Research article

Chop deletion reduces oxidative stress, improves β cell function, and promotes cell survival in multiple mouse models of diabetes

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The progression from insulin resistance to type 2 diabetes is caused by the failure of pancreatic β cells to produce sufficient levels of insulin to meet the metabolic demand. Recent studies indicate that nutrient fluctuations and insulin resistance increase proinsulin synthesis in β cells beyond the capacity for folding of nascent polypeptides within the endoplasmic reticulum (ER) lumen, thereby disrupting ER homeostasis and triggering the unfolded protein response (UPR). Chronic ER stress promotes apoptosis, at least in part through the UPR-induced transcription factor C/EBP homologous protein (CHOP). We assessed the effect of Chop deletion in multiple mouse models of type 2 diabetes and found that Chop+/− mice had improved glycemic control and expanded β cell mass in all conditions analyzed. In both genetic and diet-induced models of insulin resistance, Chop deficiency improved β cell ultrastructure and promoted cell survival. In addition, we found that isolated islets from Chop+/− mice displayed increased expression of UPR and oxidative stress response genes and reduced levels of oxidative damage. These findings suggest that CHOP is a fundamental factor that links protein misfolding in the ER to oxidative stress and apoptosis in β cells under conditions of increased insulin demand.

Introduction

Type 2 diabetes (T2D) is a world-wide disease of epidemic proportions that is estimated to afflict more than 180 million individuals, with approximately 2.9 million associated deaths per year (1, 2). Because loss of β cell function and mass are central events in the development and progression of T2D, they are key therapeutic targets for treatment of this disease (3, 4). In T2D, β cell toxicity has been linked to stimuli including glucose, lipids, proinflammatory cytokines, glycation products, and islet amyloid (4). Both signals from mitochondrial metabolism and oxidative stress are implicated in the β cell dysfunction of T2D (5–8). There is also a growing body of evidence to support the hypothesis that insulin resistance, a common underlying reason for the β cell failure that occurs in T2D, is associated with higher levels of ER stress in β cells in animal models of disease (9, 10) and also in humans (11, 12). It is proposed that increased proinsulin biosynthesis generates an unfolded/misfolded protein load in the ER lumen that activates the unfolded protein response (UPR) (9, 13). It is also hypothesized that misfolded islet amyloid precursor protein (IAPP) may accumulate in the ER lumen of β cells and cause apoptosis through induction of ER stress (11). Failure of the UPR to resolve misfolded protein and maintain ER homeostasis could lead to persistent ER stress and serve as a major determinant of β cell failure and death in diabetes.

Nonstandard abbreviations used: ATF4, activating transcription factor 4; CHOP, C/EBP homologous protein; eIF2, eukaryotic translation initiation factor 2; GSIS, glucose-stimulated insulin secretion; HF, high fat; HODE, hydroxyoctadecadienoic acid; IRE1α, inositol-requiring protein 1α; PERK, dsRNA-activated protein kinase-like ER kinase; STZ, streptozotocin; T2D, type 2 diabetes; TEM, transmission electron microscopy; UPR, unfolded protein response; XBP1, X-box binding protein 1.

Conflict of interest: The authors have declared that no conflict of interest exists.

Citation for this article: J Clin Invest. 118:3378–3389 (2008). doi:10.1172/JCI34587.
tein degradation (ERAD). However, there is less compelling evidence that supports the idea that the UPR can improve the secretion capacity of the cell. The latter conclusion is largely inferred from gene deletion studies in which defects of ER stress signaling pathways reduce survival and/or differentiation of cells that secrete large amounts of protein.

It is now evident that conditions associated with high levels of ER stress can severely compromise β cell function (15). This is apparent from the β cell failure associated with mutations in murine and human proinsulin that disrupt disulfide bond pairing and cause misfolding and accumulation of proinsulin in the ER lumen of β cells (22–26). In addition, mutations that reduce signaling through the primary sensors of the UPR or interfere with protein chaperone functions of the UPR impair β cell health (9, 13, 27–31). Finally, stimuli, such as glucose, free fatty acids, cytokines, and nitric oxide compromise β cell function and induce UPR gene expression (12, 32–37). Fundamentally, glucose regulates both PERK-mediated eIF2α phosphorylation and IRE1α-mediated Xbp1 mRNA splicing. Periodic increases in glucose, as well as chronic hyperglycemia, activate IRE1α in vivo and in isolated rat islets (29, 38). Elevated glucose concentrations increase the rate of mRNA translation through phosphatase-mediated dephosphorylation of eIF2α, and the unfolded protein load activates the UPR (38, 39). The significance of the PERK/eIF2α pathway in supporting β cell function is underscored by the observation that mice and humans harboring loss-of-function mutations in PERK develop diabetes due to loss of β cell mass (13, 27, 40). In addition, homozygous missense Ser51Ala mutation at the PERK phosphorylation site in eIF2α causes β cell deficiency in mice (28). Finally, heterozygous Ser51Ala/eIF2α mice fed a high-fat (HF) diet to induce insulin resistance develop β cell failure (9). These genetic alterations of both PERK and eIF2α increase mRNA translation so that the protein-folding load exceeds the protein-folding capacity of the ER.

Although increasing evidence indicates that ER stress occurs in β cells, possibly as a consequence of increased proinsulin synthesis to compensate for insulin resistance, it is unknown whether β cell failure and loss of glucose homeostasis in vivo result from an inadequate UPR adaptive response or activation of an apoptotic response due to chronic, unresolved ER stress. As it is not possible to address this question in simple cell culture models of β cell apoptosis, we have studied whether the absence of CHOP, a key protein implicated in UPR-induced cell death, can prevent loss of β cell function and mass due to dietary or genetically induced insulin resistance in vivo.

CHOP was identified as an ER stress–induced transcription factor that is a significant mediator of apoptosis in response to ER stress (41, 42). Chop gene induction is primarily mediated through the PERK/eIF2α/ATF4 UPR pathway, although IRE1α/XBP1 and ATF6α pathways also contribute (43–47). CHOP expression is increased in β cells from diabetic mice and humans (10–12, 48). Although Chop-null mice do not have a readily detectable phenotype under basal conditions (36, 41), β cells from Chop-null mice are protected from apoptosis caused by either nitric oxide (36) or accumulation of a folding-defective mutant of proinsulin (48). However, as CHOP is not the only death signal evoked by ER stress, the requirement for CHOP in β cell failure associated with T2D is unknown. Here, we show that Chop deletion increases the capacity of islets to produce insulin and curtails the progression of insulin resistance to diabetes. We demonstrate that deletion of Chop not only prevents β cell apoptosis, but also improves β cell function by preventing oxidative damage in response to protein misfolding in the ER. The findings demonstrate that CHOP is a fundamental factor that links protein misfolding in the ER to oxidative stress and apoptosis in β cells.

**Results**

Chop-null mutation increases obesity but prevents glucose intolerance in HF diet–fed eIF2αS/A mice. Although mice with heterozygous Ser51Ala mutation at the PERK phosphorylation site in eIF2α exhibit reduced attenuation of mRNA translation upon ER stress, they did not exhibit a readily apparent phenotype under standard conditions of diet. Analysis of glucose-stimulated translation in islets isolated from HF diet–fed heterozygous Ser51Ala mice, however, revealed an elevated rate of translation (9). These HF diet–fed mice develop diabetes and represent what we believe to be a novel model of β cell failure that results from ER stress due to elevated proinsulin biosynthesis as a consequence of interaction between genetic (eIF2αS/A allele) and environmental (HF diet) factors (9). We asked whether β cell survival and/or function are improved in these heterozygous Ser51Ala mutant mice when the CHOP-mediated death signal is absent. Deletion of the Chop gene modestly increased weight gain in HF diet–fed wild-type eIF2αS/A mice, consistent with recent observations (49). In contrast, Chop deletion significantly increased obesity in HF diet–fed eIF2αS/A mice (Figure 1A). The enhanced weight gain of eIF2αS/AChop–/– animals may be caused by accentuation of the metabolic defect previously reported for the eIF2αS/A mice (9), may be a consequence of the deletion of CHOP action as a negative regulator of adipogenesis (50, 51), or may be driven by hyperinsulinemia (see below). Glucose intolerance appeared after 5 weeks of HF diet in eIF2αS/AChop–/– mice compared with HF diet–fed control eIF2αS/AChop+/– mice. In contrast, eIF2αS/A mice with the Chop-null mutation displayed normal glucose tolerance for up to 32 weeks of HF diet despite their overt obesity (Figure 1B and Supplementary Figure 1A; supplemental material available online with this article; doi:10.1172/JCI34587DS1).

Chop-null mutation preserves β cell morphology and function in HF diet–fed eIF2αS/A mice. The improved glucose tolerance observed in the eIF2αS/AChop–/– mice was not due to increased insulin sensitivity (Supplemental Figure 2A), but rather was associated with a 6-fold increase in islet mass and pancreas insulin content (Figure 1C and Supplementary Figure 1B and C). Ultrastructural analysis was performed to monitor the distension of ER cisternae and reduction of secretory granule content characteristic of ER stress and β cell failure. Compared with β cells from HF diet–fed eIF2αS/A mice, β cells from HF diet–fed eIF2αS/AChop–/– mice displayed a significantly distended ER and reduced insulin granule number, as previously reported (9). However, strikingly, the number of dense-core insulin granules in eIF2αS/AChop–/– mice was not significantly reduced compared with those in eIF2αS/AChop+/– mice or eIF2αS/AChop+/+ mice, although ER distension was still detectable (Figure 1D and E).

The improved glucose tolerance and preserved granule content suggested that Chop deletion preserves β cell function by maintaining an adequate pool of secretory granules that were responsive to nutrient stimuli. Consistent with this theory, after 35 weeks of HF diet, the serum insulin levels were increased 2- to 3-fold in eIF2αS/AChop–/– mice compared with eIF2αS/AChop+/–, eIF2αS/AChop+/+, and eIF2αS/AChop+/+ mice (Figure 1F). Glucose-stimulated insulin secretion (GSIS) was significantly reduced in islets isolated from eIF2αS/AChop–/– mice compared with wild-type eIF2αS/AChop+/+ mice.
In contrast, islets from HF diet–fed $eIF2\alpha^{S/A}\text{Chop}^{-/-}$ mice remained glucose responsive for insulin secretion (Figure 1G). As the GSIS studies were performed with selected islets of similar size and the secretion of insulin was expressed as a percentage of total insulin content, the improved GSIS observed in $eIF2\alpha^{S/A}\text{Chop}^{-/-}$ islets was not due to increased $\beta$ cell mass, but rather reflected a genuine improvement in $\beta$ cell function. These findings show that, despite the HF diet and overt obesity, glucose homeostasis was maintained in $eIF2\alpha^{S/A}\text{Chop}^{-/-}$ mice because there was an increase in the number of functional $\beta$ cells as measured by insulin granule content and GSIS. The results suggest that $\beta$ cell failure in this $eIF2\alpha^{S/A}$ mutant mouse model is mediated through the UPR-inducible gene Chop. This conclusion is also supported by the observation that Chop deletion reduced apoptosis and increased $\beta$ cell mass in pancreata from homozygous $eIF2\alpha^{Ser51A1a}$ embryos (Supplemental Figure 3).

**Figure 1**

Chop-null mutation increases $\beta$ cell mass, improves $\beta$ cell function, and prevents glucose intolerance in HF diet–fed $eIF2\alpha^{S/A}$ mice. Mice of the indicated genotypes were fed a 45% HF diet for 35–41 weeks. (A and B) Body mass and glucose tolerance tests; $n = 8–10$ mice per condition. Significant differences between $eIF2\alpha^{S/A}\text{Chop}^{+/+}$ and $eIF2\alpha^{S/A}\text{Chop}^{-/-}$ are indicated. (C) Islet morphology shown by H&E and immunofluorescence staining. Scale bars: 400 $\mu$m (top), 50 $\mu$m (bottom). (D and E) $\beta$ cell ultrastructure from TEM and insulin granule content quantified by analysis of similar total areas from TEM images from 2 mice per condition. ER, rough ER; M: mitochondria. Scale bar: 1 $\mu$m. (F) Analysis of serum insulin levels; $n = 8–10$ mice per condition. (G) Analysis of GSIS. Islets from 2 animals per condition were analyzed in duplicate. H, high glucose (16.7 mM); L, low glucose (3.3 mM). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.  

Chop-null mutation prevents loss of $\beta$ cell mass and diabetes in a HF diet/streptozotocin model of T2D. The previous data indicated that CHOP plays a negative role in $\beta$ cell function when ER stress signaling is compromised in conjunction with a biosynthetic burden of enhanced proinsulin translation due to insulin resistance. We next evaluated the role of CHOP under conditions that combine the primary determinants of T2D, insulin resistance, and inadequate $\beta$ cell function and mass in the absence of a genetic predisposition to $\beta$ cell failure. Wild-type and Chop-null mice were fed a 60% HF diet for 5–6 weeks, and $\beta$ cell mass was reduced by administration of a moderate dose of streptozotocin (STZ). This treatment increases the proinsulin biosynthetic burden upon the remaining $\beta$ cells and challenges their ability to survive and function. This approach has been successful in evaluating therapeutic strategies that alter insulin resistance or improve $\beta$ cell function (52–54).
the absence of any additional determinant of obesity, the Chop–null mice exhibited a slight increase in weight that was not statistically different from wild-type animals (Figure 2A). However, the fed glucose levels of Chop-null mice were significantly lower than those of wild-type mice after 5–6 weeks of HF diet feeding. In addition, the overt hyperglycemia that developed in wild-type mice 4 days after administration of STZ was averted in Chop–null mice (Figure 2B).

The improved glycemic control of Chop-null HF diet–fed, STZ-treated animals was investigated by analysis of glucose tolerance, insulin tolerance, insulin secretion, insulin content, and islet morphology (Figure 2, C–J). In the absence of HF diet or with 45% HF feeding, glucose tolerance and β cell function were similar between wild-type and Chop–/– mice (Figure 1, Supplemental Figure 1, Figure 3, and Supplemental Figure 4). However, under conditions of 60% HF diet, glucose intolerance was more severe in wild-type mice compared with Chop–/– mice (Figure 2C). One week after STZ administration, the wild-type mice were severely hyperglycemic and glucose intolerant, while the Chop–/– mice were only mildly hyperglycemic and glucose intolerant (Figure 2, B and D). Blood glucose levels were 50% lower, and insulin secretion upon fasting and refeeding was signifi-
significantly attenuated in Lepr(--/--) mice compared with heterozygous Lepr(--/+), similar to recent observations (55). However, Chop deletion dramatically reduced apoptosis in the Lepr(--/--) mice to levels observed in control Lepr(+/-) mice (Figure 4A). To evaluate β cell proliferation, BrdU-containing water was administered to mice prior to the development of full islet hyperplasia (56). As the rate of proliferation is very low in adult animals, a labeling period of 23 days was implemented to ensure accurate detection of altered proliferation rates. The number of BrdU-positive cells was approximately 2-fold greater in the islets from Lepr(--/--)Chop(-/-) mice compared with those from Lepr(--/--)Chop(+/-) mice (Figure 4B). Therefore, a portion of the islet hyperplasia observed in the Chop-null mice may be attributed to increased β cell proliferation, possibly a consequence of normal β cell compensation for insulin resistance. Therefore, the islet hyperplasia in Lepr(--/--)Chop(-/-) mice was a consequence of reduced apoptosis as well as increased proliferation.

Chop-null mutation increases expression of adaptive functions and reduces expression of apoptotic functions in Lepr(--/--) mice. To provide insight into how Chop-null mutation might alter the transcriptional profile to preserve β cell function, we analyzed isolated islets for expression of genes encoding functions in the UPR, the oxidative stress response, cell death, and insulin production (Figure 5). The expression of several UPR genes encoding adaptive functions to improve ER folding capacity, such as Bip, Gpr94, Fbkp11, and p58ipk, was slightly increased in islets from Lepr(--/--)Chop(-/-) mice compared with control Lepr(--/--)Chop(+/-) mice. The expression levels of these genes were further increased in islets from Lepr(--/--)Chop(-/-) mice compared with control Lepr(--/--)Chop(+/-) mice. In addition, there was increased splicing of Xbp1 mRNA and expression of genes that encode components of the ER-associated protein degradation machinery, such as Edem and Ubc7 (Figure 5A). The expression levels of several targets of CHOP that encode proapoptotic functions, i.e., Gadd34, Dr5, and Trb3, as well as other death pathway-related genes, were reduced in Lepr(--/--)Chop(-/-) islets (Figure 5C and Supplemental Figure 5A). The RT-PCR analyses also detected slightly increased expression of genes encoding functions that prevent oxidative stress, including Sod1, Sod2, Gpx1, Ppar, and Ucp2, in Lepr(--/--)Chop(-/-) islets. However, the expression of these genes was significantly elevated in Lepr(--/--)Chop(-/-) islets (Figure 5D). In contrast, expression of CHOP-dependent ER oxidoreductase 1 (Ero1a) (42), which generates oxidizing equivalents in the ER (16, 57), was decreased upon Chop deletion. Quantitative analysis of mRNA expression of other genes encoding ER stress proteins, transcription factors, other oxidative stress-related proteins, and β cell–specific genes did not clarify the mechanism by which Lepr(--/--)Chop(-/-) cells adapt but may provide useful information for future studies (Supplemental Figure 5). As β cells express antioxidant functions at low levels (58, 59), these gene expression differences should minimize accumulation of ROS and facilitate adaptation. These findings support the hypothesis that Chop deletion improves β cell function as a consequence of increased expression of UPR adaptive and antioxidative stress response genes and reduced expression of proapoptotic functions.

Chop-null mutation reduces protein oxidation and lipid peroxidation in response to ER stress. The increased expression of genes encoding antioxidative stress responses suggested that Chop deletion improves the capacity of β cells to accommodate oxidative stress. Therefore, we measured products of protein oxidation (carbonyls) and lipid peroxidation (hydroxyoctadecadienoic acid [HODE]) in...
isolated islets. Islets from Lepr<sup>db/db</sup> diabetic mice displayed a 3-fold increase in protein carbonyls and a 2-fold increase in HODEs compared with those from control Lepr<sup>db/+</sup> mice (Figure 6A). Therefore, insulin resistance in the Lepr<sup>db/db</sup> mice was associated with oxidative stress in the islets, an observation consistent with literature indicating antioxidant molecules can improve glucose homoeostasis, restore β cell function, and reduce oxidative stress markers in the islets of Lepr<sup>db/db</sup> mice (55, 60–63). In contrast, Chop deletion significantly reduced both products of protein oxidation and lipid peroxidation in islets from these obese Lepr<sup>db/db</sup> mice.

The reduction in oxidative damage observed upon Chop deletion may be result from protection of the islets from oxidative damage caused by ROS or it may be an indirect consequence of improved glycemia (8, 64). To discriminate these possibilities, we analyzed islets in the absence of hyperglycemia and insulin resistance contributed by the Lepr<sup>db/db</sup> mutation. Islets were isolated from wild-type mice and Chop<sup>−/−</sup> mice and treated with tunicamycin to inhibit N-linked glycosylation and induce unfolded protein accumulation in vitro. Tunicamycin treatment increased protein oxidation and lipid peroxidation products 2.5- to 3-fold in wild-type islets (Figure 6B). In contrast, islets from Chop<sup>−/−</sup> mice displayed significantly reduced levels of carbonyls and HODEs after tunicamycin treatment. In addition, H<sub>2</sub>O<sub>2</sub> treatment increased levels of carbonyls and HODEs to similar extents in islets isolated from Chop<sup>−/−</sup> and Chop<sup>+/+</sup> mice (Figure 6C). Importantly, these results show that the Chop-null mutation protects β cells from oxidative damage that occurs in response to ER stress.

**Discussion**

Studies in cultured cells indicate that acute UPR activation is an adaptive response to ER stress, whereas sustained UPR activation is associated with cell death (20). Recent studies have demonstrated that insulin resistance is associated with markers of UPR activation, including CHOP induction, in murine and human islets (10–12). However, it is not known whether UPR signaling is an adaptive mechanism that sustains β cell function and survival or whether UPR signaling contributes to β cell failure and death. To study this problem, we analyzed the role of the UPR in protein secretion and cell survival in vivo under conditions that pressure the β cell to produce elevated levels of insulin. Through deletion of the CHOP-mediated death signal, we have uncovered an important adaptive function of the UPR to limit oxidative stress in response to elevated protein secretion. Surprisingly, Chop deletion not only prevented UPR-induced death, but also improved the capacity of the β cell to produce insulin in 3 models of insulin resistance–induced β cell failure: heterozygous Ser51Ala eIF2α mutant mice fed a HF diet, mice fed a HF diet in conjunction with STZ treatment, and Lepr<sup>db/db</sup> mice as a genetic model of insulin resistance. In all models, Chop deletion preserved the β cell mass and improved β cell function, monitored by glucose homoeostasis and GSIS. Finally, Chop deletion also attenuated the loss in β cell mass and apoptosis in embryonic homozygous Ser51Ala eIF2α mutant mice, a model of β cell demise that occurs in the absence of insulin resistance (Supplemental Figure 3). The sum of our findings support the idea that CHOP is a fundamental factor...
causing β cell failure and apoptosis in response to the chronic ER stress that coincides with β cell compensation for insulin resistance. Our findings indicate that in the absence of a death signal, UPR signaling can improve protein secretory capacity and preserve the functional integrity of the ER in β cells.

Our results support the hypothesis that Chop deletion improves ER function and protects against oxidative stress in response to ER stress in β cells. However, as our studies were performed in mice with Chop deletion in all tissues, the possibility exists that Chop deletion affects ER function in other tissues to alter organismal metabolism and, therefore, β cell function. Indeed, our findings as well as previously published results (49) demonstrate that Chop deletion can increase obesity. There are several possible mechanisms by which Chop deletion might increase obesity. First, Chop deletion might influence central nervous system regulation of appetite and/or metabolism. This is a topic that has not to our knowledge been explored. Second, since CHOP is highly expressed in the late stage of adipocyte differentiation and is a negative regulator of C/EBPα and C/EBPβ transcription factors, which are required for adipocyte differentiation (50), the absence of CHOP could increase signaling through the C/EBPs to increase both adipocyte differentiation and lipid biosynthesis. As a consequence of the greater adiposity in Chop-null mice, more effective fatty acid storage could reduce β cell lipotoxicity. Since palmitate can cause ER stress in β cells (12, 32, 65, 66), improving sequestration of fatty acids could improve β cell function. Recent findings also indicate that ER stress can significantly affect lipid synthesis, storage, and signaling (67, 68, 69). Therefore, the reduced ER stress as a consequence of Chop deletion could also reduce plasma lipids and lipid-induced β cell toxicity. Third, recent studies support the notion that ER stress may cause insulin resistance in liver and fat (70). Mice with reduced UPR signaling resulting from heterozygous deletion of Xbp1 developed greater insulin resistance when fed a HF diet. In addition, treatment of leptin-deficient db/db mice with chemical chaperones, which are proposed to improve protein folding in the ER, reduced insulin resistance in fat and liver (71). If Chop deletion improves ER function, it may be expected to reduce insulin resistance and thereby improve β cell function by reducing pressure on the β cell to increase insulin production. However, we believe this is unlikely because insulin resistance was not apparently reduced on the Chop deletion in other cell types, the protective effect of Chop deletion upon ER stress was observed in isolated islets (Figure 6B), indicating that the improved β cell function does not require other tissues.

Previous studies demonstrated that Chop deletion reduces apoptosis and delays glucose intolerance in heterozygous, but not homozygous, Akita mice that express Cys96Tyr misfolded proinsulin (48) and in β cells that are exposed to nitric oxide (36). However,
the molecular mechanism by which CHOP mediates β cell apoptosis under these conditions is not understood, and it is also not known whether protein misfolding in the ER contributes to death of β cells that produce wild-type proinsulin. Our findings demonstrate that Chop deletion prevents glucose intolerance by improving the β cell functional capacity of the ER to produce folded proinsulin and limit oxidative stress. This conclusion is supported by mRNA expression analysis that demonstrated Chop deletion was associated with increased expression of genes encoding antioxidative stress function, i.e., Sod1, Sod2, Gpx1, Pparγ, and Ucp2.

Presently, most data support the idea that CHOP is induced by the PERK/eIF2α/ATF4 as well as the IRE1/XBP1 and ATF6 UPR subpathways to activate proapoptotic gene expression, restore transcription initiation, and increase the oxidizing potential in the ER lumen (42, 72). CHOP induces expression of GADD34, a subunit of type 1 protein phosphatase that directs eIF2α dephosphorylation to increase mRNA translation as homeostasis in the ER is restored (42, 73). CHOP is also implicated in the induction of ERO1α, a molecule that oxidizes protein disulfide isomerase (PDI) so it can function to rearrange improperly formed disulfide bonds within unfolded proteins. Disulfide bond formation during oxidative protein folding in the ER generates oxidative stress as a consequence of electron transfer from cysteine residues through PDI and ERO1 to molecular oxygen to form hydrogen peroxide (74, 75). Future studies should elucidate whether Chop deletion protects β cells from oxidative damage through the reduced expression of GADD34 and/or ERO1.

We have shown that increased protein misfolding in the ER increases oxidative damage in wild-type islets. Oxidative stress may further accentuate protein misfolding by directly modifying protein-folding intermediates, by disturbing protein chaperone functions, or by perturbing ER Ca2+ homeostasis (76). Previous studies demonstrated that islets from Lepre/+ mice exhibit oxidative stress and neutralizing this stress can improve β cell function and prevent progression of T2D (62, 63). Indeed, antioxidant therapy has proven beneficial in diabetic animal models and possibly in humans with T2D (8, 60). Because they express low levels of antioxidant enzymes, β cells may be particularly sensitive to oxidative stress (58, 59). In addition, other stresses, such as proinflammatory cytokines, nitric oxide, hyperlipidemia, and hyperglycemia, may also produce ROS that could further disrupt protein folding in the ER lumen. We propose that oxidative damage that is caused by ER stress may be fundamental in the etiology of the β cell failure associated with both T1D and T2D.

Although the signaling pathways that are activated in response to misfolded protein accumulation in the ER have been identified, there is no evidence to support the idea that manipulation of these pathways can increase the protein folding and/or secretion potential of the ER. We believe our results, which show that deletion of the UPR-induced gene Chop improves the function of the secretory pathway, are the first example in which modulation of UPR signaling was demonstrated to preserve ER function. The improved ER function prevented β cell failure and the development of diabetes caused by insulin resistance and obesity. Although Chop was identified as an ER stress–induced transcription factor that mediates apoptosis, it is possible that changes in gene expression due to Chop deletion improve the functional capacity of the ER to reduce both protein misfolding and ER stress–mediated cell death signaling (Figure 7). Alternatively, in the absence of the CHOP-mediated death signal, UPR adaptive transcription may continue to improve the functional capacity of the ER. Further studies are required to elucidate how Chop deletion influences the transcriptional profile of the cell to preserve the functional capacity of the ER and reduce accumulation of ROS. Our findings should encourage the search for specific modulators of ER stress signaling that have the potential to improve the functional capacity of the ER for the treatment of numerous diseases associated with protein misfolding within this organelle.

Methods

Animal husbandry. Ser51Ala eIF2α mice (9) and Chop−/− mice (41) were backcrossed with C57BL/6J mice (The Jackson Laboratory). The eIF2αWT and Lepre+/+ mice (C57BKS.Cg-m+/+ Lepr+/+) JAX mice) were bred with Chop−/−.
Insulin resistance

Proinsulin

Unfolded proinsulin

ROS

ER function

Anti-ROS

CHOP

GADD34, ERO1

Apoptosis

Figure 7

Model depicting interrelationships between protein folding, UPR, CHOP, ROS, and apoptosis in β cells. The UPR induces genes to improve ER protein folding and reduce oxidative stress and also induces the proapoptotic gene Chop. CHOP enhances ROS formation, possibly through induction of GADD34 or ERO1. Chop-null mutation reduces proapoptotic gene expression to permit increased expression of UPR protective genes and antioxidative stress response genes to minimize ER stress and oxidative stress, thereby improving protein folding to support insulin production (depicted in blue). CHOP may also act, directly or indirectly, to repress transcription of some UPR protective genes or antioxidative stress response genes. Deletion of Chop in combination with insulin resistance increases β cell mass, reduces oxidative stress in islets, and preserves insulin secretion and glucose tolerance.

mice. All animals were housed at 21–23°C with 12-hour light/12-hour dark cycles in the Unit for Laboratory Animal Medicine at the University of Michigan Medical School, with free access to water and either standard rodent chow (LabDiet Formulab Diet, catalog 5008) or a 45% HF diet (Research Diets Inc.; catalog D12451) (Figure 1 and Supplemental Figures 1 and 2). Mice at 10–12 weeks of age were housed pair-wise and fed a HF diet for up to 41 weeks. All studies were performed with 8–10 male mice (except the study described in Supplemental Figure 4A, in which female mice were used) per experimental group. Body weights were measured weekly in the afternoon between 3 and 5 pm.

For all studies, age-matched littermates were utilized as controls. The effects of the Chop-null mutation were analyzed in Lepr<sup>ab</sup>+/− mice by comparison of littermates from crosses between Lepr<sup>ab</sup>/− and Lepr<sup>ab</sup>/+ mice (Figures 3–5 and Supplemental Figures 2, 4, and 5). Additional groups of Lepr<sup>ab</sup>/−Chop<sup>−/−</sup> and Lepr<sup>ab</sup>/−Chop<sup>+/−</sup> mice were included in most studies to provide a comparison with non-diabetic control animals and for evaluation of any consequences of Chop deletion.

In the HF diet–fed, STZ-treated model of T2D, standard husbandry procedures were followed as described above, and 5- to 6-week-old Chop<sup>−/−</sup> and Chop<sup>+/−</sup> male mice were fed a 60% HF diet (catalog D12494; Research Diets Inc.) for 5–6 weeks prior to i.p. administration of 150 mg/kg STZ (Research Diets Inc.; catalog D12451) (Figure 1 and Supplemental Figures 1 and 2). Mice at 10–12 weeks of age were housed pair-wise and fed a HF diet for up to 41 weeks. All studies were performed with 8–10 male mice (except the study described in Supplemental Figure 4A, in which female mice were used) per experimental group. Body weights were measured weekly in the afternoon between 3 and 5 pm.

All procedures were conducted according to the protocols and guidelines approved by the University of Michigan Committee on the Use and Care of Animals (UCCUA).

Glucose tolerance tests and blood glucose and serum insulin analysis. Glucose tolerance was measured after i.p. injection of 2 g glucose/kg body weight (e1F2xa and HF diet–fed, STZ-treated mouse studies) or 1 g/kg (Lepr<sup>ab</sup> mouse studies) to overnight-fasted animals. Blood glucose was measured using a OneTouch Ultra glucometer (LifeScan Inc.) with a sensitivity of 10 mg/dl. Serum insulin was measured with an ultrasensitive ELISA kit (Crystal Chem Inc.).

Islet morphology and immunohistochemistry. Pancreata were isolated, fixed with 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E for visualization by light microscopy. Insulin- and glucagon-containing cells were identified by immunofluorescence staining using guinea pig anti-human insulin antibody (Linco) with donkey anti–guinea pig Texas Red secondary antibody (Jackson ImmunoResearch) and rabbit anti-glucagon antibody (Linco) with goat anti-rabbit Alexa Fluor 488 secondary antibody (Invitrogen). Confocal images were recorded digitally by camera, and β cell mass was measured using Image-Pro Plus software (Media Cybernets).

Apoptosis assays and β cell proliferation. BrdU incorporation was used to analyze β cell proliferation as previously described (56). To continuously label mice with BrdU for 23 days, we substituted drinking water containing 1 mg/ml BrdU dissolved in 0.007 N NaOH. BrdU water bottles were wrapped with aluminum foil to prevent light exposure, and freshly prepared solution was provided every other day. Pancreas tissue was harvested and processed as described above for immunohistochemistry. Sections were stained with BrdU In-Situ Detection Kit (BD Biosciences—Pharmingen), and replicating cells were counted from digital photographs obtained using a phase contrast microscope. The islet areas were quantified using Image-Pro Plus software (Media Cybernets).

TUNEL assays were performed using the ApoAlert DNA Fragmentation Assay Kit (BD Biosciences—Clontech). Tissue sections from pancreata were first labeled for detection of insulin as described above, followed by TUNEL assay. Confocal images were recorded and TUNEL-positive cells were counted manually from the images. The islet areas for adult pancreatic sections were quantified using Image-Pro Plus software (Media Cybernets).

Pancreatic insulin content. Pancreata were extracted by homogenization in a cold solution of acid/ethanol containing 80% ethanol and 0.19 M hydrochloric acid, sonicated on ice, and incubated at 4°C overnight (about 16–20 h). The extracts were then centrifuged, and supernatants were diluted into sample buffer. Insulin and glucagon contents were measured by ELISA (Crystal Chem Inc.) and radioimmunoassay (Glucagon RIA GL-32K; Linco Research Inc., respectively).

β Cell ultrastructure. Transmission electron microscopy (TEM) was performed on pancreas tissue (Figure 1D) and isolated islets (Figure 3F) as previously described (9). The number of insulin granules per islet area was determined using Image-Pro Plus software (Media Cybernets).

Islet isolation. Islets of Langerhans were manually isolated after collagenase P (Roche) digestion and Ficoll gradient centrifugation (77, 78).

GSIS. Five islets of similar size were pre-incubated in basal glucose (3.3 mM) for 1 h at 37°C in Krebs-Ringer Bicarbonate HEPES buffer containing 129 mM NaCl, 5 mM NaHCO<sub>3</sub>, 4.8 mM KCl, 1.2 mM MgSO<sub>4</sub>, 1.2 mM KH<sub>2</sub>PO<sub>4</sub>, 1.0 mM CaCl<sub>2</sub>, and 10 mM HEPES at pH 7.4, plus 0.1% RIA-grade BSA (Sigma-Aldrich). Sequential static incubations were performed in low glucose (3.3 mM) for 15 or 30 min, followed by stimulatory incubation in high glucose (16.7 mM) for 30 min. Insulin release data are expressed as a percentage of total insulin content determined by a ratio of cold acid/ethanol lyss and ELISA.

Gene expression analysis. Total RNA was extracted from freshly isolated islets using RNaseasy mini kit (Qiagen) for reverse-transcription into cDNA in a 20-μl reaction using iScript cDNA Synthesis kit (BioRad). Reverse transcription reactions were incubated sequentially for 5 min at 25°C, 30 min at 42°C, and then 5 min at 85°C. CDNA products were stored at −20°C. iQ SYBR Green Supermix kit (BioRad) was used for quantitative real-time PCR (20 μl) using the iCycler iQ Real-Time PCR detection system (Bio-Rad). The thermal cycling parameters were as follows: step 1, 95°C for 10 min; step 2, 95°C for 15 s; step 3, 59°C for 1 min. Step 2 was repeated for 40 cycles. Reactions were terminated by incubation at 4°C. The relative amounts of mRNA were calculated from the Ct values using 18S rRNA for normalization. Primer sequences are presented in Supplemental Table 1.
Quantitation of oxidation products. Protein carbonyls were measured in islet extracts by ELISA (Biocell Corp.). Lipid peroxidation was quantitated by detection of HODEs as previously described (79, 80). The islet protein content was determined by Bradford assay.

Statistics. Data are represented as mean ± SEM. Statistical significance of differences between groups was evaluated using the Student t test or 1-way ANOVA test (Tukey’s test). P < 0.05 was considered statistically significant.

Acknowledgments

We gratefully thank Mary Pinter, Eric Liao, and Junying Wang for excellent technical assistance, Ming Liu and Peter Arvan for instruction in islet isolation technique, and Tom Rutkowski, Sung-Hoon Back, and David Ginsburg for critical review of the manuscript. We thank Janet Mitchell for her assistance in preparation of the manuscript and graphics. Electron, confocal, and light microscopy were performed at the University of Michigan Microscope and Image Analysis Lab (ML). We thank Chris Edwards, Bruce Donohoe, Dotty Sorenson, Sheldon Almburg, and Sasha Meshinchi of the MIL for their expertise and assistance in these studies. This work was supported in part by NIH grants DK47119 and ES086811 (to D. Ron), DK24394, HLS2173, and PO1 HL057346 (to R.J. Kaufman), and NIDDK 5P60DK020572 (to S. Pennathur) and Juvenile Diabetes Research Foundation Career Development Award 2-2003-149 (to S. Pennathur). R.J. Kaufman is an Investigator at the Howard Hughes Medical Institute.

Received for publication November 27, 2007, and accepted in revised form July 30, 2008.

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