Peptide-based sequestration of the adaptor protein Nck1 in pancreatic β cells enhances insulin biogenesis and protects against diabetogenic stresses

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One feature of diabetes is the failure of pancreatic β cells to produce insulin, but the molecular mechanisms leading to this failure remain unclear. Increasing evidence supports a role for protein kinase R–like endoplasmic reticulum kinase (PERK) in the development and function of healthy pancreatic β cells. Previously, our group identified the adaptor protein Nck1 as a negative regulator of PERK. Indeed, we demonstrated that Nck1, by directly binding PERK autophosphorylated on Tyr561, limits PERK activation and signaling. Accordingly, we found that stable depletion of Nck1 in β cells promotes PERK activation and signaling, increases insulin biosynthesis, and improves cell viability in response to diabetes-related stresses. Herein, we explored the therapeutic potential of abrogating the interaction between Nck and PERK to improve β-cell function and survival. To do so, we designed and used a peptide containing the minimal PERK sequence involved in binding Nck1 conjugated to the cell-permeable protein transduction domain from the HIV protein TAT. In the current study, we confirm that the synthetic TAT-Tyr(P)561 phosphopeptide specifically binds the SH2 domain of Nck and prevents Nck interaction with PERK, thereby promoting basal PERK activation. Moreover, we report that treatment of β cells with TAT-Tyr(P)561 inhibits glucolipotoxicity-induced apoptosis, whereas it enhances insulin production and secretion. Taken together, our results support the potential of sequestering Nck using a synthetic peptide to enhance basal PERK activation and create more robust β cells.

Promote β-cell function and survival is paramount in the development of therapies to prevent and treat both types of diabetes. Increasing evidence supports a role for protein kinase R–like endoplasmic reticulum kinase (PERK) in the maintenance of β-cell homeostasis. Indeed, PERK sustains β-cell function by enhancing proliferation (1, 2), insulin processing and secretion (1–4), antioxidant gene expression (5–7), and protection against interferon-mediated injury (8). However, the role of PERK in β-cell proliferation is far from being clearly understood. Indeed, although deficiencies in PERK expression or signaling in mice are associated with decreased β-cell mass (9, 10), half-dosage of PERK associated with Perk heterozygosity in mice increases β-cell proliferation and mass in the adult stage (11), and deletion of PERK in adult mice also increases β-cell proliferation (12). In contrast, it is clear that PERK deficiency in mice leads to defective insulin biogenesis and secretion (1, 13). In the same way, loss-of-function mutations in the Perk gene in humans have been linked with Wolcott–Rallison syndrome, a neonatal/early infancy form of diabetes characterized by a critical reduction in β-cell mass (14, 15), and pancreatic islets from types 1 and 2 diabetes patients as well as diabetic mouse models exhibit elevated levels of CHOP (CCAAT enhancer–binding protein–homologous protein), a proapoptotic transcription factor induced by aberrant or chronic PERK activation (16–19). Based on these findings, modulators of PERK activation could be potential targets of active knowledge translation for new timely preventive and therapeutic interventions to counter pancreatic β-cell failure and death in human diabetes.

We previously identified the adaptor protein Nck1 as a limiting factor of PERK activation and signaling in pancreatic β cells (20, 21). Nck proteins, a family consisting of paralogs Nck1 and Nck2, are composed exclusively of Src homology (SH) domains, each containing three N-terminal SH3 domains and a single C-terminal SH2 domain (22). Nck proteins are known to assemble molecular complexes to transduce signals from cell surface receptors to downstream effectors involved in critical biological processes, including embryonic development (23), actin cytoskeletal reorganization (24, 25), axonal guidance (26), T-cell function (27, 28), and proliferation (29). However, we promote β-cell function and survival is paramount in the development of therapies to prevent and treat both types of diabetes.

The failure of pancreatic β cells to produce insulin is well known as a central problem in both type 1 and type 2 diabetes; however, the molecular mechanisms leading to β cell failure remain unclear. Consequently, investigating mechanisms that promote β-cell function and survival is paramount in the development of therapies to prevent and treat both types of diabetes.

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The abbreviations used are: PERK, protein kinase R–like endoplasmic reticulum kinase; SH, Src homology; TAT, transactivator of transcription; 7-AAD, 7-aminoactinomycin D; FBS, fetal bovine serum.
and others have implicated Nck in other biological processes such as the unfolded protein response (20, 30–32), protein translation (31–36), and cancer progression (37, 38). Moreover, we previously reported that through its SH2 domain, Nck1 directly binds PERK autophosphorylated on Tyr561 to limit its activation (20). In agreement, stable depletion of Nck1 in the mouse insulinoma cell line MIN6 enhances basal PERK activation and signaling, a finding associated with increased proinsulin translation and a greater number of insulin secretory granules (20, 21). In a similar vein, pancreatic islets derived from NckT−/− mice display increased insulin content compared with WT mice (20). Finally, we demonstrated that Nck1 deficiency in MIN6 cells confers protection against diabetes-related stresses by promoting autophagy and expression of antioxidant genes (21). Taken together, these results strongly support the notion that Nck1 deficiency creates healthier pancreatic β cells in a PERK-dependent manner.

In the current study, we aimed to develop and characterize a synthetic cell-permeable peptide that binds Nck and abrogates the Nck/PERK interaction with the goal of phenocopying Nck1 depletion in a more therapeutically relevant manner. Here, we report that this peptide promotes basal PERK activation, creating robust β cells that exhibit enhanced insulin content and secretion and higher resistance to apoptosis induced by glucolipotoxicity.

Results

Design and biochemical characterization of an Nck-SH2 domain-binding peptide

Nck1 and Nck2 (collectively referred to as Nck) bind PERK in an SH2 domain–dependent manner (20). The interaction between Nck and PERK requires a highly conserved phosphorylated tyrosine residue (Tyr561 in mouse) located in the juxtamembrane domain of PERK (Fig. 1A). To abrogate this interaction, we designed a synthetic cell-permeable peptide, TAT-Tyr(P)561, which consists of the protein transduction domain from the HIV protein transactivator of transcription (TAT) conjugated to the amino acid sequence derived from mouse PERK that is involved in binding Nck, including the phosphorylated Tyr561 residue (Fig. 1A). Using in vitro pull-down assays, we demonstrated that GST–Nck1 and GST–Nck2 bind TAT-Tyr(P)561 (Fig. 1B). However, GST–Nck proteins containing point mutations (SH2M: Nck1 R308K or Nck2 R312K) known to abrogate SH2 domain–binding functionality (39) fail to do so, revealing that both Nck proteins interact with TAT-Tyr(P)561 through their SH2 domain. Importantly, TAT-Tyr(P)561 exhibits a relatively high specificity for binding the SH2 domain of Nck compared with other SH2 domains tested (Fig. S1). Further supporting this, MS analysis of proteins interacting with TAT-Tyr(P)561 in preliminary pull-down assays with INS-1 or HEK-293 cell lysates failed to identify SH2 domain–containing proteins other than Nck (data not shown).

To further investigate the interaction between TAT-Tyr(P)561 and Nck using an alternative approach, biotinylated TAT-Tyr(P)561 immobilized on streptavidin-coupled beads was evaluated for its ability to bind Nck from cell lysate. Nck derived from both mouse and rat cell lysates was successfully pulled down by biotin–TAT-Tyr(P)561 (Fig. 1C). Of note, the same peptide containing a phenylalanine residue rather than a phosphotyrosine residue (biotin–TAT-Phe) was unable to pull down detectable levels of Nck, demonstrating the importance of the phosphotyrosine residue in the interaction between TAT-Tyr(P)561 and Nck (Fig. 1D). In addition, the unbiotinylated TAT-Tyr(P)561 successfully competed with its biotinylated counterpart for Nck binding (Fig. S2A), whereas the unphosphorylated TAT-Tyr and mutant TAT-Phe peptides competed with biotin–TAT-Tyr(P)561 for Nck binding with a much lower affinity (Fig. S2). Finally, using isofrom-specific antibodies, we confirmed that biotin–TAT-Tyr(P)561 interacts with both human Nck homologs derived from HEK-293 cell lysates, whereas biotin–TAT-Phe does not (Fig. S3).

TAT-Tyr(P)561 abrogates the Nck/PERK interaction and promotes basal PERK activation

Given that TAT-Tyr(P)561 binds the SH2 domain of Nck in a similar manner to PERK, we next sought to determine whether it prevents Nck from interacting with PERK. To do so, we performed pulldown assays wherein the GST-fused cytoplasmic segment of PERK (cPERK) and TAT-Tyr(P)561 competed for lysate-derived Nck binding. Indeed, we found that TAT-Tyr(P)561 prevents Nck from binding GST–cPERK in a dose-dependent manner (Fig. 2A). Furthermore, we demonstrated that TAT-Tyr(P)561 directly targets the Nck/PERK interaction by demonstrating that it robustly reduces the amount of recombinant Nck1 pulled down by GST–cPERK in vitro (Fig. 2B).

We previously demonstrated that stable depletion of Nck1 in MIN6 cells promotes basal PERK activation and signaling (20, 21). Thus, we hypothesized that Nck sequestration upon treatment with TAT-Tyr(P)561 would have a similar effect in β cells. After confirming that TAT-Tyr(P)561 enters β cells using confocal microscopy (Fig. S4), we observed that treating INS-1 cells with 10 μM TAT-Tyr(P)561 for 4 days significantly elevates basal PERK activation as indicated by increased phosphorylation of PERK on its activation site Thr974 (Fig. 2, C and D). Interestingly, this increase in basal PERK activation is subtle compared with thapsigargin-induced PERK activation (Fig. S5). In agreement, we did not consistently detect significant increases in basal eIF2α phosphorylation (data not shown) or Atf4 or Chop transcription (Fig. 2E) in TAT-Tyr(P)561–treated INS-1 cells. Taken together, these results support the notion that decreasing Nck1 expression, sequestering Nck in β cells enhances basal PERK activation without considerably enhancing canonical PERK signaling.

Sequestering Nck protects β cells against glucolipotoxicity–induced apoptosis

Increased basal PERK activation associated with stable Nck1 depletion has been shown to improve β cell survival in response to various diabetes–relevant stresses (21). To assess the protective effects of sequestering Nck on β cell survival, INS-1 cells were treated with TAT-Tyr(P)561 for 4 days, including a final 24-h exposure to glucolipotoxic stresses, and apoptosis was determined by flow cytometric analysis and Western blotting. Using FITC–annexin V/7-AAD staining, we observed a
A reduced proportion of late apoptotic and dead cells and a concurrent increased proportion of healthy cells in samples pretreated with TAT-Tyr(P)561 prior to lipotoxic or glucolipotoxic stress (Fig. 3, A–D). In accordance, TAT-Tyr(P)561 protects INS-1 cells against caspase-3 cleavage, a marker of apoptosis induced by combined treatment with palmitate and glucose in...
untreated INS-1 cells (Fig. 3E). Finally, consistent with our previous findings in Nck1-depleted MIN6 cells (21), treating these cells with TAT-Tyr(P)^561 also protects against palmitate-induced caspase-3 cleavage (Fig. 3F). Taken together, our results clearly demonstrate that sequestering Nck using TAT-Tyr(P)^561 improves β cell survival in response to diabetes-relevant stresses.

Sequestering Nck enhances insulin content and secretion

We previously demonstrated that in MIN6 cells, increased basal PERK activation associated with stable Nck1 depletion correlates with increased proinsulin translation, as well as a greater number of insulin secretory granules (20, 21). To explore the possibility that TAT-Tyr(P)^561 treatment enhances insulin biogenesis, we treated INS-1 cells with 10 μM TAT-
Figure 3. TAT-Tyr(P)Y561 protects β cells against glucolipotoxicity-induced apoptosis. A, representative flow cytometry plots of INS-1 cells treated with or without 10 μM TAT-Tyr(P)Y561 for 3 days and for an additional 24 h with 2 mM palmitate (PA) and 25 mM glucose (G). Apoptosis was analyzed by FITC-annexin V and 7-AAD staining. Proportions of live (annexin V−/7-AAD−) (B), late apoptotic (annexin V+/7-AAD+) (C), and dead (annexin V−/7-AAD+) (D) cells were quantified. The data are the means ± S.D. of a typical experiment of two performed in triplicate. Statistical significance was evaluated by two-way analysis of variance with Bonferroni’s multiple comparisons test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001. E and F, lysates from INS-1 (E) or MIN6 (F) cells treated with or without 10 μM TAT-Tyr(P)Y561 for 3 days and for an additional 24 h with PA/G or PA alone were subjected to Western blotting with indicated antibodies. FL, full-length; Clv, cleaved. The data are the means ± S.D. of at least three independent experiments performed in triplicate. Statistical significance was determined using the Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.
We have previously demonstrated that a modest increase in basal PERK activation following Nck1 depletion in MIN6 cells correlates with enhanced β-cell function and survival (20, 21). Here, we demonstrated that sequestering Nck using TAT-Tyr(P)561 phenocopies the effects of reducing Nck1 expression on β-cell function and survival. Indeed, we demonstrated that treating INS-1 cells with TAT-Tyr(P)561 protects against apoptosis induced by diabetogenic stresses. Moreover, we showed that treating INS-1 cells with TAT-Tyr(P)561 enhances insulin content, as well as basal and glucose-stimulated insulin secretion. Increased basal insulin secretion following TAT-Tyr(P)561 treatment is not necessarily wanted in the context of blood glucose regulation in diabetes. However, Nck1-deficient mice display comparable glycemia and insulinemia compared with WT littermates (42), suggesting that basal insulin secretion in the perspective of Nck functional deficiency might be better controlled in the in vivo islet context.

Protein/protein interactions are involved in nearly all cellular processes and are consequently considered potential targets for therapeutics. Specifically, targeting the Nck interactome has shown promise in multiple disease models. Indeed, abrogation of the Nck/PECAM-1 interaction protects against ischemia/reperfusion injury (43), whereas targeting the interaction between Nck1 and CD3ε results in diminished T-cell receptor signaling and protects mice against autoimmune diseases (44, 45). In accordance with our current findings, these studies sup-
port the notion that targeting protein/protein interactions involving Nck is a valuable approach in the treatment of diseases. On the other hand, given that Nck proteins are expressed in multiple tissues and involved in numerous biological processes (23, 46), treatment with TAT-Tyr(P)\textsuperscript{561} in vivo could lead to unwanted responses in various physiological and pathological contexts. Notwithstanding, given that both types of diabetes are characterized by β cell death and insufficient insulin production, our study provides strong evidence that targeting the Nck/PERK interaction in the context of pancreatic islet transplantation is a viable approach of the utmost significance for the development of robust β cells, an outcome that could benefit both types of diabetes.

Experimental procedures

Cell culture and treatments

Mouse insulinoma MIN6 cells were cultured in high-glucose Dulbecco’s modified Eagle’s medium (Gibco), supplemented with 15% fetal bovine serum (FBS; Gibco), 71.5 μM β-mercaptetoethanol (Sigma), and antibiotic-antimycotic (Gibco). Rat insulinoma INS-1 cells (INS-1 832/13) were cultured in RPMI 1640 medium (Gibco) supplemented with 10% FBS, 71.5 μM β-mercaptoethanol, 10 mM HEPES (BioShop), 1 mM sodium pyruvate (Sigma), and antibiotic-antimycotic. HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS and antibiotic-antimycotic. All cells were maintained at 37 °C in a 5% CO\textsubscript{2} environment. Palmitate stock solution (15 mM) was prepared by dissolving sodium palmitate (2018) in 50% ethanol (Sigma) in 50% ethanol followed by conjugation to free fatty acid–free BSA in KRHB buffer by overnight agitation at 37 °C (free fatty acid:BSA molar ratio of 5:1). Stock solutions of synthetic TAT-conjugated peptides (Bio Basic Canada Inc.) were prepared in PBS.

Recombinant proteins and pulldown assays

Recombinant GST fusion proteins were purified from bacteria and immobilized on GSH beads. Recombinant Nck1 was obtained by thrombin cleavage of GST–Nck1 as recommended by the manufacturer. Pulldowns were performed in freshly prepared binding buffer (10% glycerol, 0.5% Triton X-100, 100 mM NaCl, 20 mM Tris, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 10 μg/μl aprotinin, 10 μg/μl leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 100 μM Na\textsubscript{3}VO\textsubscript{4}) for 3 h at 4 °C. For streptavidin pulldowns, biotinylated peptides were preincubated with high-capacity streptavidin-agarose (Thermo Scientific) or streptavidin magnetic beads (GenScript).

Western blotting and antibodies

The proteins were resolved by SDS-PAGE and transferred onto polyvinylidene difluoride membrane, which were then blocked and subsequently processed for Western blotting with the indicated antibodies. Polyclonal phosphospecific PERK Tyr\textsuperscript{561} antibody, which also recognizes TAT-Tyr(P)\textsuperscript{561}, was generated as previously described (20). Horseradish peroxidase–conjugated streptavidin was purchased from Thermo Fisher Scientific. Nck1 antibody was generated as previously described (42). Nck2 antibody (TA307351) was purchased from OriGene. Polyclonal pan-Nck antibody recognizing both Nck was generated as previously described (47). Caspase-3 antibody (9662), which recognizes both full-length and cleaved forms of the protein, was purchased from Cell Signaling Technology. Total PERK (C33E10) and phosphospecific PERK Thr\textsuperscript{168} (16F8) antibodies were purchased from Cell Signaling Technology. GST (B-14) antibody was purchased from Santa Cruz Biotechnology, and tubulin antibody was from Sigma–Aldrich.

Confocal microscopy

Cells seeded on glass-bottomed plates were treated as indicated prior to being fixed in 4% formaldehyde in PBS for 15 min at room temperature. The cells were then washed and stained with 4’,6’-diamino-2-phenylindole for 3 min at room temperature. Image acquisition was performed using the confocal laser scanning microscope LSM-510 Meta (Zeiss).

Insulin content and secretion

Cells incubated overnight in low glucose culture medium were washed and incubated with low-glucose KRHB buffer. In 1-h intervals, the cells were incubated with KRHB supplemented with 2.8 mM glucose and subsequently 8.0 mM glucose; cell media were collected after both time points. Total insulin was extracted by incubating cells in ethanol and hydrochloric acid overnight at −20 °C. Insulin secretion and content were measured using a STELLUX® chemiluminescence rodent insulin ELISA kit (Alpco) following the manufacturer’s instructions.

RNA extraction and quantitative PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) following the manufacturer’s instructions. cDNA was prepared using high-capacity cDNA reverse transcription kit (Applied Biosystems). PCRs were performed using Power SYBR Green PCR Master Mix (Applied Biosystems), and quantitative PCR was performed using the ViiA 7 real-time PCR system (Thermo Fisher Scientific).

Flow cytometry

Following indicated treatments, medium containing detached cells was collected in flow cytometry tubes, and adherent cells were trypsinized, harvested, and combined with their respective media. The cells were then stained with FITC–annexin V and 7-AAD (BioLegend) as per the manufacturer’s instructions and subsequently analyzed by flow cytometry analysis using the FACSCantoTM II system (BD Biosciences).

Statistical analysis

Statistical significance was determined as indicated in each figure (GraphPad Prism).

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