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Nck1 deficiency improves pancreatic β cell survival to diabetes-relevant stresses by modulating PERK activation and signaling

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ABSTRACT

Increasing evidence strongly supports a critical role for PERK in regulating pancreatic β cell function. In agreement, we previously reported that enhancing PERK basal activity, by silencing the SH domain-containing adaptor protein Nck1 in pancreatic β cells, increased insulin content in a PERK-dependent manner. Here we report that Nck1-deficient MIN6 cells display normal overall morphology while as expected increased number of secretory granules. Furthermore, we demonstrate that cell survival to diabetes-relevant stresses is increased, while cell viability in response to chemical endoplasmic reticulum (ER) stress inducers is not changed. In agreement, PERK activation in Nck1-depleted MIN6 cells exposed to palmitate was significantly reduced while it remained strongly induced by the ER stress inducer thapsigargin. Interestingly, silencing Nck1 in MIN6 cells results in increased PERK basal activity and expression of the PERK downstream target sestrin2, which promotes autophagy by attenuating mTORC1 activation through AMPK-dependent and -independent mechanisms. Accordingly, activated AMPK was increased, mTORC1 signaling decreased, and autophagy markers increased in Nck1-silenced MIN6 cells. Increased autophagy was recapitulated in *Nck1*$^{-/-}$ mice pancreatic β cells. In addition, basal levels of the PERK substrate Nrf2 and its antioxidant gene targets (HO-1 and Nqo1) were upregulated in Nck1-silenced MIN6 cells, revealing an active PERK-Nrf2 signaling in these cells. Finally, Akt activation was increased in Nck1-silenced MIN6 cells. Altogether, this study demonstrates that Nck1 silencing in pancreatic β cells promotes PERK activation and signaling to protect β cells against pathological stresses. These findings further provide new perspectives to advance our understanding of molecular mechanisms and signaling systems regulating pancreatic β cell fates.

1. Introduction

Increasing evidence from studies in humans, and findings from cell cultures and animal models strongly support an important role for the PKR-like endoplasmic reticulum kinase (PERK) in pancreatic β cell function and insulin biogenesis. Indeed, a role for PERK in pancreatic β cells was first highlighted by the discovery that the Wolcott–Rallison syndrome (WRS) in humans, a neonatal/early infancy form of diabetes characterized by a critical reduction in β cell mass and function is caused by loss of function mutations in the *PERK* gene [1]. Furthermore, *PERK*$^{-/-}$ mice phenocopy WRS dysfunctions and display diminished β cell mass due to reduced proliferation and differentiation during the neonatal period, consequently inhibiting postnatal gain of β cell mass [2–4]. In addition, acute inhibition of PERK through adenovirus-mediated expression of a dominant negative PERK mutant lacking its kinase domain in rat insulin-secreting β cells (INS 832/13) led to reduced cell proliferation and insulin content [5]. Surprisingly, proinsulin was abnormally retained in the ER of these cells, which implicates PERK in maturation and trafficking of proinsulin. Of interest, conditional deletion of *PERK* in young or mature adult mice significantly increased β cell death even though β cell proliferation was increased [6]. This reveals that PERK contributes to maintain β cell function also in adults, but apparently through a different mechanism than during early postnatal development.

PERK was initially defined as a serine/threonine protein kinase activated in conditions altering the endoplasmic reticulum (ER) homeostasis [7,8]. However, recent studies revealed that PERK also displays tyrosine kinase activity that mediates its autophosphorylation on tyrosine residues and contributes to regulate its activation [9,10]. ER stress-induced activated PERK phosphorylates the α-subunit of the eukaryotic initiation factor 2 (eIF2α) on Ser^{51}, resulting in attenuation of general translation, which helps to recover ER homeostasis by reducing the biosynthetic burden [11–13]. Paradoxically, this promotes translation of ATF4 (Activating Transcription Factor 4), which controls an important transcriptional program with complex outputs [12]. Under physiological conditions inducing transient PERK activation, ATF4 is protective by regulating expression of genes that contribute to the

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adaptation of cells to stress conditions created by short-lived oxygen and nutrient deprivation, and ER/oxidative stress [14–16]. Concomitantly, PERK also directly phosphorylates the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2), facilitating its nuclear translocation and subsequent transcription of target genes, whose protein products contribute to redox homeostasis and cell survival to stress [17–19], and prevent onset of diabetes [20]. In addition, Nrf2 and ATF4 were shown to dimerize and promote the expression of antioxidant genes such as heme oxygenase-1 (HO-1) [21], suggesting cooperation between PERK downstream signaling events in physiological conditions. In contrast, in pathologic settings created by the persistence of stress conditions, sustained PERK–peIF2α–ATF4 signaling induces prolonged expression of C/EBPα-homologous protein GADD153 (CHOP) [11,12], which triggers expression of genes that guide cells toward apoptosis [22]. Altogether, these studies provide evidence supporting that cellular response to stress can adopt diametrically different directions determined according to the intensity and duration of activation of the PERK–peIF2α–ATF4 signaling pathway [23].

Autophagy is a cellular process involving the formation of autophagosomes, small double layer membrane vesicles that sequestrate intracellular components to be targeted to bulk lysosomal degradation. Under physiological settings, basal levels of autophagy mediate clearance of extra or dysfunctional organelles or macromolecules, ensuring quality control essential to cellular homeostasis [24,25]. However, autophagy is enhanced in response to various stresses, providing crucial substrates for energy production and metabolic function engaged in protecting cells against deleterious conditions like nutrient-scarce conditions [26]. Interestingly, numerous studies highlighted the importance and protective role of autophagy in pancreatic β cell function and resistance to stress, and demonstrated that impairment of autophagy leads to pancreatic β cell dysfunction and death [27–30].

We previously reported that silencing Nck1 in pancreatic β cells MIN6 enhances PERK basal activity, leading to PERK-dependent increased insulin biosynthesis and content [10]. Nck (non-catalytic region of tyrosine kinase) are 47 kDa adaptor proteins containing three Src Homology (SH) 3 domains and one SH2 domain [31,32]. Nck family, composed of the highly homologous Nck1 and Nck2 proteins encoded by two different genes [32,33], mediate intracellular signal transduction by coupling cell surface receptors to specific downstream effectors [34]. Nck1 and Nck2 have been involved in critical biological processes, including embryonic development [35], actin cytoskeletal reorganization [36,37], axonal guidance [38], proliferation [39] and the unfolded protein response (UPR) [40–42]. In the present study, we report that stably silencing Nck1 in MIN6 cells is protective against cell death induced by stress of pathological relevance to diabetes. In parallel, we demonstrated that silencing Nck1 in MIN6 cells enhances PERK basal activity, phosphorylation of eIF2αSer^{51}, and ATF4 mRNA and nuclear protein levels. In accordance, we showed that in a PERK-dependent manner, autophagy and antioxidant gene expression were increased in MIN6 cells depleted of Nck1. Given that autophagy and antioxidant response clearly aid β cell survival to stress [27,29,30], our findings provide strong evidence that silencing Nck1 in MIN6 cells enables PERK activation, initiating a signaling network that contributes to improve β cell function and resistance to pathological stresses relevant to diabetes.

2. Material and methods

2.1. Cell culture

Control and shNck1 MIN6 cells were generated as previously described [10]. Cells were cultured in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 15% fetal bovine serum (FBS, Invitrogen), 0.75 mg/ml penicillin and 0.1 mg/ml streptomycin (Invitrogen), 0.55 μM β-Mercaptoethanol (Sigma) and kept at 37 °C in a 5% CO_2 environment.

2.2. Transmission electron microscopy

Control and shNck1 MIN6 cells were plated in 4-well plates under regular medium conditions. Cells were washed twice with 1× PBS and then fixed with 2.5% glutaraldehyde (Electron Microscopy Science (EMS)) in 0.1 M sodium cacodylate buffer (EMS) overnight at 4 °C. Samples were washed 3 times with 0.1 M sodium cacodylate (washing buffer) for a total of 1 h, then post-fixed with 1% aqueous tetroxide d'osmium (Mecalab) and 1.5% aqueous potassium ferrocyanide (Sigma) for 2 h, followed by washing three times with washing buffer for a total of 15 min. Samples were dehydrated with acetone (Fisher Scientific) in increasing concentrations: 30%, 50%, 70%, 80%, 90% and 3× with 100% each for 8–15 min. Infiltration was performed with Epon (Mecalab)/acetone: 1:1 overnight, 2:1 all day, 3:1 overnight and pure Epon next day for 4 h. Embedding was done with appropriate labels then polymerized in 60 °C oven 48 h. Samples were trimmed and cut in 90–100 nm thick sections with UltraCut E ultramicrotome (Reichert-Jung) and placed onto a 200 mesh copper grid (EMS). Sections were stained with Uranyl acetate (EMS) for 8 min, then Reynold's lead (EMS) for 5 min. For morphology, cells were analyzed using FEI Tecnai 12 120 kV transmission electron microscope (TEM) equipped with an AMT XR80C 8 megapixel CCD camera.

2.3. Cell treatments

Thapsigargin (Tg, Sigma) and dithiothreitol (DTT, Roche) treatments were used as indicated in figure legends. Palmitate (PA, Sigma) was prepared in 3 mM stock solution conjugated with 5% fatty acid free BSA (Roche) in Hepes-balanced Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4.74 mM KCl, 2.54 mM $CaCl_2$, 1.19 mM $MgCl_2$, 1.19 mM KH_2PO_4, 25 mM $NaHCO_3$, 10 mM Hepes, pH 7.4) by shaking overnight at 37 °C. The stock solution was then diluted at the desired concentration in culture medium. D-Glucose (Sigma) stock solution was 2 M and diluted to desired concentration in culture medium. Thiazolyl blue tetrazolium bromide (MTT, Sigma) and bafilomycin A1 (BafA1, Sigma) were used as indicated in the figure legends. Cell transient transfection with empty or myc-tagged kinase dead PERK (K618A) plasmids was performed using Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. Cells were lysed 48 h post-transfection in lysis buffer as previously described [42]. Total cell lysate was subjected to western blotting using antibodies described below. For mCherry-GFP-LC3 transient transfection, Lipofectamine 3000 was used and cells were visualized 24 h post-transfection.

2.4. MTT viability assay

Cell viability was analyzed using MTT assay. Cells were plated at 2×10^5 cells/well in 24-well plates and the next day exposed to indicated compounds for 24 h. MTT solution prepared fresh at 5 mg/ml in PBS was diluted 1:6 in regular medium and added to each well (600 μl) at the end of the treatment. Incubation was pursued at 37 °C for 3.5 h. MTT solution was then removed and 500 μl/well of DMSO was then added. Absorbance was measured at 570 nm using EnSpire Multimode plate reader (PerkinElmer).

2.5. Western blot analysis and antibodies

Cells were washed in cold PBS and lysed on ice using lysis buffer as reported above. Cell lysates were then centrifuged and supernatant used for western blot analysis. Where indicated, RIPA buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 100 mM sodium fluoride) supplemented with protease inhibitors was used to lyse cells. Where indicated, crude nuclear extracts were generated as previously described [43]. PERK and Nck1-specific polyclonal antibodies were