Reduced Insulin Signaling and Endoplasmic Reticulum Stress Act Synergistically to Deteriorate Pancreatic β Cell Function

TOMOKAZU MATSUDA, YOSHIAKI KIDO, TOHRU UCHIDA,
and MASATO KASUGA

*Department of Internal Medicine, Division of Diabetes, Metabolism, and Endocrinology,
Kobe University Graduate School of Medicine, Kobe 650-0017, Japan.*

The total pancreatic β cell mass is reduced in individuals with type 2 diabetes. We analyzed the islets of leptin receptor-deficient (Lepr$^{-/-}$) mice, a model animal for type 2 diabetes with obesity. The plasma insulin levels in Lepr$^{-/-}$ mice peaked at ~approximately 7 weeks, an age at which the animals manifest normoglycemia to moderate hyperglycemia. Consistent with this, the β cell mass was enlarged as compared with Lepr$^{+/-}$ mice, and it decreased thereafter. Thus, we focused on the islets of Lepr$^{-/-}$ mice at 7 weeks to elucidate the mechanism underlying β cell failure. Endoplasmic reticulum (ER) stress was enhanced in β cells of Lepr$^{-/-}$ mice at 7 weeks, as indicated by the increase in c-Jun and eIF2α phosphorylation. Lepr$^{-/-}$ mice also exhibited a reduction in insulin signaling in β cells at 7 weeks, as indicated by the decrease in Akt phosphorylation. These results indicate that both augmented ER stress and reduced insulin signaling occur before the onset of frank diabetes. Next, to examine the mutual effect of ER stress and insulin signaling in β cells *in vitro*, we used MIN6 insulinoma cells. Tunicamycin induced ER stress as well as inhibited insulin signaling. Conversely, the PI-3 kinase inhibitor, LY294002, enhanced ER stress. Furthermore, the reduction in insulin signaling by LY294002 facilitated the induction of ER stress with tunicamycin. Taken together, we concluded that both ER stress and reduced insulin signaling might synergistically affect pancreatic β cell dysfunction.

Type 2 diabetes is characterized by insulin resistance and β cell dysfunction. Accumulation of visceral adiposity, as observed in individuals with metabolic syndrome, induces insulin resistance. Initially, pancreatic β cells compensate for the insulin resistance associated with obesity and changes in lifestyle by up-regulating of insulin secretion. However, this period of β cell compensation is eventually followed by β cell failure—in which the pancreas fails to secrete sufficient insulin as a result of either inadequate β cell mass expansion or loss of ability of the existing β cells to respond to glucose—and diabetes ensues (6,8,11,12). Recently, it has been reported that β cell dysfunction could be detected at the early stage of the development of diabetes (12). It has also been demonstrated that the total mass of islets is reduced in individuals with type 2 diabetes, even before the onset of frank diabetes (1). The mechanism underlying β cells dysfunction at the early stage is yet unclear. Therefore, we used a leptin receptor-deficient (Lepr$^{-/-}$) mouse, as a model animal of

type 2 diabetes with obesity and focused on the periods of normoglycemia/hyperinsulinemia of the mice, around the onset of diabetes.

Here, we demonstrated that both reduced insulin signaling and augmented ER stress were observed in the islets of Lepr$^{-/-}$ mice at 7 weeks of age, which function synergistically to deteriorate β cell function and mass, leading to pancreatic β cell failure.

MATERIALS AND METHODS

Mice

We obtained Lepr$^{+/-}$ mice on the C57BL/KsJ background from Clea Japan. The animals were maintained and their blood glucose and plasma insulin concentrations were determined, as described previously (4,7).

Only male mice were used for the experiments. This study was performed according to the guidelines of the Animal Ethics Committee of the Kobe University Graduate School of Medicine.

Immunoblot analysis.

The lysates were probed with GRP78 or CHOP (Santa Cruz Biotechnology) antibodies; Akt, phosphorylated Akt, c-Jun, phosphorylated c-Jun, eIF2α, or phosphorylated eIF2α (Cell Signaling) antibodies; β-actin antibodies (Sigma-Aldrich); and PCNA antibodies (Dako).

Immunostaining and morphometric analysis of islets

We subjected 3 to 5 mice of each genotype at the indicated age subjected to morphometric analysis. Pancreatic sections were subjected to two-color immunofluorescence staining with antibodies to insulin and glucagon (both from Dako). For morphometric analysis, islets images were manually traced and analyzed using WinROOF software (Mitani). The cross-sectional area of islets with more than 5 insulin-positive cells was measured in at least 3 sections separated by 200 μm. Islet density was determined as the number of islets divided by the total area of the pancreas. The total β cell mass was calculated as the total β cell area expressed as a percentage of the total area of the pancreas. The size of individual β cells was determined as the total β cell area divided by the total number of β cells that yielded results similar to those obtained by direct β cells tracing. The number of β cells was expressed per mm^2 of the total area of the pancreas.

Quantitation of mRNA by real time RT-PCR

Total cellular RNA was isolated from MIN6 cells by using RNeasy kit (QIAGEN Science). Real-time RT-PCR analysis of the total RNA was performed with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems). Each reaction was performed in triplicate.

Statistical analysis

Data are presented as means ± S.E.M. and compared by analysis of variance. A *P* value of <0.05 was considered statistically significant.

RESULTS

We examined leptin receptor-deficient (Lepr$^{-/-}$) mice as a model of type 2 diabetes with obesity. As a control, we used heterozygous leptin receptor-deficient (Lepr$^{+/-}$) mice, which do not develop obesity. Lepr$^{-/-}$ mice started to exhibit obesity from 5 weeks, followed by hyperinsulinemia. Interestingly, the plasma insulin concentrations reached a peak at ~approximately 7 weeks, which then gradually decreased. Consistent with the decrease in the plasma insulin levels, blood glucose concentrations were greatly elevated (Fig. 1). Next, we measured the β cell mass of Lepr$^{-/-}$ mice. Immunofluorescence analysis revealed that at 7

weeks, the β cell mass in Lepr$^{-/-}$ mice enlarged by approximately 3-fold compared with that in Lepr$^{+/-}$ mice. However, as the development of diabetes progressed, the β cell mass decreased in Lepr$^{-/-}$ mice by 75% at 12 weeks as compared with that at 7 weeks (Fig. 2A, B). There were no significant differences in the β cell mass of Lepr$^{+/-}$ mice between 7 and 12 weeks. We evaluated β cell mass by measuring β cell number, islet density, and individual β cell size. The β cell number significantly decreased and the islet number tended to decrease in Lepr$^{-/-}$ mice at 12 weeks as compared with those at 7 weeks, while the individual β cell sizes were comparable between both mice (Fig. 2C). Furthermore, we examined β cell proliferation by immunostaining with antibodies to proliferating cell nuclear antigens (PCNAs). The number of PCNA-positive β cells significantly decreased in Lepr$^{-/-}$ mice at 12 weeks compared with that at 7 weeks (Fig. 2D). Thus, plasma insulin concentrations peaked even before the onset of frank diabetes in Lepr$^{-/-}$ mice, suggesting that β cell failure might occur at the early stage of the development of diabetes. Moreover, the decrease in the β cell mass could be mainly attributable to the reduction of β cell number, not β cell size.
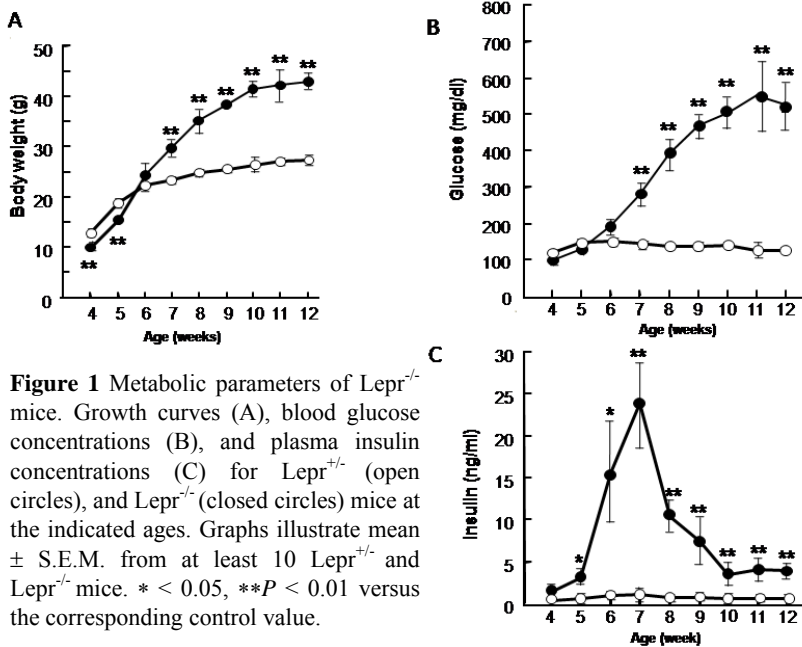

Figure 1 Metabolic parameters of Lepr$^{-/-}$ mice. Growth curves (A), blood glucose concentrations (B), and plasma insulin concentrations (C) for Lepr$^{+/-}$ (open circles), and Lepr$^{-/-}$ (closed circles) mice at the indicated ages. Graphs illustrate mean ± S.E.M. from at least 10 Lepr$^{+/-}$ and Lepr$^{-/-}$ mice. ∗ < 0.05, ∗∗P < 0.01 versus the corresponding control value.

Next, we analyzed the status of ER stress in pancreatic β cells of Lepr$^{-/-}$ mice. Immunoblot analysis using isolated islets revealed that a molecular chaperone, GRP78/Bip, and phosphorylated eIF2α were increased at 8 weeks in the β cells of Lepr$^{-/-}$ mice, indicating the augmentation of ER stress (Fig. 3). Electron microscopy revealed that secretory granules were lower in β cells in which the ER was well arranged in the peripheral region. However, its cisternae were enlarged especially near the Golgi complex in Lepr$^{-/-}$ mice at 8 weeks of age (Matsuda et al. unpublished data), suggestive of ER stress. These results suggested that ER stress might be implicated in the development of pancreatic β cell failure of Lepr$^{-/-}$ mice.

We have previously reported that insulin signaling in β cells might be indispensable for the maintenance and regulation of pancreatic β cell mass (5). Thus, we examined the status of insulin signaling in β cells of Lepr$^{-/-}$ mice. Immunoblot analysis demonstrated that the form of Akt with phosphorylated Ser473 was less abundant in islets of Lepr$^{-/-}$ mice than in

those of Lepr$^{+/-}$ mice (Fig. 3). Therefore, ER stress and reduced insulin signaling may be the mechanism underlying pancreatic β cell failure in Lepr$^{-/-}$ mice.
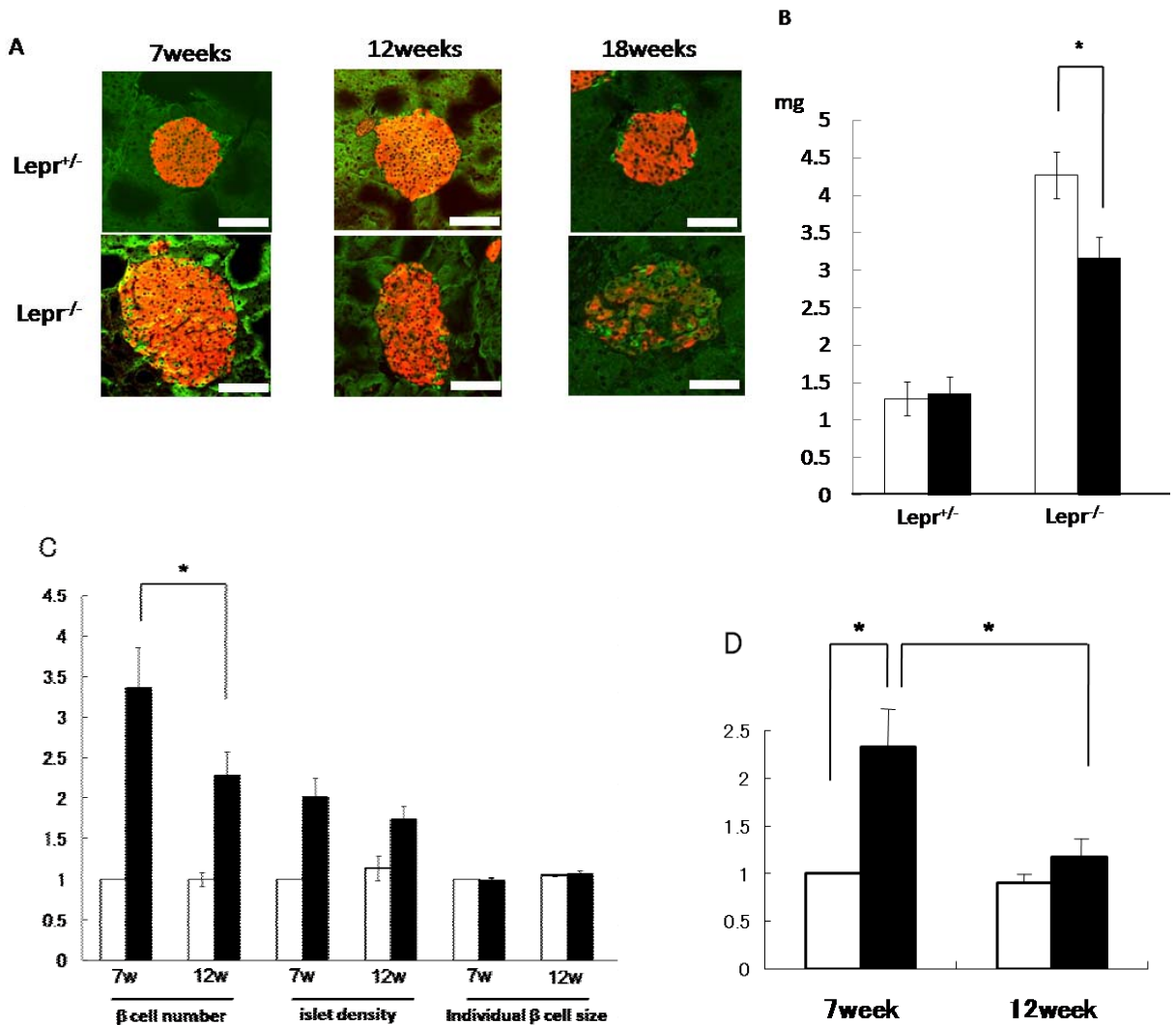
Figure 2 Islet characteristics of Lepr$^{-/-}$ mice. (A) Immunostaining of pancreas sections from Lepr$^{+/-}$ and Lepr$^{-/-}$ mice at the indicated ages with antibodies to insulin (red) and glucagon (green). Scale bars, 100μm. (B) β Cell mass in 7- (open bars) and 12-week (closed bars)-old Lepr$^{+/-}$ and Lepr$^{-/-}$ mice. (C) The number of β cells was determined by the number of β cells in pancreatic sections divided by the total pancreatic area. Islet density was determined as the number of islets divided by the total area of the pancreas. The sizes of individual β cells were determined from the total area of insulin-positive cells in the pancreatic sections divided by the number of nuclei in insulin positive cells. All the values of the Lepr$^{-/-}$ mice (closed bars) were expressed as the ratio to those of Lepr$^{+/-}$ mice (open bars). (D) Pancreatic sections were stained with antibodies to PCNA, and the number of PCNA-positive β cells was determined. Graphs indicate mean ± S.E.M.
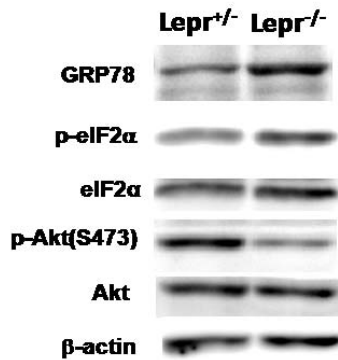
Figure 3 Immunoblot analysis in islets of Lepr^{+/-} and Lepr^{-/-} mice. Islets isolated from 8-week-old mice of the indicated genotypes were subjected to immunoblot analysis with antibodies to the indicated proteins. p-eIF2α and p-Akt represent phosphorylated forms of eIF2α and Akt, respectively.

Based on the results shown above, we examined the *in vitro* involvement of ER stress with insulin signaling in β cells using MIN6 insulinoma cells. Tunicamycin induces ER stress by inhibiting N-linked glycosylation in the ER. Incubation of MIN6 cells with tunicamycin for various periods resulted in an increase in eIF2α and c-Jun phosphorylation, and XBP-1, and CHOP expression, indicative of ER stress. Tunicamycin-loaded MIN6 cells also exhibited a decrease in insulin signaling, such as reduced AKT and GSK3β phosphorylation (Fig. 4A). Conversely, the PI-3K inhibitor LY294002 enhanced the phosphorylation of eIF2α and c-Jun and the expression of CHOP, indicative of augmented ER stress (Fig. 4B). Furthermore, loads of both tunicamycin and LY294002 induced ER stress to a great extent than tunicamycin or LY294002 alone, indicating that the reduction of insulin signaling by LY294002 facilitated the induction of ER stress with tunicamycin (Fig. 4C). Taken together, both ER stress and reduced insulin signaling might synergistically affect pancreatic β cell dysfunction.

To analyze the mechanism underlying the synergy, we next performed RT-PCR using RNA extracted from MIN6 cells stimulated with tunicamycin and/or LY294002. Tunicamycin induced the expression of GRP78, Chop, and XBP-1 in MIN6 cells. Addition of LY294002 further enhanced the expression of Chop and XBP-1, but not the expression of GRP78, although LY294002 alone did not alter their expression as compared with the control (Fig. 5). Thus, the inhibition of insulin signaling dampened ER function by inhibiting the induction of the molecular chaperone GRP78, leading to MIN6 cells that were vulnerable to ER stress. In fact, the overexpression of GRP78 in MIN6 cells inhibited the induction of ER stress markers such as phosphor-eIF2α, phosphor-c-Jun, and Chop by tunicamycin (data not shown).
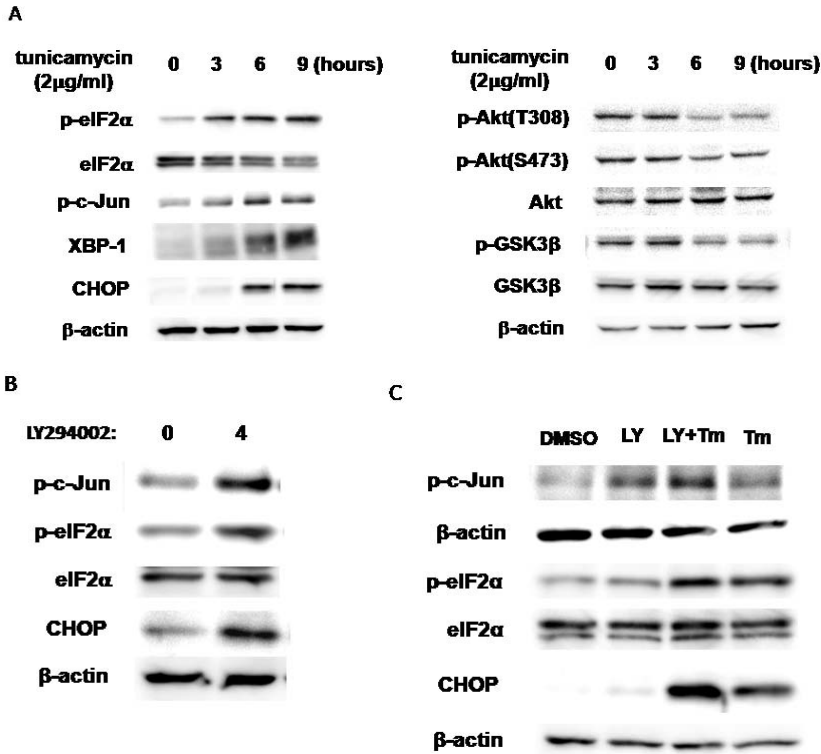
Figure 4. Immunoblot analysis in MIN6 cells incubated with tunicamycin and/or LY294002. (A) MIN6 cells were incubated with 2μg/ml tunicamycin for the indicated time and then subjected to immunoblot analysis with the antibodies to the indicated proteins. (B) MIN6 cells were incubated with ot without LY294002, PI-3 kinase inhibitor, for 8 hours and then subjected to immunoblot analysis with the antibodies to the indicated proteins. (C) MIN6 cells were incubated with LY294002 and/or tunicamycin, and then subjected to immunoblot analysis with antibodies to the indicated proteins.
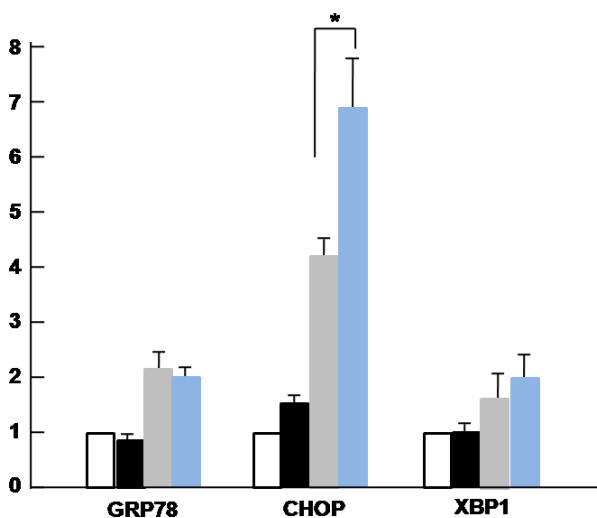
Figure 5. Altered gene expression in MIN6 cells incubated with tunicamycin and/or LY294002. The abundance of mRNAs for the indicated proteins was determined by real-time RT-PCR analysis of total RNA isolated from MIN6 cells. The amount of mRNAs in MIN6 cells are expressed relative to those in control animals. Incubation with DMSO (open bars), LY294002 (closed bars; black), tunicamycin (cloosed bars; grey), and LY294002+tunicamycin (closed bars; blue). Data are means ± SE of tripicates. ∗ $P < 0.05$ (ANOVA) versus the corresponding value (1.0) for MIN6 cells incubated with DMSO as the control.

DISCUSSION

In this study, we demonstrate that the induction of GRP78, the main molecular chaperone, and the activation of PERK were apparent at the early stage of diabetes in the islets of db/db mice, an animal model of type 2 diabetes with obesity. Db/db mice also exhibit a decrease in phosphorylation of Akt, the main molecule of the insulin-signaling pathway in islets. Furthermore, we indicated that the inhibition of insulin signaling might result in ER stress in pancreatic β cells. Thus, both insulin resistance and ER stress might present in pancreatic β cells and act synergistically to deteriorate β cell function. This synergism between insulin resistance in β cells and ER stress might culminate in the decrease in β cell mass and insulin synthesis, leading to pancreatic β cell failure.

Pancreatic β cell dysfunction can be observed in all cases of type 2 diabetes, even at the prediabetic or impaired glucose tolerance (IGT) stage. Recently, it has been reported that β cell mass was reduced in type 2 diabetes. The reduction in β cell mass is also observed at the prediabetic stage (1). Thus, it is important to elucidate the mechanism underlying pancreatic β cell dysfunction and β cell loss before the onset of frank diabetes for prevention of diabetes progression.

Pancreatic β cells secrete sufficient amounts of insulin for insulin resistance, and contain developed ER. On the other hand, β cells are vulnerable to various factors that elicit ER stress. Recently, it has been reported that augmented ER stress might underlie the pathogenesis of pancreatic β cell failure (3,13) as well as the induction of peripheral insulin resistance (9). Others have demonstrated that chemical chaperones reduced the peripheral insulin resistance due to the enhanced folding capacity of ER, resulting in the inhibition of the onset of diabetes (10).

Thus far, augmented ER stress has been demonstrated to reduce insulin signaling via serine-phosphorylated IRS-1 through JNK activation (9). Other studies have shown that ER stress-induced Chop inhibits Akt activity by the induction of TRB3 (2). Conversely, we demonstrated that reduced insulin signaling might enhance ER stress. Taken together, in the state of insulin resistance, both ER stress and reduced insulin signaling might occur and affect each other, culminating in the loss of β cell mass.

ACKNOWLEDGEMENTS

REFERENCES

1. **Butler, A.E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R.A., Butler, P.C.** 2003 β cell deficit and increased β-cell apoptosis in human with type 2 diabetes. Diabetes **52**:102-110.

2. **Du, K., Herzig, S., Kulkarni, R.N., Montominy, M.** 2003 TRB3: A tribbles homolog that inhibits Akt/PKB activation by insulin in liver. Science **300**:1574-1577.

3. **Harding, H.P., Zeng, H., Zhang, Y., Jungries, R., Chung, P., Plesken, H., Sabatini, D.D., Ron, D.** 2001 Diabetes mellitus and exocrine pancreatic dysfunction in *Perk*$^{-/-}$ mice reveals a role for translational control in secretory cell survival. Mol. Cell **7**: 1153-1163.

4. **Hashimoto, N., Kido, Y., Uchida, T., Matsuda, T., Suzuki, K., Inoue, H., Matsumoto, M., Ogawa, W., Maeda, S., Fujihara, H., Ueta, Y., Uchiyama, Y., Akimoto, K., Ohno, S., Noda, T., Kasuga, M.** 2005 PKCλ regulates glucose-induced insulin secretion through modulation of gene expression in pancreatic β cells. J. Clin. Invest. **115**:138-145.

5. **Hashimoto, N., Kido, Y., Uchida, T., Asahara, S., Shigeyama, Y., Matsuda, T., Takeda, A., Tsuchihashi, D., Nishizawa, A., Ogawa, W., Fujimoto, Y., Okamura, H., Arden, K.C., Herrera, P.L., Noda, T., Kasuga, M.** 2006 Ablation of PDK1 in pancreatic β cells induces diabetes as a result of loss of β cell mass. Nat. Genet. **38**:589-593.

6. **Kasuga, M.** 2006 Insulin resistance and pancreatic β cell failure. J. Clin. Invest. **116**:1756-1760.

7. **Kido, Y., Burks, D.J., Withers, D., Bruning, J.C., Kahn, R.C., White, M.F., Accili, D.** 2000 Tissue-specific insulin resistance in mice with mutations in the insulin receptor, IRS-1, and IRS-2. J. Clin. Invest. **105**:199-205.

8. **Leahy, J.L.** 1990 Natural history of beta-cell dysfunction in NIDDM. Diabetes Care **13**:992-1010.

9. **Ozcan, U., Cao, Q., Yilmaz, E., Lee, A.H., Iwakoshi, N.N., Ozdelen, E., Tuncman, G., Gorgun, C., Glimcher, L.H., Hotamisligil, G.S.** 2004 Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. Science **306**:457-461.

10. **Ozcan, U., Yilmaz, E., Ozcan, L., Furuhashi, M., Vaillancourt, E., Smith, R.O., Gorgun, C.Z., Hotamisligil, G.S.** 2006 Chemical Chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science **313**:1137-1140.

11. **Rhodes, C.J.** 2005 Type 2 diabetes – a matter of β-cell life and death? Science **307**:380-384.

12. **Weyer, C., Bogardus, C., Mott, D.M., Pratley, R.E.** 1999 The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. J. Clin. Invest. **104**:787-794.

13. **Zhang, P., McGrath, B., Li, S., Frank, A., Zambito, F., Reinert, J., Gannon, M., Ma, K., McNaughton, K., Cavener, D.R.** 2002 The PERK eukaryotic factor 2α 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.