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Uncoupling Proteostasis and Development *in Vitro* with a Small Molecule Inhibitor of the Pancreatic Endoplasmic Reticulum Kinase, PERK*

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Background: PERK controls unfolded protein load in the ER and promotes a latent gene expression program whose relative contributions to cell physiology are incompletely understood.
Results: Acute PERK inhibition deregulates protein synthesis and promotes accumulation of misfolded pro-insulin.
Conclusion: PERK contributes to proteostasis acutely.
Significance: The proteostatic activity of PERK can be uncoupled from its latent role in gene expression.

Loss-of-function mutations in *EIF2AK3*, encoding the pancreatic endoplasmic reticulum (ER) kinase, PERK, are associated with dysfunction of the endocrine pancreas and diabetes. However, to date it has not been possible to uncouple the long term developmental effects of PERK deficiency from sensitization to physiological levels of ER unfolded protein stress upon interruption of PERK modulation of protein synthesis rates. Here, we report that a selective PERK inhibitor acutely deregulates protein synthesis in freshly isolated islets of Langerhans, across a range of glucose concentrations. Acute loss of the PERK-mediated strand of the unfolded protein response leads to rapid accumulation of misfolded pro-insulin in cultured beta cells and is associated with a kinetic defect in pro-insulin processing. These *in vitro* observations uncouple the latent role of PERK in beta cell development from the regulation of unfolded protein flux through the ER and attest to the importance of the latter in beta cell proteostasis.

PERK/PEK[3] was initially identified as a pancreas-enriched kinase of the α subunit of translation initiation factor 2 (eIF2α) (1). Its physiological significance became evident with the discovery of its localization to the membranes of the endoplasmic reticulum (ER) and the key role of unfolded protein stress in the ER lumen in PERK activation (2, 3). PERK deletion disrupts a negative feedback loop whereby mounting levels of unfolded protein stress in the ER lumen enhance eIF2α phosphorylation (4). The consequent attenuation of protein synthesis matches the flux of proteins into the ER lumen with the capacity of the organelle to fold and process this biosynthetic load. Thus, PERK activity establishes a translational arm to the metazoan unfolded protein response that favors protein folding homeostasis (proteostasis) (5).

Loss-of-function mutations in *EIF2AK3*, the gene encoding PERK, result in a syndromic form of human diabetes mellitus, the Walcott-Rallison syndrome (6), which is closely recapitulated by the mouse knock-out (7, 8) and partially captured by a mutation in its substrate, EIF2A^{S51A}, that prevents phosphorylation by PERK (9). The destructive consequences of PERK deficiency in the insulin-producing beta cells of the islets of Langerhans fit well with evidence that pro-insulin biosynthesis is deregulated in islets explanted from PERK knock-out mice and from mice with the aforementioned eIF2α^{S51A} mutation (7, 10).

These observations were consistent with the primacy of the PERK role in regulating the flux of unfolded proteins into the ER lumen at the level of ER client protein translation. However, alternative explanations for the PERK role in maintaining beta cell function and survival arose: whereas phosphorylation of eIF2α attenuates translation initiation of most mRNAs thereby favoring proteostasis, rare mRNAs, exemplified by those encoding the transcription factor ATF4, are exempt from this fate and are rather translationally up-regulated by eIF2α phosphorylation (11). Thus, regulated translational reinitiation of ATF4 (12, 13) and ATF5 (14) couples ER stress to a PERK-dependent gene expression program with complex outputs (15–17).

Furthermore, careful analysis of PERK knock-out mice and cells with genetic lesions compromising PERK activity uncovered functional defects that were also consistent with a role for PERK-mediated gene expression in defective islet development (8, 18). Normal development is critical to the metabolic coupling that drives many aspects of beta cell physiology and is required for glycemic control. Furthermore, an early study of beta cell conditional deletion of PERK suggested that the deleterious effects of *EIF2AK3* knock-out are played out selectively during pancreatic development (19) and not in the adult. These observations led some to question the role of PERK in moderating the secreted protein load in the endocrine pancreas (20). Their question gains further legitimacy by the fact that the key

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[3] The abbreviations used are: PERK, pancreatic endoplasmic reticulum kinase; ER, endoplasmic reticulum; KRH, Krebs-Ringer-HEPES; MEF, mouse embryonic fibroblast; NTD, N-terminal domain; Tricine, *N*-tris(hydroxymethyl)methylglycine.

insights into the PERK role in proteostasis were derived from study of cells and tissues with loss of function or interfering genetic lesions, all of inherent latency.

Here, we exploit a recently discovered highly specific small molecule inhibitor of PERK kinase to study PERK-mediated regulation of protein synthesis in an otherwise *EIF2AK3* wild-type background, temporally uncoupling PERK developmental and proteostatic roles.

EXPERIMENTAL PROCEDURES

Materials—GSK2606414 (PERKi) was a gift from Jeffrey Axten, GalxoSmithKline, Collegeville, PA. The 10 mM stock solution in dimethyl sulfoxide was diluted in buffer or cell culture media immediately before use.

In Vitro eIF2α Phosphorylation Assay—PERK kinase domain and N-terminal lobe of eIF2α (eIF2α^{NTD}) were expressed from plasmids PerkKD-pGEX4T-1 and eIF2α^{NTD} −2aOPTx3M(1–185)pET-30a(+) in bacteria and purified by GST and Ni affinity chromatography respectively (2, 21). Phosphorylation reactions containing final amounts of 5 nM PERK, 2.68 μM eIF2α^{NTD}, and the indicated concentration of PERKi or 0.02% dimethyl sulfoxide in reaction buffer (20 mM HEPES, pH 7.5,50 mM KCl, 2 mM MgOAc, 2 mM $MnCl_2$, 1.5 mM DTT) were started by the addition of 10 μM or 1 mM ATP and stopped after 60 min by the addition of SDS-PAGE loading dye. Proteins were quantified following scanning of Coomassie Blue-stained 12% PAGE gels on a Licor Odyssey scanner, and nonlinear regression analysis to determine the IC_{50} was performed using Prism (GraphPad) software.

Cell Culture and Immunoblot Analysis—Wild-type, *Perk*$^{-/-}$, and *eIF2a*$^{A/A}$ immortalized MEFs as well as Min6 cells were cultured in DMEM supplemented with 10% Fetalclone II (Hyclone), 1× Pen/Strep, 1× nonessential amino acids (Invitrogen), and 55 μM β-mercaptoethanol (9, 16), Cells were treated with vehicle (dimethyl sulfoxide) or the indicated compounds for the periods indicated in the figure legends. For puromycin labeling, 10 μg/ml puromycin was added 10 min prior to harvest. Cytoplasmic extracts were prepared in Triton X-100 buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA, 10 mM phenylmethylsulfonyl fluoride, 15 μg/ml aprotonin, and 6 μg/ml pepstatin A, 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate), and total eIF2α, eIF2α^{P}, PDI (Protein Disulfide Isomerase), and puromycinylated proteins were detected by immunoblotting as described previously (7, 22, 23). Pro-insulin was detected by incubation with SC-9168 (Santa Cruz Biotechnology) anti-insulin antibody. High molecular weight complex analysis was conducted as described previously with a few modifications (24). Briefly, after treatment cells were washed with ice-cold PBS, 2 mM EDTA, and 20 mM *N*-ethylmaleimide, incubated on ice in the same buffer for 20 min, and then collected by scraping in the same buffer. The cells were then lysed in Triton X-100 buffer with 250 mM sucrose instead of glycerol and supplemented with 20 mM *N*-ethylmaleimide. After clearing at 20,000 × *g* for 15 min the lysate was layered over a cushion consisting of 20% glycerol, 20 mM HEPES, pH 7.5, 100 mM NaCl and centrifuged at 100,000 × *g* for 45 min. The upper phase was removed and the tubes inverted to remove all liquid. The pellets were dissolved in 1× urea-PAGE sample buffer (9.6 M urea, 12% glycerol, 1.36% SDS, 40 mM Tris, pH 6.8) with or without 100 mM DTT and separated on 18% Tris-Tricine-urea gels. Immunoblot detection was conducted using IR800 conjugated secondary antisera followed by scanning on a Licor Odyssey scanner. The primary scans were quantified using ImageJ software. For Fig. 2*C* the PERKi-dependent reversal of phosphorylation was calculated by adjusting the raw values to a scale from 0 to 100 (thapsigargin induced level = 100) and then calculating the reversal by subtracting each value from 100 (thapsigargin induced level = 0).

Rat Islet Isolation, Culture, and Metabolic Labeling—Islets were isolated in batches from pancreases from three rats on histopaque gradients following digestion with Liberase TL (Roche Applied Science) essentially as described for mice with RPMI 1640 medium used in place of DMEM and solution volumes increased 3-fold (25). Following gradient isolation and washing in RPMI 1640 medium, islets <200 μm in diameter were hand-picked and cultured over night in RPMI 1640 medium supplemented with 10% heat-inactivated serum and 1× Pen/Strep and 37 °C, 5% CO_2. For ^{35}S pulse-labeling experiments, pools of 60 size-matched islets were washed twice in Krebs-Ringer-HEPES (KRH) buffer (115 mM NaCl, 5 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 20 mM HEPES, 7.4, 10 mM $NaHCO_3$, pH 7.4) supplemented with 0.1% BSA and 2.8 mM glucose and resuspended in 1 ml of the same buffer. After 1.5 h of culture, the medium was changed to 200 μl of fresh KRH-0.1% BSA supplemented with 1 μm of PERKi or vehicle and the indicated concentration of glucose. After 0.5 h EXPRESS^{35}S label (PerkinElmer Life Sciences) was added to 0.229 μCi/μl, and the islets were cultured a further 20 min washed in ice-cold PBS and lysed in Triton X-100 buffer. For pulse chase analysis, the pretreatment was as above, but following a shorter 15-min ^{35}S pulse the medium was removed and new KRH-0.1% BSA containing 0.13 mg/ml cystine-2H20, 0.06 mg/ml methionine, and the 16.8 mM glucose was added for the indicated chase period prior to washing in ice-cold PBS and lysis in Triton X-100 buffer. The lysates were cleared at 20,000 × *g* for 15 min, precleared with a nonspecific antiserum, and pro-insulin and insulin were then immunoprecipitated on protein A/G beads using a mixture of 5 μl of SC-9168 (Santa Cruz Biotechnology) and 0.75 μl of I2018 (Sigma) antisera for each point. Proteins were separated on 18% Tris-Tricine-urea PAGE gels, stained with Instantblue, and dried between cellulose sheets prior to exposure to Phosphor storage plates. Quantification of the detected signal by Storm PhosphorImager was done using ImageJ software.

RESULTS AND DISCUSSION

GSK2606414 Potently Inhibits PERK-mediated eIF2a Phosphorylation and Regulation of Protein Synthesis in Vivo—PERK is a bipartite transmembrane protein with a luminal unfolded protein stress-sensing domain and a cytosolic kinase domain (2) that is activated by oligomerization and trans-autophosphorylation (3, 26). GSK2606414 (henceforth referred to as PERKi) is a high affinity ligand of the PERK kinase domain that interferes with kinase activity by competing for ATP (27). The competitive nature of the inhibitory effect was confirmed by

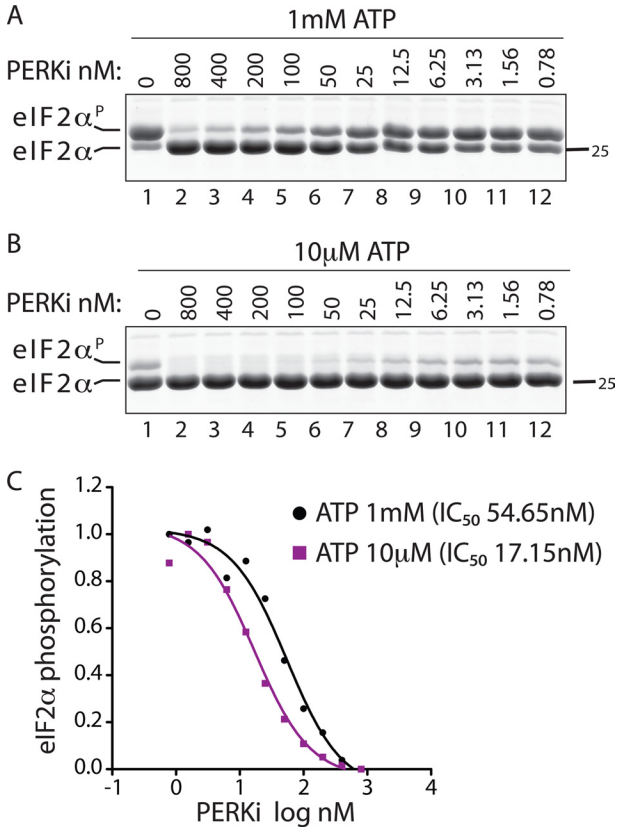

FIGURE 1. **GSK2606414 (PERKi) inhibits PERK kinase activity in the presence of physiological levels concentrations of ATP.** *A*, SDS-PAGE analysis of reactions containing 5 nM PERK kinase domain, 2.5 μM eIF2α N-terminal lobe, and 1 mM ATP, incubated for 60 min in the presence of the indicated concentrations of PERKi. The phosphorylated (eIF2α^P) and unphosphorylated forms of eIF2α are noted. *B*, as in *A* except the reactions contained 10 μM ATP. *C*, nonlinear regression analysis of the relationship between eIF2α phosphorylation and PERKi concentration in *A* and *B*.

measuring the phosphorylation of PERK substrate, eIF2α, *in vitro* in the presence of varying concentrations of PERKi. The concentration of PERKi that inhibited eIF2α phosphorylation to 50% (IC_{50}) was 17 nM when the assay was performed in the presence of 10 μM ATP (approximately at the K_m) and 54 nM when performed in the presence of 1 mM ATP (well above PERK K_m for ATP) (Fig. 1).

To gauge the inhibitory effect *in vivo* we measured the ability of PERKi to reverse the PERK-mediated inhibition of protein synthesis in cells exposed to the ER stress-causing agent thapsigargin (4). Punctual rates of protein synthesis were estimated by flooding cells with puromycin and quantifying the mass of puromycinylated nascent peptides by immunoblotting with a selective antibody (22). Thapsigargin led to marked inhibition of the puromycinylated signal in lysates of Min6 pancreatic β cells, as expected (Fig. 2*A*, *lane 3*). PERKi reversed both the thapsigargin-mediated inhibition of protein synthesis and the enhanced eIF2α phosphorylation, with 50% reversal (EC_{50}) noted at 373 nM (95% confidence limits 299–464 nM) (Fig. 2, *B* and *C*). In considering the lower efficacy of PERKi in the *in vivo* assay compared with the *in vitro* assay is worth noting that the *in vitro* assay measures eIF2α phosphorylation, whereas the *in vivo* assay relies on recovery of protein synthesis from eIF2(αP)-mediated inhibition. Thus, in addition to pharmacokinetic factors the ~6-fold lower efficacy of PERKi *in vivo* may also be due to the fact that protein synthesis is inhibited by substoichiometric levels of eIF2(αP) (28). This conjecture is supported by the observation that the dose-response curve to PERKi measured in terms of reversing thapsigargin-mediated eIF2α phosphoryla-

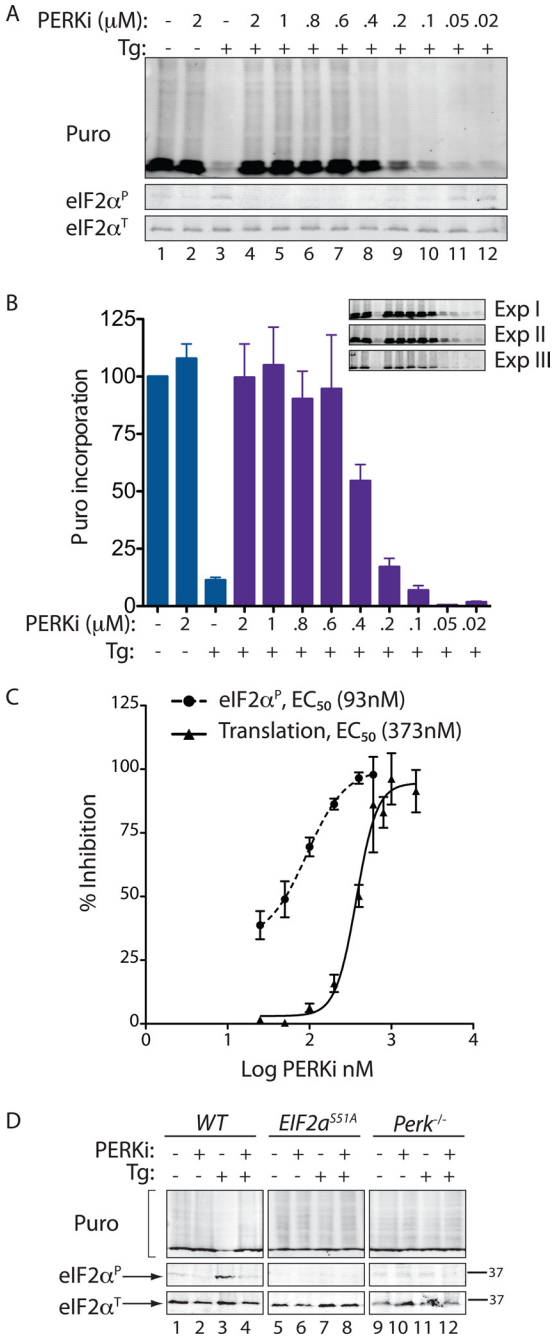

FIGURE 2. **PERKi blocks ER stress-inducible PERK kinase activity *in vivo*.** *A*, immunoblot analysis of puromycin incorporation into total protein in untreated Min6 beta cells or cells exposed to thapsigargin (0.5 μM for 30 min) and the indicated concentrations of PERKi. Puromycin (10 μg/ml) was added during the last 10 min, immediately before harvest. *B*, quantification of the signal from three replicates of the experiment (shown in the *inset*) of which *A* is a typical representative. *C*, nonlinear regression analysis of the concentration-dependence of PERKi reversal of thapsigargin-mediated translational repression ($n = 3$, shown in *A* and *B*) and phosphorylation of eIF2α ($n = 4$, one of which is shown in *A*). *D*, immunoblot of total puromycinylated proteins, phosphorylated (eIF2α^P), and total eIF2α^T in wild-type (WT), *EIF2α*^{S51A}, and *Perk*$^{-/-}$ MEFs exposed to PERKi (2 μM) and thapsigargin (0.5 μM) as indicated.

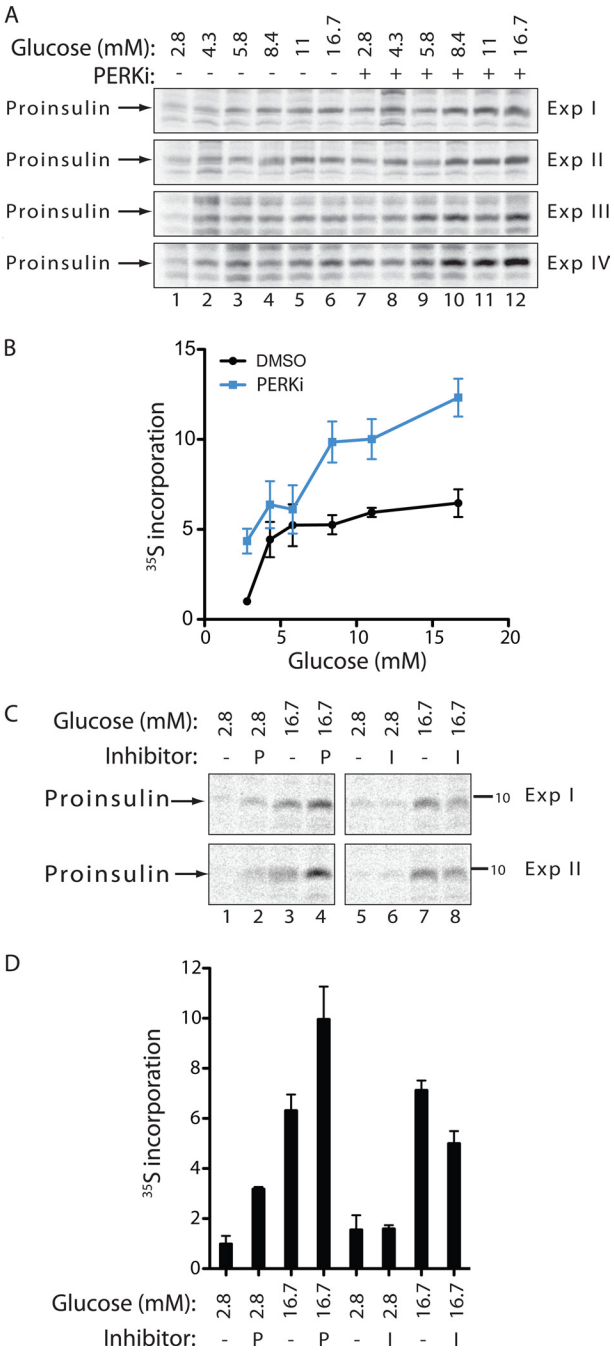

FIGURE 3. **PERK regulates insulin biosynthesis at both low and high glucose concentrations.** *A*, autoradiographs of metabolically labeled pro-insulin immunopurified from isolated rat pancreatic islets incubated for 30 min at the indicated glucose concentration in the absence or presence PERKi (1 μM) and pulse-labeled with [^{35}S]methionine/cysteine for last 10 min before harvest. Four representative experiments are shown. *B*, plot of the mean ± S.E. of the signal from the samples shown in *A*. *C*, autoradiographs of metabolically labeled pro-insulin immunopurified from isolated rat pancreatic islets exposed to the PERKi (*P*, 1 μM) or the IRE inhibitor 4μ8C (*I*, 30 μM) in the presence of the indicated concentration of glucose. Two representative experiments are shown. *D*, plot of the mean ± S.E. of the signal from the samples shown in *C*.

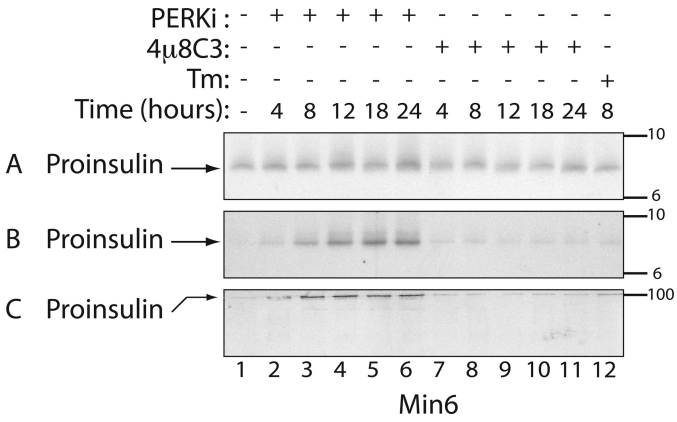

FIGURE 4. **Accumulation of insoluble aberrantly disulfide-bonded pro-insulin in PERKi-treated Min6 cells.** Immunoblots of pro-insulin recovered in lysates of untreated Min6 cells or cells exposed to PERKi (1 μM), the IRE1 inhibitor 4μ8C (30 μM), or the ER stress-inducing agent tunicamycin (*Tm*, 3 μg/ml) for the indicated period prior to lysis are shown. *A*, immunoblot of pro-insulin content of the total lysate, resolved on a reducing SDS-PAGE. *B*, immunoblot of pro-insulin recovered from the pellet fraction of the lysate in *A* following ultracentrifugation through a glycerol cushion, solubilization in 8 M urea, and resolution by reducing SDS-PAGE. *C*, the samples in *B* resolved by nonreducing SDS-PAGE.

tion is shifted to the right with an EC_{50} of 93 nM compared with the curve based on reversing the inhibition in protein synthesis (Fig. 2*C*).

PERKi also reversed the ER stress-mediated repression of protein synthesis in wild-type mouse embryonic fibroblasts, but had no measurable effect on protein synthesis rates or eIF2α phosphorylation in MEFs homozygous for the *EIF2A*^{S51A} mutation that blocks regulatory phosphorylation (9) or in *Perk*$^{-/-}$ MEFs that lack the target kinase (Fig. 2*D*). Together, these observations point to the selectivity of PERKi/GSK2606414 *in vivo* effects to the inhibition of PERK-mediated eIF2α phosphorylation.

PERK Modulates Physiological Levels of Pro-insulin Synthesis in Wild-type Islets of Langerhans—The role of PERK in promoting islet health is undisputed. However, past experiments measuring PERK-mediated regulation of protein synthesis in islets and beta cells were conducted by comparing wild-type tissues and cells with those harboring genetic lesions in *EIF2AK3*, the gene encoding PERK. Thus, it was impossible to deconvolute the latent, developmental effects of PERK deficiency from the acute effects of loss of the proteostatic role of PERK in matching ER load and capacity. This inherent limitation in past studies has led Cavener and colleagues to question whether PERK has any important proteostatic role in insulin producing cells (18).

The rapid onset action of PERKi permits, for the first time, the study of the role of PERK in regulating protein synthesis in an otherwise developmentally wild-type context. Fully developed, wild-type adult rat islets of Langerhans were exposed to PERKi at different ambient glucose concentrations, and pro-insulin translation was quantified by pulse-labeling with [^{35}S]methionine/cysteine followed by immunoprecipitation. Pro-insulin translation increased with glucose concentration, as noted previously. In untreated islets the effect of glucose were near maximal at physiologically high glucose levels (*i.e.* above 5 mM). PERKi enhanced pro-insulin biosynthesis both at physiologically low glucose levels (2.5 mM) and at physiologically high levels, with a shallow plateau in between (Fig. 3, *A* and *B*). The impact of PERKi on pro-insulin biosynthesis was not shared by 4μ8C, an inhibitor of the IRE1 strand of the unfolded protein response (29) (Fig. 3, *C* and *D*), arguing for a direct and

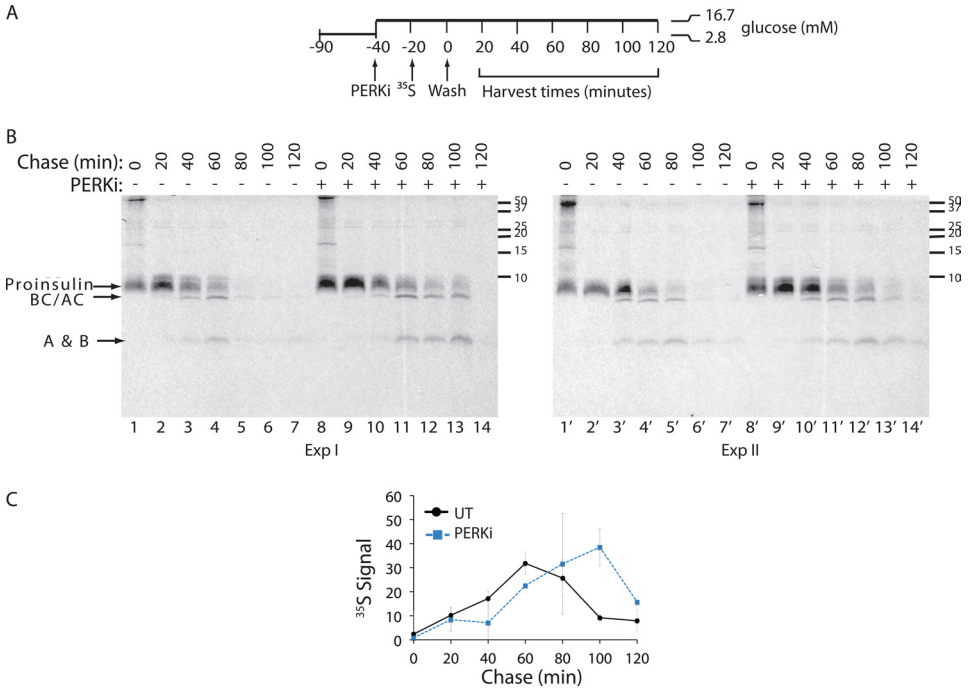

FIGURE 5. **Delayed insulin maturation in PERKi-treated cells.** *A*, schema of design of the pulse chase labeling experiment, showing the temporal relationship among glucose concentration, addition of inhibitors, metabolic label, washout, and harvest. *B*, autoradiographs of pro-insulin, insulin, and biosynthetic intermediates immunopurified from lysates of pulse chase metabolically labeled isolated rat islets of Langerhans in the absence or presence of PERKi (1 μM). Shown is a representative experiment conducted in duplicate. *C*, quantification of mature insulin signal from *B*.

specific proteostatic effect of PERK under physiological circumstances.

Of note, the conspicuous dip in PERKi action at physiological glucose concentrations (evinced by the convergence of the *blue* and *black traces* in Fig. 3*B*) is concordant with the discontinuous PERK-dependent effects of ambient glucose on protein synthesis in insulin-producing beta cells initially suggested by Jonas and colleagues; with PERK activity noted at both low and high glucose levels with relative silence at euglycemia (30). In the later study concordant changes were also observed in the levels of phosphorylated eIF2α. Unfortunately, our anti-eIF2a-P immunoblots lack the sensitivity to measure this parameter in lysates of isolated islets of Langerhans. However, given the robustness of the effects of PERKi on protein synthesis in islets and given the strength of the evidence that the inhibitor works by changing levels of phosphorylated eIF2α in systems where this can be measured reliably (Fig. 2, *A*, *C*, and *D*), there is good reason to believe that in islets, too, the inhibitor is exerting its effects by inhibiting the canonical PERK pathway.

PERK Inhibition Acutely Compromises ER Proteostasis—To gauge the consequence of acute loss of PERK-mediated proteostasis in insulin-producing cells we turned to Min6 cells, a beta cell line that can be cultured in large quantities (unlike isolated islets) and are thus better suited to large scale biochemical studies. Pro-insulin is a transient species, as it is rapidly converted to mature insulin; nonetheless, Min6 cell lysates have a readily detectable pro-insulin immunoblot signal at steady state. Normally, pro-insulin is soluble, as it escapes pelleting with high molecular weight species in a previously described assay for detecting insoluble ER localized proteins (24) (Fig. 4, *A* and *B*, *lane 1*). However, exposure of Min6 cells to PERKi resulted in a time-dependent increase of insoluble high molecular weight pro-insulin; which is found largely as an aberrantly disulfide bonded oligomeric species (Fig. 4, *B* and *C*, *lanes 2–6*). By contrast, inhibition of the IRE1 strand of the unfolded protein response did not lead to pro-insulin redistribution into a high molecular weight fraction (*lanes 7–12*). These observations are consistent with a rapid compromise in ER proteostasis upon PERK inhibition affecting quality control in the organelle.

Cavener and colleagues had previously uncovered a kinetic defect in ER to Golgi transport in islet cells following persistent compromise of PERK activity (effected by siRNA or by a dominant negative allele) (18). To determine whether acute interruption of proteostasis mediated by short term inhibition of PERK could contribute to this functional defect, we followed pro-insulin maturation (a marker for efferent ER transport) by pulse chase labeling and immunoprecipitation of isolated rat islets of Langerhans that were stimulated to synthesize pro-insulin by exposure to high glucose in the presence and absence of the PERK inhibitor (Fig. 5*A*). Deregulated pro-insulin biosynthesis was noted at the end of the pulse, as expected (Fig. 5*B*, compare *lanes 1* and *1′* and *lanes 8* and *8′*) and was associated with a reproducible, albeit subtle, kinetic defect in pro-insulin maturation in islets exposed to the PERK inhibitor (Fig. 5, *B* and *C*).

The study by Gupta *et al.* (18) lacked a quantitative component, thus it is impossible to compare the kinetic defect in efferent ER transport between our study and theirs. Nonetheless, the emergence of a kinetic defect in pro-insulin maturation, minutes after exposure to a PERK inhibitor, argues in favor of a role for deregulated protein synthesis and impaired proteostasis in the genesis of this manifestation of compromised PERK function.

CONCLUDING REMARKS

The rapid onset of PERK inhibition in cells exposed to GSK2606414 has enabled experiments that inform the debate on the genesis of the consequences of defective PERK function in secretory cells. A direct role for PERK in ER proteostasis is clearly established, one that is likely independent of downstream developmental effects. This study, which utilizes an orthogonal pharmacological approach, provides critical confirmation to previous genetic experiments documenting the role of PERK in moderating unfolded protein load in the ER of beta cells exposed to glycemic excursions in the physiological range (7, 10, 30).

This study also favors a role for compromised proteostasis in the genesis of the phenotype of loss-of-function lesions in the PERK strand of the unfolded protein response, as defective proteostasis is predicted to affect fully differentiated secretory cells. It is noteworthy that a recent study has shown that loss of regulated eIF2α phosphorylation (by introduction of S51A mutation) also compromises the viability and function of beta cells in the fully developed adult endocrine pancreas (31) and is not restricted to compromising development and proliferation of islets of Langerhans in the pre- and perinatal period, as had been previously suggested (19).

The focus on early consequences of PERK inhibition and the use of explanted adult tissue (islets of Langerhans) both serve to uncouple changes in protein synthesis from developmental changes in a robust isolated *in vitro* system. It seems likely that acute inhibition of PERK will have the same effect *in vivo*, although this remains to be determined experimentally. Furthermore, the findings reported here do not in any way detract from the importance of the gene expression program downstream of PERK, nor from the role of PERK in beta cell development. However, we believe our findings should temper a recent revisionist trend (20) that denies a role for regulated protein synthesis in PERK function.

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REFERENCES

1. Shi, Y., Vattem, K. M., Sood, R., An, J., Liang, J., Stramm, L., and Wek, R. C. (1998) Identification and characterization of pancreatic eukaryotic initiation factor 2 α-subunit kinase, PEK, involved in translational control. *Mol. Cell. Biol.* **18,** 7499–7509
2. Harding, H. P., Zhang, Y., and Ron, D. (1999) Translation and protein folding are coupled by an endoplasmic reticulum resident kinase. *Nature* **397,** 271–274
3. Bertolotti, A., Zhang, Y., Hendershot, L. M., Harding, H. P., and Ron, D. (2000) Dynamic interaction of BiP and the ER stress transducers in the unfolded protein response. *Nat. Cell Biol.* **2,** 326–332
4. Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., and Ron, D. (2000) *Perk* is essential for translational regulation and cell survival during the unfolded protein response. *Mol. Cell* **5,** 897–904
5. Balch, W. E., Morimoto, R. I., Dillin, A., and Kelly, J. W. (2008) Adapting proteostasis for disease intervention. *Science* **319,** 916–919
6. Delépine, M., Nicolino, M., Barrett, T., Golamaully, M., Lathrop, G. M., and Julier, C. (2000) EIF2AK3, encoding translation initiation factor 2-α kinase 3, is mutated in patients with Wolcott-Rallison syndrome. *Nat. Genet.* **25,** 406–409
7. Harding, H. P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D. D., and Ron, D. (2001) Diabetes mellitus and exocrine pancreatic dysfunction in Perk$^{-/-}$ mice reveals a role for translational control in survival of secretory cells. *Mol. Cell* **7,** 1153–1163
8. Zhang, P., McGrath, B., Li, S., Frank, A., Zambito, F., Reinert, J., Gannon, M., Ma, K., McNaughton, K., and Cavener, D. R. (2002) The PERK eukaryotic initiation factor 2 α kinase is required for the development of the skeletal system, postnatal growth, and the function and viability of the pancreas. *Mol. Cell. Biol.* **22,** 3864–3874
9. Scheuner, D., Song, B., McEwen, E., Liu, C., Laybutt, R., Gillespie, P., Saunders, T., Bonner-Weir, S., and Kaufman, R. J. (2001) Translational control is required for the unfolded protein response and *in vivo* glucose homeostasis. *Mol. Cell* **7,** 1165–1176
10. Scheuner, D., Vander Mierde, D., Song, B., Flamez, D., Creemers, J. W., Tsukamoto, K., Ribick, M., Schuit, F. C., and Kaufman, R. J. (2005) Control of mRNA translation preserves endoplasmic reticulum function in beta cells and maintains glucose homeostasis. *Nat. Med.* **11,** 757–764
11. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) Regulated translation initiation controls stress-induced gene expression in mammalian cells. *Mol. Cell* **6,** 1099–1108
12. Vattem, K. M., and Wek, R. C. (2004) Reinitiation involving upstream ORFs regulates ATF4 mRNA translation in mammalian cells. *Proc. Natl. Acad. Sci. U.S.A.* **101,** 11269–11274
13. Lu, P. D., Harding, H. P., and Ron, D. (2004) Translation re-initiation at alternative open reading frames regulates gene expression in an integrated stress response. *J. Cell Biol.* **167,** 27–33
14. Zhou, D., Palam, L. R., Jiang, L., Narasimhan, J., Staschke, K. A., and Wek, R. C. (2008) Phosphorylation of eIF2 directs ATF5 translational control in response to diverse stress conditions. *J. Biol. Chem.* **283,** 7064–7073
15. Ma, Y., Brewer, J. W., Diehl, J. A., and Hendershot, L. M. (2002) Two distinct stress signaling pathways converge upon the CHOP promoter during the mammalian unfolded protein response. *J. Mol. Biol.* **318,** 1351–1365
16. Harding, H. P., Zhang, Y., Zeng, H., Novoa, I., Lu, P. D., Calfon, M., Sadri, N., Yun, C., Popko, B., Paules, R., Stojdl, D. F., Bell, J. C., Hettmann, T., Leiden, J. M., and Ron, D. (2003) An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11,** 619–633
17. Lu, P. D., Jousse, C., Marciniak, S. J., Zhang, Y., Novoa, I., Scheuner, D., Kaufman, R. J., Ron, D., and Harding, H. P. (2004) Cytoprotection by pre-emptive conditional phosphorylation of translation initiation factor 2. *EMBO J.* **23,** 169–179
18. Gupta, S., McGrath, B., and Cavener, D. R. (2010) PERK (EIF2AK3) regulates proinsulin trafficking and quality control in the secretory pathway. *Diabetes* **59,** 1937–1947
19. Zhang, W., Feng, D., Li, Y., Iida, K., McGrath, B., and Cavener, D. R. (2006) PERK EIF2AK3 control of pancreatic β cell differentiation and proliferation is required for postnatal glucose homeostasis. *Cell Metab.* **4,** 491–497
20. Cavener, D. R., Gupta, S., and McGrath, B. C. (2010) PERK in beta cell biology and insulin biogenesis. *Trends Endocrinol. Metab.* **21,** 714–721
21. Marciniak, S. J., and Ron, D. (2006) Endoplasmic reticulum stress signaling in disease. *Physiol. Rev.* **86,** 1133–1149
22. Schmidt, E. K., Clavarino, G., Ceppi, M., and Pierre, P. (2009) SUnSET, a nonradioactive method to monitor protein synthesis. *Nat. Methods* **6,** 275–277
23. Zito, E., Chin, K. T., Blais, J., Harding, H. P., and Ron, D. (2010) ERO1β, a pancreas-specific disulfide oxidase promotes insulin biogenesis and glucose homeostasis. *J. Cell Biol.* **188,** 821–832
24. Marciniak, S. J., Yun, C. Y., Oyadomari, S., Novoa, I., Zhang, Y., Jungreis, R., Nagata, K., Harding, H. P., and Ron, D. (2004) CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes Dev.* **18,** 3066–3077
25. Yesil, P., Michel, M., Chwalek, K., Pedack, S., Jany, C., Ludwig, B., Bornstein, S. R., and Lammert, E. (2009) A new collagenase blend increases the number of islets isolated from mouse pancreas. *Islets* **1,** 185–190
26. Liu, C. Y., Schröder, M., and Kaufman, R. J. (2000) Ligand-independent

dimerization activates the stress-response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. *J. Biol. Chem.* **275,** 24881–24885
27. Axten, J. M., Medina, J. R., Feng, Y., Shu, A., Romeril, S. P., Grant, S. W., Li, W. H., Heerding, D. A., Minthorn, E., Mencken, T., Atkins, C., Liu, Q., Rabindran, S., Kumar, R., Hong, X., Goetz, A., Stanley, T., Taylor, J. D., Sigethy, S. D., Tomberlin, G. H., Hassell, A. M., Kahler, K. M., Shewchuk, L. M., and Gampe, R. T. (2012) Discovery of 7-methyl-5-(1-{[3-(trifluoromethyl)phenyl]acetyl}-2,3-dihydro-1H-indol-5-yl)-7H-pyrrolo[2,3-d]pyrimidin-4-amine (GSK2606414), a potent and selective first-in-class inhibitor of protein kinase R (PKR)-like endoplasmic reticulum kinase (PERK). *J. Med. Chem.* **55,** 7193–7207
28. Matts, R. L., Levin, D. H., and London, I. M. (1983) Effect of phosphorylation of the α-subunit of eukaryotic initiation factor 2 on the function of reversing factor in the initiation of protein synthesis. *Proc. Natl. Acad. Sci. U.S.A.* **80,** 2559–2563
29. Cross, B. C., Bond, P. J., Sadowski, P. G., Jha, B. K., Zak, J., Goodman, J. M., Silverman, R. H., Neubert, T. A., Baxendale, I. R., Ron, D., and Harding, H. P. (2012) The molecular basis for selective inhibition of unconventional mRNA splicing by an IRE1-binding small molecule. *Proc. Natl. Acad. Sci. U.S.A.* **109,** E869–878
30. Elouil, H., Bensellam, M., Guiot, Y., Vander Mierde, D., Pascal, S. M., Schuit, F. C., and Jonas, J. C. (2007) Acute nutrient regulation of the unfolded protein response and integrated stress response in cultured rat pancreatic islets. *Diabetologia* **50,** 1442–1452
31. Back, S. H., Scheuner, D., Han, J., Song, B., Ribick, M., Wang, J., Gildersleeve, R. D., Pennathur, S., and Kaufman, R. J. (2009) Translation attenuation through eIF2α phosphorylation prevents oxidative stress and maintains the differentiated state in beta cells. *Cell Metab.* **10,** 13–26