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**Original Paper**

# Hyperglycemic Conditions Promote Rac1-Mediated Serine536 Phosphorylation of p65 Subunit of NFκB (RelA) in Pancreatic Beta Cells

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**Key Words**
p65 • RelA • NFκB • Rac1 • Metabolic stress • Islet β-cell • Small molecular inhibitors for Rac1

**Abstract**
***Background/Aims:*** We recently reported increased phosphorylation (at S536) of the p65 subunit of NFκB (Rel A) in pancreatic beta (INS-1 832/13) cells following exposure to hyperglycemic (HG) conditions. We also demonstrated that HG-induced S536 phosphorylation of p65 is downstream to the regulatory effects of CARD9 since deletion of CARD9 expression significantly attenuated HG-induced S536 phosphorylation of p65 in beta cells. The overall objective of the current investigation is to identify putative mechanisms underlying HG-induced phosphorylation of p65 in islet beta cells following exposure to HG conditions. ***Methods:*** INS-1 832/13 cells were incubated in low glucose (LG; 2.5 mM) or high glucose (HG; 20 mM) containing media for 24 hours in the absence or presence of small molecule inhibitors of G protein prenylation and activation. Non-nuclear and nuclear fractions were isolated from INS-1 832/13 cells using a commercially available (NE-PER) kit. Degree of S536 phosphorylation of the p65 subunit was quantified by western blotting and densitometry. ***Results:*** HG-induced p65 phosphorylation was significantly attenuated by inhibitors of protein prenylation (e.g., simvastatin and L-788,123). Pharmacological inhibition of Tiam1-Rac1 (e.g., NSC23766) and Vav2-Rac1 (e.g., Ehop-016) signaling pathways exerted minimal effects on HG-induced p65 phosphorylation. However, EHT-1864, a small molecule compound, which binds to Rac1 thereby preventing GDP/GTP exchange, markedly suppressed HG-induced p65 phosphorylation, suggesting that Rac1 activation is requisite for HG-mediated p65 phosphorylation. Lastly, EHT-1864 significantly inhibited nuclear association of STAT3, but not total p65, in INS-1 832/13 cells exposed to HG conditions. ***Conclusion:*** Activation of Rac1, a step downstream to HG-induced activation of CARD9, might represent a requisite signaling

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step in the cascade of events leading to HG-induced S536 phosphorylation of p65 and nuclear association of STAT3 in pancreatic beta cells. Data from these investigations further affirm the role(s) of Rac1 as a mediator of metabolic stress- induced dysfunction of the islet beta cell.



## Introduction

It is well established that exposure of pancreatic islet beta cells to metabolic stress conditions (e.g., high glucose, saturated fatty acids, biologically active sphingolipids, and pro-inflammatory cytokines) results in significant alterations in cellular function, including induction of oxidative and endoplasmic reticulum (ER) stress, stress kinase activation, mitochondrial dysfunction, and nuclear collapse leading to cell demise [1-10]. Several underlying signaling pathways have been proposed, including induction of apoptotic genes, in the cascade of events leading to dysfunction of the islet beta cells under metabolic stress [11-16]. Along these lines existing evidence supports key roles for NFκB, a transcription factor, in the regulation of cellular function under conditions of stress, inflammation and pathology of various diseases [17-23]. Published evidence also implicates NFκB in regulation of islet beta cell function in health and diabetes [19, 24-26].

NFκB is localized, in its inactive state, in the cytosolic compartment as a p65/p50 heterodimer *via* complexation with IκB proteins. Under conditions of increased intracellular stress and inflammation, NFκB gains its active conformation following a signaling step involving phosphorylation of IκB, which, in turn, releases p65 (encoded by the RelA gene) leading to translocation of NFκB to the nuclear compartment for induction of specific genes involved in stress/inflammation-mediated cellular dysregulation and demise [18, 20, 27]. Besides IκB, the p65 subunit of NFκB is functionally regulated *via* phosphorylation at its critical S276 and S536 residues. Evidence in multiple cell types suggests that phosphorylation of p65 at Ser276 is mediated by protein kinase A and ribosomal protein S6 kinase alpha-5 (MSK1) kinase in the cytosolic and nuclear compartments, respectively. IκB kinase (IKK), TANK-binding kinase 1 (TBK1), and 90 kDa ribosomal S6 kinase (RSK1) have been identified as putative kinases that control the phosphorylation of p65 at the S536 residue [27-32]. Lastly, it is widely felt that phosphorylation at S276 promotes half-life of p65, while activation of S536 results in increased proteasomal degradation of NFκB; based on these conclusions, it is postulated that phosphorylation of p65 at S276 contributes to cell survival, whereas phosphorylation at S536 accelerates cell death *via* apoptosis [33].

Recent investigations from our laboratory revealed that caspase recruitment domain family member 9 (CARD9) mediates metabolic dysfunction of the pancreatic beta cell *via* activation of a Rac1-mediated signaling cascade involving S536 phosphorylation of p65 [16]. The aim of the current investigation is to further identify putative mechanisms underlying Rac1-mediated effects on p65/RelA phosphorylation at the S536 residue in pancreatic beta cells exposed to HG conditions. To address this question, we have employed a pharmacological approach to determine relative contributory roles of protein prenylation and asked if inhibition of Rac1 halts HG-induced p65 phosphorylation and subcellular distribution (e.g., targeting to the nuclear compartment) in INS-1 832/13 cells. Specifically, we employed five structurally distinct inhibitors (Supplementary Table S1) to accomplish our goals stated in the current investigations (for all supplementary material see www.cellphysiolbiochem.com). The first two inhibitors are simvastatin and L-788,123, which inhibit biosynthesis of substrates required for protein prenylation and protein prenyl transferases, respectively. In addition, we utilized NSC23766 and Ehop-016, which inhibit Tiam1-Rac1 and Vav2-Rac1-mediated signaling steps, respectively. Lastly, we tested the effects of EHT-1864, which has been shown to inhibit Rac1 activation and function *via* inhibition of nucleotide binding to Rac1 (Supplementary Table S1 for additional information). We present data to further validate our original hypothesis that Rac1 mediates metabolic stress- induced dysfunction of the islet beta cell.

## Materials and Methods

*Materials*

Antibodies directed against phospho-p65 (S536; 93H1), total p65 (D14E12), STAT3 (124H6; Mouse mAb #9139) and Rabbit HRP-conjugated secondary antibodies were from Cell Signaling Technology, Inc (Danvers, MA, USA). Simvastatin, L-788,123, Ehop-016 and EHT-1864 were from Cayman Chemicals (Ann Arbor, MI, USA). NSC23766 was from Tocris (Minneapolis, MN, USA). The protease and phosphatase inhibitor cocktails were from Thermo Scientific (Waltham, MA; catalog # 78430) and Santa Cruz Biotechnology (Dallas, TX; catalog # sc-45045), respectively.

*Culture of insulin-secreting INS-1 832/13 cells*

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) was used to culture INS-1 832/13 cells (passage numbers 50-60). Cells were treated overnight with low serum (2.5% fetal bovine serum)/ low glucose (2.5mM) media prior to each experiment. They were incubated further in either low glucose (LG; 2.5 mM) or high glucose (HG; 20 mM) containing media for 24 hours in the presence or absence of small molecule inhibitors (Supplementary Table S1) as indicated in the text.

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

INS-1 832/13 cells were incubated under LG (2.5mM) or HG (20mM) exposure conditions for 24 hrs. To obtain the nuclear and non-nuclear fractions, cell fractionation was conducted using NE-PER Nuclear and Cytoplasmic Extraction kit according to our published method [34-36]. The purity of these fractions was assessed using specific protein markers (GAPDH, Lamin B and Histone H3).

*Western Blotting*

Cell lysates (~40-50 μg protein) were prepared using RIPA buffer supplemented with protease and phosphatase inhibitors. The protease inhibitor cocktail consisted of 4-benzenesulfonyl fluoride hydrochloride, aprotinin, bestatin, leupeptin, pepstatin A, and E-64 Protease Inhibitor. The phosphatase inhibitor cocktail is consisted of imidazole, sodium fluoride, sodium molybdate, sodium orthovanadate and sodium tartrate dihydrate. These lysates were resolved by SDS-PAGE gels (10% gels, 120V for 1.5-2 hours. at room temperature) and transferred onto nitrocellulose membranes (at 110V for 1hr at $4^0$C). Membranes were blocked in 3% BSA for 1 hour at room temperature and probed overnight with primary antibody (1:1,000 dilution) in 1.5% BSA in PBS-T. Following three 5 min washes with PBS-T, the blots were probed with secondary antibody (1:2,000) for 1 hour. Following three 10 min washes, the western blot bands were then detected using ECL detection kit (ThermoScientific, Waltham, MA, USA) and X- ray imaging. The band intensities were quantified using Image Studio Lite imaging software (LiCOR Biosciences, Lincoln, NE, USA).

*Statistical analysis*

Data are presented as mean ± SEM or mean ± SD from multiple experiments as indicated in figure legends. Statistical analysis was done using the student's t-test. A $p$-value of $< 0.05$ was considered statistically significant.

## Results

Data accrued from our earlier investigations suggested that incubation of INS-1 832/13 cells with HG (20mM; 24 h) results in significant alterations in mitochondrial (caspase-3 activation) and nuclear (Lamin-B degradation) functions leading to impaired GSIS and beta cell demise [16, 35-37]. We utilized this experimental model in the following studies to identify putative mechanisms underlying HG-induced phosphorylation of p65 at S536.

*Protein prenylation plays a regulatory role in HG-induced phosphorylation of p65 in beta cells*

We recently reported a significant increase in S536 phosphorylation of p65 in insulin-secreting INS-1 832/13 cells following exposure to HG conditions [16]. In the current investigation, we undertook a pharmacological approach to further decipher the mechanisms underlying HG-induced phosphorylation of p65. A variety of small molecule inhibitors (Supplementary Table S1) were employed to accomplish our objective. At the outset, we used two structurally distinct inhibitors to assess the roles of protein prenylation in the cascade of events leading to HG-induced phosphorylation of p65. The first is Simvastatin (SMV), a known inhibitor of the cholesterol biosynthetic pathway, which depletes the intracellular pools of mevalonic acid, and its downstream intermediates, such as isoprenyl pyrophosphates that are required for protein prenylation (i.e., farnesylation and geranylgeranylation) [36, 38, 39]. The second inhibitor that we employed is L-788,123, which inhibits G protein (e.g., Rac1) prenylation *via* inhibition of farnesyl transferase (FTase) and geranylgeranyl transferase (GGTase) [40, 41].

Data depicted in Fig. 1 (Panel A) demonstrate increased phosphorylation of p65 (lane 1 *vs*. lane 3) in INS-1 832/13 cells following incubation with HG. Co-provision of SMV markedly attenuated HG-induced phosphorylation of p65 (lane 3 *vs.* lane 4). These data indicate requirement for intermediate(s) of the cholesterol biosynthetic pathway (e.g., isoprenyl pyrophosphates) in HG-induced S536 phosphorylation of p65. Pooled data from six independent studies are graphed in Fig. 1 (Panel B). It is noteworthy that, SMV treatment significantly increased phosphorylation of p65 under LG conditions (lane 1 *vs.* lane 2), suggesting key roles for intermediates of the cholesterol biosynthetic pathway in retaining p65/RelA in its dephosphorylated state under basal glucose conditions (see below). Furthermore, incubation of INS-1 832/13 cells with SMV, but not the diluent (DMSO) induced clear morphological changes (cell rounding) in these cells (Fig. 1; Panel C and D); these data further validate the hypothesis that protein prenylation plays key roles in cell morphology and cytoskeletal arrangements and architecture [38].

We next assessed the roles of protein prenylation in HG-mediated phosphorylation of p65/RelA by employing L-788,123, a known inhibitor of FTase and GGTase [42, 43]. Data shown in Fig. 2 (Panel A) demonstrate a robust increase in the phosphorylation of p65 under HG exposure conditions (lane 1 *vs*. lane 3). Co-provision of L-788,123 to the incubation medium markedly suppressed HG-induced phosphorylation of p65 (lane 3 *vs*. lane 4). Interestingly, unlike SMV, exposure of cells to L-788,123 under LG conditions did not significantly affect the phosphorylation of p65 (lane 1 *vs.* lane 2). Based on our findings shown in Fig. 1 and 2 we conclude that a protein prenylation-dependent step might be involved in HG-induced S536 phosphorylation of p65. We also propose that increased S536 phosphorylation of p65 seen under LG conditions in the presence of SMV (Fig. 1; lane 1 *vs.* lane 2) may not be due to a prenylation-dependent step but might be under the regulatory control of other intermediates of the cholesterol biosynthetic pathway. Pooled data from four independent experiments are shown in Fig. 2 (Panel B).

*Tiam1-Rac1 and Vav2-Rac1 signaling modules may not contribute to HG-induced p65 phosphorylation in INS-1 832/13 cells*

Numerous investigations in a variety of cell types, including retinal endothelial cells [44, 45] and pancreatic beta cells [10, 46-49] have implicated Rac1, a small molecular weight G protein, in metabolic stress-induced cell dysfunction. More importantly, these studies have identified Tiam1 and Vav2 as putative guanine nucleotide exchange factors (GEFs) involved in HG-induced activation of Rac1 [49-51]. In addition, post-translational geranylgeranylation is necessary for appropriate targeting of Rac1 to relevant subcellular compartments for optimal regulation of its effector proteins [39, 47, 52]. In light of our findings that a prenylation-dependent signaling step is necessary for HG-induced phosphorylation of p65, we tested the effects of NSC23766 (inhibitor of Tiam1-Rac1 signaling axis; [48]) and Ehop-016 (inhibitor of Vav2-Rac1 signaling module; [48]) on HG-induced p65 phosphorylation. Data depicted

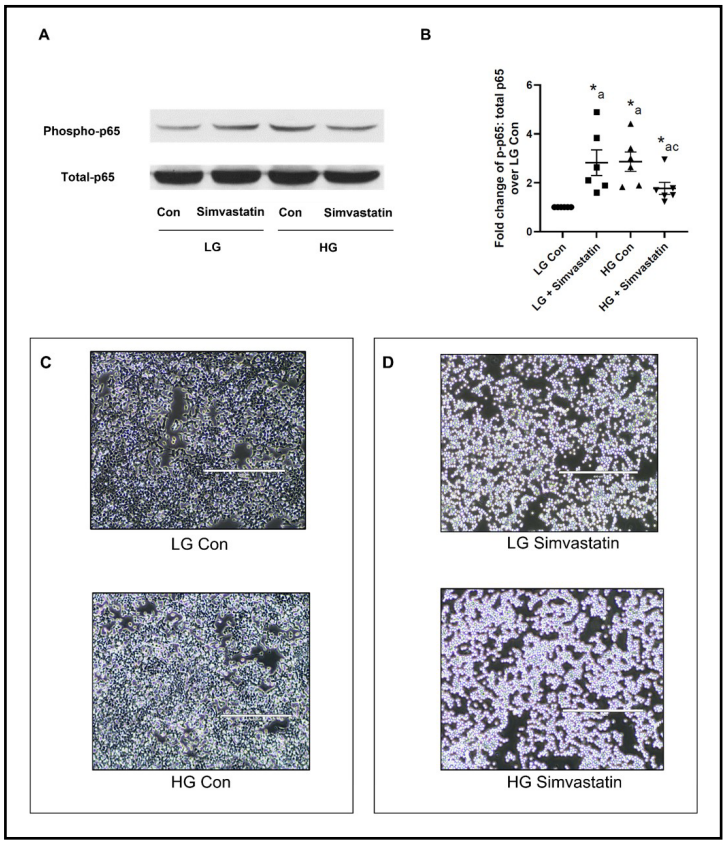


**Fig. 1.** SMV, a known inhibitor of mevalonic acid and its downstream intermediates including FPP and GGPP, inhibits HG-induced p65 phosphorylation in INS-1 832/13 cells. Panel A: INS- 1 832/13 cells were treated with LG or HG without or with SMV (15μM) or DMSO (diluent control) for 24 hours. Cell lysate proteins were separated on SDS-PAGE gels and transferred onto nitrocellulose membranes as described in the Methods section. Blots were probed with (S536) phospho-p65 antibody and subsequently with total p65 antibody for quantification of phospho- to total p65 ratios. A representative blot from six independent studies is shown here. Panel B: Densitometry was done on phospho- and total p65 bands from the six independent experiments described in Panel A. Pooled data from these experiments were plotted as fold change from the LG Control treated group (LG Con). The results are indicated as mean ± SEM. (Comparisons: a: significant compared with LG Con; c: significant compared with HG + SMV; *p<0.05). Panel C: Shows representative microscopic images obtained from INS-1 832/13 cells treated for 24 hours with diluent/DMSO under LG (LG Con) and HG (HG Con) conditions, representing control groups of cells in the experiments described above (Bar- 400μm). Panel D: Representative microscopic images where INS-1 832/13 cells were treated for 24 hours with LG or HG with SMV 15 μM (respectively, LG Simvastatin and HG Simvastatin), representing treatment groups of cells in the experiments described above. (Bar- 400μm) Rounding up of INS-1 832/13 cells are visible with SMV 15 μM treatment.

in Fig. 3 (Panel A) demonstrate no significant effects of NSC23766 on phosphorylation of P65 under LG conditions (lane 1 *vs.* Lane 2). As above, we noted a significant increase in p65 phosphorylation under HG conditions (lane 1 *vs.* lane 3). Further, HG-induced phosphorylation of p65 was resistant to NSC23766 (lane 3 *vs.* lane 4). Pooled data from three independent experiments are provided in Fig. 3 (Panel B). Based on these data we conclude that Tiam1-Rac1 signaling step may not be involved in HG-induced p65 phosphorylation.

To assess putative roles of Vav2-Rac1 signaling pathway in HG-induced phosphorylation of p65, we determined the effects of Ehop-016 on HG-induced phosphorylation of p65. Data shown in Fig. 4 (Panel A) demonstrate minimal effects of Ehop-016 on basal (lane 1 *vs.* lane 2) or HG-induced phosphorylation (lane 3 *vs.* lane 4) of p65 in INS-1 832/13 cells. Pooled data from six independent studies are included in Fig. 4 (Panel B). Based on the findings in Fig. 3 and 4, we conclude that Rac1 activation mediated by Tiam1 and Vav2 may not mediate HG-induced phosphorylation of p65 in INS-1 832/13 cells.

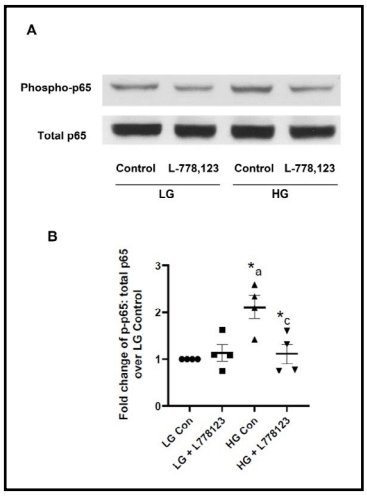


**Fig. 2.** L-788,123, an inhibitor of protein prenylation (i.e., FTase and GGTase), attenuates HG-induced p65 phosphorylation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were incubated under LG or HG conditions with or without L-788,123 (20μM) or DMSO (diluent control) for 24 hours. Lysates from these cells were separated by SDS-PAGE and the relative abundance of total and phospho-p65 were determined by western blotting. A representative blot from four independent studies is shown here. Panel B: Densitometry analysis was performed on total and phospho-p65 from studies described in Panel A, and pooled data were plotted as fold change from phospho-p65:total p65 ratios of LG Con. The results are mean ± SEM. (Comparisons: a: significant compared with LG Con; b: significant compared with LG + L-788,123; c: significant compared with HG + L788123; *p<0.05).

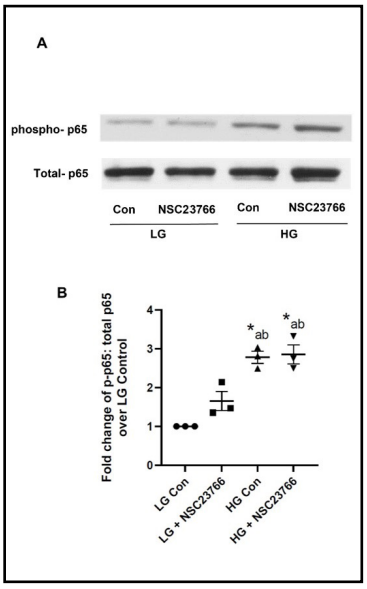


**Fig. 3.** NSC23766, an inhibitor of Tiam1-mediated activation of Rac1, exerts minimal effects on HG-induced p65 phosphorylation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were incubated under LG and HG conditions without or with NSC23766 (20μM) for 24 hours. Relative abundance of total and phospho-p65 in cell lysates was determined by western blotting. A representative blot from three independent experiments is shown here. Panel B: Band intensities of total and phospho-p65 from studies in Panel A were quantified by densitometry and pooled data from three studies are provided herein. The results are expressed as mean ± SEM. (Comparisons: a: significant compared with LG Con; b: significant compared with LG + NSC23766; *p<0.05).

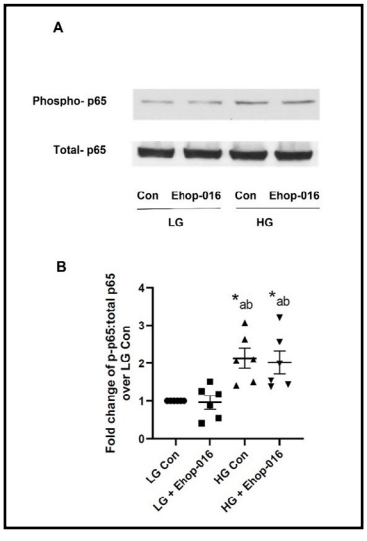


**Fig. 4.** Ehop-016, an inhibitor of the Vav2-mediated activation of Rac1, elicits no significant effects on HG-induced p65 phosphorylation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were incubated under LG and HG conditions without or with Ehop-016 (5 μM) or DMSO (diluent control) for 24 hours. The cell lysates were separated by SDS-PAGE and relative abundance of total- or phospho-p65 was determined by western blotting. A representative blot from six independent experiments is shown here. Panel B: Band intensities of total or phospho-p65 were quantified by densitometry. Pooled data from six experiments, expressed as mean ± SEM, are depicted in this figure (Comparisons: a: significant compared with LG Con; b: significant compared with LG + Ehop-016; *p<0.05).

*Direct inhibition of Rac1 attenuates p65 phosphorylation under HG-exposure conditions in INS-1 832/13 cells*

Next, we sought to further assess the roles of Tiam1/Vav2-independent effects of Rac1 in HG-induced p65 phosphorylation in INS-1 832/13 cells. Désiré and coworkers developed EHT-1864, a small molecular weight compound, which inhibited Rac1 function *in vivo* [53]. Mechanistic *studies* have revealed that EHT-1864 binds to Rac1 with high affinity, thereby retaining Rac1 in an inert and inactive state by preventing displacement of pre-bound guanine nucleotides (GDP/GTP) [54, 55]. Earlier studies from our laboratory reported significant inhibition of HG-induced p38MAPK, p53 phosphorylation and cell demise by EHT-1864 in insulin-secreting cells [35, 37, 56]. Therefore, we determined GEF-independent regulatory effects of EHT-1864 on HG-induced phosphorylation of p65 in INS-1 832/13 cells. Data shown in Fig. 5 (Panel A) suggest a significant increase in p65 phosphorylation under basal conditions in the presence of EHT-1864 (lane 1 *vs.* lane 2). The degree of stimulation by EHT-1864 was comparable to HG-induced effects of p65 phosphorylation (lane 2 *vs.* lane 3). Interestingly, HG-induced phosphorylation of p65 was significantly inhibited by provision of EHT-1864 (lane 3 *vs.* lane 4). Pooled data are included in Fig. 5 (Panel B). Based on these data described in Figures 3-5, we conclude that Tiam1-Rac1 and Vav2-Rac1 signaling steps may not underlie HG-induced p65 phosphorylation, and that direct inactivation of Rac1 (by EHT-1864) might exert differential effects on basal and HG-induced phosphorylation of p65. It is also noteworthy that identical effects by SMV (Fig. 1) and EHT-1864 (Fig. 5) were seen on S536 phosphorylation of p65 under LG (stimulation of phosphorylation) and HG (inhibition of phosphorylation) conditions in INS-1 832/13 cells. It is conceivable that SMV elicited effects are mediated *via* a Rac1-dependent mechanism (see Discussion).

*Rac1 activation is necessary for the targeting/association of STAT3, but not p65, with the nuclear fraction in INS-1 832/13 cells under HG exposure conditions*

Published evidence in other cell types provide evidence suggesting key regulatory roles for Rho GTPases in the activation of STAT transcription factors [57]. Specifically, studies of Kim and Yoon demonstrated that Rac1 activation is necessary for translocation of NFκB and STAT3 complexes in starved cancer cells [58]. Furthermore, STAT3 has been implicated in beta cell function in health and diabetes [59-61]. Therefore, we undertook a study to quantify relative abundance of total p65 and STAT3 in the nuclear fractions isolated from INS-1 832/13 cells following exposure to LG and HG in the absence and presence of EHT-1864.

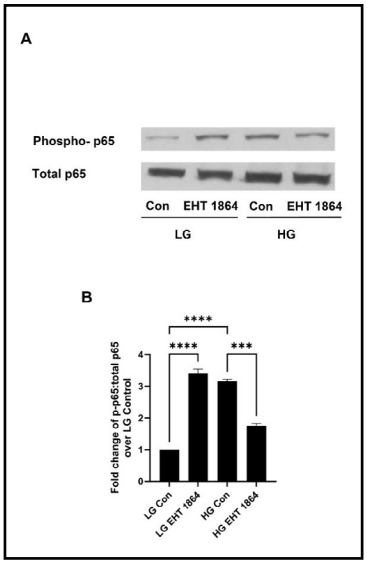


**Fig. 5.** EHT-1864, a known inhibitor of Rac1 family of GTPases, significantly attenuates HG-induced p65 phosphorylation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were incubated under LG and HG conditions in the presence or absence of EHT-1864 (10 μM) for 24 hours. Cell lysates were subjected to SDS-PAGE and the relative abundance of total and phospho-p65 was determined by western blotting and quantified by densitometry. A representative blot from two independent studies is shown here. Panel B: Band intensities of total or phospho-p65 were quantified by densitometry. Pooled data from two independent experiments, expressed as mean ± SD, are depicted here. (Comparisons: p-values: 0.0017 LG vs. LG with EHT-1864; 0.0005 LG vs. HG; and 0.0025 HG vs. HG with EHT-1864).

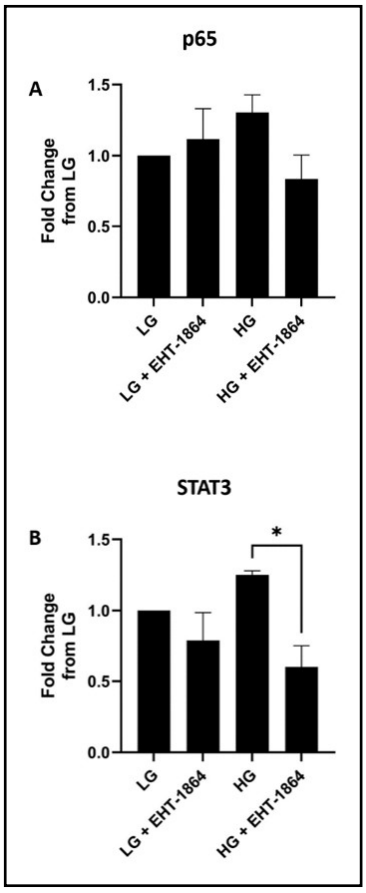


**Fig. 6.** EHT-1864, a specific inhibitor of Rac1, attenuates nuclear association of STAT3 in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were incubated under LG and HG conditions in the absence or presence of EHT-1864 (10 μM) for 24 hours and subjected to subcellular fractionation yielding non-nuclear and nuclear fractions. Western blot analysis was done on the nuclear fractions, and relative abundance of total p65 was quantified by densitometry in these fractions. Pooled data from three independent experiments, expressed as mean ± SEM, are depicted here. Data are expressed as fold change relative to LG Con. Panel B: INS-1 832/13 cells were incubated under LG and HG conditions in the absence or presence of EHT-1864 (10 μM) for 24 hours and subjected to subcellular fractionation yielding non-nuclear and nuclear fractions. Western blot analysis was done on the nuclear fractions, and relative abundance of STAT3 was quantified by densitometry in these fractions. Pooled data from three independent experiments, expressed as mean ± SEM, are depicted here. Data are expressed as fold change relative to LG Con. (Comparisons: * significant compared with HG Con; *p<0.05).

Data depicted in Fig. 6 indicated no significant effects of Rac1 inhibition on the association of p65 with the nuclear fraction (Panel A). However, EHT-1864 significantly inhibited nuclear accumulation of STAT3 in INS-1 832/13 cells exposed to HG conditions. A modest inhibition of STAT3 association with the nuclear fraction was noted in LG treated cells in the presence of EHT-1864, but such an effect did not reach statistical significance. Taken together, these data suggest that Rac1 might contribute to STAT3 translocation to the nuclear fraction to modulate functions of apoptotic proteins, such as p53 [62], which are known to contribute to HG-induced, Rac1-mediated beta cell dysfunction [35, 37, 63].

## Discussion

Recent findings from our laboratory demonstrated a significant increase in S536 phosphorylation of the p65 subunit (RelA) of NFκB in pancreatic beta cells exposed to HG conditions. Furthermore, we reported that HG-induced S536 phosphorylation of p65 is downstream to HG-induced activation of CARD9 since siRNA-mediated knockdown of CARD9 significantly attenuated HG-induced S536 phosphorylation of p65 [16]. As a logical extension to these studies, we undertook the current investigation to further decipher mechanisms underlying HG-induced phosphorylation of p65 at S536. Salient features of the current investigations include: [i] protein prenylation plays a key regulatory role in HG-induced phosphorylation of p65; [ii] activation of Rac1 mediated by GEFs, such as Tiam1 and Vav2, may not contribute to HG-induced p65 phosphorylation, rather direct inhibition of Rac1 by "locking " the G protein in its inactive conformation (with EHT-1864) attenuates HG-induced p65 phosphorylation; and [iv] activation of Rac1 is necessary for the association of STAT3, but not p65, with the nuclear fraction in INS-1 832/13 cells under HG exposure conditions.

These data provide the first evidence that activation of Rac1, a step downstream to HG-induced activation of CARD9 [16] represents a requisite step in the cascade of events leading to S536 phosphorylation of p65 in pancreatic beta cells. Our studies also provide evidence to suggest that Rac1 activation is necessary for association of STAT3, a transcriptional factor, which has been implicated in cellular dysfunction in a variety of pathologies including neurodegeneration, cancer and diabetes [64], and importantly to islet beta cell function in health and inflammation [59].

Corry et al. recently reviewed novel modulatory roles for Rho subfamily of G proteins in the intracellular activation of STAT transcription factors under conditions of cell proliferation, invasion, and metastasis [57]. Using a model system involving HEp-2 cells, Boyer and coworkers have demonstrated novel modulatory roles for Rac1 in the activation of NFκB *via* targeting of the Skp, Cullin, F-box containing complex (SCF complex) IkBα to the ruffling membranes [65]. Recent studies by Kim and Yoon provided compelling evidence to implicate activated Rac1 in the degradation of IκBα, and translocation of STAT3-NFκB complexes to the nuclear compartment in starved cancer cells [58]. These investigations revealed that Rac1 is activated in starved cancer cells and that activated Rac1 coexisted with STAT3 and NFκB. Furthermore, shRNA-mediated depletion of Rac1 and overexpression of a dominant-negative mutant of Rac1 (Rac1N19) attenuated the degradation of IκBα, and subsequent nuclear translocation of STAT3-NFκB complexes, suggesting key regulatory roles for Rac1 in the translocation of STAT3-NFκB complexes to the nuclear compartment. Using retinal micro vessels derived from streptozotocin-induced diabetic rats, Kowluru and coworkers demonstrated increased binding of p65 at the Rac1 promoter [45]. Mechanistically, these studies revealed that overexpression of Sirtuin 1, a known histone deacetylase, markedly suppressed hyper-acetylation of p65, decreased its binding at the Rac1 promoter and ameliorated Rac1-Nox2 mediated mitochondrial damage. Based on these findings, these researchers proposed that, under diabetic conditions, the transcriptional activation of Rac1 in the retina is mediated *via* acetylation of p65, and that modulation of acetylation during the early stages of diabetic retinopathy provides an opportunity to halt the development of the disease [45]. Lastly, investigations by Sobuz and associates in fibroblasts suggested additional roles for protein acetylation-deacetylation signaling steps in the nuclear export of p65 [66]. They demonstrated that Sirtuin 7, deacetylates Ran, a small GTPase at lysine37 leading to the export of p65. Based on additional findings from complementary studies, these investigators proposed that nuclear export of p65 is mediated via Sirtuin7-mediated deacetylation of Ran.

Recent evidence in pancreatic beta cells further affirms the regulatory roles of NFκB signaling pathway in the induction of metabolic dysfunction. For example, using INS-1 cells exposed to high glucose (33 mM), Ganesan and coworkers reported significant increase in the expression of members of the NFκB signalome (RelA, RelB, p50/p105, and IκB), leading to increased apoptosis of these cells. Interestingly, vitexin, an apigenin flavone glycoside, significantly attenuated high glucose-mediated cell dysfunction and apoptosis. Lastly, data accrued in these studies also demonstrated a significant increase in the expression of Nrf2, a transcriptional factor involved in the upregulation of antioxidant proteins thereby offering protection against oxidative stress, in beta cells incubated with vitexin [67]. Along these lines, studies by Darwish and coworkers demonstrated significant inhibition of macrophage infiltration of pancreatic islets in streptozotocin-induced type 1 diabetic mice following treatment with Resveratrol, a polyphenol with antioxidant properties. Mechanistic studies revealed significant attenuation of CXCL16/NFκB signaling axis in animals treated with Resveratrol [68]. Together, data from studies of Ganesan et al. [67] and Darwish et al. [68]. appear to suggest critical regulatory roles for intracellular oxidative stress as a contributor for NFκB-mediated cell dysfunction of the islet beta cell.

What is known about potential contributory roles of S536 phosphorylation of p65 in islet beta cell dysfunction? Using insulin-secreting clonal HIT-T15 cells, Puddu and coworkers investigated beneficial effects of pioglitazone on cytotoxic effects induced by advanced glycation end products (AGEs) [69]. Data from these investigations revealed significant

protection by pioglitazone against AGE-induced indices of metabolic dysfunction, including alterations in intracellular redox status, increased S536 phosphorylation of p65 and downregulation of IκBα expression. Investigations by Nano et al. have documented protective effects of islet neogenesis associated protein (INGAP) against proinflammatory cytokine-induced metabolic defects, including accelerated NFκB signaling S536 phosphorylation and nuclear accumulation of p65 [70]. Novoselova and coworkers studied potential protective effects of peroxiredoxin 6 (Prx6) against metabolic dysfunction in rat insulinoma RIN-m5F cells following exposure to high glucose or proinflammatory cytokines [71]. Based on data accrued from a series of complementary studies, these investigators surmised that NF-κB signaling module, specifically S536 phosphorylation of p65, as a target for Prx6 mediated protective effects. Studies by Fløyel and coworkers have suggested novel roles for S536 phosphorylated p65 in Src kinase-associated phosphoprotein 2 (SKAP2) proinflammatory cytokine-induced effects in clonal beta (INS-1E) cells, rat islets and human islets [72]. They reported that depletion of SKAP2 resulted in increased cytokine-induced apoptosis in INS-1E cells and primary rat islets, thus implicating an antiapoptotic role for SKAP2. Furthermore, forced expression of SKAP2 exerted protective effects against cytokine-induced apoptosis, a significant reduction in the levels of S536-phosphorylated p65, nitric oxide production, and CHOP expression. Based on these findings, these authors concluded that SKAP2 controls beta cell sensitivity to cytokines via its regulation of NFkB-inducible nitric oxide synthase-ER stress pathway. Together, the above studies provide groundwork for S536 phosphorylation and nuclear association of p65 as one of the modules that can be targeted for alleviating dysregulation of pancreatic beta cells under metabolic stress. Our current observations further affirm support to this postulation in that suppression of sustained activation of Rac1 that we reported earlier in pancreatic beta cells under the duress of metabolic stress, might aid in restoring islet beta cell dysfunction via inhibition of S536 phosphorylation of p65. Additional studies are needed to further substantiate this postulation.

The findings from our current investigations indicate divergent effects of Rac1 inhibitors on HG-induced S536 phosphorylation of p65 in that NSC23766 and Ehop-016 exerted no effects while EHT-1864 elicited inhibitory effects on HG-induced S536 phosphorylation of p65. These data indicate that Tiam1-Rac1 (NSC23766-sensitive) and Vav2-Rac1 (Ehop-016-sensitive) signaling pathways may not be involved in this signaling cascade. In contrast, EHT-1864, a small molecule compound that targets nucleotide binding pocket of Rac1 thereby "locking" the G protein in its inactive conformation, inhibited HG-induced Rac1-mediated p65 phosphorylation. Published evidence from our laboratory in pancreatic beta cells suggests that EHT-1864 significantly attenuates HG-induced activation of p38 MAPK, p53 and ATM kinase and cell death [35, 37, 56]. Together, these observations provide convincing evidence in support of the model that sustained activation of Rac1, which is known to occur under conditions described in this investigation, is necessary for HG-induced S536 phosphorylation of p65. It should be noted that earlier studies from our laboratory demonstrated significant inhibitory effects of EHT-1864 on GSIS [73]. Furthermore, we have been able to decipher novel regulatory roles of Rac1 in high glucose-induced activation of several proapoptotic signaling pathways, including Nox2, p53, p38MAPK and cell demise [35, 37, 56], based on which we concluded that Rac1-Nox2 signaling module plays novel regulatory roles in HG-induced p38MAPK/p53 activation and loss in GSIS culminating in metabolic dysfunction and the onset of diabetes. Our observations also raise an interesting possibility that EHT-1864 represents a small molecule compound that can be used to document roles of Rac1 in the pathogenesis of beta cell dysfunction and demise under metabolic stress. Efforts to utilize this compound to prevent islet dysfunction in animal models of impaired insulin secretion and diabetes are underway in our laboratory.

Lastly, it may be germane to point out that while the current studies were aimed at identifying mechanisms underlying HG-induced S536 phosphorylation of p65, a large body of evidence (recently reviewed in [27]) suggests that p65 undergoes a variety of post-translational modifications including phosphorylation-dephosphorylation, acetylation, oxidation, nitration, ubiquitination and methylation. Interestingly, p65 has been shown to

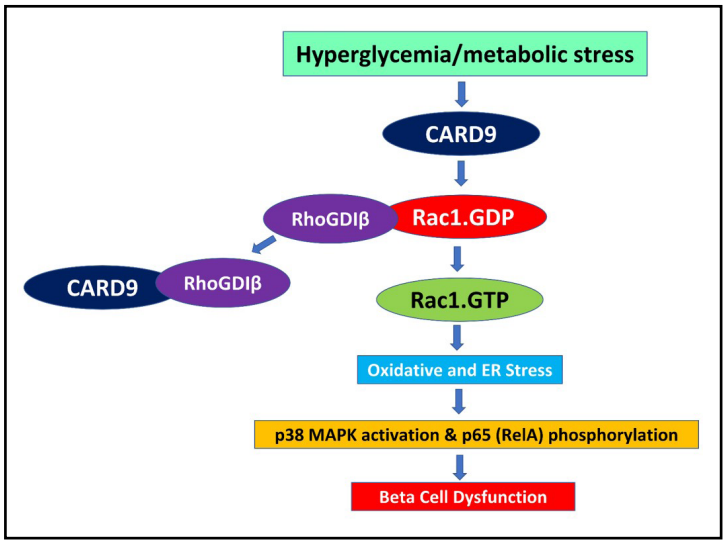


**Fig. 7.** Proposed model for HG-induced, CARD9-mediated metabolic dysregulation of the islet beta cell. Based on the data accrued from our recent [16] and current investigation, we propose that HG (metabolic stress) conditions promote activation of a signaling pathway involving CARD9. Exposure of pancreatic beta cells to HG promotes dissociation of RhoGDIβ-Rac1 complex, via increased association between RhoGDIβ and CARD9. This, in turn, leads to the activation of Rac1, and associated downstream signaling steps including activation of oxidative (Nox2-mediated) and ER stress, and activation of stress kinases (p38MAPK). Data accrued from the current investigation demonstrate that Rac1 activation step is necessary for S536 phosphorylation of p65 (RelA), and potential translocation of p65-STAT3 complex to the nuclear fraction under these conditions. Additional studies are needed to further assess roles of this signaling pathway and identification of other regulatory proteins/factors that require signaling steps (e.g., protein prenylation) that are necessary for HG-induced p65 phosphorylation, as demonstrated in the current study.

undergo phosphorylation at multiple Ser and Thr residues by various kinases [27]. Potential alterations in these post-translational modifications in the pancreatic beta cell exposed to severe metabolic stress conditions culminating in beta cell dysregulation and demise need to be addressed in depth to further assess the contributory roles and involvement of p65 (RelA) in the pathogenesis of metabolic defects in the islet beta cell under these conditions.

Based on data accrued from our recent studies [16] and our current investigations, we propose a model implicating HG-induced CARD9-mediated metabolic dysregulation of the islet beta cell (Fig. 7). It is noteworthy that our findings highlighted in [16] and in the present study affirm further support to recent observations by Kaur et al. identifying CARD9 as one of the 9 genes identified in the "T1D-T2D islet expression quantitative trait locus interaction network" in human islets [74]. Additional studies are warranted to precisely identify roles of these genes (e.g., CARD9) in islet beta cell function in health and diabetes.

## Conclusion

Based on data accrued in our current investigation, we propose that a protein prenylation-dependent, Tiam1/Vav2-independent, and Rac1-mediated signaling step might underlie HG-induced S536 phosphorylation of p65 in pancreatic beta cells. Since it has been postulated that S536 phosphorylation of p65 triggers apoptotic signaling, the current studies further affirm roles of Rac1 in HG-induced metabolic dysregulation of the pancreatic beta cell. Another important outcome of our current study is the observation of GEF-independent regulatory control of Rac1 in the NFkB signaling pathway. Additional studies are needed to further validate this hypothesis. While our data lend support that a prenylation dependent pathway is necessary for HG-mediated effects, specifically in studies involving the use of the inhibitor of FTase/GGTase, potential regulatory effects of SMV needs to be examined further, including reversal of SMV-mediated effects by exogenous mevalonic acid, farnesyl and geranylgeranyl pyrophosphates [38, 39, 49, 52].

## Abbreviations

CARD9 (Caspase recruitment domain containing protein 9); EHop-016 ($N^4$-(9-ethyl-9H-carbazol-3-yl)-$N^2$-[3-(4-morpholinyl) propyl]-2,4-pyrimidinediamine); EHT-1864 (2-(4-morpholinylmethyl)-5-[[5-[[7-(trifluoromethyl)-4-quinolinyl] thio] pentyl] oxy]-4H-pyran-4-one dihydrochloride); ER stress (endoplasmic reticulum stress); FPP (farnesyl pyrophosphate); FTase (farnesyl transferase); GEFs (guanine nucleotide exchange factors); GGPP (geranylgeranyl pyrophosphate); GGTase (geranylgeranyl transferase); GSIS (glucose-stimulated insulin secretion); GDP/GTP (guanosine diphosphate/guanosine triphosphate); HG (high glucose); IKK (IκB kinase); INGAP (islet neogenesis associated protein); L-778,123 (4-[[5-[[4-(3-chlorophenyl)-3-oxo-1-piperazinyl] methyl]-1H-imidazol-1-yl] methyl]-benzonitrile, monohydrochloride); LG (Low glucose); MSK1 (ribosomal protein S6 kinase alpha-5 kinase); NFκB (nuclear factor κB); Nfr2 (nuclear factor erythroid 2-related factor 2); Nox2 (phagocyte-like NADPH oxidase 2); NSC23766 (N6-[2-[[4-(diethylamino)-1-methylbutyl] amino]-6-methyl-4-pyrimidinyl]-2-methyl-4,6-quinolinediamine, trihydrochloride); P65/RelA (p65 subunit of NFkB); Prx6 (peroxiredoxin 6); Rac1 (ras-related C3 botulinum toxin substrate 1); RSK1 (90 kDa ribosomal S6 kinase); SCF complex (Skp, Cullin, F-box containing complex); SKAP2 (Src kinase-associated phosphoprotein 2); SMV (simvastatin); STAT3 (signal transducer and activator of transcription 3); T1D-T2D (Type 1 diabetes - Type 2 diabetes); TBK1 (TANK-binding kinase 1); Tiam1 (T-lymphoma invasion and metastasis-inducing protein 1); Vav2 (vav guanine nucleotide exchange factor 2).

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*Author Contributions*

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

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## Disclosure Statement

The authors declare that no conflicts of interest exist.

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