Impact of Endoplasmic Reticulum Stress Pathway on Pancreatic β-Cells and Diabetes Mellitus

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Diabetes is caused by impaired insulin secretion in pancreatic β-cells and peripheral insulin resistance. Overload of pancreatic β-cells leads to β-cell exhaustion and finally to the development of diabetes. Reduced β-cell mass is evident in type 2 diabetes, and apoptosis is implicated in this process. One characteristic feature of β-cells is highly developed endoplasmic reticulum (ER) due to a heavy engagement in insulin secretion. The ER serves several important functions, including post-translational modification, folding, and assembly of newly synthesized secretory proteins, and its proper function is essential to cell survival. Various conditions can interfere with ER function and these conditions are called ER stress. Recently, we found that nitric oxide (NO)-induced apoptosis in β-cells is mediated by the ER-stress pathway. NO causes ER stress and leads to apoptosis through induction of ER stress-associated apoptosis factor CHOP. The Akita mouse with a missense mutation (Cys96Tyr) in the insulin 2 gene has hyperglycemia and a reduced β-cell mass. This mutation disrupts a disulfide bond between A and B chains of insulin and may induce its conformational change. In the development of diabetes in Akita mice, mRNAs for an ER chaperone Bip and CHOP were induced in the pancreas. Overexpression of the mutant insulin in mouse MIN6 β-cells induced CHOP expression and led to apoptosis. Targeted disruption of the CHOP gene did not delay the onset of diabetes in the homozygous Akita mice, but it protected islet cells from apoptosis and delayed the onset of diabetes in the heterozygous Akita mice. We conclude that ER overload in β-cells causes ER stress and leads to apoptosis via CHOP induction. These results highlight the importance of chronic ER stress in β-cell apoptosis in type 2 diabetes, and suggest a new target to the management of the disease. Exp Biol Med 228:1213–1217, 2003

Key words: diabetes mellitus; endoplasmic reticulum stress; apoptosis; CHOP/GADD153; nitric oxide

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Diabetes mellitus is caused by impaired insulin signaling and decreased insulin secretion. Pancreatic β-cell is the only cell that secrets insulin, and therefore it plays an important role in glucose homeostasis. Excessive loss of β-cells is a cause of Type I diabetes, and dysfunction of β-cells is a common feature of Type II diabetes (1, 2). In Type I diabetes, the β-cell is selectively disrupted by autoimmune and/or inflammatory processes, which results in insulin deficiency and hyperglycemia. In Type II diabetes, insulin resistance, which is often associated with obesity, is a major factor in progression of the disease. However, hyperglycemia occurs only when β-cells fail to compensate for the increased demand for insulin secretion.

Apoptosis in β-cells is involved not only in Type I diabetes but also in Type II diabetes (3). Apoptosis in β-cells is induced by various stimuli such as activation of death receptors and DNA damage. Recent studies have revealed that the endoplasmic reticulum (ER) is the organelle that can sense the various stresses and transmit apoptotic signals. In this study, we focus on ER stress-mediated apoptosis in β-cells and evaluate how ER stress is involved in the development of both Type I and Type II diabetes.

Endoplasmic Reticulum Function and Endoplasmic Reticulum Stress

The ER is one of the important organelles, and it serves several important functions, including post-translational modification, folding and assembly of newly synthesized secretory proteins, and a cellular calcium store. Evidence is accumulating that defective folding and rapid degradation of mutant proteins is one of the causes of various disease including amyloidosis, cystic fibrosis, and neurodegenerative disorders such as Prion disease, Alzheimer's disease and Parkinson's disease (4). Therefore, proper function of the ER is essential to cell survival. Various conditions can disturb ER functions, including inhibition of protein glycosylation, reduction of formation of disulfide bonds, calcium depletion from the ER lumen, impairment of protein transport from the ER to the Golgi, expression of malfolded

proteins, etc. Such ER dysfunction causes proteotoxicity in the ER, collectively termed "ER stress" (5–7).

To survive under ER stress conditions, cells have a self-protective mechanism against ER stress, which is termed the ER stress response (5–7). At least four functionally distinct responses have been identified so far (Fig. 1).

The first response involves upregulation of the genes encoding ER chaperone proteins including Bip/GRP78 and GRP94, which increase protein-folding activity and prevent protein aggregation. These proteins share a consensus sequence in their promoters, which is termed as the *cis*-acting ER stress response element (ERSE) or the unfolded protein response element (UPRE). Three distinct ER-membrane localized upstream components, Ire1 (inositol requiring 1), PERK (RNA-dependent protein kinase-like endoplasmic reticulum eIF2α kinase), and ATF6 (activating transcription factor 6), transmit stress signals from the ER to nucleus in response to perturbation of protein folding in the ER.

The second response consists of translational attenuation to reduce the synthesis of new protein and to prevent further accumulation of unfolded proteins. This phenomenon occurs at the level of translational initiation via phosphorylation of eIF2α that regulates the binding of the initiator Met-tRNA to the ribosome. Phosphorylation of eIF2 at Ser51 of its α subunit blocks this step and thus inhibits protein synthesis. PERK is the essential kinase responsible for this phosphorylation during ER stress.

The third response is degradation of misfolded proteins in the ER, which is called ER-associated degradation (ERAD). Misfolded proteins, which are not refolded in the ER, can be detected by the ER quality control system, retrotransported from the ER to the cytosol, and degraded by the 26S proteasome.

The fourth response is apoptosis, which occurs when severe and prolonged ER stress extensively impairs the ER functions. Apoptosis is necessary not only for removing the cells that threaten the integrity of the organism but also for proper development and differentiation.

Endoplasmic Reticulum Stress-Mediated Apoptosis Pathway

There are three known apoptosis pathways that are triggered by ER stress. The first is transcriptional induction of the gene for CHOP (C/EBP homologous protein)/GADD153, a member of the C/EBP family of transcription factors (8). CHOP is barely detected under physiological conditions, but is strongly induced in response to ER stress (9). Overexpression and targeted disruption of the CHOP gene has demonstrated that CHOP promotes apoptosis in response to ER stress (10–14). Transcriptional activation of the CHOP gene is mediated by three distinct upstream components of ER stress, Ire1 (15), PERK (16), and ATF6 (17). On the other hand, little is known about the downstream target of CHOP leading to apoptosis.

The second apoptotic pathway is activation of the cJUN NH2-terminal kinase (JNK) pathway. JNKs constitute a family of signal transduction proteins, regulating gene expression and participating in determination between apoptosis and survival in response to stresses. Activated Ire1 recruits the TRAF2 and ASK1, which results in formation of the Ire1-TRAF2-ASK1 complex leading to JNK activation (18, 19).

The third pathway is activation of the ER-localized cysteine protease, caspase-12 (20). Caspase-12 is activated by ER stress, but apparently not by death receptor-mediated or mitochondria-targeted apoptotic signals. It was reported that caspase-12 is activated through activation of m-calpain (21), IRE1α/TRAF2 (22), or caspase-7 (23). Although targeted disruption of the caspase-12 gene in mice clearly demonstrated that caspase-12 mediates ER stress-induced apoptosis, the role of caspase-12 in humans is not yet clear (24).

Endoplasmic Reticulum Stress-Mediated Apoptosis in Pancreatic β-cells

As mentioned previously, β-cells play a central role in glucose homeostasis *in vivo*, and β-cell dysfunction leads to

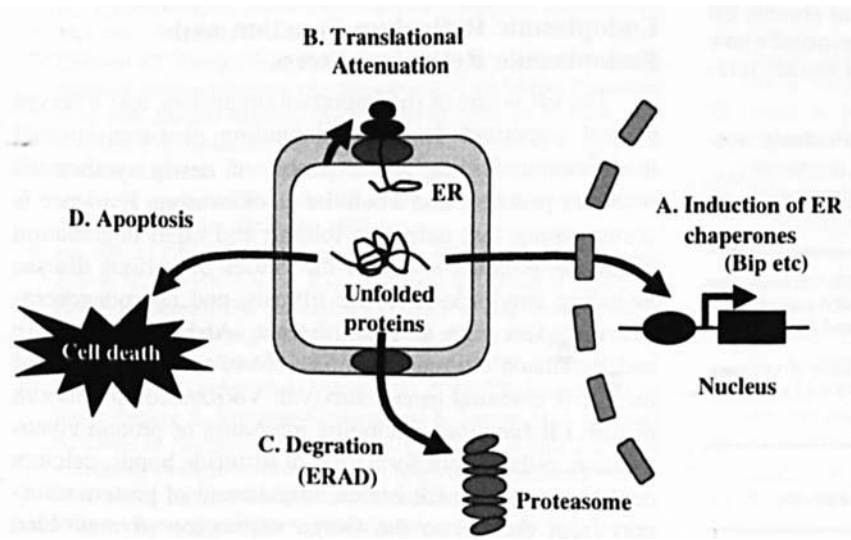

Figure 1. The ER stress response pathway (7). Accumulation of unfolded proteins in the ER activates four distinct cellular responses. (A) Transcriptional induction of ER chaperones increases protein folding activity and prevents protein aggregation. (B) Translational attenuation reduces the load of new protein synthesis and prevents further accumulation of unfolded proteins. (C) The ER-associated degradation (ERAD) pathway eliminates misfolded proteins by the ubiquitin-proteasome system. (D) When functions of the ER are severely impaired, apoptosis is induced to destroy the cell.

the development of diabetes. One of the characteristic features of β-cells is a highly developed ER, which can be important for insulin secretion. ER stress transducer proteins, including Ire1α, PERK, and Bip are highly expressed in pancreatic β-cells, probably reflecting the fact that β-cells are highly engaged in protein secretion. High expression of these ER stress transducer proteins could be necessary for strict quality control of secretory proteins, including insulin, in β-cells. Severe disturbance in protein folding in the ER leads to cell death, but susceptibility toward ER stress differs from cell to cell. Recent findings including our studies have revealed that β-cells represent one of the most susceptible tissues for ER stress, and ER stress-mediated apoptosis in β-cells can be a cause of diabetes *in vivo*.

MIN6 Cell, a β-Cell Line, Sensitive for NO-Induced Apoptosis

Nitric oxide (NO) has been reported to serve as a mediator of β-cell failure in Type I diabetes. NO is produced by inflammatory cytokines including interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and interferon-γ (IFN-γ) (2). The β-cells are highly vulnerable to NO but the mechanism underlying this phenomenon is unclear. It is generally believed that NO induces DNA damage leading to cell death through the poly (ADP-ribose) polymerase (PARP) pathway or p53 pathway (Fig. 2A). Studies on islet cells lacking PARP and p53-negative cell have suggested that there is another apoptosis pathway distinct from the DNA damage. Thus, we hypothesized that NO disturbs ER function and leads to apoptosis through the ER stress response pathway in β-cells.

We therefore studied the effect of NO on MIN6 cell, a mouse β-cell–derived cell line. When MIN6 cells were treated with an NO donner, S-nitroso-Nacetyl-D, L-penicillamine (SNAP), apoptosis of the cells occurred at 0.2 mM or higher concentrations of SNAP (Fig. 3A). However,

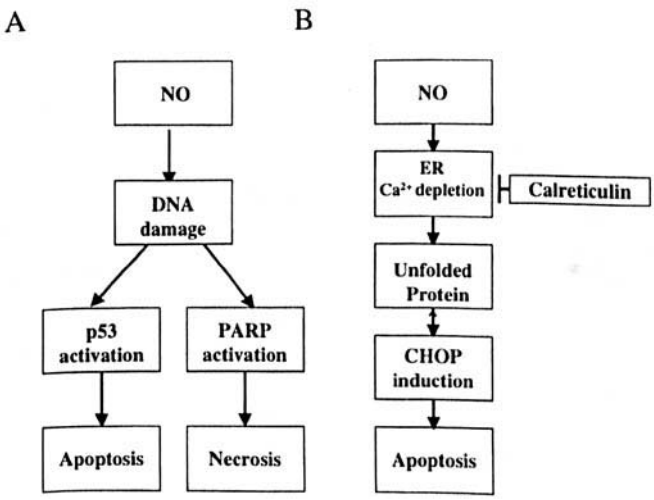

Figure 2. Proposed mechanisms of NO-induced cell death (13). (A) DNA damage pathway. (B) ER stress pathway.

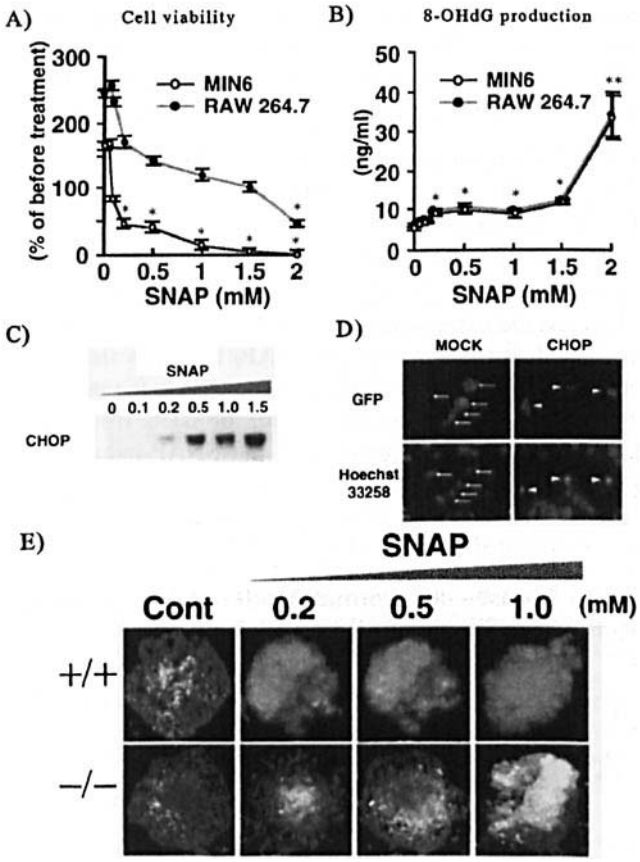

Figure 3. Induction of ER stress by NO in pancreatic β-cells (13). (A and B) MIN6 cells or RAW 264.7 cells were treated with various concentrations of SNAP. Cell viability (A) and 8-OHdG production (B) were examined. * Indicates significant decrease ($P < 0.05$) compared with before treatment (A). *$P < 0.05$, **$P < 0.001$ vs. non-treated cells (B). (C) MIN6 cells were treated with indicated concentrations of SNAP, and CHOP mRNA expression was detected by Northern blot analysis. Induction of CHOP mRNA was detected at 0.2 mM or higher concentrations of SNAP. (D) Overexpression of CHOP introduces apoptosis in MIN6 cells. MIN6 cells were co-transfected with pEGFP and pOPRSVI-CAT (Mock) or pOPRSVI-CHOP (CHOP; CHOP expression vector). Transfected cells and apoptotic cells were detected by GFP fluorescence and by Hoechst 33258 staining, respectively. Cells overexpressing CHOP (shown by arrowhead) but not those with Mock transfection (shown by arrows) were apoptotic. (E) Islets from CHOP knockout mice were resistant to NO-induced apoptosis. Islets were isolated from wild-type (+/+) or CHOP knockout (−/−) mice, and were stimulated with indicated concentrations of SNAP. The red fluorescence represents normal cells, and green fluorescence represents apoptotic cells.

the concentration of DNA damage marker, 8-hydroxy-2'-deoxyguanosine (8-OHdG), increased only very slightly from 0.2 mM to 1.5 mM and markedly increased from 2 mM or higher concentrations of SNAP (Fig. 3B). On the other hand, RAW 264.7 cells, a mouse macrophage-derived cell line, exhibited inhibition of cell proliferation but not apoptosis at 0.5 to 1.5 mM, and showed apoptosis only at 2 mM or higher concentrations of SNAP, which paralleled with the increase in 8-OHdG (Fig. 3A, 3B). The apoptosis of the MIN6 cell by SNAP was paralleled with the induction of CHOP mRNA, which occurred at 0.2 mM SNAP (Fig. 3C). Therefore, the apoptosis of MIN6 cells by low con-

centration of SNAP seemed to be mediated through the ER stress pathway. The involvement of CHOP in apoptosis of MIN6 cell was further confirmed by introduction of apoptosis in the cells by overexpression of CHOP (Fig. 3D).

C/EBP Homologous Protein Knockout Mouse—Resistant to NO-Induced β-Cell Apoptosis

To further confirm that the β-cell apoptosis caused by NO is induced by the ER stress-mediated pathway, impact of NO on the islets isolated from CHOP knockout mice was evaluated. By treatment with SNAP, the islets from wild-type mice showed dose-dependent increase in the number of apoptotic cells. On the other hand, the islets from CHOP knockout mice were resistant to the SNAP-induced apoptosis (Fig. 3E), suggesting that the apoptosis of β-cells induced by NO was, at least in part, induced through the ER stress-mediated pathway (Fig. 2B).

Akita Mouse—an Animal Model of Endoplasmic Reticulum Stress-Mediated Diabetes

The Akita mouse is a spontaneously diabetic model reported by Yoshioka *et al.* (25), and is characterized by progressive hyperglycemia with reduced β-cell mass without insulitis or obesity (26). Genetic analysis revealed that a mutation in the insulin 2 gene (*Ins2*) (Cys96Tyr) is responsible for the diabetic phenotype in this mouse (27). This mutation changes cysteine to tyrosine of proinsulin and thus disrupts a disulfide bond formation between its A and B chains, and is considered to induce a drastic conformational change of this molecule (Fig. 4A).

The mouse has two non-allelic insulin genes (*Ins1* and *Ins2*) and *Ins2* transcripts represent the majority of total insulin in wild-type mice. Although heterozygous Akita mice develop severe diabetes, single *Ins1* or *Ins2* knockout mutant mice do not develop diabetes (28). Therefore, the phenotype of Akita mice does not merely reflect the production of non-functional insulin but rather indicates that the mutant insulin of Akita mice itself has significant impact on β-cell dysfunction. We hypothesized that ER stress in β-cells could cause apoptosis and lead to the development of diabetes *in vivo*, and the Akita mouse could serve as a good model of ER stress-mediated diabetes.

In fact, we found that progressive hyperglycemia in the Akita mouse is accompanied by CHOP induction and β-cell

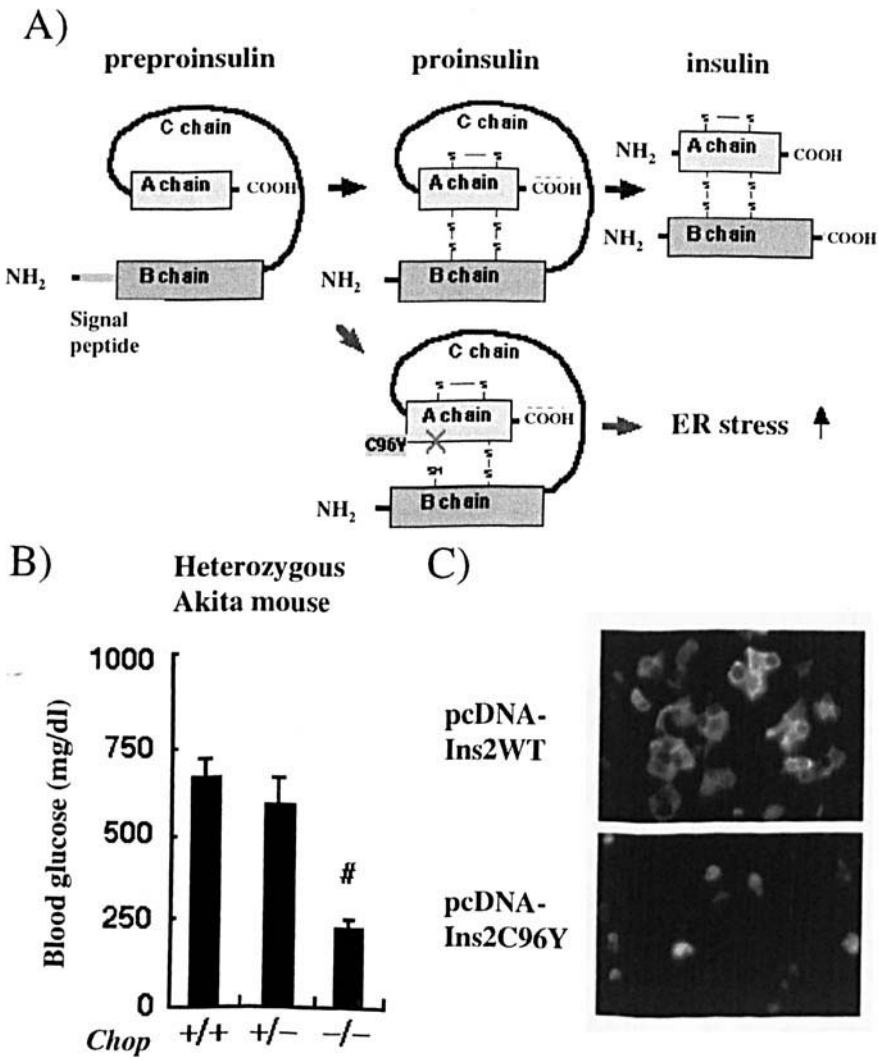

Figure 4. Studies in the Akita mouse (14). (A) Processing of insulin and its mutation in the Akita mouse. In the ER, preproinsulin is processed to proinsulin by cleavage of signal peptide and disulfide bonds are formed between A and B chains. In the Akita mouse, missense mutation in the insulin 2 gene, which replaced cysteine by tyrosine at position 96, disrupts a disulfide bond formation and induces a drastic conformational change of this molecule. This mutant insulin could cause ER stress leading to β-cell death. (B) Hyperglycemia was prevented by disruption of CHOP gene in heterozygous Akita mice. The Akita mouse was crossed with the CHOP knockout mouse. Fasting blood glucose levels were measured in each indicated genotype mouse groups (male mice) at 8 weeks of age. (C) MIN6 cells that overexpressed mutant insulin of Akita mouse showed apoptosis. MIN6 cells were cotransfected with pEYFP (an ER localization vector that expresses enhanced yellow fluorescent protein) and either pcDNA-$Ins2^{WT}$ (that expresses wild type insulin 2) or pcDNA-$Ins2^{C96Y}$ (that expresses mutant insulin 2 of Akita mouse). The transfected cells were observed under a fluorescence microscope (× 200). Cells expressing $Ins2^{WT}$ showed ER-like fluorescence, whereas cells expressing $Ins2^{C96Y}$ became round and detached from dishes.

apoptosis (14). In the development of diabetes in the mice, mRNAs for Bip and CHOP were induced in the pancreas. Overexpression of the mutant insulin of Akita mouse in MIN6 cells induced CHOP expression and led to apoptosis (Fig. 4C).

To examine if CHOP is involved in the development of diabetes *in vivo* in the Akita mouse, we crossed the Akita mouse with the CHOP knockout mouse and studied the effect of CHOP knockout in the Akita mouse. As expected, disruption of the CHOP gene delayed the onset of diabetes in heterozygous Akita mice (Fig. 4B). However, in homozygous Akita mice, disruption of the CHOP gene did not delay the onset of diabetes. Therefore, it is confirmed that CHOP is involved in the development of diabetes in the Akita mouse and other pathways such as the JNK pathway and the caspase-12 pathway may also be involved in the apoptosis.

Conclusions and Implications of the Results

Our recent findings suggest the involvement of the ER stress in β-cells apoptosis, which could modify the development of both Type I and Type II diabetes. The protein folding is easily perturbed by physiological and various pathological conditions. Increased demand for insulin secretion under certain conditions, such as obesity, insulin resistance, and long-term treatment with sulfonylureas, may result in β-cell overload. β-cell dysfunction under these conditions is often termed "pancreatic β-cell exhaustion". Overload of β-cells may induce chronic ER stress, cause β-cell dysfunction, and finally lead to a reduction in β-cell mass via apoptosis.

A comprehensive understanding of the mechanisms of protein folding and how they relate to the development of diabetes will contribute to provide new targets for the prevention and management of this disease.

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