Original Paper

Phagocytic NADPH Oxidase Links ARNO-Arf6 Signaling Pathway in Glucose-Stimulated Insulin Secretion from the Pancreatic β -Cell

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Key Words

Pancreatic islet • Insulin secretion • NADPH oxidase • ADP-ribosylation factor-6 • Reactive oxygen species

Abstract

Background: Recent findings from our laboratory have demonstrated that glucose-stimulated insulin secretion (GSIS) involves interplay between a variety of small G proteins belonging to the Rho (e.g., Cdc42 and Rac1) and ADP-ribosylation factor (e.g., Arf6) subfamilies. Using immunological, pharmacological and molecular biological approaches, we have also identified guanine nucleotide exchange factors (GEFs) for Rac1 (e.g., Tiam1) and Arf6 (e.g., ARNO) in clonal INS-1 832/13 cells, normal rat islets and human islets. As a logical extension to these studies, we investigated, herein, potential downstream signaling steps involved in Arf6/ARNO-mediated GSIS. **Methods:** Using a selective pharmacological inhibitor of ARNO/Arf6 signaling axis (e.g., secinH3) we assessed regulatory roles for Arf6/ARNO in promoting phospholipase D (PLD), phagocytic NADPH oxidase (Nox2), reactive oxygen species (ROS), extracellular-regulated kinases (ERK 1/2) and cofilin (actin-severing protein) signaling steps in clonal INS-1 832/13 cells. **Results:** Our data suggested a marked inhibition by secinH3 of glucose-induced PLD activation, ERK1/2 phosphorylation and dephosphorylation of cofilin, suggesting that Arf6/ARNO signaling mediates PLD, ERK1/2 and cofilin activation in beta-cells. In addition, secinH3 blocked glucose-induced Nox2 activation and associated ROS generation, thus placing Nox downstream to Arf6/ARNO signaling step. Lastly, we also demonstrate a significantly higher cofilin phosphorylation (inactive) in islets derived from type 2 diabetic human donors as well as the Zucker Diabetic Fatty (ZDF) rat, a model for type 2 diabetes. **Conclusion:** Together, our current findings identify signaling steps downstream to ARNO/Arf6 axis leading to insulin secretion.

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Introduction

Islet β -cell function is regulated by glucose concentration, the major physiological insulin secretagogue. Rapid metabolism of glucose within the β -cell leads to the generation of second messengers as well as cationic events that convert extracellular stimulus to intracellular signals and the eventual release of insulin. Activated signal transducers co-ordinate to bring about cytoskeletal remodeling and direct the insulin-laden granules to the plasma membrane for fusion [1]. It is becoming increasingly clear that signals translated from glucose metabolism to small G proteins direct the trafficking of insulin-laden granules to the plasma membrane for fusion and release of insulin into circulation. Furthermore, recent findings from multiple laboratories suggested that remodeling of cytoskeleton is under the control of small G proteins, including those belonging to the Arf (i.e. Arf6) and Rho (i.e. Cdc42/Rac1) families of proteins [2].

The ADP-ribosylation factor (Arf) is a subset of the Ras-superfamily of small G proteins. Among the six isoforms of Arf, Arf6 is shown to be an essential regulator of cytoskeletal remodeling [3]. Initial evidence supporting a role for Arf6 in regulated-secretion came from the neuroendocrine chromaffin cell [4]. Recent reports from multiple laboratories including our own established a role for Arf6 in regulation of GSIS from the pancreatic beta cell [5, 6]. Characteristic to all GTPases, Arf6 cycles between an active GTP-bound and inactive GDP-bound conformations. This dynamic process is catalyzed by guanine-nucleotide exchange factors (GEFs) and GTPase activating factors (GAPs). The widely implicated GEF for Arf6 in secretory cell types including pancreatic beta cell, is ARNO, a member of the cytohesin family. Cytohesin family comprises of four members, cytohesin-1, ARNO/cytohesin-2, GRP1/cytohesin-3 and cytohesin-4. Cytohesins share a central Sec7 domain that is responsible for the GEF activity on Arfs [7]. Until recently, BFA was the only available inhibitor to study the functional roles of Arf which was insensitive to cytohesins. But with recent development of secinH3, a selective inhibitor for cytohesins, has been instrumental in dissecting a role for ARNO in GSIS. SecinH3 gets its name for its high affinity to the functional domain of cytohesins, sec7.

ARNO-mediated activation of Arf6 has been shown to be a direct regulator of PLD [8, 9]; generating lipids that facilitate alterations in membrane composition thus preparing the membrane for exocytosis. Biologically active lipids have a partial role to play in the activation of Rho GTPase, Rac1, by facilitating the dissociation of Rac1 from Rho-GDI [10]. In tumor cells, Arf6-GTP regulates ERK1/2 activation in invasion of tumor cells, which in turn activates Rac1 [11]. Rac1-GTP acts as a direct link regulating the translocation of NADPH oxidase subunit to the plasma membrane and their assembly [12]. ROS generated from activated Nox assembly is also required for cytoskeletal remodeling [13]. Therefore, all the above molecular signals co-ordinate to bring about insulin release in response to glucose stimulation.

More recent studies from many laboratories, including our own suggested novel regulatory roles from phagocyte-like NADPH oxidase (Nox2) in the cascade of events leading to GSIS [14, 15]. Specifically, it has been shown that Nox2-derived generation of reactive oxygen species (ROS) regulate GSIS, albeit precise cellular mechanisms underlying ROS-dependent signaling steps leading to GSIS remain elusive. Along these lines, ROS generation has been linked to ERK1/2 activation [16] and cytoskeletal remodeling in other cell types [13]. The overall goal of the current study is to investigate putative signaling modules downstream to ARNO/Arf6 that would promote GSIS. Using secinH3, a specific cytohesin inhibitor [17] of ARNO/Arf6, we demonstrate that glucose-induced Arf6 activation modulates the PLD/ERK1/2/ROS/cofilin signaling axis leading to insulin secretion.

Materials and Methods

Materials

SecinH3 was from Tocris Biosciences (Ellisville, MO). Antiserum directed against total p47^{phox} was from Santa Cruz Biotechnology (Santa Cruz, CA). Antiserum against phospho-p47^{phox} was from Abcam

(Cambridge, MA). Phospho- and total antibodies for ERK1/2 and cofilin were purchased from Cell Signaling (Boston, MA). PLD assay kit (A12219) was from Invitrogen Molecular probes (Carlsbad, CA). All other reagents used in these studies were from Sigma Aldrich Co. (St. Louis, MO) unless stated otherwise.

Insulin-secreting cells

INS-1 832/13 cells were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum supplemented with 100 IU/ml penicillin and 100 IU/ml streptomycin, 1 mM sodium pyruvate, 50 μ M 2-mercaptoethanol, 11 mM glucose, and 10 mM HEPES (pH 7.4).

All animal protocols were reviewed and approved by the Wayne State University Institutional Animal Care and Use Committee. Islets were isolated from pancreas of male Sprague-Dawley rats (Harlan Laboratories, Oxford, MI), using collagenase digestion and a ficoll gradient as we described previously [18]. Male ZDF and lean control rats were procured from Charles River laboratories (Wilmington, MA) and maintained in a 12-h light/dark cycle with free access to water and food (Diet no. 5008). Islets were isolated from both lean control and ZDF rats by collagenase digestion [18]. Briefly, Hank's buffered saline solution (HBSS) containing collagenase (type V, 1.8 mg/mL) was injected through the pancreatic duct, excised, incubated for 19 min at 37°C and then filtered through a 500 μ wire mesh. The digested pancreas was rinsed with HBSS and islets were separated by ficoll-gradient centrifugation. After several washes in HBSS, islets were handpicked under an inverted light microscope.

Human islets from a normal and T2DM donor Human pancreatic islets were obtained from Prodo Laboratories, Inc. (Irvine, CA). Control islets were from a 54 year old male (85-90% purity) and diabetic islets were from a 45 year old male donor purity (~60% purity) were homogenized with Tris-HCl buffer (50 mM, pH 7.4) containing sucrose (250 mM), EDTA (1 mM), DTT (1 mM), and protease inhibitor cocktail. Protein content was measured, resolved on 12% SDS-PAGE, and used for Western blot analysis.

Quantitation of PLD activity

The Amplex Red phospholipase D (PLD) assay kit provides for a sensitive method of measuring PLD activity in vitro. An enzyme-coupled assay, PLD activity is measured by using Amplex Red reagent. First, PLD cleaves phosphatidyl choline (PC) to choline and PA. The choline generated is oxidized by choline oxidase to hydrogen peroxide. Finally, hydrogen peroxide along with horseradish peroxidase present in the reagent mix reacts with Amplex Red and generates highly fluorescent resorufin. We used the protocol for PLD enzyme activity at near neutral pH 8. Essentially treated cells were collected and lysate prepared in buffer (Tris 50 mM, pH 8.0). Cells were broken by three freeze-thawing cycles and clarified to remove debris (unbroken cells). Supernatant was used for protein estimation. 100 μ g of lysate was made to 100 μ L total volume and to which 100 μ L of the prescribed reagent mix was added and incubated for 30 min at 37 °C. Hydrogen peroxide was used as a control and a standard curve was generated. Fluorescence was measured in a microplate reader (Synergy Biotek Instrument) at E_x 530 nm and E_m 590 nm.

Translocation and membrane association of p47^{phox}

Translocation of p47^{phox} method was adapted from [19]. Briefly, INS-1 832/13 cells were stimulated with low (2.5 mM) and high (20 mM) glucose as indicated in the figure. Lysates were prepared using homogenization buffer containing 20 mM Tris, 0.5 mM EDTA, 0.5 mM EGTA, 250 mM sucrose, 1 mM DTT, 100 μ M PMSF and protease inhibitor. After 40 strokes, the lysate was clarified by centrifugation at 1300 rpm for 10 min at 4 °C. Supernatant was removed and further subjected to differential centrifugation at 40000 rpm for 30 min at 4 °C. The resulting pellet (membranic fraction) was resuspended in homogenization buffer. Protein estimation was performed and 20 μ g of proteins were loaded onto SDS-PAGE. Proteins were further transferred onto nitrocellulose membrane and probed for p47^{phox}.

Quantitation of ROS generation

INS-1 832/13 cells or rat islets were treated with secinH3 and stimulated with glucose for 60 min. Following incubation, medium was removed, and cells were further incubated with DCHFDA (10 μ M) at 37 °C for 30 min in PBS. DCHFDA is a non-polar compound, diffuses rapidly into the cells and hydrolyzes cellular esterases into a polar compound, 2', 7'-dichlorofluorescein. In the presence of ROS, 2', 7'-dichlorofluorescein oxidizes readily to form fluorescent compound dichlorofluorescein. After 30 min, cells were washed with ice-cold PBS and harvested. Protein estimation was done and equal amounts of protein were added into 96-

Fig. 1. SecinH3 inhibits glucose-induced activation of PLD. INS-1 832/13 cells were starved in low serum-low glucose overnight $\pm$ secinH3 (50 μ M) and stimulated with either low glucose (LG, 2.5 mM) or high glucose [HG, 20 mM] $\pm$ secinH3 (50 μ M) for 30 min. PLD activity was determined by Amplex red PLD assay kit. 50 μ g protein was made up to 100 μ L volume with 1x reaction buffer (kit). 100 μ L of working reagent [kit components] was added and incubated for 30 min at 37 °C. Fluorescence was measured at 530/590 nm and hydrogen peroxide was used as a positive control. Data shown above are expressed as fold change over basal and mean $\pm$ SEM from three independent experiments. * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly.

well black bottomed plates. Fluorescence was measured at E_m 485 nm and E_x at 535 nm using luminescence spectrophotometer (Perkin Elmer).

Preparation of cell lysates for phosphoprotein analysis

INS-1 832/13 cells or isolated islets were lysed in 50 mM Tris buffer pH 8.0 containing 10 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, 0.1% SDS, 1mM EDTA, 1mM PMSF, 10 μ g/mL leupeptin, 10 μ g/mL aprotinin, 5 mM EGTA, 5 mM EDTA, 10 mM NaF and 1 mM sodium orthovanadate. After incubation for 10 min in lysis buffer on ice, samples were clarified to remove debris and supernatant collected. Protein estimation was done using Pierce BCA 660 nm protein assay. Equal amounts of protein was loaded and separated by SDS-PAGE. Resolved proteins were transferred onto nitrocellulose membrane and probed for phosphoproteins as indicated in text. Western blot protein bands were visualized by Kodak Imaging System (Rochester, NY) and analyzed densitometrically with the provided software.

Statistical analyses

The statistical significance of the difference between the experimental conditions was determined by Student's t test and One way ANOVA as applicable. p values < 0.05 were considered significant.

Results

ARNO-Arf6 signaling axis is necessary for glucose-mediated activation of PLD

Several lines of evidence implicate Arf6 activation to be requisite for reorganization of biological membranes. Membrane trafficking involves the input of lipid-modifying enzymes which either help to form micro-domains and/or supply secondary messengers. Phospholipase D (PLD) which specifically catalyzes the breakdown of phosphatidylcholine to phosphatidic acid (PA) and choline has been shown to be modulated via Arf6 activation in other cell types [8, 20, 21]. More importantly, activation of PLD has been implicated in physiological insulin secretion [22]. Therefore, we sought to assess if PLD activity was dependent on activation of Arf6 in the pancreatic β -cell. As depicted in Figure 1, glucose treatment of INS-1 832/13 cells resulted in a significant increase in PLD activity. Such an increase in PLD activation in the glucose-stimulated β -cell was attenuated significantly by secinH3. These data indicate that glucose-induced PLD activation is downstream to ARNO/Arf6 signaling step.

Fig. 2. SecinH3 inhibits glucose-induced translocation of p47^{phox} to membrane. Panel A: INS-1 832/13 cells were starved in low serum-low glucose overnight $\pm$ secinH3 (50 μ M) and stimulated with either low glucose (LG, 2.5 mM) or high glucose [HG, 20 mM] $\pm$ secinH3 (50 μ M) for 60 min. At the end of stimulation, cells were lysed in homogenization buffer. Lysates were then differentially centrifuged [methods] to separate cytosol and membranous fractions. Total amount of proteins were quantitated in membrane fractions and separated onto SDS-PAGE. Proteins transferred onto nitrocellulose membrane were probed for p47^{phox}. A representative blot from three independent experiments is shown here. Panel B: Data shown above are expressed as fold change over basal and mean $\pm$ SEM from three independent experiments. In Panel A, * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly.

Fig. 3. SecinH3 inhibits glucose-induced phosphorylation of p47^{phox}. Panel A: INS-1 832/13 cells were starved in low serum-low glucose overnight $\pm$ secinH3 (50 μ M) and stimulated with either low glucose (LG, 2.5 mM) or high glucose (HG, 20 mM) $\pm$ secinH3 (50 μ M) for 60 min. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-p47^{phox}. The blot was reprobed for total p47^{phox}. A representative blot from three independent experiments is shown here. Panel B: Data shown above are expressed as fold change over basal and mean $\pm$ SEM from three independent experiments. In Panel A, * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly.

Inhibition of ARNO-Arf6 signaling pathway attenuates glucose-induced signaling steps involved in the activation of Nox2

As stated above, recent studies from multiple laboratories have implicated novel roles for Nox2 activation and associated ROS generation in GSIS. Furthermore, in the context of our current investigation, activation of Nox2 has been linked to Arf6-PLD signaling axis in polymorphonuclear leukocytes [23]. Therefore, we next determined if glucose-induced phosphorylation and membrane association of p47^{phox}, a member of the cytosolic core of Nox2 [24] are regulated by a signaling step involving activation of Arf6 by ARNO. Data in Figure 2 (Panel A) suggested an increase in the translocation and membrane association of p47^{phox} in INS-1 832/13 cells incubated with stimulatory concentrations of glucose (lane 3 vs. lane 1). Furthermore, secinH3 significantly inhibited glucose-induced translocation of this subunit (lane 4 vs. lane 3). Pooled data from multiple experiments are shown in Figure 2; Panel B.

Emerging evidence also implicates that phosphorylation of p47^{phox} is necessary for its translocation to the membrane, and the Nox2 holoenzyme assembly [25]. Therefore, we examined if glucose induces phosphorylation of this protein, and if so, whether it is

Fig. 4. SecinH3 inhibits glucose-induced generation of ROS. Panels A and B: INS-1 832/13 cells (A) or rat islets (B) were starved in low serum-low glucose overnight $\pm$ secinH3 (50 μ M) and stimulated with either low glucose (LG, 2.5 mM) or high glucose (HG, 20 mM) $\pm$ secinH3 (50 μ M) for 60 min. At the end of stimulation, cells were incubated with DCFHDA (10 μ M; 30 min) and harvested for quantitation of DCF fluorescence. Data shown above are expressed as fold change over basal and mean $\pm$ SEM from three independent experiments. In Panel A, * represents $p < 0.05$ vs. low glucose without secinH3; high glucose with secinH3 and low glucose with secinH3. Similar symbols do not differ significantly. Panel B, * represents $p < 0.05$ vs. low glucose without secinH3; low glucose with secinH3 and high glucose with secinH3. Similar symbols do not differ significantly.

Fig. 5. Pharmacological inhibition of ARNO attenuates glucose-induced ERK1/2 activation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were starved overnight in the presence or absence of secinH3 (50 μ M) and were stimulated with low glucose (LG, 2.5 mM) and high glucose [HG, 20 mM] for 30 min in the continuous presence or absence of secinH3. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-ERK 1/2 (p-44/42). The blot was reprobed for total ERK 1/2. Panel B: Data were densitometrically analyzed and is expressed as fold change in ratio of p- ERK 1/2 over total ERK 1/2 and are mean $\pm$ SEM of three independent experiments yielding similar results. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. high glucose without secinH3 and *** $p < 0.05$ vs. low glucose without secinH3.

dependent upon an ARNO/Arf-6-dependent signaling step. Data in Figure 3 indicated a significant increase in the phosphorylation of p47^{phox} in INS-1 832/13 cells following exposure to glucose (lane 3 vs. lane 1). Furthermore, inhibition of ARNO-mediated activation of Arf6 markedly attenuated glucose-induced phosphorylation of p47^{phox}. Taken together, data provided in Figures 2 and 3 support a role for ARNO/Arf6 axis in glucose-mediated phosphorylation and membrane association of p47^{phox}.

We next assessed if glucose-induced phosphorylation and translocation of p47^{phox} to the membrane translates into functional activation of Nox2. Compatible with our recently published findings [14], glucose significantly induced activation of Nox2 and associated generation of ROS in INS-1 832/13 cells (Fig. 4; Panel A) and normal rat islets (Fig. 4; Panel B). More importantly, and in support of the data described under Figures 2 and 3, secinH3 markedly attenuated glucose-induced ROS generation in both cells studied thereby suggesting that glucose-induced Nox2 activation is dependent upon the Arf6/ARNO signaling module.

Fig. 6. Pharmacological inhibition of ARNO function attenuates glucose-induced cofilin activation in INS-1 832/13 cells. Panel A: INS-1 832/13 cells were starved overnight in the presence or absence of secinH3 (50 μ M) and were stimulated with low glucose (LG, 2.5 mM) and high glucose (HG, 20 mM) for 30 min in the continuous presence or absence of secinH3. Lysates were prepared in RIPA lysis buffer substituted with appropriate phosphatase and protease inhibitors. Quantitated amounts of proteins were loaded and probed for p-cofilin (Ser 3). The blot was reprobed for total cofilin. A representative blot from five independent experiments is shown here. Panel B: Data were densitometrically analyzed and is expressed as fold change in ratio of p-cofilin over total cofilin and are mean $\pm$ SEM of five independent experiments yielding similar results. * represents $p < 0.05$ vs. low glucose without secinH3; ** $p < 0.05$ vs. low glucose without secinH3 and *** $p < 0.05$ vs. high glucose without secinH3.

Fig. 7. Evidence for sustained phosphorylation of cofilin in ZDF rat and type 2 human islets. Panel A: Isolated islets from lean control (Lean ctrl) and ZDF rats were lysed using RIPA buffer. An equal amount of lysate protein was resolved by SDS-PAGE. Western blotting determined the levels of phosphorylated and total cofilin. A representative blot from six sets of lean control and ZDF rats is shown here. Panel B: Isolated islets from normal (Ctrl) and diabetic (T2D) humans were lysed using RIPA buffer. An equal amount of lysate protein was resolved by SDS-PAGE. Western blotting determined the levels of phosphorylated and total cofilin. A blot from one experiment is shown here.

Glucose-induced ERK1/2 activation requires ARNO-mediated activation of Arf6

As a logical extension to the studies described above, we next evaluated the regulatory effects of ARNO/Arf6 signaling pathway on glucose-induced ERK1/2 activation. The premise underlying these studies is to further examine the regulatory roles of Arf6/ARNO as an upstream modulatory step to ERK1/2 activation and cytoskeletal remodeling steps that we described recently. Specifically, we have been able to identify ERK1/2 as one of the intermediate steps involved in glucose-induced Rac1 activation, which is necessary for insulin secretion [26]. With this in mind, we determined if glucose-mediated activation of ERK1/2 is sensitive to inhibition of ARNO/Arf6 signaling pathway. As shown in Figure 5 (Panel A), and compatible with our recent data [26], high glucose markedly enhanced the phosphorylation of ERK1/2 (Lane 2 vs. lane 1). But co-treatment with secinH3 significantly reduced glucose-induced ERK1/2 activation in these cells (Fig. 5; lane 4 vs 2). Pooled data from multiple studies are provided in Figure 5 (Panel B). In summary, our findings described in Figure 5 implicate regulatory roles for ARNO/Arf6 signaling pathway in glucose-mediated regulation of ERK 1/2/ and associated increase in ROS levels, which are necessary for insulin secretion as demonstrated in clonal β -cells [27], normal rat islets [14, 28] and human islets [29].

Selective inhibition of ARNO-Arf6 signaling step attenuates glucose-induced dephosphorylation of cofilin

Next, a series of investigation aimed at further understanding ARNO/Arf6 signaling has demonstrated many roles in regulation of actin cytoskeleton. Particularly in neuroendocrine cells, cortical actin is shown to act as a barrier to preformed dense core granules. Upon stimulation, the actin network is dissolved thus allowing the granules to traverse the cell in order to access the plasma membrane. In order to study organization of actin cytoskeleton, we investigated one of the actin-binding agents, cofilin. Inactive cofilin is defined by its phosphorylated state. Activation of a phosphatase in response to stimulus, dephosphorylates cofilin and thus enabling it to bind actin. First we determined the time-kinetics for the dephosphorylation or phosphorylation cycle of cofilin upon glucose stimulation. When INS-1 832/13 cells were stimulated with glucose for 0, 15, 30 and 60 min, dephosphorylation of cofilin increased after 30 min time-point (data not shown). This is in line with second-phase of insulin secretion where the reserve pool of granules needs to access the plasma membrane. To imply a role for active Arf6 in actin remodeling we tested the effect of secinH3 on glucose-induced dephosphorylation of cofilin. Cells incubated with secinH3 failed to dephosphorylate cofilin upon glucose stimulation (Fig 6). This establishes a regulatory role for ARNO/Arf6 in cytoskeletal remodeling.

Preliminary evidence to implicate altered phosphorylation-dephosphorylation of cofilin in islets from type 2 diabetic human donors and ZDF rats

Type 2 diabetes is characterized by defective insulin secretion and one reason might be due to abnormal functioning of proteins involved in vesicular transport. A study by Parton LE in ZDF rats demonstrated the 57 genes implicated in glucose metabolism, membrane trafficking and vesicle exocytosis were differentially regulated owing to elevated levels of fatty acids and glucose [30]. Among the genes screened, mRNA levels for actin modulators were elevated in ZDF rats than in lean controls. We wondered what would be the effect of prolonged exposure to high levels of glucose on activity of cofilin. To investigate this, we used islets from ZDF rats and type 2 diabetic humans and compared the levels of p-cofilin to respective controls (Fig. 7 A-B). As seen in figure 6, phosphorylated cofilin levels were high in diabetic condition; possibly attributing defective insulin secretion to loss in actin remodeling.

Discussion

Recent advances in cell biology have identified key signaling molecules responsible for transportation and fusion of vesicles in exocytotic pathway. Available models of secretory pathway from published literature elucidate a crosstalk between lipid-modifying enzymes, kinases, small G proteins and cytoskeletal remodeling agents. Over decades, Arf proteins have been widely accepted as regulatory molecules involved in membrane traffic in multiple cell types. In support of this, we recently determined a positive modulatory role for ARNO/Arf6 in GSIS [6]. We demonstrated that dominant-negative mutant and siRNA of Arf6 attenuated GSIS in the pancreatic β -cells. Typical to G proteins, Arf6 cycles between a GDP- to GTP-bound form which are tightly regulated by GEFs and GAPs. Many GEFs have been identified to regulate the GTP/GDP cycle of Arf6. In this context, one member of the cytohesin family, ARNO, to be the designated activator of Arf6 in regulated secretion. Recently published data from our laboratory have implicated novel cross-talk between ARNO and Arf6 leading to insulin secretion. We recently reported a marked inhibition in GSIS by either secinH3, or siRNA-ARNO, thus suggesting key roles for this axis in physiological insulin secretion. Furthermore, we have been able to demonstrate that glucose-induced activation of Rho G proteins (Cdc42 and Rac1) lies downstream to ARNO/Arf6 activation, thus establishing key

Fig. 8. A model depicting signaling events downstream to ARNO/Arf6 activation leading to GSIS in pancreatic β -cells. Based on data herein and available literature, we investigated a regulatory role for ARNO/Arf6 in signaling events leading to GSIS. In our recently published data, we demonstrated the requirement of ARNO to activate Arf6 upon glucose stimulation and thereby activate downstream effectors (i.e., Cdc42 and Rac1). Activated Arf6 interacts with multiple effectors to carry out cellular functions. In the current study, we tested the effect of secinH3 on the activity of plausible effectors of Arf6 that were implicated in GSIS. The ERK1/2 kinase and the lipid-generator, PLD are important downstream effectors of Arf6 modulating vesicular transport by activating Rac1 and thus aiding in the assembly of NADPH oxidase (Nox). Activation of Nox leads to the generation of ROS which in turn facilitates activation of cofilin, an actin-severing agent. Dephosphorylated cofilin binds to actin filaments thus severing them and remodeling the cytoskeleton to direct the insulin-laden secretory granules towards plasma membrane for release of insulin.

roles for both Arf and Rho subfamily of GTPases in physiological insulin secretion [6]. The overall goal of the currently described studies is to further understand the modulatory roles of ARNO/Arf6 in GSIS, specifically at the level of activation of phospholipases, such as PLD, the products of which have been shown to uniquely regulate Rho G proteins, specifically by virtue of their ability to dissociate G proteins (e.g., Rac1) from their regulatory molecules (e.g., GDI). Indeed, our current findings suggest that glucose-induced PLD activation is mediated by ARNO/Arf6 signaling step.

Another signaling molecule ERK1/2 was identified as an important downstream effector of Arf6-GTP. In Madin-Darby canine kidney cells (MDCK), Arf6-GTP regulated the activation of ERK1/2 during tubule development. Activated ERK1/2 remodeled the cytoskeleton by further activating Rac1 [31]. Recent studies from our own laboratory, presented an indispensable role for ERK1/2-regulated Rac1 activation in GSIS [26]. The above evidences prompted us to investigate whether ARNO was an upstream regulator of ERK1/2. Blocking ARNO with secinH3 down-regulated the activation of ERK1/2 in response to glucose. Thus, we were convinced that ARNO regulated ERK1/2-mediated activation of Rac1 in pancreatic β -cells. There are several reports suggesting ERK1/2 regulates the activation of PLD [32] while there are others which supported PLD as an upstream activator of ERK1/2 [33]. The link between PLD and ERK1/2 is yet to be established with regards to GSIS in pancreatic β -cells. Indeed, our findings appear to suggest that both PLD and ERK1/2 lie downstream to Arf6/ARNO.

Having determined a role for PLD and ERK1/2 in ARNO/Arf6 signaling axis, we focused on the downstream signaling events to Rac1 [34]. Nox2 is a highly regulated protein complex

formed by association of membrane (gp91^{phox}, p22^{phox} and Rap1) and cytosolic (p47^{phox}, p67^{phox}, p40^{phox} and Rac1) components upon stimulation. A stimulatory effect of glucose generates Rac1-GTP which is pivotal in driving the cytosolic components to associate with membrane components thus mediating the formation and activation of Nox holoenzyme. Several studies including our own have demonstrated contributory roles for Nox-mediated generation of ROS in insulin secretion [14, 15]. An elegantly described study in human neutrophils, demonstrated the regulation of Arf6-PLD signaling in superoxide production by cytohesin-1, another family member of ARNO [23]. As a logical connection to all the evidences, we tested the effect of secinH3 on the translocation of p47^{phox} to the membrane. Upon stimulation with glucose, p47^{phox} translocated to the membrane which was effectively blocked by the ARNO-inhibitor. Compatible with our recent findings demonstrating a role for Nox2-generated ROS in insulin secretion [14, 27], the current data indicates a regulatory role for ARNO/Arf6 upstream to Nox2 activation

GSIS is a dynamic process that is well-orchestrated by various proteins to regulate actin remodeling. Some of the key regulators of actin rearrangement are Cdc42/Rac1, PAK1 and cofilin. Cofilin is an actin severing agent whose activity is determined by its phosphorylated state. Contrary to popular convention, cofilin's activity is enhanced when dephosphorylated. Many upstream regulators including Rac1 and Nox control the activation cycle of cofilin. In pancreatic beta cells, emerging evidence suggests the requirement of ROS in addition to elevated ATP/ADP ratio for the release of insulin in response to glucose stimuli [35]. ROS acts as second messengers to facilitate cytoskeletal remodeling. In INS 832/13 cells, glucose induced dephosphorylation of cofilin and secinH3 inhibited this event (Fig. 6). This clearly indicates cofilin as an effector in the ARNO/Arf6 signaling axis. In cultured pig proximal tubular epithelial cells (PTEC), Ishibashi evidently demonstrated the inactivation of cofilin under high glucose conditions for prolonged periods [36]. It is noteworthy that we also found a similar increase in phosphorylated cofilin (inactive) in both ZDF rat and diabetic human islets, consistent with findings described in PTEC preparations (Fig. 7). Whether a loss in the activity of cofilin could explain impaired insulin secretion in type 2 diabetes remains to be investigated further.

Based on the data from our current studies and the published evidence along these lines, we propose that GSIS involves a significant cross talk between members of the Arf and Rho GTPases (Fig. 8). We propose that glucose-induced activation of PLD leads to the generation of biologically active lipids, which, in turn, regulate G-protein (e.g., Rac1) function by dissociating Rac1 from its inhibitory GDI [10]. It is also likely that glucose-induced, Arf6-mediated ERK1/2 activation facilitates Rac1 activation as we have demonstrated recently [6, 26]. Activated Rac1 and phosphorylated p47^{phox} translocate to the membrane for the assembly and functional activation of Nox2, which, in turn, generate ROS transiently. The latter has been shown to control cytoskeletal re-arrangements as demonstrated by increased dephosphorylation (and activity) of cofilin in the current study. Furthermore, we also present data to indicate sustained phosphorylation (and inactivation) of cofilin in islets derived from human and animal models of diabetes. Future studies will be dedicated towards understanding potential defects involved in the signaling steps under conditions of glucolipotoxicity and diabetes.

Abbreviations

Arf6 (ADP-ribosylation factor 6); ARNO (Arf-nucleotide binding site opener); DCHFDA (2',7'-dichlorodihydrofluorescein diacetate); ERK1/2 (Extracellular-regulated kinases 1/2); GEFs (Guanine nucleotide exchange factors); GSIS (Glucose-stimulated insulin secretion); Nox2 (NADPH oxidase 2); PLD (Phospholipase D); ZDF rat (Zucker-diabetic fatty rat).

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