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A farnesylated G-protein suppresses Akt phosphorylation in INS 832/13 cells and normal rat islets: Regulation by pertussis toxin and PGE_2

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ABSTRACT

Protein isoprenylation constitutes incorporation of either 15-carbon farnesyl or 20-carbon geranylgeranyl derivative of mevalonic acid onto the C-terminal cysteine, culminating in increased hydrophobicity of the modified proteins for optimal membrane anchoring and interaction with their respective effectors. Emerging evidence confirms the participatory role of prenylated proteins in pancreatic β-cell function including insulin secretion. Herein, we investigated the putative regulatory roles of protein farnesylation in cell survival signaling pathways in insulin-secreting INS 832/13 cells and normal rodent islets, specifically at the level of protein kinase-B/Akt phosphorylation induced by insulin-like growth factor [IGF-1]. Selective inhibitors of farnesylation [e.g., FTI-277 or FTI-2628] or knockdown of the β-subunit of farnesyl transferase by siRNA significantly increased Akt activation under basal and IGF-1-stimulated conditions. Consequentially, the relative abundance of phosphorylated FoxO1 and Bad were increased implicating inactivation of critical components of the cell death machinery. In addition, FTI-induced Akt activation was attenuated by the PI3-kinase inhibitor, LY294002. Exposure of INS 832/13 cells to pertussis toxin [PTx] markedly potentiated Akt phosphorylation suggesting involvement of a PTx-sensitive G-protein in this signaling axis. Furthermore, prostaglandin E_2, a known agonist of inhibitory G-proteins, significantly attenuated FTI-induced Akt phosphorylation. Taken together, our findings suggest expression of a farnesylated G-protein in INS 832/13 cells and normal rat islets, which appear to suppress Akt activation and subsequent cell survival signaling steps. Potential regulatory roles of the islet endogenous protein kinase-B inhibitory protein [Probin] in islet function are discussed.

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1. Introduction

Protein prenylation comprises of incorporation of either a 15-carbon farnesyl group or a 20-carbon geranylgeranyl group derived from mevalonic acid onto the C-terminal cysteine residues of proteins with conserved-CAAX signature at the C-termini. Post-translational prenylation is felt to increase the hydrophobicity of the modified proteins thus enabling appropriate targeting of the prenylated proteins to relevant subcellular compartments for optimal interaction with their respective effectors. Prenylation is catalyzed by two specific enzymes *viz*., farnesyl transferase [FTase] or geranylgeranyl transferase [GGTase], which are heterodimeric with a common α-regulatory and dissimilar β-catalytic subunits [1,2]. Prenylated proteins have been shown to regulate various cellular functions including physiological insulin secretion from pancreatic β-cells ([3,4] for recent reviews).

Farnesyl transferase inhibitors [FTIs] are a class of antineoplastic drugs that are targeted to downregulate the function of Ras-oncoproteins by inhibition of the farnesylation step [5–7]. FTI-277 and FTI-2628, are well known CAAX-peptidomimetics, that inhibit farnesylation of Ras- and Rho-family of G-proteins and regulate their functional competence [8]. Employing these inhibitors, we recently demonstrated roles for farnesylated G-proteins in the cascade of events leading to glucose-stimulated insulin secretion [GSIS] from pancreatic β-cells ([9] and references therein). Specifically, we have been able to demonstrate that pharmacological and molecular biological inhibition of FTases lead to significant inhibition in glucose-induced Erk1/2 activation, Rac1 activation and insulin secretion in INS 832/13 cells and normal rat islets. Along these lines, it is widely accepted that Erk/MAP-kinase and protein kinase-B [PKB]/Akt pathways represent the two major

Abbreviations: Erk, extracellular signal regulated kinase; FoxO1, Forkhead box protein O1; FTI, farnesyl transferase inhibitor; FTase, farnesyl transferase; GTP, guanosine triphosphate; IGF-1, insulin-like growth factor-1; MEK, mitogen activated extracellular kinase; MPA, mycophenolic acid; PI3-kinase, phosphoinositide 3 phosphate kinase; PKB, protein kinase B; PGE_2, prostaglandin E_2; PTx, pertussis toxin; siRNA, small interfering RNA.

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intracellular signaling modules that regulate growth and proliferation of pancreatic β-cells. It is widely recognized that activation of Erk signaling pathway stimulates cell proliferation while PKB/Akt signaling axis promotes cell survival [10,11].

Earlier studies have demonstrated pleiotropic actions of insulin-like growth factor [IGF-1] in most mammalian cells including pancreatic islets [12–14]. The cell survival/proliferative effects of IGF-1 is attributed to the activation of MEK/Erk and phosphoinositide 3-phosphate kinase [PI3K]/Akt pathways that regulate cellular physiological events at transcriptional level [15–17]. Given that cell cycle events are tightly regulated, and that inhibition of protein farnesylation downregulates Erk signaling pathway in the islet [9], a comprehensive investigation pertaining to the signaling pathways that are intimately associated with and involved in cell cycle regulation appears worthy of investigation. With this in mind, we undertook the current investigation to determine potential roles for protein farnesylation in the activation of PKB/Akt signaling pathway in insulin-secreting INS 832/13 cells and in normal rat islets in the absence or presence of IGF-1, one of the well-studied stimulator of Akt. Using pharmacological [i.e., FTI-277 and FTI-2628] and molecular biological [i.e., siRNA–FTase β-subunit] approaches we provide the first evidence for a suppressive [i.e., negative modulatory] role of a farnesylated protein in the functional activation of Akt in pancreatic β-cells.

2. Research design and methods

2.1. Materials

FTI-277 and FTI-2628 was procured from Calbiochem [San Diego, CA]. Pertussis toxin [PTx] and prostaglandin E_2 [PGE_2] were from Sigma Chemical [St. Louis, MO]. Small interfering RNA [siRNA, a pool of 3 target specific 21 nucleotide length with $5' \rightarrow 3'$ UCACGUFACUUCUACCAUAtt in the sense strand and UAUGGUAGAAGUCACGUGAct in the antisense strand] designed to knockdown gene expression of FTase β-subunit and scrambled-siRNA [negative control] were from Ambion [Foster City, CA]. HiPerfect transfection reagent was from Qiagen [Valencia, CA]. Insulin-like growth factor [IGF-1], LY294002, antibodies for phospho-Akt [Ser-473], phospho-FoxO1 [Ser-256], total Akt and total FoxO were from Cell Signaling Technology [Beverly, MA]. Phosphorylated Bad [Ser-136] and total Bad antisera were purchased from Santa Cruz Biotechnology [Santa Cruz, CA]. All other reagents used in these studies were from Sigma Aldrich Co. [St. Louis, MO] unless stated otherwise.

2.2. Stock solutions

Stock solutions of FTI-277 or FTI-2628 were made in dimethyl sulphoxide. IGF-1 was dissolved in ultra pure water containing 0.1% bovine serum albumin. Necessary dilutions were made with fresh Krebs–Ringer bicarbonate buffer [KRB, pH 7.4] and used immediately. For all the compounds prepared in alcohol and DMSO, the final concentration of in the medium was kept less than 0.1%.

2.3. Insulin secreting cell lines and pancreatic islets

INS 832/13 cells [provided by Dr. Chris Newgard, Duke University Medical Center, Durham, NC] were cultured in RPMI 1640 medium containing 10% heat inactivated fetal bovine serum supplemented with 100 IU/mL penicillin and streptomycin, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol and 10 mM HEPES [pH 7.4] at 37 °C in a 5% CO_2 incubator. The cultured cells were subcloned twice weekly following trypsinization and passages 53–57 were used for the study.

Islets were isolated from 6 week-old male Sprague–Dawley rats [Harlan Laboratories, Oxford, MI] by collagenase digestion of the pancreas, purified by Ficoll density gradient and then handpicked [9,18]. All experiments were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee. Islets were cultured in RPMI-1640 medium supplemented with 11 mmol/L glucose, 0.2% $NaHCO_3$, 10% fetal calf serum and 0.1% penicillin/streptomycin for 6 h prior to exposure with various inhibitors.

2.4. Farnesylation inhibition studies

INS 832/13 cells were cultured to sub confluence [~80%] in RPMI-1640 medium supplemented with 10% fetal calf serum prior to inhibitor exposure. Cells were then incubated with low serum–low glucose media containing either diluent [DMSO] or in the presence or absence of FTI-277 [5 μM] or FTI-2628 [10 μM] for 12 h. For studies involving growth factor, the incubation medium was replaced with fresh KRB, and incubated further for an additional 1 h prior to stimulation with IGF-1 [10 nM] in the continuous presence or absence of inhibitors as indicated in the text. A similar protocol was followed for isolated islets except that the incubations were carried out with FTI-277 [10 μM].

2.5. Knockdown of FTase β-subunit in INS 832/13 cells

This was carried out according to the method we reported recently [9]. Briefly, INS 832/13 cells were cultured in 24-well plates to sub confluence a day before transfection. Endogenous knockdown of FTase β-subunit was carried out by transfecting cells with siRNA duplexes that target FTase β-subunit mRNA [5′ UCACGUFACUUCUACCAUAtt 3′, 100 nmol/L] using HiPerfect transfection reagent for two days. To assess the specificity of RNA interference, cells were transfected with non-targeting scrambled siRNAs that includes at least four nucleotide mismatches with all known mouse, rat and human gene duplexes which do not lead to specific degradation of any known cellular mRNA [negative control]. The cells were incubated overnight with low serum–low glucose media followed by fresh KRB for 1 h prior to stimulation with IGF-1 [10 nM] for indicated time points.

2.6. Studies with LY294002, pertussis toxin and mycophenolic acid

INS 832/13 cells or islets were cultured overnight with FTI-277 [5–10 μM], mycophenolic acid [MPA, 3.3–25 μg/mL], LY294002 [10 μM] or PTX [100 ng/mL] as indicated in the text. Following incubation, cells were harvested and lysed for Western blotting.

2.7. Studies of G_i-protein activation with prostaglandin E_2 [PGE_2]

INS 832/13 cells were serum–glucose starved overnight and subsequently treated with PGE_2 [10 μM, 1 h]. For studies, to ascertain the effects of G_i-protein activation under conditions of farnesylation inhibition, INS 832/13 cells were pretreated overnight with FTI-277 [5 μM] and then exposed to PGE_2 [10 μmol] for 1 h; for experiments involving growth factors, the cells were preincubated with PGE_2 [10 μmol, 1 h] and subsequently stimulated with IGF-1 [10 nM] for 15 or 60 min in the continuous presence or absence of the PGE_2. After the incubation period, cells were harvested, lysed and processed for Western blotting as described below.

2.8. Preparation of cell lysates and immunoblotting

INS 832/13 cells or isolated islets were lysed in 50 mM Tris buffer pH 8.0 containing 10 mM NaCl, 1% NP-40, 5% deoxycholic

acid, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 5 mM EGTA, 5 mM EDTA, 10 mM NaF and 1 mM sodium orthovandate. Following sonication [20 cycles/min], cell lysates were centrifuged at 1000 × *g* for 5 min and the supernatant was collected. Protein content was determined by Bradford method and an equal amount was separated by 10% SDS–PAGE. The resolved proteins were transferred onto polyvinylidene fluoride membrane, blocked with 5% BSA for 1 h and incubated overnight at 4 °C with anti-phospho Akt [Ser-473]/Akt [1:1000], phospho-FoxO [Ser-256]/FoxO and phospho-Bad [Ser-136]/Bad antibody [1:500] followed by horseradish peroxidase-conjugated secondary anti rabbit IgG [1:1000] for 1 h at room temperature. The immunoreactive bands were detected by the enhanced chemiluminescence system [ECL, Amersham Biosciences, Little Chalfont, UK] and the intensity was quantified using Kodak imaging software.

2.9. Statistical analysis

Results are expressed as mean ± S.E. One-way ANOVA was performed to analyze the data for statistical significance and Student–Newman–Keuls method was employed to test the difference between the groups. p values <0.05 were considered significant.

3. Results

3.1. Selective inhibitors of FTase induce Akt phosphorylation in INS 832/13 cells and normal rat islets

Using pharmacological and molecular biological approaches, we recently demonstrated novel regulatory roles for protein farnesylation in glucose-induced phosphorylation of Erk1/2, Rac1 activation and associated insulin secretion in clonal INS 832/13 cells and normal rat islets [9]. As a logical extension to these studies, we undertook the current investigations to further examine the roles of protein farnesylation in the signaling steps leading to the activation of protein kinase-B/Akt, which has been implicated in cell survival pathways in many cell types, including the pancreatic islet [19]. This was accomplished by incubation of cells in the absence or presence of FTI-277 or FTI-2628 [5–10 μM; 12 h] followed by quantitation of the phosphorylated Akt by Western blotting. Data in Fig. 1 [Panels A and B] suggested a significant increase in the phosphorylation of Akt following inhibition of protein farnesylation in INS 832/13 cell. In addition, we also noticed a marked stimulation in Akt phosphorylation in isolated islets incubated with FTI-277 [Fig. 1; Panels C and D]. Total levels of Akt were not affected by inhibitors of farnesylation [Fig. 1; Panels A and C] in either cell type studied. Taken together, these

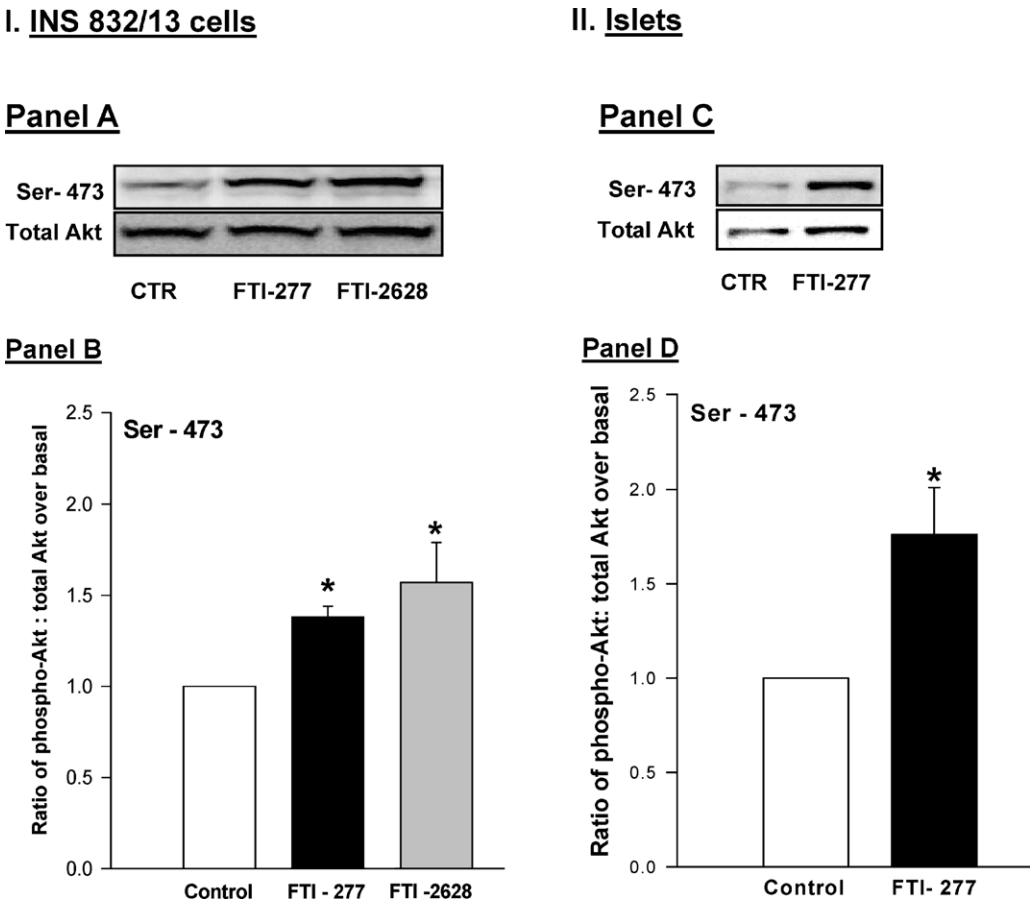

Fig. 1. Selective inhibitors of protein farnesylation promote Akt phosphorylation in INS 832/13 cells and normal rat islets. (I) INS 832/13 cells incubated with either vehicle control [DMSO] or FTI-277 [5 μM]/FTI-2628 [10 μM] overnight were harvested and equal quantity of lysates were resolved by SDS–PAGE, blot transferred and incubated with phosphorylated and total Akt antibody [see Section 2]. Following incubation with secondary HRP-conjugated secondary antibody the immune complexes were detected using ECL kit. *Panel A:* a representative blot from three experiments yielding similar results is shown. *Panel B:* densitometric analysis of the protein bands were carried out and expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^*p < 0.05$ vs. control. (II) Lysates from normal rat islets incubated with diluent or FTI-277 [10 μM] were resolved by SDS–PAGE and blot transferred for total and phosphorylated Akt. The immune complexes were detected using ECL kit as described above. *Panel C:* a representative blot from three independent experiments is shown. *Panel D:* protein bands were quantitated densitometrically and expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^*p < 0.05$ vs. control.

findings suggested a potential possibility for the expression of one [or more] farnesylated protein[s] in insulin-secreting cells, which plays a negative modulatory role[s] in the functional activation of Akt. We will refer to this protein as Probin [protein kinase-B inhibitory protein] in the current study.

3.2. Selective inhibitors of protein farnesylation potentiate IGF-1 induced phosphorylation of Akt in insulin-secreting cells

It is well established that Akt serves as one of the target proteins for the action[s] of growth factors, including IGF-1 in insulin-secreting cells. The above findings prompted us to examine potential roles of Probin in Akt phosphorylation induced by IGF-1 in INS 832/13 cells in the absence or presence of FTIs. Data from optimization experiments revealed increase in Akt phosphorylation by IGF-1 within 5 min of exposure that peaked at 15 min and remained stable for at least 30 min [additional data not shown]. Data from IGF-1 concentration-dependency for Akt activation suggested robust activation of Akt in INS 832/13 cells and rat islets in the range of 10–100 nM. Data in Fig. 2 [Panels A and B] suggested a marked potentiation of IGF-1-induced phosphorylation of Akt following inhibition of Probin by FTI-277. Likewise, inhibition of Probin's farnesylation by FTI-2628 markedly stimulated IGF-1-induced phosphorylation of Akt [Fig. 2; Panels C and D]. Taken together, data in Figs. 1 and 2 implicate negative modulatory roles for Probin in Akt activation.

3.3. siRNA mediated gene silencing of FTase β-subunit also potentiates basal and IGF-1-induced Akt phosphorylation

Protein farnesyl transferase and geranylgeranyl transferases are heterodimeric in nature. They share the same α-subunit [often referred to as FTase/GGTase α-subunit]. However, they have distinct β-subunits, which dictate their substrate specificity and catalytic function [1,3,4]. To further confirm the pharmacological observations reported in Figs. 1 and 2, we quantitated Akt phosphorylation in insulin-secreting cells following knockdown of the FTase β-subunit. This was accomplished according to the procedure we described recently [9], under which conditions ~65% reduction in the expression of FTase β-subunit was observed [Fig. 3; Panel A]. Further, data in Fig. 3 [Panels B and C] also suggested a marked stimulation of basal Akt phosphorylation at Ser-473 residue in cells in which FTase β-subunit was compromised *via* siRNA–FTase β-subunit. These data complement with observations accrued using FTI-277 and FTI-2628 [Figs. 1 and 2]. In addition, IGF-1-induced Akt phosphorylation was further potentiated in cells where FTase-β expression was knockdown [Fig. 3; Panels B and C]. Together, these data support our overall

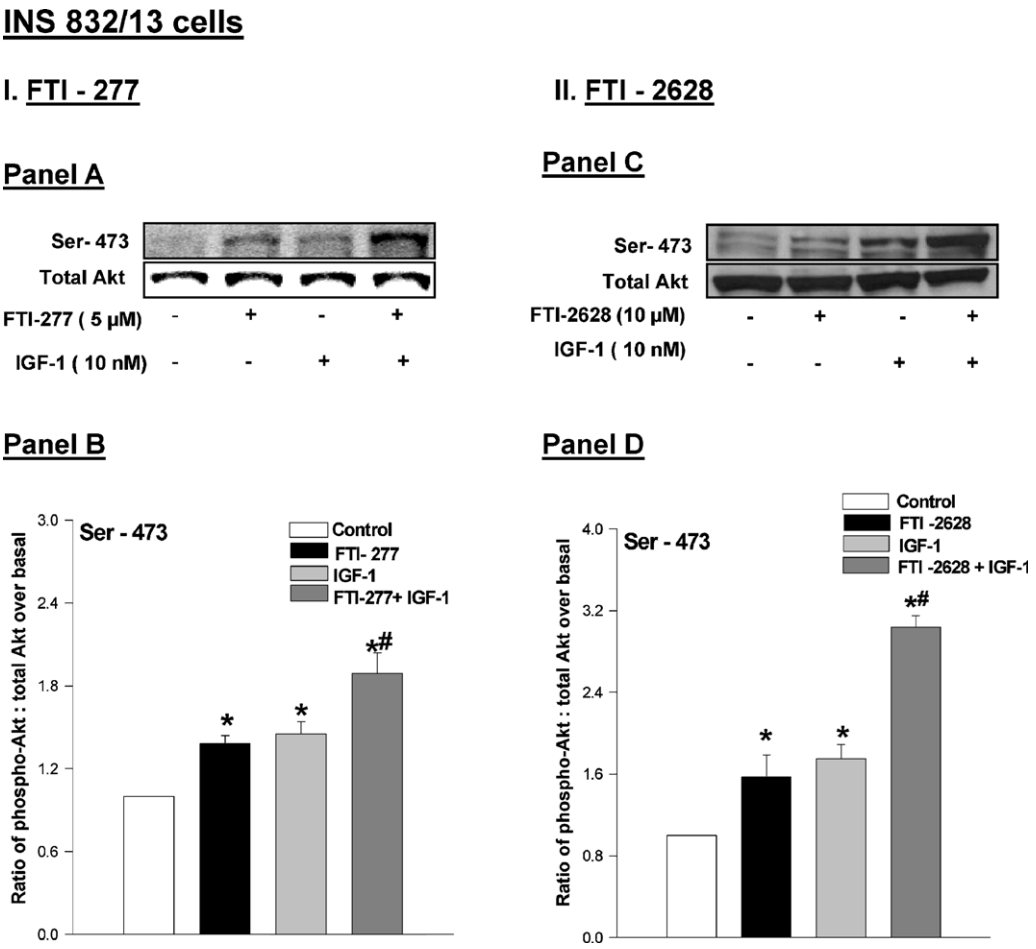

Fig. 2. FTI-277 or FTI-2628 potentiates IGF-1 induced Akt phosphorylation. (I) INS 832/13 cells treated with FTI-277 [5 μM] overnight were further incubated in the absence or presence of IGF-1 [10 nM] for 15 min. Lysate proteins were resolved by SDS–PAGE, blot transferred and probed for phosphorylated- and total Akt as described in Fig. 1. *Panel A:* a representative blot from three experiments yielding similar results is shown. *Panel B:* protein bands were quantitated and expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. FTI-277 or IGF-1. (II) Lysates from INS 832/13 cells treated with FTI-2628 [10 μM] and subsequently stimulated with IGF-1 [10 nM] were resolved by electrophoresis and blot transferred. The membranes were probed for phosphorylated/total Akt and developed as described above. *Panel C:* a representative blot from three experiments yielding similar results is shown. *Panel D:* protein bands were quantitated densitometrically and expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. FTI-2628 or IGF-1.

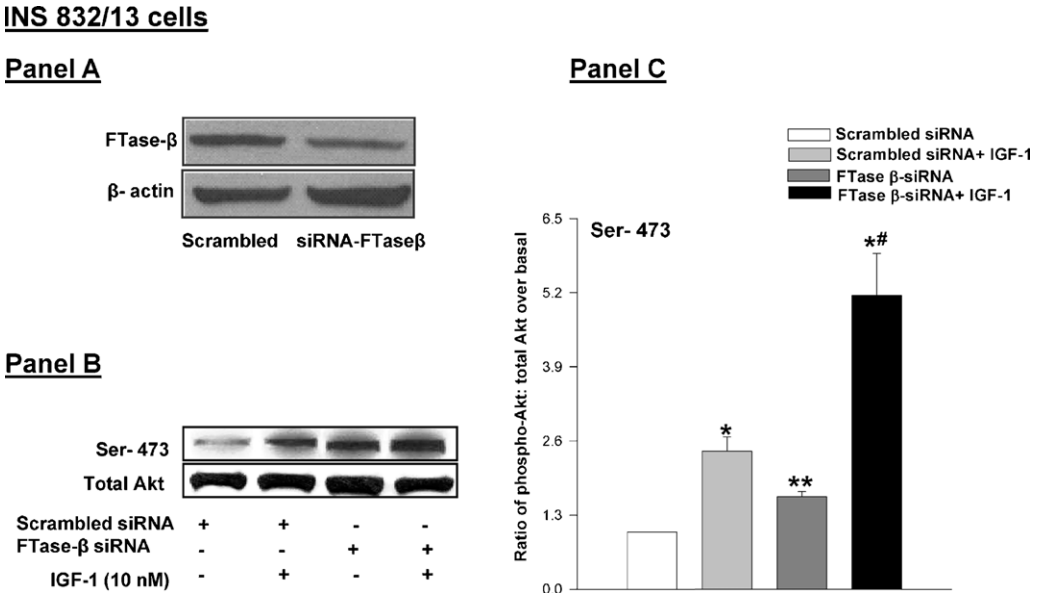

Fig. 3. siRNA-mediated knockdown of FTase β-subunit potentiates basal and IGF-1-induced phosphorylation of Akt. INS 832/13 cells were transfected with either scrambled siRNA or siRNA specific for FTase β-subunit [see Section 2 for additional details]. *Panel A:* expression of the FTase β-subunit was verified by Western blotting and a representative blot from three experiments yielding similar results is shown. *Panel B:* transfected cells were further incubated with IGF-1 [10 nM] for an additional 15 min. Cell lysates were resolved by SDS–PAGE and blot transferred. The membranes were incubated with phosphorylated and total Akt antibody and developed using ECL kit. A representative blot from three experiments yielding similar results is shown. *Panel C:* protein bands were quantitated densitometrically and data are expressed as ratio of phosphorylated Akt:total Akt normalized to scrambled siRNA treatment. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. scrambled siRNA, $^{**}p < 0.05$ vs. scrambled siRNA, $^{\#}p < 0.05$ vs. FTase-β knockdown cells stimulated with IGF-1.

hypothesis that inhibition of Probin function [*via* inhibition of its farnesylation by pharmacological or molecular biological approaches] leads to augmentation of Akt phosphorylation.

3.4. FTI-induced inhibition of Probin leads to Akt phosphorylation via a PI3-kinase-sensitive mechanism

Akt phosphorylation induced by growth factors typically involves PI3 kinase-dependent mechanisms. Therefore, we investigated if FTI-induced phosphorylation of Akt is mediated by a PI3-sensitive mechanism in normal rat islets. To address this, Akt phosphorylation was measured in rat islets incubated in the absence or presence of FTI-277 and LY294002 [10 μM], a selective inhibitor of PI3 kinase. Data in Fig. 4 [Panel A] indicated a marked inhibition FTI-277-induced phosphorylation of Akt. Pooled data from multiple studies are provided in Fig. 4 [Panel B]. It should be noted that a modest, but significant inhibition of FTI-277-induced phosphorylation of Akt was also demonstrable in INS 832/13 cells

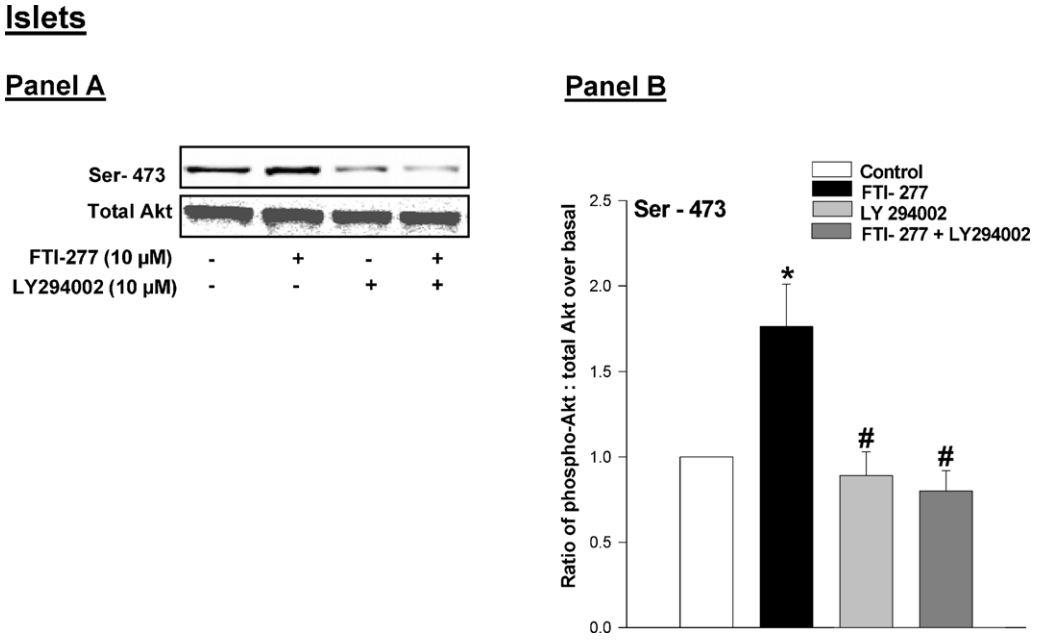

Fig. 4. FTI-277 induces Akt phosphorylation is mediated through PI 3-kinase pathway in normal rat islets. Lysates from islets cultured with FTI-277 [10 μM] in the presence or absence of LY294002 [10 μM] were resolved by electrophoresis and blot transferred for phosphorylated and total Akt as described earlier [see Fig. 1]. *Panel A:* a representative blot from three experiments is shown. *Panel B:* protein bands were quantitated densitometrically expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. FTI-277.

[1.31 ± 0.02 units in the presence of FTI-277 alone vs. 1.01 ± 0.02 units in the presence of FTI-277 plus LY294002, $p < 0.05$, $n = 3$ studies]. Together, these data suggested potential involvement of a PI3 kinase-sensitive mechanism underlying the Akt phosphorylation under conditions of inhibition of Probin functions by inhibition of its farnesylation.

3.5. Inhibition of Probin leads to inactivation of FoxO1

Forkhead transcription factors of the FoxO family are important downstream targets of PKB/Akt, whose phosphorylation leads to their inactivation and subsequent sequestering in the cytosol. Given that inhibition of Probin farnesylation [by FTIs or FTase-β siRNA] activates Akt, our next set of experiments were focused to understand whether such an experimental manipulation have any effects on the functional status of FoxO1 in insulin-secreting cells. This was accomplished by quantitating phosphorylation of FoxO1 at Ser-256 residue in INS 832/13 cells and islets treated with FTI-277 and subsequently stimulated with IGF-1 [10 nM; 15 min, see Section 2 for additional details]. Western blot analysis [Fig. 5; Panels A and B] indicated a modest, but significant increase in FoxO phosphorylation in cells treated with FTI-277 and/or IGF-1 in INS 832/13 cells. Likewise, we also noticed a significant increase in the phosphorylation status of FoxO1 in normal rat islets incubated with FTI-277, under which conditions it promoted the activation of Akt [Fig. 5; Panels C and D]. Taken together, data described above further support our hypothesis that inhibition of Probin's farnesylation leads to activation of Akt in a PI3 kinase-sensitive fashion. Further, such conditions lead to inactivation of FoxO in INS 832/13 cells, thereby promoting cell survival signaling mechanisms.

3.6. Inhibition of Probin leads to phosphorylation and inactivation of Bad

The cell survival effects of Akt are mainly attributed to its transcriptional regulation of proapoptotic signaling proteins. The Bcl-2 family of proteins, including Bad is generally regarded as important mediators of apoptosis whose activity is regulated by their phosphorylated/dephosphorylated states. For example, Bad protein has been shown to be inactive in its phosphorylated state [20]. To further understand the downstream effect of inhibition of Probin on Akt-induced phosphorylation of Bad, INS 832/13 cells were incubated in the absence or presence of FTI-277 and/or IGF-1 and relative degrees of phosphorylation of Bad was quantitated by Western blotting [see Section 2 for additional details]. Data in Fig. 6

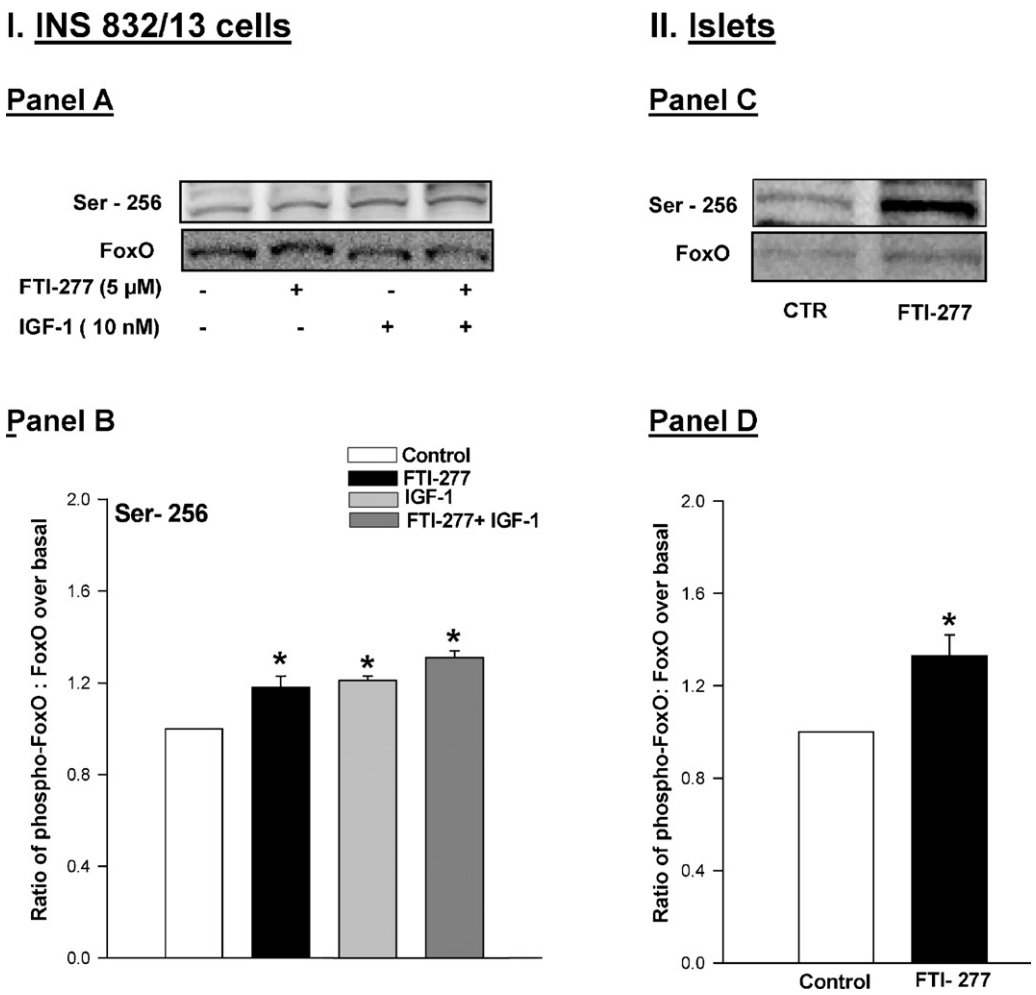

Fig. 5. FTI-277 promotes inactivation of FoxO1 in INS 832/13 cells and normal rodent islets. (I) INS 832/13 cells treated with FTI-277 [5 μM] overnight were further incubated in the absence of presence of with IGF-1 [10 nM] for 15 min. Lysate proteins were resolved by SDS–PAGE and probed for phosphorylated and total FoxO by Western blotting. The protein bands were detected by using ECL kit as described earlier [see Fig. 1]. *Panel A:* a representative blot from three experiments yielding similar results is shown. *Panel B:* intensity of the protein bands was quantitated densitometrically and expressed as the ratio of phosphorylated FoxO:FoxO normalized to basal levels. Data are mean ± S.E. from three experiments. $^*p < 0.05$ vs. control. (II) Lysates from islets cultured with FTI-277 [10 μM] were resolved by SDS–PAGE and blot transferred for phosphorylated and total FoxO. Immune complexes were detected and quantitated densitometrically. *Panel C:* a representative blot from three experiments is shown. *Panel D:* protein bands were quantitated densitometrically and expressed as the ratio of phosphorylated FoxO:FoxO normalized to basal levels. Data are mean ± S.E. from three experiments. $^*p < 0.05$ vs. control.

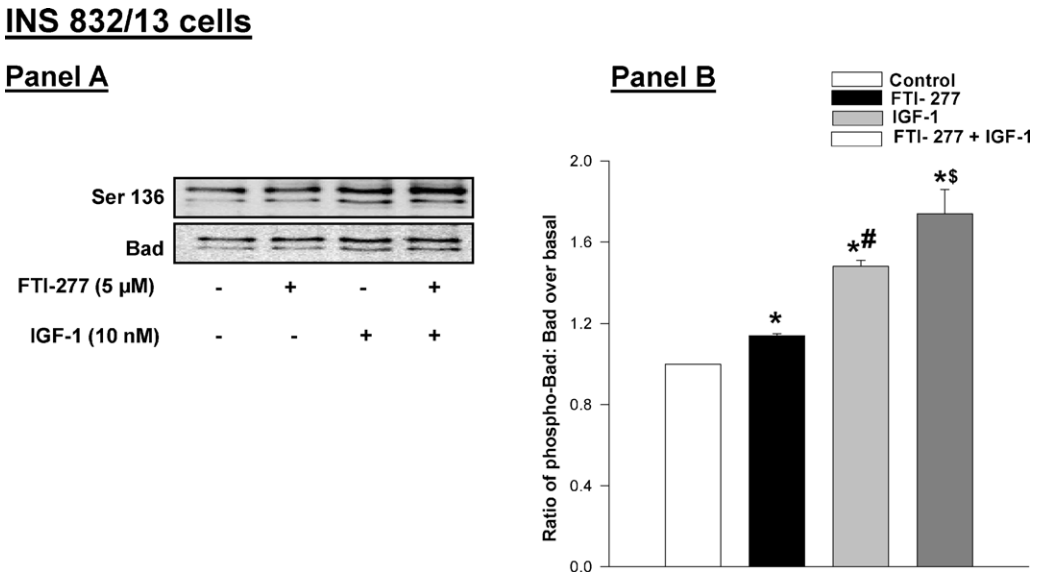

Fig. 6. FTI-277 potentiates IGF-1-induced phosphorylation of Bad in INS 832/13 cells. INS 832/13 cells cultured overnight with FTI-277 [5 μM] were further incubated in the absence or presence of IGF-1 [10 nM] for 15 min. Lysates prepared thereof were resolved by SDS–PAGE, blot transferred and probed for phosphorylated and total Bad expression. Immune complexes were identified using ECL kit and quantitated as described earlier [see Fig. 1]. *Panel A:* a representative blot from three experiments is shown. *Panel B:* protein bands were quantitated densitometrically expressed as the ratio of phosphorylated Bad:Bad normalized to basal levels. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. FTI-277, $^{\$}p < 0.05$ vs. FTI-277 or IGF-1.

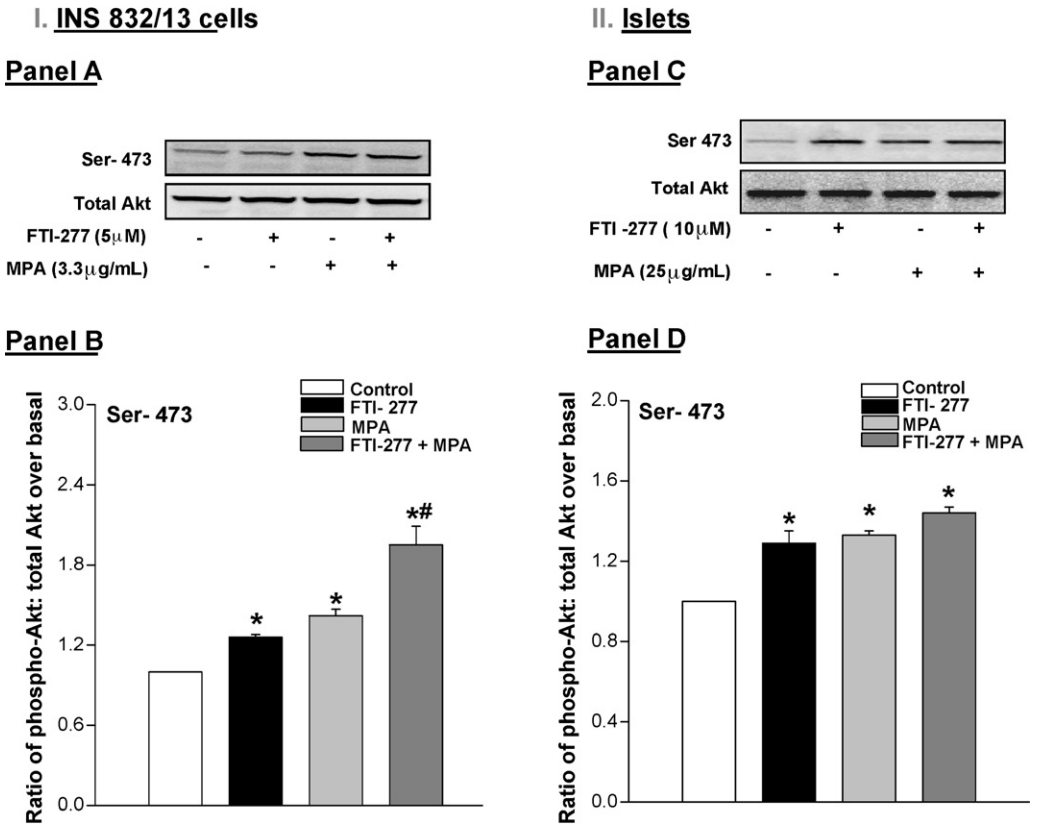

Fig. 7. Depletion of GTP further potentiates FTI-277-induced Akt phosphorylation in INS 832/13 cells and normal rat islets. (I) INS 832/13 cells were cultured overnight with FTI-277 [5 μM] in the absence or presence of MPA [3.3 μg/mL]. Lysate proteins were resolved by SDS–PAGE, blot transferred for phosphorylated and total Akt. The immune complexes were detected using ECL kit. *Panel A:* a representative blot from three experiments is shown. *Panel B:* protein bands were quantitated densitometrically expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. FTI-277 and or MPA. (II) Lysates from islets cultured with FTI-277 [10 μM] in the absence or presence of MPA [25 μg/mL] were resolved by SDS–PAGE and blot transferred for phosphorylated and total Akt. The immune complexes were detected and quantitated densitometrically. *Panel C:* a representative blot from three experiments is shown. *Panel D:* protein bands were quantitated densitometrically and expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. control.

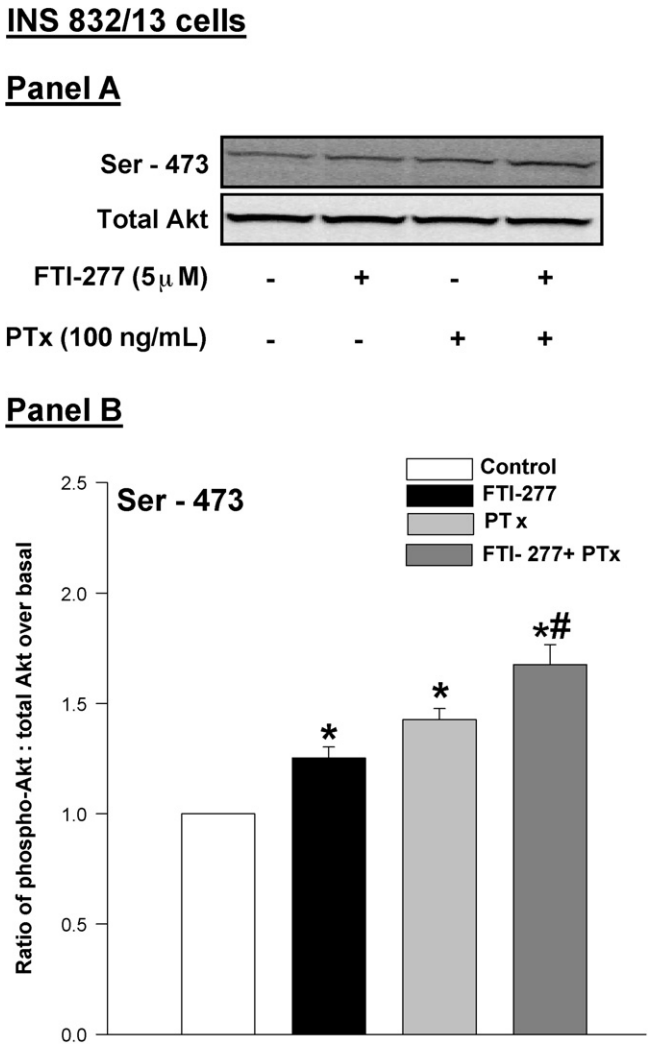

Fig. 8. Exposure of INS 832/13 cells to PTx leads to increased Akt phosphorylation, which is potentiated by FTI-277. INS 832/13 cells were cultured overnight with FTI-277 [5 μM] and in the absence or presence of PTx [100 ng/mL]. Lysate proteins were resolved by SDS–PAGE, blot transferred for phosphorylated and total Akt. Immune complexes were detected using ECL kit. *Panel A:* a representative blot from three experiments is shown. *Panel B:* protein bands were quantitated densitometrically and expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments. $^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. FTI-277.

suggested a significant increase in Bad phosphorylation following inhibition of farnesylation of Probin with FTI-277. Furthermore, IGF-1 alone promoted Bad phosphorylation which was further stimulated in the presence of FTI-277. Taken together, data depicted in Figs. 5 and 6 suggested a marked stimulation in the phosphorylation of FoxO and Bad proteins following inhibition of Probin prenylation; these signaling steps, which are under the control of activated Akt, are expected to promote cell survival events.

3.7. GTP depletion potentiates Akt phosphorylation induced by inhibitors of farnesylation

Using mycophenolic acid [MPA], a selective inhibitor of inosine monophosphate dehydrogenase, which catalyzes the *de novo* biosynthesis of GTP, Metz and associates [21,22] and Straube and coworkers [23] have provided evidence for a permissive role for GTP in insulin secretion. Further, studies from our laboratories have demonstrated a critical requirement for endogenous GTP in glucose-mediated activation of endogenous G-proteins and associated insulin secretion [24,25]. Herein, we investigated potential consequences of depletion of intracellular GTP pools on Akt phosphorylation in INS 832/13 cell and normal rat islets. Data in Fig. 7 demonstrated a significant increase in Akt phosphorylation following GTP depletion. Interestingly, FTI-277 coprovision markedly potentiated the Akt phosphorylation. Based on this evidence, we conclude a critical requirement for endogenous GTP for the regulatory effects of Probin on Akt phosphorylation in INS 832/13 cells and normal rat islets.

3.8. FTI-mediated activation of Akt in INS 832/13 cells is potentiated by pertussis toxin (PTx)

We next asked the question if Probin' function is regulated by inhibitory class of heterotrimeric G-proteins [e.g., Gi/Go]. To determine this, we quantitated Akt phosphorylation in INS 832/13 cells exposed to PTx, which ADP ribosylates and inactivate inhibitory class of G-proteins. Data shown in Fig. 8 [Panels A and B] suggested increased phosphorylation of activated Akt in PTx pre-treated cells. Furthermore, we also noticed that the Akt phosphorylation induced by FTI-277 is additive to a maximally active concentration of PTx. Taken together, our findings suggest that Probin function might be further regulated by PTx-sensitive inhibitory class of G-proteins. Such an experimental formulation was further tested by quantitating FTI-induced phosphorylation by a known agonist of inhibitory class of G-proteins.

3.9. PGE_2, a known activator of inhibitory class of trimeric G-proteins, markedly inhibits FTI-induced Akt phosphorylation in INS 832/13 cells

Several lines of experimental evidence implicate expression of functionally regulable inhibitory class of trimeric G-proteins [e.g., Gi/Go] in insulin-secreting cells ([4,26] for reviews). For example, earlier studies from our laboratory have demonstrated the presence of PTx-sensitive high affinity GTPase activities in the membrane and secretory granule fractions derived from normal rat and human islets. We also reported activation of such a GTPase activity by PGE_2 [27]. In addition, we have reported activation of the carboxylmethylation of Gγ subunits by PGE_2 in clonal HIT-T15 cells and normal rat islets [25]. Based on our data that demonstrate potentiation of FTI-induced Akt phosphorylation by PTx [Fig. 8], we examined if pre-treatment of INS 832/13 cells with PGE_2 inhibits FTI-induced Akt phosphorylation. Data in Fig. 9 [Panel A] suggested a significant inhibition of both the basal and FTI-induced phosphorylation of Akt by PGE_2. Interestingly however, IGF-1-induced Akt phosphorylation was resistant to PGE_2-treatment [Fig. 9; Panel B]. Pooled data from multiple studies is provided in Fig. 9 [Panel C]. Together, these data suggest distinct regulatory mechanisms underlying FTI and IGF-1-mediated activation of Akt [see Section 4 for a working model].

4. Discussion

Akt is a serine/threonine kinase the activation of which is linked to extracellular stimuli and the activation of PI3 kinase signaling step. Activation of Akt is consequent to phosphorylation at multiple sites including Thr-308 residue at T-loop and Ser-473 residue at C-terminal hydrophobic motif [28]. Phosphorylation of Akt at Thr-308 promotes catalysis by inducing conformational changes, while phosphorylation at Ser-473 increases the substrate binding affinity to multiple folds. Although, for the complete catalytic competence, phosphorylation at these two sites is necessary, it is widely felt that phosphorylation at Ser-473 brings about maximal Akt activity. The multiphosphorylated Akt then dissociates from the membrane and target substrates located in the cytoplasm and nucleus.

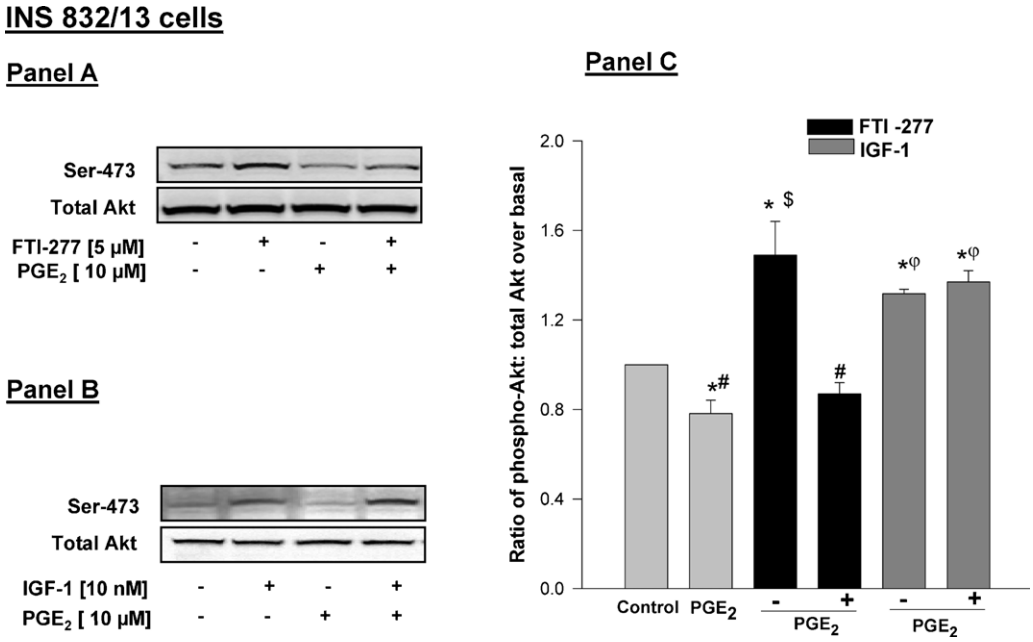

Fig. 9. Differential effects of PGE2 on Akt phosphorylation induced by FTI-277 or IGF-1 in INS 832/13 cells. Cultured INS 832/13 cells were cultured overnight with FTI-277 [5 μM] and then treated with PGE_2 for 60 min [*Panel A*] or incubated with either PGE2 [10 μM, 60 min] and then stimulated with IGF-1 [10 nM, 15 or 60 min] [*Panel B*] or Lysate proteins were resolved by SDS–PAGE, blot transferred and probed for phosphorylated and total Akt. The immune complexes were detected using ECL kit and a representative blot from three experiments is shown. *Panel C:* protein bands were quantitated expressed as the ratio of phosphorylated Akt:total Akt normalized to basal levels. Data are mean ± S.E. from three experiments.*$p < 0.05$ vs. control; $^{\$}p < 0.05$ vs. PGE_2 or FTI plus PGE_2; $^{\Phi}p < 0.05$ vs. PGE_2 or PGE_2 plus IGF-1.

The overall objective of the current study was to determine potential roles of protein farnesylation in the activation of Akt. Data accrued from the present work provided evidence to suggest the expression of a farnesylated protein [i.e., Probin] in insulin-secreting INS 832/13 cells and normal rat islets, which appears to suppress the basal and IGF-1-stimulated Akt phosphorylation in these cells. Further, our findings also suggest that Probin regulates Akt phosphorylation in a PI3-kinase sensitive fashion and that inhibition of Probin function by pharmacological and molecular biological approaches leads to activation of Akt and associated phosphorylation of FoxO1 and Bad. These signaling steps would retain the β-cell in the pro-survival mode for optimal function.

The growth promoting effects of various growth factors are attributed principally to their association with and regulation of Erk and Akt signal pathways [17]. Upon binding to their cognate receptors, growth factors activate tyrosine kinases that promote association between adaptor proteins including Shc and Grb2 culminating in the activation of Ras at the plasma membrane. The sequential activation of Raf → MEK → Erk1/2 thereafter to regulate diverse cellular processes has been reported in multiple cell types [10,29]. In contrast to the Ras/Raf-1/MEK/Erk1/2 pathway, the PKB/Akt pathway represents the single most signaling module that regulate cellular responses to growth factors [e.g., insulin, IGF-1] as well as nutrients to elicit cell survival effects [10,30]. Published evidence from multiple laboratories suggests that small G-proteins play a pivotal role in the functional regulation of Erk and Akt signaling pathways [9,31]. Indeed, post-translational prenylation of the candidate G-proteins appear to dictate its precise targeting to subcellular compartments and facilitate their effects on aforementioned signaling pathways. Along these lines, we recently demonstrated specific roles for protein farnesylation in glucose-mediated activation of a Raf-1 → Erk1/2 → Rac1 signaling pathway leading to insulin secretion [9]. With increasing number of studies that support a cross talk between MEK/Erk1/2 and PKB/Akt pathways [28,32,33], herein we investigated the likely effect on the PI3K/PKB/Akt pathway following farnesylation inhibition in insulin secreting INS 832/13 cells and isolated rat islets. Using pharmacological [e.g., FTI-277 and FTI-2628], we have been able to demonstrate negative modulatory roles for the farnesylated Probin in Akt phosphorylation in insulin-secreting cells. Pharmacological observations were further supported by studies involving the use of siRNA–FTase β-subunit. Together, based on our findings it appears that protein farnesylation plays regulatory roles in cell proliferation and survival pathways.

In the present study, we demonstrated that FTIs negatively regulate FoxO activity [i.e., inhibition through phosphorylation] by signaling through PI3 kinase/Akt pathway implicating transcriptional regulation of cell survival genes. Available evidence implicates that, PKB/Akt signaling axis mediates many of the antiapoptotic effects of PI3-kinase by phosphorylation and inactivation of cell death associated proteins. Among several substrates, the winged-helix family of transcription factors (FKHRL1 or FoxO) are regulated negatively by the PI3K/Akt by phosphorylation at three conserved amino acid residues i.e., Thr-24, Ser-256 and Ser-319 that restricts FoxO to the cytoplasm and susceptible for proteosomal degradation [34]. Of the three sites, PKB-specific phosphorylation of FoxO1 at Ser-256 has been considered crucial for transcriptional regulation of apoptotic genes as it prevents reentry into the nucleus by introducing a negative charge in the basic stretch of residues that forms the nuclear localization signal [35,36].

The cell survival effects of growth factors essentially involve activation of PI3 kinase/Akt signal pathway, which eventually culminates in the phosphorylation of Bad, a Bcl-2 family member [20,37,38]. Bcl-2 family proteins exist either as homo- or hetero-dimers and a precise ratio between the forms generally determines cell fate. The proapoptotic Bad being a direct substrate for PKB/Akt has two conserved amino acid sites i.e., Ser-136 and Ser-112 that are phosphorylated by Akt and Ras–MAPK, respectively. Bad in the dephosphorylation state, induce cell death by complexing with Bcl-XL and generating Bax homodimers, however in phosphorylated form, Bad remains inactive, binds with 14-3-3 and promote cell survival [38]. In the present study, Akt phosphorylation observed as a consequence of inhibition of farnesylation is coupled

with phosphorylation of Bad implicating inactivation of critical components of the cell-intrinsic death machinery thus increasing the chances of β-cell survival. Our findings are consistent with the observations of Du and Prendergast [39], which demonstrated activation of PI3K–Akt pathway, masks the proapoptotic effects of FTIs in Rat1/Ras cell system.

Our current findings also suggest that Akt phosphorylation is under the control of PTx-sensitive inhibitory class of trimeric G-proteins. However, it is not clear at present, if Probin is downstream to a PTx-sensitive G-protein, or if Probin itself represents such a G-protein. This is based on our observation that the effects of FTI and PTx on Akt phosphorylation are additive. Therefore, additional studies are needed to further confirm the identity of Probin. Our findings also suggest that both the basal and FTI-induced phosphorylation of Akt were attenuated by PGE_2, a known activator of inhibitory class of G-proteins; such effects are mediated *via* PGE_2 receptor 3 (*Ptger3* [40]). Several lines of evidence suggest regulatory roles for PGE_2 in islet function. For example, using HIT-T15 cells, Robertson and associates have demonstrated the presence of PGE_2 receptors in the cell membrane fraction, which is regulated by inhibitory component of adenylate cyclase [41,42]. Studies from Laychock's laboratory have demonstrated that PGE_2-receptor is coupled to PTx-sensitive G-protein and that the later was able to reverse the PGE_2-inhibition of glucose-induced insulin secretion [43]. In addition, studies from our laboratory have demonstrated expression of functionally regulable high-affinity GTPase activities in the membrane and secretory granule fractions derived from normal rat and human pancreatic islets that is stimulated by PGE_2 [27]. Lastly, we have also demonstrated regulation of the carboxylmethylation of Gγ-subunits by PGE_2 *via* a PTx-sensitive mechanism [25]. Taken together, our findings implicate inhibitory class of G-proteins in the signaling axis leading to FTI-mediated activation of Akt in INS 832/13 cells and normal rat islets.

Our findings also suggest that such IGF-1 can directly activate Akt phosphorylation presumably *via* a mechanism not requiring inhibitory G-proteins since IGF-1-mediated activation of Akt appears to be resistant to PGE_2 at least under our current experimental conditions using INS 832/13 cells. Interestingly however, recent studies by Meng and coworkers in HIT-T15 cells demonstrated a significant reduction in basal Akt phosphorylation by PGE_2, which was reversed by IGF-1 [40]. Based on these observations these authors concluded that PGE_2 induces metabolic defects in β-cells primarily by upregulating the *Ptger3* gene expression and associated down regulation of Akt/FoxO signaling cascade. Our findings are compatible with these observations since PGE_2 markedly reduced basal Akt phosphorylation. Reversal of inhibition of Akt phosphorylation by IGF-1 is difficult to assess since the later significantly increased phosphorylation of Akt. Nonetheless, our data clearly implicate potential roles for inhibitory class of trimeric G-proteins in FTI-induced phosphorylation of Akt. As in the case of Probin, additional studies are needed to determine precise identity of these G-proteins. Our findings of potential regulatory cross-talk between PTx-sensitive Gi/Go proteins and PI3 kinase/Akt signaling mechanisms are compatible with recent observations of Hayakawa and colleagues who demonstrated involvement of Gi/Go class of proteins in prolactin-releasing peptide mediated activation of Akt signaling pathway in GH3 rat pituitary cells [44]. More recent investigations by Mo and coworkers [45] have also implicated PTx-sensitive G-proteins in atypical cannabinoid-induced Akt phosphorylation in human umbilical vein endothelial cells.

The cell survival effects of FTIs are still controversial as they are primarily designed to inhibit tumorigenesis by decreasing the functional competence of Ras oncoproteins. Sun and associates reported resistance of Lonafarnib [an FTI] to induce growth arrest or apoptosis in human non-small lung cancer cells, which was independent of Akt activation [46]. Significant neuroprotective effects of FTIs [e.g., FTI-277 and chaetomellic acid] have been reported against N-methyl-D-aspartic acid-induced striatal brain damage [47]. These observations raise important questions regarding achievement of complete inhibition of Ras function with FTIs. This may, in part, be due to the fact that the three isoforms of Ras [i.e., K-, H- and N-Ras] are differentially sensitive to prenyltransferase inhibitors. In this context, studies by Ashar and associates have demonstrated that K-Ras remains bound to the membrane following exposure to SCH6636 [an FTI] for subsequent activation of PI3 kinase [48]. Another possible candidate that is likely to activate PI3-kinase/Akt signal axis, is RhoB because of its ability to switch from the farnesylated to a geranylgeranylated form following inhibition of farnesylation by FTIs [49]. Therefore, the ability of some of these G-proteins to switch between farnesylation and geranylgeranylation makes the validation of FTI effects on cell survival/function difficult. Nonetheless, the above findings allow us to consider these potential candidate G-proteins for their involvement in Probin-mediated regulation of Akt activation and function in the islet β-cell.

Based on the current findings, we propose a model for Probin-mediated regulation of Akt signaling axis in the pancreatic β-cell [Fig. 10]. Pharmacological [e.g., FTIs] or molecular biological [e.g.,

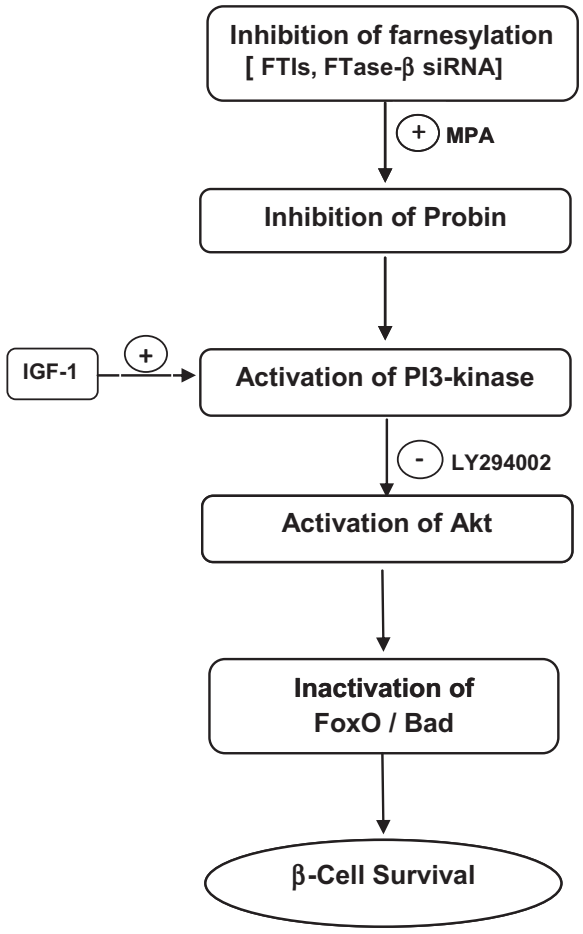

Fig. 10. A model for Probin-mediated suppression of PKB/Akt pathway in pancreatic β-cells. We propose a model for Probin-mediated regulation of Akt signaling axis in the pancreatic β-cell. Pharmacological or molecular biological inhibition of protein farnesylation in isolated β-cells leads to inhibition of Probin function, which, in turn, regulates Akt phosphorylation and prosurvival pathways in a PI3 kinase-dependent fashion. Not shown here are the PTx-sensitive Gi/Go class of proteins, which are coupled to PGE_2 receptors [see Section 4 for a complete description of the model].

siRNA–FTase β-subunit] treatment of pancreatic β-cells leads to inhibition of farnesylation of Probin culminating in inhibition; such an effect can also be seen to a large degree following depletion of intracellular GTP pools by MPA. Inhibition of Probin leads to PI3 kinase-mediated phosphorylation of Akt. Such a signaling step is inhibited by LY294002. Activation of Akt phosphorylation leads to inactivation of FoxO/Bad, which is conducive for β-cell survival. Not depicted in this model are the PTx-sensitive Gi/Go class of proteins, which are coupled to PGE_2 receptors. Our data also is suggestive of a distinct regulation of PI3 kinase-mediated activation of Akt exerted by IGF-1. Additional studies are needed to determine the precise identity of Gi/Go class of proteins and Probin. Further understanding of the identity and nature of these proteins is critical to develop novel tools to boost β-cell survival signaling pathways to maintain β-cell mass, which is known to decrease under various pathological conditions including the duress of glucotoxicity, lipotoxicity [or both] or elevated cytokines.

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