained (slow) phase, not early phase of GSIS [27]. Taken together, these findings implicate that Rac1 activation may not be obligatory for CARD9-mediated effects on insulin secretion. What then are some potential mechanisms whereby CARD9 might mediate GSIS?

First, our findings do not preclude regulation, by CARD9, of other mediators of G protein functions, including GEFs and GDIs in eliciting indirect effects on islet beta cell function, including insulin secretion. In this context, earlier investigations by Jia and coworkers have suggested modulatory roles of CARD9 in the regulation of ERK activation *via* regulation of Ras-GRF1, a known GEF for H-Ras [41]. Using a variety of complementary experimental methods, these investigators have reported that Dectin-1, a spleen tyrosine kinase (Sky)-coupled C-type lectin receptor, promotes innate immunity response in a CARD9-dependent fashion. Specifically, their investigations revealed that Dectin-1 promotes Sky-mediated

tyrosine phosphorylation of Ras-GRF1, which in turn promotes complexation between active (GTP-bound) H-Ras and CARD9 and subsequent activation of downstream signaling pathways, including ERK activation. Several earlier investigations have demonstrated roles for GRF-1 in islet beta cell function, including activation of ERK1/2 [42, 43]. In this context, published evidence provided direct evidence for key contributory roles of ERK1/2 activation in GSIS [36, 38]. However, potential roles of H-Ras-GRF-1-Raf1-ERK1/2 signaling module in CARD9-mediated GSIS may be unlikely since our findings suggested no significant effects of CARD9 knockdown on glucose-induced ERK1/2 activation.

Second, besides GRFs, some GEFs have been shown to contribute to regulation of CARD9 signaling pathways. For example, Futosi and Mocsai [44] have highlighted critical interaction between PLCγ2, Vav-family members and CARD9 in mediation of tyrosine kinase signaling pathways in neutrophils. Furthermore, recent studies of Roth and coworkers [45] have implicated direct roles for Vav-family of GEFS (Vav-1, −2 and −3) as mediators of CARD9 signaling for innate antifungal immunity. Specifically, they demonstrated activation of CARD9-Dectin-1/2 signaling module in the regulation of downstream signaling events leading to pro-inflammatory gene transcription. Based on data accrued from complementary investigations, these investigators provided a compelling mechanism for Syk-coupled C-type lectin receptors (CLRs)-mediated regulation of CARD9 and down-stream signaling events for innate antifungal immunity. It has been speculated [45] that Vav family members, by virtue of their scaffolding properties, could facilitate appropriate subcellular localization (i.e., proximity) of CARD9 for optimal interaction with its key upstream regulators, including PKCδ, which has been shown to be the putative kinase responsible for phosphorylation and activation of CARD9 [46]. Such regulatory effects of Vav might represent non-G protein dependent mechanisms; these need to be verified experimentally. It may be germane to point out that earlier studies from our laboratory have reported key roles for Vav2 in GSIS from pancreatic beta cells. In these investigations, we have also demonstrated glucose-induced tyrosine phosphorylation of Vav2 in pancreatic beta cells [47, 48]. Even though, the current studies do not provide evidence for Vav2-Rac1 signaling axis in CARD9-mediated GSIS, it is likely that specific GEFs endogenous to the pancreatic beta cell could provide precise control of CARD9-mediated signaling steps leading to GSIS. Additional investigations are needed to further support this formulation.

Third, in addition to regulation of GRFs/GEFs, CARD9 has also been implicated in regulation of cellular activation and function *via* its interaction with GDIs. For example, investigations by Wu and coworkers have demonstrated novel regulatory roles for CARD9 in the regulation of LyGDI-Rac1 signaling module in the generation of reactive oxygen species (ROS) in macrophages following microbial attack [49]. Specifically, they reported complexation of CARD9 with LyGDI following cellular activation leading to dissociation of LyGDI-Rac1 complex culminating in the activation of Rac1 and associated downstream cellular signaling pathways leading to ROS generation. We propose that such a mechanism might not be operating in the islet beta cell in the context of potential roles of LyGDI in GSIS since we recently demonstrated that siRNA-mediated knockdown of LyGDI in INS-1 832/13 cells resulted in significant reduction in glucose-induced Rac1 activation without affecting GSIS [24]. In the light of our current observations indicating that CARD9 regulates

GSIS in a Rac1-independent fashion, we conclude that CARD9-mediated effects on GSIS might not underlie LyGDI-Rac1 signaling module.

Lastly, it is noteworthy that while the current investigations have revealed critical roles for CARD9 in physiological insulin secretion, it is likely that CARD9 could elicit regulatory roles in the pathogenesis of islet beta cell dysregulation under conditions of metabolic stress, including chronic exposure to hyperglycemic conditions (i.e., glucotoxicity). Along these lines, we have proposed key roles for sustained activation of Rac1-p38MAPK signaling module in the onset of cell dysregulation under the duress of glucotoxicity [39, 50]. Recent evidence from our laboratory indicated that CARD9-si significantly (~50%) suppressed sustained activation of Rac1 under glucotoxic conditions followed by inhibition (~40%) of p38MAPK. Based on these findings we concluded that CARD9 mediates activation of Rac1-p38MAPK signaling pathway in pancreatic beta cells exposed to chronic glucotoxic conditions [51]. Our findings add strength to the emerging body of evidence in *in vitro* and *in vivo* model systems that highlights regulatory roles for CARD9 in the onset of metabolic and cardiovascular diseases [19, 22, 23, 52]. Studies are in progress in our laboratory to further affirm roles of CARD9-Rac1-stress kinase signaling axis in the onset of metabolic dysregulation of the islet beta cell under metabolic stress.

In conclusion, we present the initial evidence for the expression of CARD9 in human islets, rat islets, mouse islets and insulin-secreting INS-1 832/13 cells. We propose that CARD9 contributes to GSIS *via* a p38-sensitive mechanism, which is distinct from ERK1/2-Rac1 signaling pathway(s). In the light of existing evidence implicating various MAPKs (e.g., p38 MAPK) in cell function in health and disease [53, 54], additional investigations, including CARD9's role in the regulatory control of glucose-induced p38 MAPK pathway and insulin secretion, remain logical and fertile avenues for future research.

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Abbreviations used

BRD5529	a known inhibitor of CARD9-E3 ubiquitin ligase TRIM62 protein-protein interaction
CARD9	caspase recruitment domain containing protein 9
ERK	extracellular signal-regulated kinase
GAP	GTPase-activating protein
GDI	guanine nucleotide dissociation inhibitor
GEF	guanine nucleotide exchange factor
GRF-1	guanine nucleotide releasing factor-1
GSIS	glucose-stimulated insulin secretion

LyGDI	Rho-GDP dissociation inhibitor-β
Mas	mastoparan
PPRs	pattern recognition receptors
p38MAPK	p38 mitogen-activated protein kinase
β-PIX	p21-activated protein kinase exchange factor alpha (ARHGEF7)
P-Rex1	Phosphatidylinositol-3, 4, 5-trisphosphate-dependent Rac exchange factor 1
Rac1	Ras-related C3 botulinum toxin substrate 1
RacGAP1	Rac GTPase activating protein 1
ROS	reactive oxygen species
siRNA	small interfering RNA
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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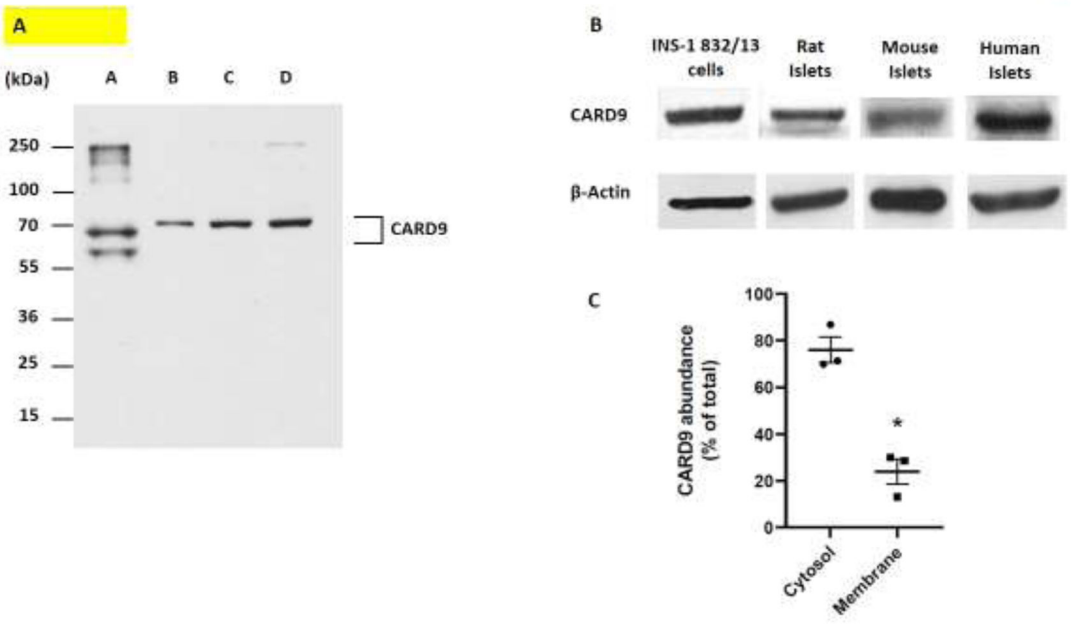

Figure 1: Evidence suggesting that CARD9 is expressed in pancreatic beta cells, and that it is predominantly cytosolic in distribution

Panel A: A representative western blot depicting the specificity of the CARD9 antibody employed in the current studies. (A) THP-1 cell lysate (positive control; 50 μg/lane), (B) 15 μg INS-1 832/13 cell lysate protein, (C) 30 μg INS-1 832/13 cell lysate protein and (D) 45 μg INS-1 832/13 cell lysate protein.

Panel B: Western blot data demonstrating expression of CARD9 in lysates derived from INS-1 832/13 cells, rat islets, mouse islets and human islets. Corresponding actin levels are provided in this blot.

Panel C: A graphical representation for relative abundance of CARD9 in the membrane and cytosolic fractions in INS-1 832/13 cells. Total membrane and cytosol fractions were isolated from INS-1 832/13 cells using the Mem-PER Plus Membrane Extraction kit, and relative abundance of CARD9 in these fractions was determined by western blotting. Intensity of the bands was quantified by densitometry. Data are means ± SEM from three independent experiments (*$p<0.05$ *vs.* cytosol).

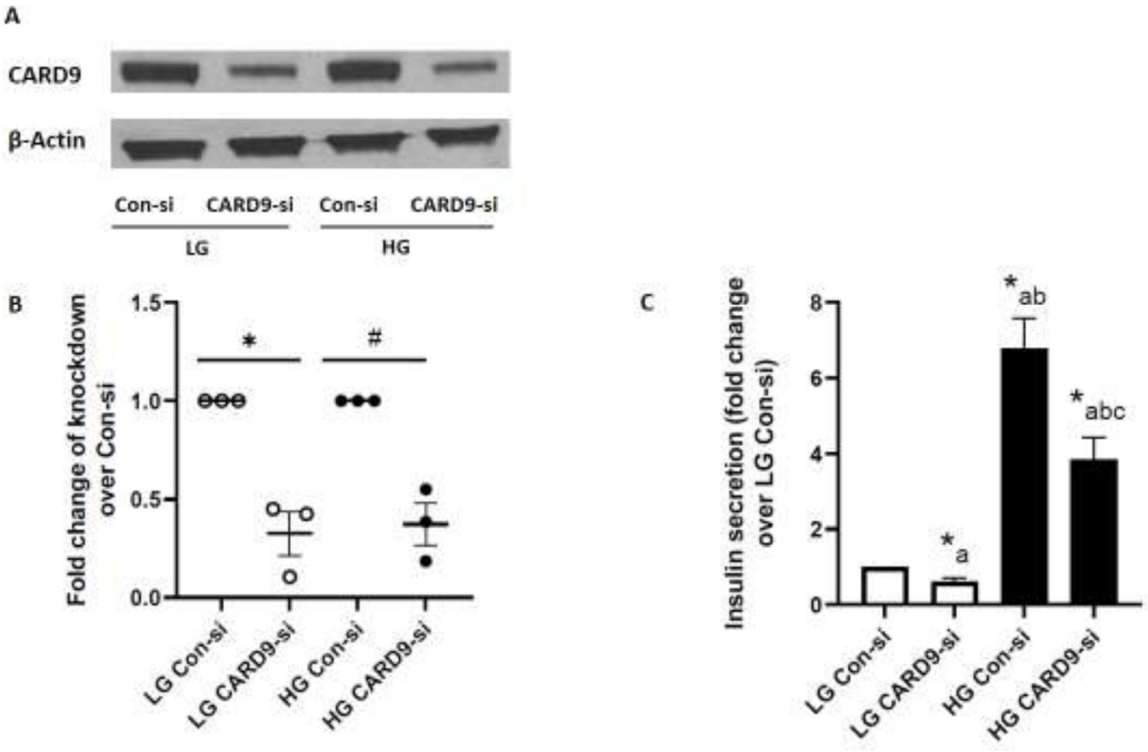

Figure 2: Knockdown of CARD9 expression results in inhibition of GSIS in INS-1 832/13 cells.

Panel A: Lysates from Con-si or CARD9-si transfected INS-1 832/13 cells exposed to LG and HG conditions were probed for CARD9 by western blotting. These data indicated significant knockdown of endogenous expression in cells transfected with CARD9-si under both LG and HG conditions. β-Actin was used as loading control. A representative blot from three experiments is shown here.

Panel B: Shows a graphical representation of the degree of CARD9 knockdown under our current experimental conditions. (Data are mean ± SEM from 3 independent experiments; Comparisons: *: significant *vs.* LG Con-si; #: significant *vs.* HG Con-si (*p<0.05).

Panel C: Con-si or CARD9-si transfected INS-1 832/13 cells were exposed to LG or HG for 45 minutes and insulin secreted into the media was quantified using ELISA. Data are mean ± SEM from three independent experiments. Comparisons: a: significant *vs.* LG Con-si; b: significant compared with LG CARD9-si; c: significant *vs.* HG Con-si. (*p<0.05).

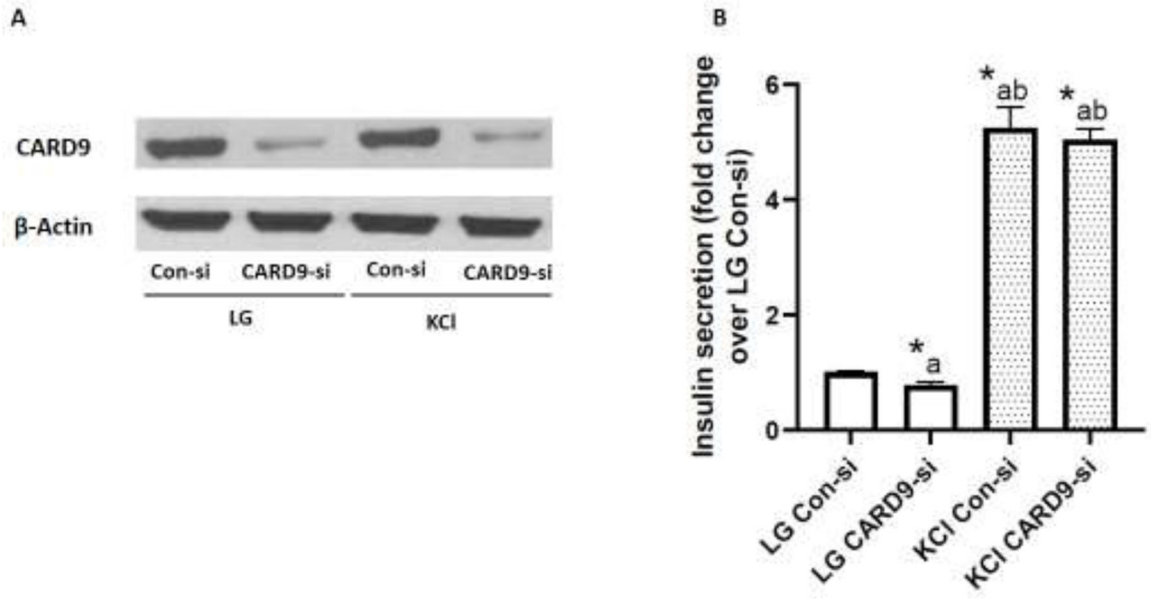

Figure 3: CARD9 knockdown elicits no significant effects on insulin secretion promoted by KCl in INS-1 832/13 cells

INS 832/13 cells were transfected with Con-si or CARD9-si and treated with LG or KCl (60mM; concentration of NaCl was reduced in the medium to maintain osmolality) for 60 min. Insulin secreted into the media was quantified using ELISA.

Panel A: a representative western blot depicting the degree of CARD9 knockdown under conditions employed in these studies. β-Actin was used as loading control.

Panel B: Shows a graphical representation of insulin secreted into the medium in INS-1 832/13 cells exposed to LG or KCl is shown herein. Data are mean ± SEM and is representative of two independent experiments. Comparisons: a: significant *vs.* with LG Con-si; b: significant *vs.* with LG CARD9-si (*p<0.05).

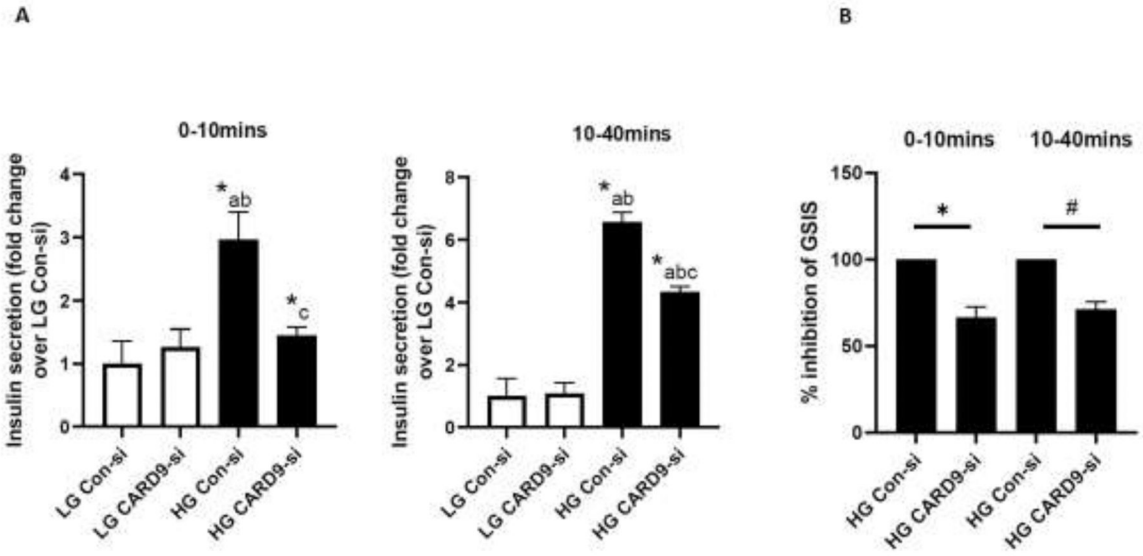

Figure 4: Depletion of CARD9 in INS-1 832/13 results in inhibition of both rapid and slow phases of GSIS as determined by a static phase secretion assay

INS-1 832/13 cells were transfected with either Con-si or CARD9-si (as above). Forty eight hours post transfection, the cells were exposed to LG or HG for 10 minutes (representing the rapid phase of insulin secretion) and the supernatant was completely removed after this incubation. The cells were immediately exposed to the same stimuli for an additional 30 minutes (representing the slow phase of insulin secretion) and the supernatant was removed following this incubation. Amount of insulin released into the media was quantified by ELISA.

Panel A: Data from a representative experiment (n=4) demonstrating the regulatory roles of CARD9 in rapid (0–10 minutes) and slow (10–40 minutes) phases of GSIS in INS-1 832/13 cells. Data are mean ± SEM. Comparisons: a: significant *vs.* LG Con-si; b: significant compared with LG CARD9-si; c: significant *vs.* HG Con-si. (*$p<0.05$).

Panel B depicts the percent inhibition of GSIS in INS-1 832/13 cells transfected with CARD9-si (indicated in Panel A). The degree of GSIS observed in INS-1 832/13 cells transfected with Con-si was taken as 100%. Data are mean ± SEM (n=4 independent experiments). Comparisons: *: significant *vs.* HG Con-si in the rapid phase of secretion; #: significant *vs.* HG Con-si in the slow phase of secretion (*,# $p<0.05$).

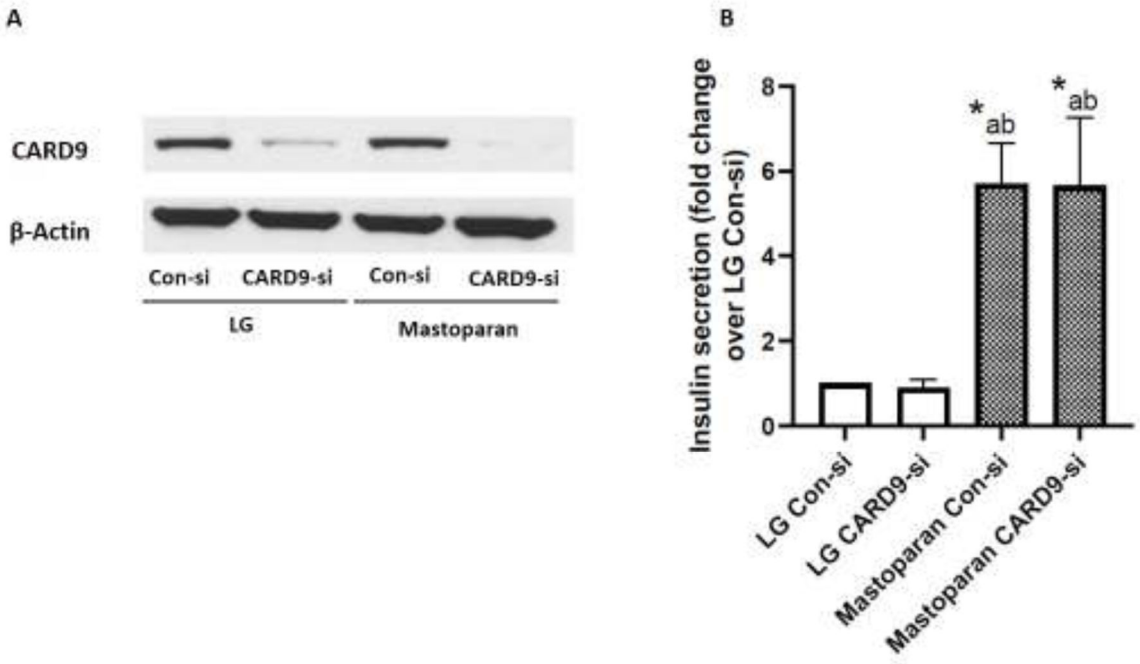

Figure 5: Mastoparan-induced insulin secretion is not affected by depletion of CARD9 expression in INS-1 832/13 cells

INS-1 832/13 cells were transfected with Con-si or CARD9-si and 48 hours post-transfection, the cells were treated with LG or Mas (30 μM) for 45 minutes. Insulin secreted into the media was quantified by ELISA.

Panel A: A representative western blot showing the degree of CARD9 knockdown under conditions employed in these studies. β- Actin was used as loading control.

Panel B: Graphical representation of insulin secretion in mastoparan-stimulated INS-1 832/13 cells (fold change over LG Con-si). Pooled data from 3 independent experiments is given here. Data are mean ± SEM. Comparisons: a: significant *vs.* with LG Con-si; b: significant *vs.* LG CARD9-si (*$p<0.05$).

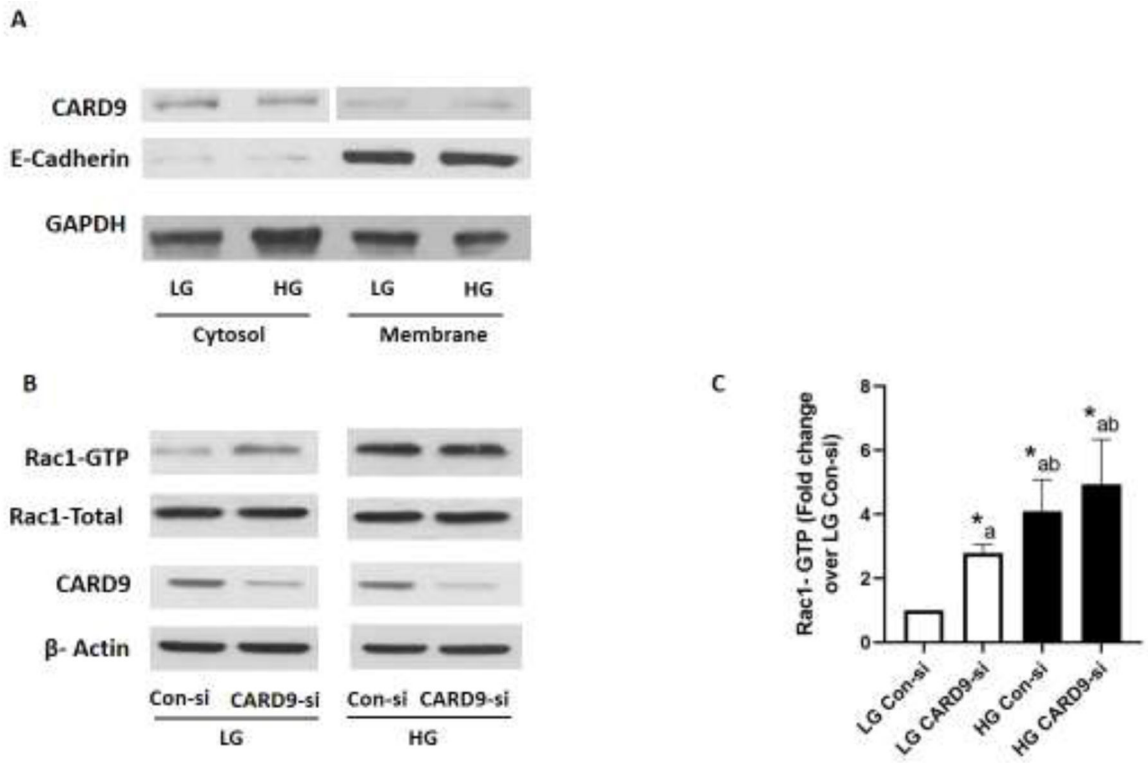

Figure 6: Subcellular distribution and Rac1 activation are not altered in CARD9-depleted INS-1 832/13 cells following exposure to stimulatory glucose

Panel A: Total membrane and cytosol fractions were isolated from INS-1 832/13 cells exposed to either LG or HG for 45 minutes as described under Methods section. Relative abundance of CARD9 in these fractions was determined by western blotting. Purity of cytosolic and membrane fractions was assessed by determining relative abundance of GAPDH and E-Cadherin, respectively. These data suggest no significant alterations in the distribution of CARD9 between the cytosolic and membrane fractions under conditions conducive to GSIS. A representative blot from three independent experiments is shown here.

Panel B: INS-1 832/13 cells transfected with Con-si or CARD9-si were exposed to LG or HG for 15 minutes, and the degree of Rac1 activation (GTP-bound conformation; Rac1-GTP) was determined by a pull-down assay as described under Methods. Abundance of total Rac1 in the cell lysates, and the degree of CARD9 knockdown obtained is also shown alongside Rac1 activation data. β-Actin was used as loading control. These data indicated no significant effects of CARD9 knockdown on glucose-induced Rac1 activation in INS-1 832/13 cells. A representative blot from three independent experiments is shown here.

Panel C: Densitometric quantification of active Rac1 (Rac1-GTP) from studies depicted in Panel B is provided here. Data are expressed as mean ± SEM from three independent experiments. Comparisons: a: significant *vs.* LG Con-si; b: significant compared with LG CARD9-si (*$p<0.05$).

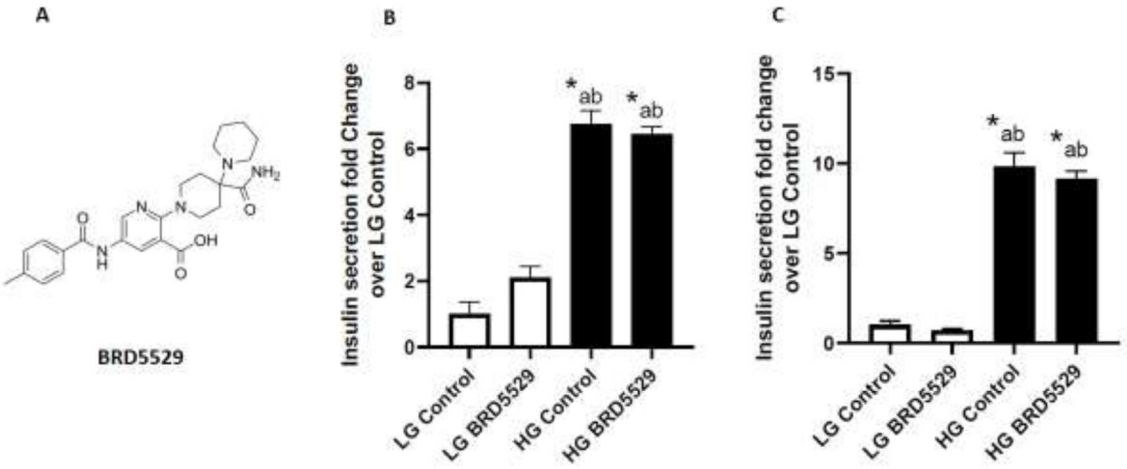

Figure 7: BRD5529, a known inhibitor of CARD9, exerts no significant effects on GSIS in INS-1 832/13 cells

Panel A: Structure of BRD5529.

Panels B and C: GSIS was quantified in INS-1 832/13 cells incubated with BRD5529 either for 60 minutes (**Panel B**) or overnight (**Panel C**) as described under Methods. Data given in **Panel B** are from one experiment expressed as mean ± SEM (quadruplicate measurements). Data given in **Panel C** are representative of three independent experiments with comparable effects on insulin secretion. Comparisons: a: significant *vs.* LG Control; b: significant *vs.* LG BRD5529. (*p<0.05).

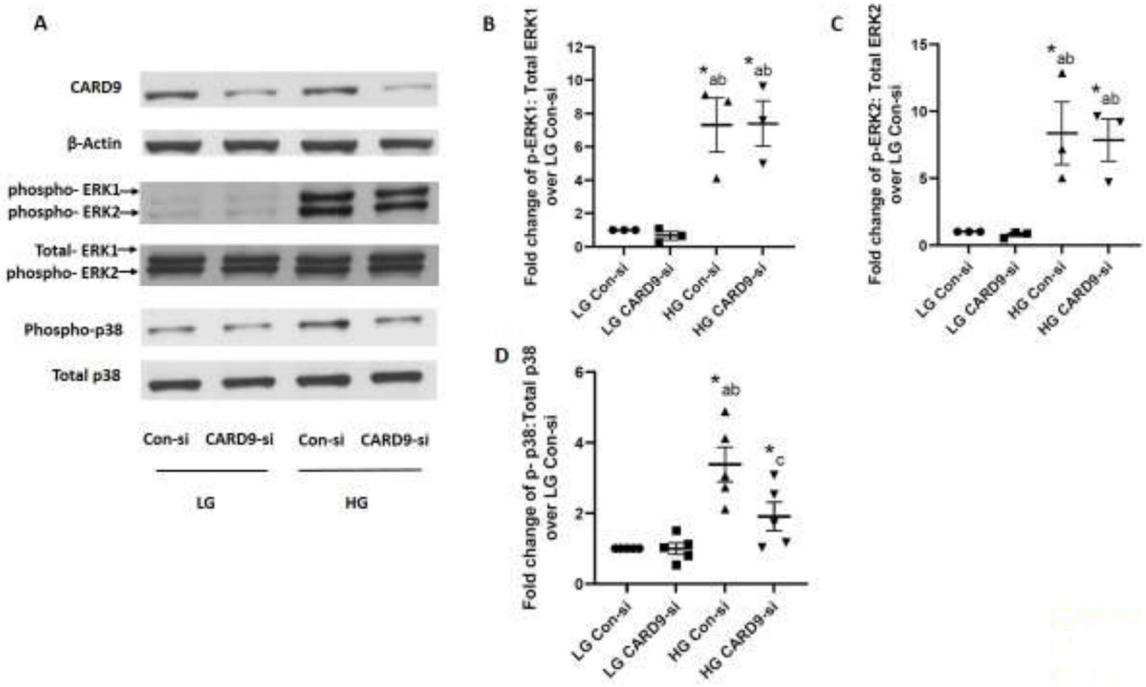

Figure 8: siRNA-mediated knockdown of CARD9 inhibits glucose-induced p38 phosphorylation, but not ERK1/2 phosphorylation in INS- 1832/13 cells

INS-1 832/13 cells transfected with either Con-si or CARD9-si followed by incubation with either LG or HG for 30 minutes. Cell lysates were prepared in RIPA lysis buffer containing protease and phosphatase inhibitors. Levels of phospho-ERK1/2 or phospho-p38 were determined by western blotting. The same blots were stripped and re-probed for total ERK1/2 and total p38.

Panel A: shows a representative blot demonstrating the degree of knockdown of CARD9 in these experiments. β-Actin was used as loading control. Representative blots indicating the relative abundance of total and phospho-ERK1/2 and p38 are also shown here.

Panel B: shows pooled data (n=3 independent experiments) on the degree of glucose-induced phosphorylation of ERK1 in INS-1 cells transfected with either Con-si or CARD9-si. Comparisons: a: significant *vs.* LG Con-si; b: significant compared with LG CARD9-si. (*p<0.05).

Panel C: shows pooled data (n=3 independent experiments) on the degree of glucose-induced phosphorylation of ERK2 in INS-1 cells transfected with either Con-si or CARD9-si. Comparisons: a: significant *vs.* LG Con-si; b: significant compared with LG CARD9-si. (*p<0.05).

Panel D: shows pooled data (n=5 independent experiments) on the degree of glucose-induced phosphorylation of p38 in INS-1 cells transfected with either Con-si or CARD9-si. Comparisons: a: significant *vs.* LG Con-si; b: significant compared with LG CARD9-si; c: significant *vs.* HG Con-si. (*p<0.05).