Experimental Diabetes Research

Mitochondria and Endoplasmic Reticulum in Diabetes and Its Complications

Guest Editors: Ki-Up Lee, Robert A. Harris, In-Kyu Lee, and Sayon Roy

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Editorial Board

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Editorial

Mitochondria and Endoplasmic Reticulum in Diabetes and Its Complications

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Recent studies have provided evidence that mitochondrial dysfunction and endoplasmic reticulum (ER) stress are major pathogenic factors for diabetes and its complications. Mitochondria are the most important source of the chemical energy required by cells. Regulation of the role that mitochondria play in the metabolism of glucose and fatty acids, the primary fuels used by cells to produce ATP, has been the subject of intensive research for several decades. In spite of this, much remains to be learned about tissue-specific fuel selection and the pathogenesis of diabetes and its complications. Mitochondria also play an important role in the generation of reactive oxygen species (ROS) and cell apoptosis. The ER is a major subcellular compartment involved in calcium homeostasis, lipid synthesis, and protein folding and maturation. Various factors that interfere with ER function lead to accumulation of unfolded proteins. This triggers downstream signaling pathways, which is called the unfolded protein response (UPR). Although this is an adaptive mechanism to resolve ER stress, chronic UPR activation may lead to cell injury. Cellular homeostasis also depends upon the functional relationship between mitochondria and the ER. Propagation of calcium signaling from ER to mitochondria is involved in both ATP production and cell death. On the other hand, the ER requires ATP to function properly, which may make it the best site for sensing metabolic stress.

In this special issue of the journal we have assembled several invited reviews, from well-recognized investigators, on the roles of mitochondrial dysfunction and ER stress in the pathogenesis of diabetes and its complications. Some papers also deal with important issues like mitochondrial biogenesis, mitochondrial fusion/fission, and autophagy in the diabetic state.

Dr. A. Naudi et al. reviewed the mechanism of cellular dysfunction in response to mitochondrial oxidative stress. Increases in plasma glucose and free fatty acid (FFA) cause mitochondrial overproduction of ROS. This leads to several maladaptive responses including blockade of glycolysis and accumulation of upstream glycolytic metabolites, PARP activation and consequent increases in the production of inflammatory mediators, and protein oxidative damage. They also suggested the use of antioxidants, uncouplers, or PARP inhibitors for the prevention or reversal of diabetic complications.

Dr. Z. A. Ma et al. discussed the molecular mechanism of mitochondrial dysfunction-induced cell injury. In pancreatic beta cells, mitochondrial ROS produced by metabolic stress activates UCP2, which leads to proton leak across the mitochondrial inner membrane. This reduces beta cell synthesis of ATP and reduces glucose-stimulated insulin secretion. In addition, ROS oxidizes polyunsaturated fatty acids in mitochondrial membrane phospholipids (cardiolipin), and this impairs membrane integrity and leads to cytochrome c release into the cytosol and apoptosis. Group VIA phospholipase A_2 ($iPLA_2$beta) appears to provide a mechanism for repairing mitochondrial phospholipid damage. The authors suggested that interventions that attenuate the adverse effects of ROS on beta-cell mitochondrial phospholipids may represent a means for preventing the development of type 2 diabetes.

Dr. B. Ponugoti et al. reviewed the role of the FOXO family of forkhead transcription factors in the regulation of cellular oxidative stress response pathways. FOXO proteins are known to play an important role in protection of cells against oxidative stress. However, in response to certain ROS levels, FOXO proteins switch from prosurvival to proapoptotic signaling, resulting in cell death. In the diabetic state, the induction of FOXO by hyperglycemia plays an important role in the generation of proinflammatory cytokines. On the other hand, insulin signaling inactivates FOXO1. The authors suggested that activated FOXO1 disrupts the mitochondrial electron transport chain, negatively affecting fatty acid oxidation.

Dr. A.-M. Joseph et al. reviewed skeletal muscle mitochondrial metabolism with special emphasis on mitochondrial biogenesis, mitochondrial fusion/fission, and autophagy. Mitochondrial biogenesis is induced by numerous physiological, environmental, and pharmacological stimuli and is regulated by various factors including PGC-1, NRF 1/2, and SIRT1–7. In the diabetic state, these processes become deregulated and the ability of the cell to respond to environmental changes is diminished. The potential to stimulate mitochondrial biogenesis through physiological interventions such as exercise, caloric restriction, or pharmacological mimetics of mitochondrial biogenesis can be promising in improving insulin sensitivity. The paper also described mitochondrial dynamics (fusion/fission) and autophagy. Levels of the fusion proteins Mfn2 and OPA1 are reduced in skeletal muscles of diabetic patients, suggesting mitochondrial fusion is an important signaling event for mitochondrial biogenesis in muscle.

Dr. S. H. Back et al., Dr. U. Karunakaran et al., Dr. B. Basha et al., and Dr. J. Xu et al. separately reviewed the roles of ER stress in the pathogenesis of beta-cell failure, endothelial dysfunction, and diabetic cardiomyopathy, respectively. ER plays a central role in protein folding and in quality control of newly synthesized proteins. The ER also serves as an essential site for synthesis of lipids and for high-capacity buffering of intracellular calcium. Increased demand or decreased ability to fold proteins in the ER leads to accumulation of unfolded proteins in the ER, a state called "ER stress." In this state, a series of compensatory mechanisms are induced, collectively termed UPR, that include inhibition of protein translation, increased expression of ER chaperones, ER-associated degradation, and cell apoptosis. Although this is an adaptive mechanism to resolve ER stress, chronic UPR activation may lead to cell injury. In the diabetic state, high plasma glucose and FFA levels are well known to cause cellular dysfunction and injury, which is called "glucotoxicity," "lipotoxicity," and "glucolipotoxicity" when the two are combined. These metabolic stresses are known to induce ER stress and UPR.

Dr. Y. Tanaka et al. reviewed the role of autophagy in the pathogenesis of diabetic nephropathy. Autophagy is a major catabolic pathway involved in degrading and recycling macromolecules and damaged organelles to maintain intracellular homeostasis. This dynamic process involves membrane formation and fusion and includes autophagosome formation, autophagosome-lysosome fusion, and the degradation of intra-autophagosomal contents by lysosomal hydrolases. The authors summarized recent studies showing that autophagy in podocytes and renal tubular epithelial cells plays a renoprotective function under several pathologic conditions.

Finally, Dr. J. Leem and Dr. E. H. Koh reviewed the functional relationship between mitochondria and the ER. Recent studies using electron tomography demonstrated that the outer mitochondrial membrane and the ER are joined by tethers, enabling ER proteins to associate directly with proteins and lipids of the outer mitochondrial membrane. Many papers to date have shown that ER stress induces mitochondrial dysfunction to cause cell apoptosis. In addition, recent lines of evidence suggest that the reverse is also happening; mitochondrial dysfunction induces ER stress to decrease adiponectin synthesis in adipocytes and to cause hepatic insulin resistance. Thus, it appears that bidirectional communications exist between these two organelles. Future studies are needed to carefully dissect the interactions between mitochondria and ER.

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Review Article

Endothelial Dysfunction in Diabetes Mellitus: Possible Involvement of Endoplasmic Reticulum Stress?

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The vascular complications of diabetes mellitus impose a huge burden on the management of this disease. The higher incidence of cardiovascular complications and the unfavorable prognosis among diabetic individuals who develop such complications have been correlated to the hyperglycemia-induced oxidative stress and associated endothelial dysfunction. Although antioxidants may be considered as effective therapeutic agents to relieve oxidative stress and protect the endothelium, recent clinical trials involving these agents have shown limited therapeutic efficacy in this regard. In the recent past experimental evidence suggests that endoplasmic reticulum (ER) stress in the endothelial cells might be an important contributor to diabetes-related vascular complications. The current paper contemplates the possibility of the involvement of ER stress in endothelial dysfunction and diabetes-associated vascular complications.

1. Introduction

Diabetes mellitus (DM) is a growing metabolic disease that continues to be a leading health problem worldwide. The World Health Organization (WHO) has estimated that there are currently 346 million people affected by diabetes worldwide and anticipates that diabetes-related deaths would double by 2030 [1]. These figures highlight the importance of continued research and the need for novel methods to both prevent and treat this pandemic.

The adverse long-term effects of DM involve many organ systems and are associated with a complex pathology involving a large number of secondary cellular and subcellular changes. While diabetes management has largely focused on control of hyperglycemia, the rising burden of this disease is mainly correlated to its vascular complications [2, 3]. This is reflected by a 4-fold increase in the incidence of coronary artery disease, a 10-fold increase in peripheral vascular disease, and a 3- to 4-fold higher mortality rate with as much as 75% of diabetics ultimately dying from vascular disease [4]. Type II diabetes differs principally from type I diabetes in that it is accompanied by a period of hyperinsulinemia and is characterized by late as opposed to early onset of hyperglycemia. In type I DM, vascular involvement (through endothelial dysfunction) occurs as a result of metabolic insult/hyperglycemia, while in type II DM, endothelial dysfunction plays a more direct role, and is aggravated by, rather than caused by, hyperglycemia [5].

The high glucose-induced "*oxidative stress*" and "*endoplasmic reticulum (ER) stress*" (which might be dependent on oxidative stress) of the endothelium may play major roles in the initiation and progression of cardiovascular clinical manifestations in diabetes. The following sections of this paper will provide evidence on the molecular mechanisms of endothelial dysfunction in diabetes with special reference to the role of hyperglycemia/oxidative stress-induced ER stress in the endothelium.

2. Endothelial Dysfunction and Vascular Complications in Diabetes

Endothelial cells line the internal lumen of all the vasculature and serve as an interface between circulating blood and vascular smooth muscle cells (VSMCs) [6]. Apart from being the key participant during the process of angiogenesis, these dynamic structures can actively regulate basal vascular tone and vascular reactivity in physiological and pathological conditions. They respond to mechanical forces

and neurohumoral mediators by releasing a variety of relaxing and contracting factors such as nitric oxide (NO) and prostacyclin [7]. The balance between the vasodilatation and vasoconstriction is maintained by the endothelium, and the disruption of this balance leads to endothelial dysfunction and causes damage to the arterial wall [8, 9]. Endothelial cells are also responsible for the maintenance of blood fluidity and restoration of vessel wall integrity (when injured) to avoid bleeding [7]. Endothelial cell-derived factors also are critical mediators of VSMC growth and inflammation [10].

Loss of function/regulation of function of the endothelium (endothelial dysfunction) in a basal state or after activation may be a critical and initiating factor in the development of diabetic micro- and macrovascular disease [2, 11]. Blood glucose levels continue to be the principal link between diabetes and vascular disease. In diabetics and normal subjects, results of glucose tolerance tests have shown rapid loss of brachial artery endothelium-dependent vasodilatation—with fast recovery in normal subjects and a slower recovery in diabetics [12]. This emphasizes that even in nondiabetics, postprandial exposure to elevated blood glucose can disturb the endothelium-dependent regulation of blood flow [13].

The microvascular complications in diabetes include retinopathy, nephropathy, and neuropathy, while macrovascular complications are manifested in ischemic heart disease, stroke, and peripheral vascular disease [3, 14]. "*Aberrant*" angiogenesis (one of the main functions of endothelial cells) is one of the main reasons for these vascular complications associated with diabetes [15]. However, it must be noted that diabetic vascular complications associated with aberrant/altered angiogenesis may either be caused by excessive angiogenesis (retinopathy, nephropathy) or deficient angiogenesis (impaired wound healing, impaired collateral vessel formation, neuropathy, embryonic vasculopathy, and transplant rejection) [15].

Several diabetes-related studies have identified an array of molecular entities and pathways that influence endothelial function and reported how altered endothelial function and changes in endothelial cell-derived factors can lead to vascular complications in a hyperglycemic setting. There is, however, no distinct pathway that causes vascular disease in diabetes, due to the simple fact that the dysregulation of glucose homeostasis (the defining feature of diabetes) can in itself occur either in the absence or presence of insulin, not to mention other frequent comorbid diseases such as hypertension and obesity. Therefore, the cellular processes underlying endothelial dysfunction are not yet clearly understood, and multiple mechanisms are probably involved. A better understanding of endothelial dysfunction in a diabetic setting would aid in the search for novel approaches in the prevention and treatment of diabetes vascular disease.

3. Etiology of Endothelial Dysfunction in Diabetes

Type II diabetes is characterized by three main metabolic disturbances (triggers): (1) *hyperlipidemia*, (2) early *hyperinsulinemia,* and (3) hyperinsulinemia followed by pancreatic β-cell failure leading to *hyperglycemia* [16]. Each of these metabolic disturbances acts as "triggers" eventually causing endothelial dysfunction through the influence of different "mediator" molecules [17–19]. In a clinical setting it might be difficult to validate how much damage is caused in terms of endothelial dysfunction by each of these metabolic changes. However, several lines of evidence point to the fact that "oxidative stress" caused by these metabolic changes plays a key role in endothelial dysfunction [7, 20].

Among these metabolic changes hyperglycemia has been recognized as the primary cause in the pathogenesis of diabetic vascular disease and other complications. Hyperglycemia-induced increase in glucose oxidation and mitochondrial generation of superoxide anion ($O_2^{\bullet -}$) in turn leads to DNA damage and activation of poly (ADP ribose) polymerase (PARP) as a reparative enzyme [16, 21]. PARP-induced ADP ribosylation of glyceraldehyde phosphate dehydrogenase (GAPDH) then diverts glucose from its glycolytic path into alternative biochemical pathways leading to increase in advanced glycation end products (AGEs), hexosamine and polyol flux, and activation of classical isoforms of protein kinase C, that are considered the mediators of hyperglycemia-induced cellular injury [16, 21–25]. Though there are several different pathways involved in hyperglycemia-mediated endothelial dysfunction, evidence suggests that all these different pathways/mechanisms that are induced by hyperglycemia lead to considerable generation of reactive oxygen species (ROS), which is responsible for the oxidative stress; however, metabolism of glucose may not be a requirement for the generation of ROS [26]. Oxidative stress or the increase in the levels of ROS in the biological system is known to occur due to increased activity of the ROS-generating systems and/or decreased antioxidant defense mechanisms [27]. The excessive ROS so formed can then aggravate cellular injury by promoting activation of the biochemical pathways (autooxidation of glucose, AGE formation, activation of the polyol pathway, and stimulation of the eicosanoid metabolism) that initiate ROS generation in the first place as a response to hyperglycemia, thus completing a vicious cycle [28] (Figure 1).

The excessive ROS generation is known to impair endothelial nitric oxide synthase (eNOS) activity and NO production thereby affecting endothelium-dependent vasodilation [23]. Hyperglycemia-induced oxidative stress has also been associated with increased endothelial cell apoptosis *in vitro* and *in vivo* [29]. Several antioxidant therapies were found to improve or normalize the endothelium-dependent responses in different models of diabetes and hyperglycemia as well as significantly decrease the hyperglycemia-induced apoptosis [28–30]. Both experimental and clinical evidence thus suggest compromised endothelial function in diabetic conditions and identified hyperglycemia and/or hyperglycemia-mediated oxidative stress as the major causative factor for the cardiovascular pathological conditions that follow [7, 27, 28].

4. Molecular Basis of Endothelial Dysfunction in Diabetes—Current Understanding

Endothelial cells have multiple physiological functions, and therefore alterations in endothelial cell function may affect

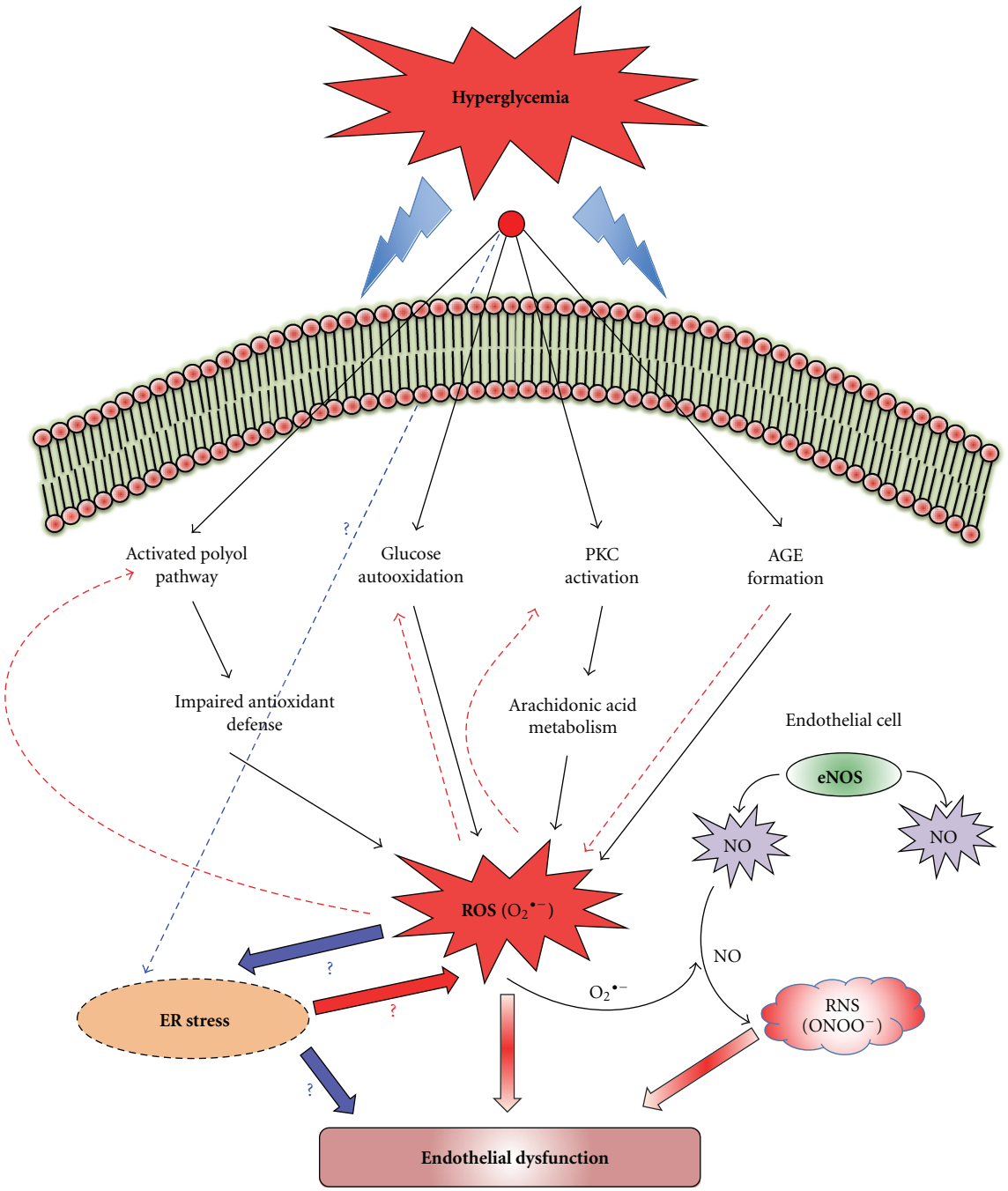

Figure 1: *Hyperglycemia-induced oxidative stress and endothelial dysfunction (possible role of ER stress)*. High glucose levels in circulation can divert glucose into alternative biochemical pathways leading to the increase in advanced glycation end products (AGEs), glucose autooxidation, hexosamine and polyol flux, and activation of classical isoforms of protein kinase C, that are considered to be the mediators of hyperglycemia-induced cellular injury. Many different pathways involved in hyperglycemia-mediated endothelial dysfunction induced by hyperglycemia lead to considerable generation of reactive oxygen species (ROS), which is responsible for the oxidative stress. The excessive ROS so formed can then aggravate cellular injury by promoting activation of the biochemical pathways (red dotted arrows) that initiate ROS generation in the first place as a response to hyperglycemia, thus completing a vicious cycle. Superoxide anion ($O_2^{\bullet -}$) can also react with NO to yield peroxynitrite which is also known to be a mediator of endothelial dysfunction. It is still unclear whether the ER stress response can be initiated as a direct response to the increasing load on protein synthesis and maturation due to hyperglycemia or due to the hyperglycemia-associated oxidative stress. The possibility that ER stress response also can lead to excessive ROS formation cannot be ruled out.

one or more of these systems, either simultaneously or at distinct time periods [7]. Thus, either an increase or a decrease in any of the endothelial cell-related chemical messengers and/or alterations in any of the endothelial cell-related functions may contribute to the development of endothelial dysfunction [7].

In type I diabetic patients, reports of poor vasodilatation, increased blood levels of von Willebrand factor (vWF), thrombomodulin, selectins, type IV collagen, and so forth are indicators of endothelial cell dysfunction [7]. These pathological changes can then induce alterations in the vasculature and is known to support the progression of the disease condition. endothelin-1 (ET-1), a potent vasoconstrictor and mitogen produced by endothelial cells that act on VSMCs, is significantly increased in hyperglycemic/diabetic conditions and plays a critical role in the development of vascular diseases [31, 32]. Additionally, increased activation of protein kinase C (PKC), angiotensin II (ANG-II), increased levels of advanced glycation end products (AGEs), increased plasminogen activity inhibitor-1 (PAI-1), and so forth have been related to endothelial dysfunction in diabetes.

Increased inflammatory activation has also been linked to diabetic vascular complications [5]. Human aortic endothelial cells (HAECs) exposed to transient hyperglycemia have exhibited epigenetic changes in the p65 subunit of NFκB gene, with increased expression of monocyte chemoattractant protein -1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), interleukin-6 (IL-6), and inducible nitric oxide synthase (iNOS) [33, 34]. Also, a rise in the soluble forms of VCAM-1 and ICAM-1 (leukocyte adhesion molecules) has been observed in diabetic patients and is associated with an increased cardiovascular disease risk [35, 36]. Furthermore, there is a moderate elevation in plasma C-reactive protein levels in diabetic and atherosclerotic patients, which could be a manifestation of low-grade chronic inflammation of vessels [37–40].

In vitro and *in vivo* studies demonstrated that the activity of eNOS and generation of nitric oxide (NO) are significantly reduced in endothelial cells exposed to a hyperglycemic environment [7, 9, 23, 41]. It has been demonstrated that mouse microvascular endothelial cells (MMECs) develop oxidative stress after exposure to high glucose [42]. NADPH oxidase is activated by high glucose and acts as the primary source of ROS (specifically $O_2^{\bullet -}$), and this activation promotes eNOS uncoupling and higher production of H_2O_2 and superoxide. The excess $O_2^{\bullet -}$ may also quench NO thereby reducing its bioavailability leading to reduced endothelium-dependent vasodilatation [43–45]. Reports have also suggested that hyperglycemia-induced $O_2^{\bullet -}$ production is accompanied by an increase in NO formation as a result of the activation of inducible NOS (iNOS) [21]. The $O_2^{\bullet -}$ and NO react to yield peroxynitrite ($ONOO^-$), a strong oxidant, which in turn is known to contribute significantly to endothelial dysfunction [46–48] (Figure 1). Peroxynitrite oxidizes tetrahydrobiopterin (BH_4), an essential cofactor in the regulation of eNOS and iNOS levels in endothelial cells, and its levels are affected by acute rises in plasma glucose [21, 42, 49–51]. BH_4 is known to stabilize the dimeric forms of NOS, which would otherwise remain as a monomer [21, 52–54]. BH_4 is therefore of prime importance in the maintenance of endothelial function, and it has been shown to be affected early in both humans and mice in type II diabetes [44, 55].

Recent evidence suggests that hyperglycemia-induced endothelial cell dysfunction may occur secondary to increased oxidant stress and a concomitant increase in ER stress [56, 57]. Although oxidative stress can induce ER stress, it is still unclear whether these cellular responses are causally linked or two independent effects of high glucose exposure. The following sections of this paper dwell upon the possible role of ER stress in endothelial dysfunction in a diabetic milieu.

5. ER Stress: General Overview

Apart from playing a key role as a central eukaryotic membranous organelle, formed in continuity with the outer membrane of the nuclear envelope and responsible for synthesis, folding, and maturation of membrane and secreted proteins, lipid biosynthesis, and calcium storage, the endoplasmic reticulum (ER) acts as the prime quality control centre and signal transducing entity that can sense and respond to changes in cellular homeostasis [58, 59]. The intra-ER milieu provides a Ca^{2+}- rich, unique oxidizing environment, which is critical for the formation of disulphide bonds and for protein folding and assembly into its precise native conformations prior to transport to the Golgi compartment [58, 59]. In addition, accuracy in protein folding is also monitored by the many Ca^{2+}-dependent molecular chaperones, which stabilize the protein folding intermediates [60].

The entry of unfolded polypeptide chains, as they are synthesized, into the ER remains largely variable, changing rapidly in response to the different ongoing cellular process, the environment, and the physiologic state of the cell [61, 62]. As such the ER must be highly receptive to these changes and critically respond in the best interest of maintaining cell integrity and normal function. The normal physiological state of the ER is challenged when the influx of the nascent unfolded polypeptides exceeds the processing capacity of the ER (ER stress) [63]. In such a scenario the cell responds to the increasing presence of unfolded proteins within the ER through the activation of an intricate set of integrated signaling pathways that relay information from the ER to the cytosol and the nucleus, aiming to restore normal ER and cellular functions [63–65]. In fact, a cell's life and death decisions are made by these pathways depending on whether the ER stress is resolved or not. These pathways, known as the *unfolded protein response (UPR)* or *ER stress response* are thus critical for normal cellular homeostasis, development of the organism, and are also known to play major roles in the pathogenesis of many diseases such as diabetes, obesity, inflammation, cardiovascular disorders, viral infections, neurodegeneration, and cancer [64, 66–71].

The UPR engages to alleviate ER stress by (1) transcriptional induction of UPR genes (e.g.: chaperones to enhance folding capacity), (2) translational attenuation of global protein synthesis (to reduce the ER workload and

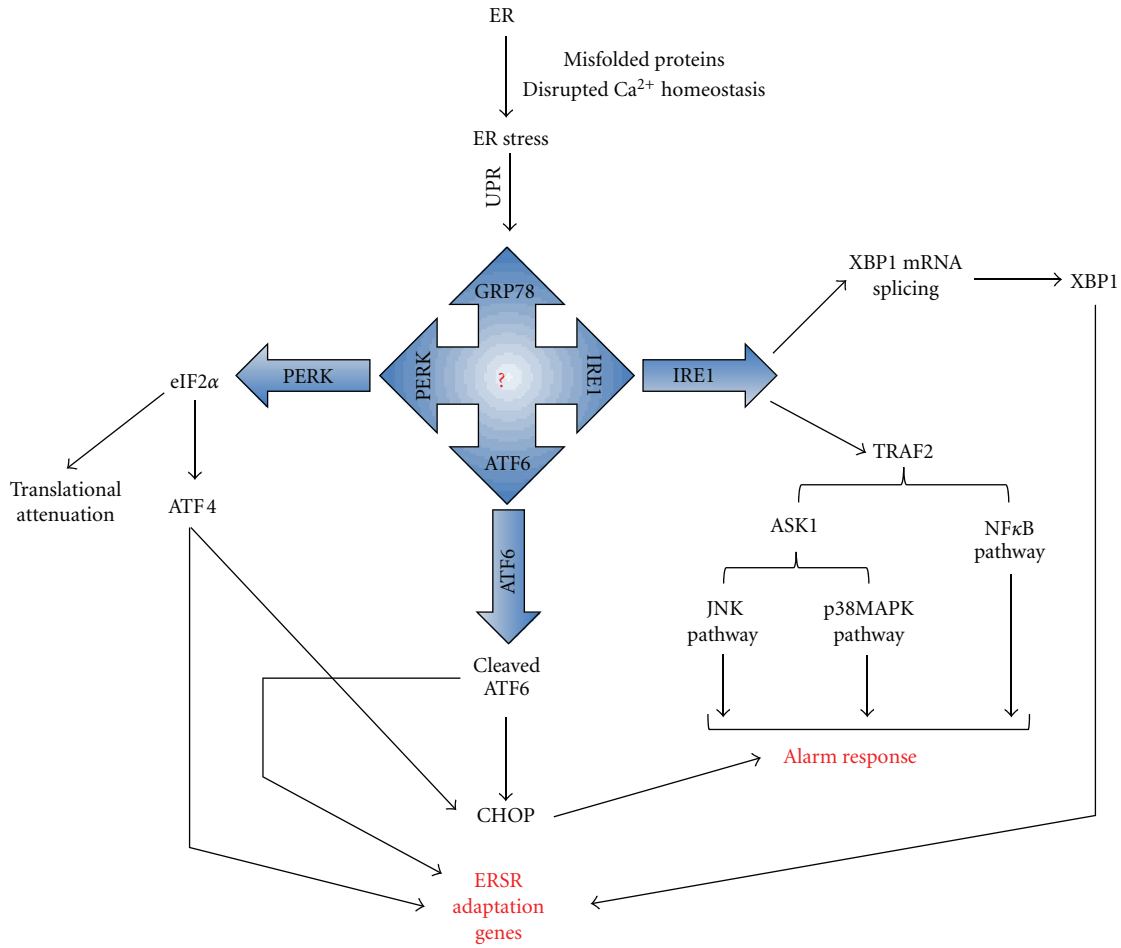

Figure 2: *Signal transduction events associated with ER stress.* The accumulation of misfolded proteins and disruption of Ca^{2+} homeostasis in the ER disrupt ER function leading to ER stress. The unfolded protein response (UPR) is initiated as a response to this stress, where GRP78, PERK, IRE1, and ATF6 play a central role. However, there might be more unknown mediators of ER stress. The cell initially tries to resolve the ER stress and restore normal cell function by halting protein synthesis and activation of several ER Stress Response (ERSR) adaptation genes, which include chaperones and proteins of the ER-associated degradation (ERAD) system. However, prolonged ER stress leads to the activation of the "alarm response", leading to cell damage, dysfunction, and finally apoptosis.

thus decreases further production of misfolded proteins), and finally (3) ER-associated degradation (ERAD, to remove and clear unfolded proteins from the ER lumen) [65]. This tripartite mode of response ensures that, in the event that the stress responses are insufficient to restore the ER environment (ER stress remains unresolved with an environment suboptimal for proper protein folding), the cell is destined for programmed cell death/apoptosis [66, 72–74]. At the molecular level the three main proximal transducers of ER stress are: (1) protein-kinase-like ER Kinase (PERK), (2) inositol requiring protein-1 (IRE1), and (3) activating transcription factor-6 (ATF6) [65] (Figure 2).

PERK, an ER transmembrane, dimerizes and undergoes trans-autophosphorylation upon ER stress. The active phosphorylated from of PERK phosphorylates the α-subunit of eukaryotic translation initiation factor 2-alpha (eIF2α), leading to reduced recognition of AUG initiation codons, translational attenuation, and thus reduction of unfolded proteins in the ER [53, 60, 63]. To ensure that the translational attenuation is reversed once the ER stress is relieved, the translation of mRNA encoding genes such as activating transcription factor-4 (ATF4), which have a lower requirement of eIF2, is enhanced [61]. ATF4 then induces growth arrest and DNA damage-inducible protein-34 (GADD34), which in turn recruits protein phosphatase-1 (PP1) to dephosphorylate the PERK-phosphorylated eIF2α thus reversing translational attenuation [61, 75] (Figure 3).

IRE1, a ubiquitously expressed ER transmembrane glycoprotein, is unique in the fact that it contains both kinase and endoribonuclease activities in the cytoplasmic domain [76]. Oligomerization and trans-autophosphorylation of IRE1 in response to ER stress activates its endoribonucleolytic activity, which then specifically excises an intron of the X-box binding protein-1 (XBP1) mRNA to generate a mature XBP1 mRNA [77]. This XBP1 mRNA with a new open reading frame encodes for a 376aa protein and a C-terminal

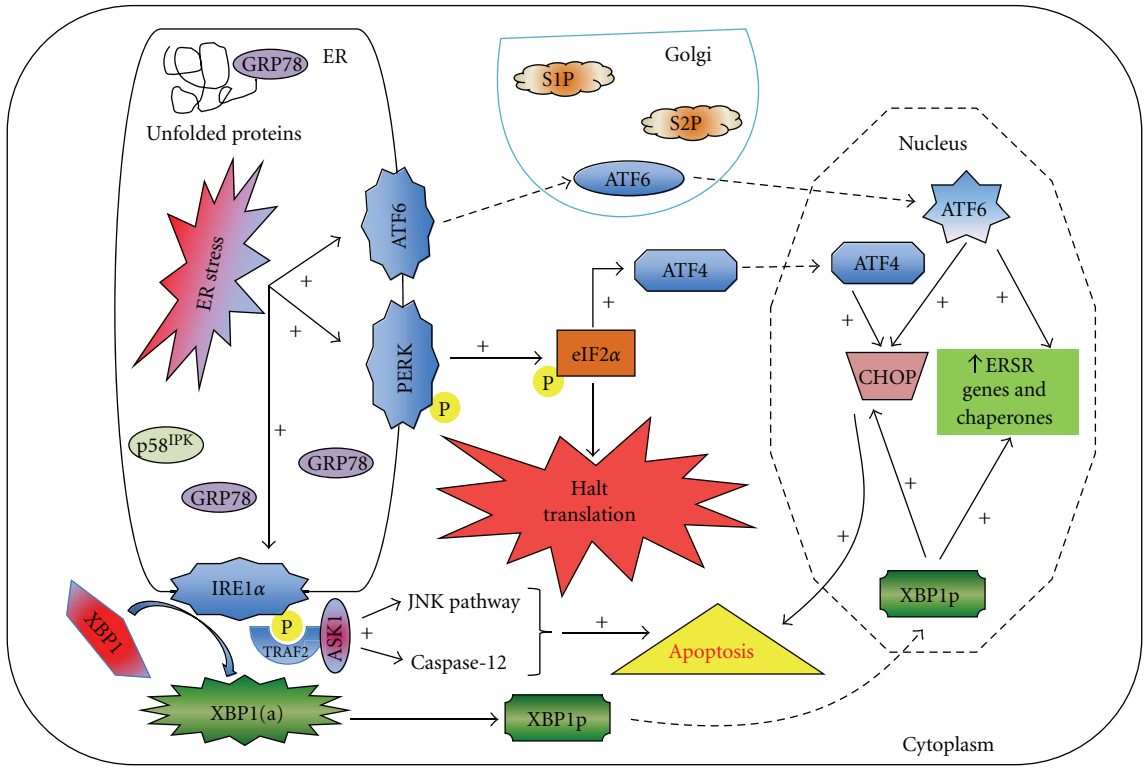

Figure 3: *Signaling by PERK, IRE1, and ATF6.* In an unstressed condition, GRP78, the primary sensor of ER stress binds to transmembrane ER proteins, PERK, IRE1, and ATF6, preventing their activation. The accumulation of unfolded proteins leads to dissociation of GRP78 from PERK, IRE1, and ATF6. GRP78, however, binds to the unfolded protein. GRP78 dissociation leads to PERK and IRE1 oligomerization and trans-autophosphorylation of their cytosolic domains. The active phosphorylated PERK (p-PERK, active) in turn phosphorylates eIF2α (p-eIF2α, inactive), leading to attenuation of global protein synthesis. Under these conditions, selected mRNAs, such as ATF4, are translated, which induces the expression of genes involved in restoring ER homeostasis. ATF4 can activate GADD34, which recruits a phosphatase to dephosphorylate p-PERK and reverse translational attenuation. ER-resident $p58^{IPK}$s is an inhibitor of PERK. Phosphorylation of IRE1 (p-IRE1) activates its endoribonucleolytic activity, which then excises an intron of the XBP1 mRNA to generate a mature XBP1 mRNA, which then encodes for the active protein, XBP1. The XBP1 protein then translocates into the nucleus and supports the ER stress response. Interaction of p-IRE1 with TRAF2 can elicit the activation of the JNK pathway and caspases leading to apoptosis. GRP78 release from the dimeric ATF6 leads to the translocation of its monomeric form to the Golgi, where it undergoes proteolytic processing by the proteases (S1P and S2P). The cleaved ATF6 then translocates to the nucleus where it activates the ERSR genes and chaperones. Alternatively, CHOP/GADD153 can be activated by ATF4, ATF6, or XBP1, which promotes apoptosis by decreasing the levels of antiapoptotic Bcl-2 in the cell. The dotted arrows denote translocation. XBP1 (a) denotes the mature XBP1 mRNA, while XBP1p denotes the protein product of XBP1 (a). The "+" sign signifies activation.

transcriptional activation domain and translocates to the nucleus which then induce numerous ER stress response genes [61, 76–78] (Figure 3).

ATF6 (670aa), though an ER transmembrane like PERK and IRE1, exists as a dimer (α and β) in the absence of ER stress [61–63, 65]. ER stress facilitates the translocation of the monomeric forms of ATF6 (~90 kDa) to the Golgi lumen where they are cleaved by the S1P and S2P proteases, yielding N-terminal cytosolic (~400aa, 50 kDa) fragments, which translocate to the nucleus and drive the activation of many ER stress response genes [61–63, 65] (Figure 3).

Although, PERK, IRE1, and ATF6 efficiently relay information to the nucleus regarding the accumulation of misfolded proteins in ER stress, the master regulator and sensor or ER stress seems to be the glucose regulated protein-78 (GRP78) [65]. GRP78 serves as a negative regulator of PERK, IRE1, and ATF6 by interacting with the luminal domains of these proteins in the absence of stress. The accumulation of unfolded proteins in the ER leads to the release of GRP78 (which binds to the unfolded proteins) from the transducers followed by subsequent activation of the UPR [61, 66]. While GRP78 dissociation form PERK and IRE1 permits their dimerization and activation, the release of GRP78 from the dimeric ATF6 permits its translocation to the golgi lumen where it is subjected to intramembrane proteolysis as mentioned earlier [79, 80] (Figure 3).

In conditions where the stress causing the UPR cannot be resolved, leading to the continuous accumulation of unfolded proteins in the ER, the aim of the UPR switches gears from a prosurvival mode to that of a proapoptotic mode [65]. Although well-known pathways such as the NFκB signaling, the JNK signaling pathway, and the p38MAPK pathway are known to play important roles in IRE1-mediated initiation of apoptosis, the contribution of

each of these pathways or crosstalk between them requires further experimental clarification [59]. The proapoptotic CHOP/GADD153 is upregulated downstream of ATF4, which in turn downregulates the antiapoptotic Bcl-2, favoring mitochondrial cytochrome c release, caspase activation, and eventually apoptosis [81].

6. ER Stress and Endothelial Dysfunction in Diabetes—Possible Links

ER stress and the UPR play important roles in the pancreatic islet cell survival and function [82]. Accumulation of misfolded mutant insulin (in type I diabetes) in the ER of pancreatic β-cells causes chronic ER stress [83]. PERK overactivation in such cases and lack of $p58^{IPK}$ (a PERK inhibitor), which is necessary in derepression of translation attenuation caused by PERK, are known to cause pancreatic β-cell apoptosis, leading to type I diabetes [84, 85]. Additionally, defective islet proliferation and increased ER stress-induced apoptosis were observed in PERK-deficient mice which are known to develop severe hyperglycemia soon after birth [86]. While mice on a high fat diet with heterozygous-targeted mutation of the PERK substrate, eIF2α, exhibit glucose intolerance, mice with homozygous mutation of the eIF2α phosphorylation site (Ser51Ala) die prenatally with diabetes and pancreatic β-cell deficiency [70, 87]. Obesity, one of the leading cause of type II diabetes, evokes ER stress in the peripheral tissues by activation of IRE1-induced JNK-dependent serine phosphorylation of IRS1, leading to insulin resistance [88]. While IRE1$\alpha-/-$ and XBP$-/-$ knockout mice are not viable, XBP$+/-$ mice are known to exhibit insulin resistance and type II diabetes [88–90].

The above-mentioned evidence, though suggestive of the possible role of ER stress in the development of diabetes, does not shed light on the role of ER stress and UPR in the vascular complications that follow hyperglycemia. The ER, exquisitely sensitive to glucose availability, depends on the blood glucose levels for the energy supply required for the protein folding process [91]. Endothelial cells, always exposed to elevations and reductions of blood nutrients, are very dynamic, metabolically active cells, with a high volume of protein synthesis, which predispose them to ER stress [92]. Endothelial cells in particular cannot tolerate the continued high glucose exposure, and thus ER stress is initiated in a diabetic milieu [93]. However, it is yet unclear whether endothelial dysfunction in response to high glucose levels occurs secondary to increased oxidative stress and a concomitant increase in ER stress or vice versa.

6.1. High Glucose-Induced Oxidative Stress and ER Stress—Double Edged Sword—Which Side Is Sharper? Several studies suggest that hyperglycemia-induced oxidative stress contributes to significant endothelial dysfunction and vascular complications in diabetes (briefly reviewed under the subheadings Etiology of Endothelial Dysfunction in Diabetes and Molecular Basis of Endothelial Dysfunction in Diabetes—Current Understanding, in this paper). Although there are some studies suggesting that glucose-induced ER stress is independent of oxidative stress in endothelial cells [94], a large body of evidence suggest that oxidative stress induces ER stress and vice versa [91, 95]. Since the ER maintains an oxidative environment which favours protein folding and maturation, increase in the protein synthesis and protein folding load in response to hyperglycemia can lead to the accumulation of ROS [96]. In order to limit the accumulation of ROS, the PERK pathway is known to activate an antioxidant program (including the GSH) though ATF4 and NRF2 activation [96]. Hyperglycemia-mediated changes in the PERK pathway may thus contribute to ROS accumulation due to impaired antioxidant response. Recently, paraoxonase-2 (PON2), an ER-resident enzyme, is known to reduce ROS generation in the ER, thus moderating ROS-activated ER stress and reducing apoptosis of endothelial cells [97, 98].

As mentioned earlier, UPR first aims to restore cellular homeostasis by controlling the accumulation of misfolded proteins in the ER. In particular, GRP78, the ER stress-induced chaperone, is sensitive to glucose concentration and shown to be induced in endothelial cells [99]. ER stress inducers such as thapsigargin and tunicamycin are known to induce GRP78 expression in endothelial cells [92]. GRP78 induction may also protect human endothelial cells from oxidative stress-induced cellular damage [100].

NADPH oxidase, the prime source of ROS in endothelial cells is activated in presence of high glucose with a concomitant decrease in the generation of NO [26, 42–44, 101, 102]. Nox1/Nox2 activation (produce superoxide anions) upon high glucose exposure leads to the formation of detrimental levels of ROS which can salvage NO to form $ONOO^-$ and cause eNOS uncoupling [103]. On the other hand, Nox4 (protective and produces H_2O_2 constitutively) activation leads to controlled ROS production which is known to play a significant role on endothelial cell signaling and vasodilatation [103]. NADPH oxidase (Nox1/Nox2) activation might link ER stress and oxidative stress to the high glucose-induced apoptosis of endothelial cells [104]. Nox2 activation and oxidative stress further amplify CHOP/GADD153 induction, which in turn promotes apoptosis. Reports suggest that CHOP induction and apoptosis as a response to ER stress is reduced in Nox2-deficient mice thereby preventing renal dysfunction [104]. This might be true for high glucose-exposed endothelial cells where Nox2 activation has shown to induce apoptosis [105] which might be possibly through the activation of CHOP-mediated ER stress response.

High glucose-induced ROS generation disrupt Ca^{2+} homeostasis leading to leakage of Ca^{2+} from the ER lumen [95, 106]. As mentioned earlier, intra-ER Ca^{2+} levels are critical, since many ER chaperones depend on Ca^{2+} for their activity [60]. A decrease in intra-ER Ca^{2+} can thus impair the UPR leading to a situation where the ER stress is not resolved when the cell must finally decide to undergo apoptosis [105]. In endothelial cells exposed to high glucose, disrupted Ca^{2+} homeostasis, due to ROS generation, can thus evoke an apoptotic response which may be induced by ER stress response mediators [107]. Additionally, increase in the cytosolic Ca^{2+} concentration can also stimulate mitochondrial ROS generation through various mechanisms

[95]. Increased mitochondrial Ca^{2+} loading can generate ROS as byproduct of the electron transport chain and can mediate cytochrome c release and apoptosis [108]. Ca^{2+} may also stimulate the Krebs cycle in endothelial cells thereby increasing O_2 consumption and ROS generation [109]. Ca^{2+}-induced stimulation of eNOS can yield large amounts of NO, which can react with the superoxide anion to form $ONOO^-$, thereby oxidizing BH_4 and promoting eNOS uncoupling [106].

6.2. Hyperglycemia-Induced Endothelial Cell Apoptosis—Role of ER Stress. ER stress response transducers initiate apoptosis once resolution of the stress has not been achieved. However, the point at which the "apoptotic switch" is activated in response to ER stress has not yet been elucidated. ER responses to unfolded proteins (adaptation, alarm, and apoptosis) are primarily mediated through IRE1 [59]. IRE1 activation leads to the activation of XBP1 (adaptation), tumor necrosis factor receptor-associated factor 2 (TRAF2) TRAF2 (alarm through NFκB), and JNK and p38MAPK pathways (apoptosis through ASK1 and caspase-12) [59, 73]. High glucose exposure of endothelial cells and high glucose-mediated oxidative stress in endothelial cells are known to activate ASK1 [110, 111]. ASK1 can also cause NO deficiency (a hallmark of endothelial cell dysfunction in diabetes) by regulating eNOS [112]. Oxidant stress-mediated ASK1 activation can cause downregulation of antiapoptotic Bcl-2, disruption of the mitochondrial membrane potential, and activation of a caspase cascade [113]. As mentioned earlier CHOP/GADD153 activates apoptosis by downregulating Bcl-2 [81]. Bcl-2 is known to be reduced in high glucose-exposed endothelial cells which may lead to the increase in intracellular AGE (characteristic of endothelial cell dysfunction in diabetes levels) [114, 115]. Linking the modulation of ASK1 and Bcl-2 levels in hyperglycemia, ER stress response might play a critical role in endothelial cell dysfunction in diabetes.

It has been shown that high glucose induces apoptosis of human endothelial cells through sequential activation of JNK and caspase-3 [116]. Studies suggest that caspase-12, an ER stress response mediator of apoptosis, can activate caspase-3 [73]. Blocking JNK and caspase-3 activity in high glucose-exposed human endothelial cells in culture prevented high glucose-induced apoptosis [116]. Additionally, vitamin C treatment (antioxidant) reduced JNK levels in these high glucose-exposed cells [116]. Given the role of ER stress response in JNK caspase-12 activation, the high glucose-induced ROS generation-mediated increase in endothelial cell apoptosis due to the activation of JNK and caspase-3 activity and its reversal with blockade of JNK/caspases-3 or antioxidant treatment indicate that ER stress might play a significant role in endothelial cell dysfunction in diabetes.

Tumor necrosis factor-α (TNFα), another known activator of the JNK pathway and inducer of apoptosis of endothelial cells, is known to be activated by high glucose-induced oxidative stress [117–120]. TNFα can in turn generate ROS and reduce activation of eNOS [121]. TNFα activation and its binding to its receptor TNFR1 recruit TRAF2 leading to downstream activation of NFκB, JNK and caspases which in turn decides cell death and survival decisions [120]. Increase in the levels of TRAF2 has been shown in high glucose-exposed endothelial cells [122]. TRAF2, known to be essential for ER stress induced IRE1 mediated UPR, can be an important link for the role of ER stress in endothelial cell dysfunction in diabetes.

$p58^{IPK}$ inhibits PERK, thereby inhibiting eIF2α phosphorylation and returning ER homeostasis in stressed cells [84]. $p58^{IPK}$ transfection may have significantly reduced apoptosis of retinal endothelial cells by decreasing both mRNA and protein levels of CHOP and TNFα in the retina of diabetic rats, thereby reducing retinal blood vessel leakage [123, 124]. As in the case of pancreatic β-cells [85], prolonged exposure of endothelial cells to high glucose levels might decrease $p58^{IPK}$ leading to ER stress-mediated endothelial cell dysfunction in diabetes.

6.3. Endothelial Cell Response to Insulin in Diabetes—Role of ER Stress. Cellular insulin resistance may be selective in terms of its nature and extent with respect to certain cell systems and may vary in terms of the metabolic, mitogenic, pro-survival, and vascular actions of insulin [16]. Protective effects of insulin include its ability to protect against apoptosis and to stimulate the production of NO [125, 126]. The role of insulin resistance at the level of the endothelial cell in vascular diseases is rather unclear.

The increase in the protein-folding demand and the signaling involving calcium and ROS induce the UPR, leading to the transcription of genes whose products mount an inflammatory response. An excess of glucose can further boost the UPR and inflammation, contributing to insulin resistance and apoptosis. The ER stress response-conferred insulin resistance in endothelial cells could also further promote inflammatory stress signaling and contribute to the metabolic deterioration that is associated with type II diabetes and vascular diseases. JNK and IκB kinase (IKK) are activated (through an IRE-1-alpha dependent fashion), in addition to proinflammatory genes, in response to ER stress in these cells as well [82, 127]. Activated JNK phosphorylates serine residues on IRS-1, thereby inhibiting it, and the overall insulin-signaling pathway. This in turn leads to insulin resistance through defective downstream signaling (causing less AKT activation and lower NO production) [82, 128].

7. Potential Therapeutic Strategies

Studies have brought to light the possible link between hyperglycemia-induced ER stress and endothelial cell dysfunction. Given the relationship between oxidative stress and ER stress, the probable first line of therapy would be the use of biologically known and pharmacologically available antioxidants. Thioredoxin-1 (Trx1), an extensively studied antioxidant, growth regulator, and antiapoptotic protein, is known to interact with and inhibit ASK1 activity [27], one of the prime mediators of ER stress-related apoptotic response. Recently, it was reported that hyperglycemia-induced oxidative stress was primarily due to the induction of Trx1 inhibitory protein (TXNIP), an endogenous inhibitor of Trx1 activity, leading to the inhibition of the antioxidant

function of Trx1 [129]. TXNIP binding to Trx1 further reduces the ability of Trx1 to bind efficiently with its other protein partners, such as ASK-1 thereby reducing the antiapoptotic property of Trx1 [129]. Studies are warranted to investigate the role of Trx1 and TXNIP in the induction of ER stress response to hyperglycemia in endothelial cells.

Preserving or restoring ER function might be therapeutic. Certain small molecules (chemical chaperones) such as 4-phenylbutyric acid and taurine-conjugated ursodeoxycholic acid were found to significantly reduce phosphorylation of PERK and IRE1α to improve glucose tolerance and insulin sensitivity [130]. A tetrameric form of resveratrol (vaticanol B) exhibited the capacity to inhibit UPR and inflammatory response by reducing protein-folding load and maintaining membrane integrity thereby preventing ER stress-induced apoptosis [131]. Salvianolic acid B was recently shown to protect endothelial cells from oxidative stress damage through the induction of GRP78 [100]. Salubrinal-induced dephosphorylation of eIF2α has shown to protect cells from ER stress-induced apoptosis [132].

ERAD involves the Ubiquitin-26S proteasome degradation pathway in the tagging and degradation of terminally unfolded proteins. Efficiency of the ubiquitin-proteasome system pathway in eliminating the unfolded proteins probably decides the fate of the cell [133]. A compromised ERAD/ubiquitin-proteasome system would mean that even though there has been a check on the synthesis of unfolded proteins through ER stress response, the cell is unable to get rid of accumulated misfolded proteins. This would mean that since the ER stress cannot be resolved the cell has to undergo apoptosis. Hyperglycemia is known to impair proteasome function [134]. The loss of function of the ubiquitin-proteasome system and continuing accumulation of unfolded proteins in the ER might be the "apoptotic switch" which when turned on demands the cell to switch gears from prosurvival mode to proapoptotic mode. Hence impaired ERAD/ubiquitin-proteasome system would play a significant role in hyperglycemia-induced ER stress and endothelial cell dysfunction. Therapeutic strategies to improve the efficacy of the ubiquitin-proteasome system thus could possibly confer protection from diabetes-associated endothelial cell dysfunction and oxidative stress.

8. Conclusion

In conclusion, the current paper suggests the role of diabetes-mediated ER stress in the endothelial cell dysfunction and accompanying cardiovascular diseases in diabetes. These possible links between hyperglycemia-induced ER stress and oxidative stress might offer the premises for conducting additional experiments to establish a unified molecular mechanism and thereby identify a potential therapeutic target in reducing vascular complications in diabetes. Elucidation of this molecular mechanism could also extend this to other pathological conditions where ER stress and oxidative stress might play a causal role. Thus a direct response to the current paper would be to test potential therapeutic agents (antioxidants and/or chemical chaperones) that might aid in long-term restoration of the endogenous capacity of endothelial cells to initiate and ER stress response in response to high glucose-induced protein misfolding, restore ER homeostasis, and escape apoptosis.

More studies are required to establish a time-dependent variation in the expression of ER stress proteins in a diabetic milieu. It is possible that in early stages of diabetes the endothelial cell may compensate for the ER stress that is associated with hyperglycemia while in later stages of advanced diabetes these compensatory mechanisms fail resulting in endothelial cell dysfunction and associated complications. Moreover, studies are required with adequate glycemic controls to examine whether the extent or duration of hyperglycemia could possibly regulate the expression of ER stress transducers.

Abbreviations

AGEs: Advanced glycation end products
ANG-II: Angiotensin II
ASK1: Apoptosis signal regulating kinase-1
ATF4: Activating transcription factor-4
ATF6: Activating transcription factor-6
BH_4: Tetrahydrobiopterin
CHOP: CCAAT/-enhancer-binding protein homologous protein/GADD153
DM: Diabetes mellitus
eIF2α: Eukaryotic translation initiation factor 2-alpha
eNOS: Endothelial nitric oxide synthase
ER: Endoplasmic reticulum
ERAD: ER-associated degradation
ERSR: ER stress response
ET-1: Endothelin-1
GADD153: Growth arrest and DNA Damage-inducible protein-153/CHOP
GADD34: Growth arrest and DNA Damage-inducible protein-34
GAPDH: Glyceraldehyde phosphate dehydrogenase
GSH: Glutathione
HAECs: Human aortic endothelial cells
ICAM-1: Intercellular adhesion molecule-1
IKK: I kappa B kinase
iNOS: Inducible nitric oxide synthase
IRE1: Inositol-requiring protein-1
JNK: c-Jun N terminal kinase
MCP-1: Monocyte chemoattractant protein-1
NFκB: Nuclear factor kappa B
NO: Nitric oxide
$O_2^{\bullet -}$: Superoxide anion
$ONOO^-$: Peroxynitrite
PAI-1: Plasminogen activity inhibitor-1
PARP: Poly(ADP) ribose polymerase
PERK: Protein-kinase-like ER kinase
PKC: Protein kinase C

PP1: Protein phosphatase-1
ROS: Reactive oxygen species
TNFR1: TNFα receptor-1
TNFα: Tumor necrosis factor alpha
TRAF2: TNF receptor-associated factor-2
Trx1: Thioredoxin-1
TXNIP: Trx interacting protein
UPR: Unfolded protein response
VCAM-1: Vascular cell adhesion molecule-1
VSMCs: Vascular smooth muscle cells
vWF: von-Willebrand Factor
WHO: World Health Organization.

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Review Article

Role of Forkhead Transcription Factors in Diabetes-Induced Oxidative Stress

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Diabetes is a chronic metabolic disorder, characterized by hyperglycemia resulting from insulin deficiency and/or insulin resistance. Recent evidence suggests that high levels of reactive oxygen species (ROS) and subsequent oxidative stress are key contributors in the development of diabetic complications. The FOXO family of forkhead transcription factors including FOXO1, FOXO3, FOXO4, and FOXO6 play important roles in the regulation of many cellular and biological processes and are critical regulators of cellular oxidative stress response pathways. FOXO1 transcription factors can affect a number of different tissues including liver, retina, bone, and cell types ranging from hepatocytes to microvascular endothelial cells and pericytes to osteoblasts. They are induced by oxidative stress and contribute to ROS-induced cell damage and apoptosis. In this paper, we discuss the role of FOXO transcription factors in mediating oxidative stress-induced cellular response.

1. Introduction

Diabetes mellitus is a chronic disease characterized by elevated blood sugar levels resulting from either lack of insulin production or resistance to insulin. In 2010, there were nearly 230 million individuals with diabetes worldwide which is estimated to reach 430 million by 2030 [1]. Recently, a study conducted by the U.S. Centers for Disease Control and Prevention (CDC) indicated that 25.8 million Americans or 8.3% of its population were affected by diabetes in 2010 [2]. Diabetes has severe health consequences associated with numerous diabetic complications including retinopathy, neuropathy, and nephropathy [3–5]. Accumulating evidence suggests that hyperglycemia-induced production of free radicals and the subsequent oxidative stress contributes to the development and progression of diabetes and related complications [6–8].

Reactive oxygen species (ROS) are oxygen free radicals that are generated as by-products of mitochondrial metabolism and function as signaling molecules in various intracellular processes including cell proliferation, migration, and apoptosis [9]. ROS produced during normal metabolic processes are removed rapidly with the help of various endogenous detoxifying enzymes. While normal cellular ROS concentrations are necessary for proper functioning of cells, excessive, non-physiological concentrations of ROS result in oxidative stress. ROS such as superoxide (O_2^-) and hydroxyl radicals ($HO^\bullet$), and hydrogen peroxide (H_2O_2), are highly reactive and can cause damage to biological macromolecules such as DNA, proteins, and lipids [9]. Major sources of oxidative stress during diabetes include glucose autooxidation, overproduction of ROS by mitochondria, non-enzymatic glycation, and the polyol pathway [6, 10]. In the polyol pathway, aldose reductase converts glucose into sorbitol with NADPH as a coenzyme. In diabetes, increased flux through the polyol pathway enhances oxidative stress because of increased consumption of NADPH by aldose reductase. Since NADPH is required for generation of endogenous antioxidant glutathione (GSH), reduced NADPH availability depletes GSH leading to greater oxidative stress [6]. Other mechanisms through which high glucose levels can lead to advanced glycation endproducts are discussed below.

ROS leads to the generation of intracellular signals that stimulate inflammation and cell death. They include protein kinase C (PKC), c-Jun-N-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) [11–15]. In many

cell types, ROS lead to the activation of the forkhead box O (FOXO) transcription factors that include FOXO1, FOXO3, and FOXO4, which can mediate the effects of ROS through regulation of gene transcription. These transcription factors have been implicated in diverse cellular processes ranging from glucose metabolism to cell behavior including cell cycle and apoptosis [16, 17]. In addition to being activated by ROS, FOXO proteins play a critical role in oxidative stress by upregulating expression of antioxidant genes [9]. However, FOXO proteins are involved in many other processes and can have apparently contradictory effects in different cell types [18]. FOXO proteins are transcription factors but also have important function as corepressors or coactivators so that direct DNA binding is not a prerequisite for modulating the transcription of gene targets [19]. For simplicity, we will use the term FOXO for all or any of the FOXO transcription factors throughout this paper, unless otherwise specified.

2. Regulation of FOXO by Oxidative Stress

FOXO transcription factors are critical mediators of oxidative stress and are activated by various kinds of cellular stress stimulus. Oxidative stress regulates FOXO activity through various posttranslational modifications including phosphorylation, acetylation, and ubiquitination, which in turn regulate the subcellular localization of FOXOs, protein-protein interactions, and transcriptional activity of FOXO proteins. While some of these modifications promote FOXO transcriptional activity, others are inhibitory. For example, stress-activated kinase JNK directly phosphorylates FOXO4 at residues Thr447 and Thr451, which leads to its nuclear translocation and induces FOXO4 transcriptional activity [20]. Another kinase implicated in oxidative stress-induced phosphorylation of FOXO is mammalian Ste20-like protein kinase 1 (MST1). During oxidative stress, MST1 phosphorylates FOXO3 at residue Ser207, which results in FOXO3 release from binding protein, 14-3-3. This release allows FOXO3 to translocate to the nucleus thereby modulating target gene expression [21].

FOXO transcriptional activity is also regulated by acetylation. The effects of oxidative stress-induced acetylation on FOXO function vary based upon the experimental conditions. Sirtuins (SIRTs), mammalian homologs of the yeast silent information regulator 2 (sir2) deacetylase, are critical regulators of FOXO transcriptional activity and are induced by oxidative stress [22, 23]. It has been reported that acetylation by cAMP-response-element-binding-protein (CREB-) binding protein (CBP)/P300 positively regulates FOXO transcriptional activity during oxidative stress, while SIRT1-mediated deacetylation represses the activity of FOXO transcription factors (FOXO1, FOXO3, and FOXO4) [24]. Other reports suggest that oxidative stress-induced FOXO4 acetylation negatively regulates its transcriptional activity, and deacetylation by SIRT1 counteracts the acetylation-mediated FOXO4 inhibition [25]. Furthermore, studies from Brunet et al. suggest that SIRT1 differentially affects FOXO3 function in response to oxidative stress [23]. SIRT1 associates with and deacetylates FOXO3 both *in vitro* and *in vivo*. SIRT1 deacetylation of FOXO3 increases expression of its target genes involved in cell cycle arrest and DNA repair such as p27 and GADD45. In contrast, SIRT1 deacetylation reduces expression of FOXO3 proapoptotic target genes such Bim and Fas ligand. These results indicate that deacetylation can both enhance and reduce FOXO3-induced activity depending upon the target gene.

Besides phosphorylation and acetylation, FOXO proteins are further regulated by ubiquitination during oxidative stress. In response to insulin or growth factor signaling, FOXO transcription factors are phosphorylated, polyubiquitinated, and degraded [26]. It has been reported that AKT-dependent phosphorylation is required as a prerequisite for ubiquitin-mediated degradation of FOXO1 and FOXO3. FOXO ubiquitination is mediated by F-box protein Skp2, a subunit of the SCF (Skp1/Cul1/F-box) E3 ubiquitin ligase protein complex [27, 28]. In contrast to insulin/growth factor signaling, upon oxidative stress, FOXO4 becomes monoubiquitinated and translocated into the nucleus, resulting in its increased transcriptional activity. Monoubiquitination of FOXO4 is mediated by E3 ubiquitin ligase murine double minute 2 (MDM2) [28]. Figure 1(a) shows the effect of ROS-induced oxidative stress that regulates FOXO by altering its phosphorylation or acetylation status. In contrast, growth factor-mediated induction of AKT which phosphorylates FOXO at specific amino acids leads to its export from the nucleus and Skp2 which leads to its ubiquitination and degradation (Figure 1(b)).

3. Role of FOXO in Oxidative Stress

FOXO proteins play an important role in protection of cells against oxidative stress. Oxidative stress is caused by overproduction of ROS or inefficient breakdown of ROS. Efficient detoxification of ROS by cellular detoxification systems protects cells against oxidative damage. The levels and enzymatic activities of various antioxidant enzymes such as manganese superoxide dismutase (MnSOD), catalase, and glutathione peroxidase are decreased during hyperglycemia-induced oxidative stress [11]. It is now well established that cells activate FOXO transcription factors to reduce the level of oxidative stress by the induction of enzymes that breakdown ROS such as MnSOD and catalase [29, 30]. For example, FOXO3 directly binds to MnSOD promoter at FOXO binding elements to increase its expression. Activation of MnSOD in mitochondria protects cells from ROS-mediated injury by converting superoxide radicals to oxygen and hydrogen peroxide (H_2O_2). Enzymes catalase and glutathione peroxidase further breakdown H_2O_2 into water and oxygen [30, 31]. The functional significance of FOXO in regulating oxidative stress is further revealed by gene deletion studies. Mice lacking FOXO factors (FOXO 1/3/4) in hematopoietic stem cells (HSCs) exhibit decreased self-renewal, leading to defective repopulating activity [32]. Consistent with this, FOXO-deficient HSCs showed increased ROS levels, decreased expression of antioxidant proteins, and increased apoptosis, suggesting critical role of FOXOs in stress resistance. Recent evidence suggests that FOXO factors

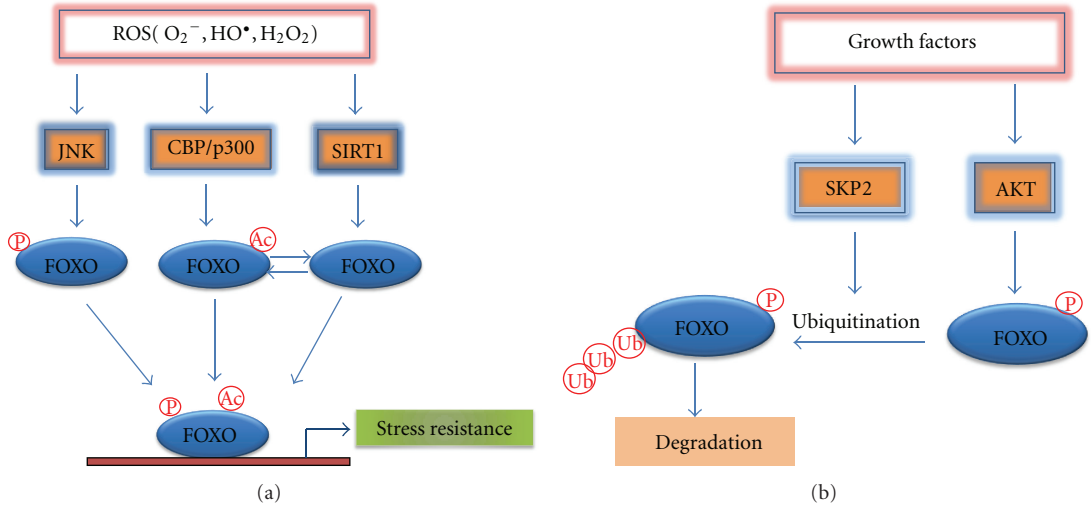

Figure 1: (a) Model of FOXO regulation during ROS-induced oxidative stress. In response to ROS-induced oxidative stress, the activity of FOXO proteins is modulated by various posttranslational modifications including phosphorylation and acetylation. The stress-activated kinase JNK phosphorylates FOXO leading to its nuclear translocation and activation. FOXO is acetylated by acetyltransferase CBP/p300 upon oxidative stress stimuli and deacetylated by SIRT1 deacetylase. Change in the acetylation status may activate or inhibit FOXO activity depending on the target genes and experimental conditions. Activation of FOXO by various posttranslational modifications leads to the induction of stress response genes such as MnSOD, catalase, and GADD45α. (b) Negative regulation of FOXO by growth factor signaling. Upon growth factor stimulation, AKT phosphorylates FOXO proteins on conserved residues, leading to their nuclear exclusion. SKP2-dependent ubiquitination, which may be induced by Akt, leads to its subsequent degradation. P: phosphorylation; Ac: acetylation; Ub: ubiquitination.

play a fundamental role in skeletal homeostasis by upregulating antioxidant enzymes [33, 34]. Deletion of FOXO1 in osteoblasts results in decreased expression of antioxidants such as glutathione. The resulting increased oxidative stress reduces osteoblast numbers and bone formation. Consistent with this, conditional deletion of FOXO factors (FOXO 1/3/4) in bone results in increased oxidative stress, loss of osteoblasts, and decreased bone mass, suggesting, FOXO factors are indispensable for skeletal homeostasis because of their antioxidant defense properties [33, 34]. It was also found that FOXO1 deletion in osteoblasts is associated with decreased protein synthesis. FOXO1 promotes protein synthesis in osteoblasts through direct regulation of ATF4, a transcription factor required of amino acid import and protein synthesis [33]. In the previous example, FOXO1 is protective through the induction of antioxidants. However, under conditions where inflammation is high, FOXO1 may also have a direct effect on osteoblasts by mediating inflammation-induced apoptosis [35]. In this case, FOXO1 is thought to induce expression of proapoptotic factors and exert an apoptotic rather than a protective effect. Under conditions of bone formation, FOXO1 may exert another set of effects. It has been reported that FOXO1 is needed for differentiation of osteoblast precursors to osteoblasts and that overexpression of FOXO1 interferes with progression of osteoblast precursors through the cell cycle [36]. Thus, the impact of FOXO1 on osteoblasts or their precursors may be highly dependent upon the context and microenvironment.

4. Role of FOXO in Cell Proliferation and Survival

FOXO transcription factors play a role in cell proliferation and survival by regulating the expression of genes involved in a number of cellular processes including cell cycle arrest, DNA repair, and apoptosis. In response to certain levels of oxidative stress, FOXO factors induce expression of target genes that control cell cycle progression and DNA repair, including p27Kip1, retinoblastoma-like protein p130, and cyclin D1/2, growth arrest, and DNA damage-inducible gene 45α (GADD45α) [37–41]. For example, FOXO causes cell-cycle arrest in G1 phase by inducing negative cell-cycle regulators such as cdk inhibitor $p27^{kip1}$ [37] and by repressing the expression of G1 cyclins D1 and D2 [40]. Besides promoting cell cycle arrest, FOXO also plays a critical role in stress resistance by facilitating repair of damaged DNA. FOXO3 induces cell cycle arrest at G2-M checkpoint and triggers DNA repair by inducing expression of the DNA damage response gene GADD45α [41]. Although in most cases FOXO proteins are associated with cell cycle arrest, in some cases FOXO proteins appear to promote cell cycle progression.

FOXO transcription factors typically induce either cell death by regulating proapoptotic genes but depending upon the context can enhance survival. In response to certain ROS levels, FOXO transcription factors switch from prosurvival to proapoptotic signaling leading to cell death. However,

the exact molecular mechanisms by which FOXO switches from prosurvival to prodeath signaling remain unknown. In diabetes, chronic hyperglycemia-induced mitochondrial ROS stimulate various signaling pathways leading to activation of FOXO, which in turn activates several proapoptotic factors. FOXO1 activation is elevated in diabetic connective tissue and mediates advanced glycation endproduct and TNF-alpha-induced apoptosis both of which are elevated in diabetic connective tissue [42–44]. It has been proposed that diabetes-enhanced activation of FOXO1 limits wound healing by enhancing fibroblast apoptosis and proliferation [43]. FOXO1 regulates genes of both the extrinsic and intrinsic apoptotic pathways [42]. FOXO3 and FOXO4 induce apoptosis by directly binding Bcl-6 promoter and enhancing its expression and negatively regulate expression of an antiapoptotic protein BCL-X_L [45]. It was further shown that silencing endogenous FOXO3 or overexpression of a dominant negative mutant of FOXO3 resulted in decreased expression of a variety of proapoptotic genes, including Bcl-6 and Bim, in response to hydrogen peroxide-induced oxidative stress. We have recently shown that hyperglycemia during diabetes stimulates microvascular endothelial cell and pericyte apoptosis leading to early stages of diabetic retinopathy [46]. High glucose leads to ROS generation that enhances FOXO1 activation and induction of several classes of genes that regulate endothelial cell behavior including proapoptotic and proinflammatory factors. These results suggest that FOXO1 plays an important role in the development of diabetic retinopathy due to its effect on inflammatory and apoptotic gene expression in microvascular cells [46]. Moreover, high glucose and advanced glycation endproducts that are elevated in diabetes stimulate loss of microvascular retinal pericytes through a process that involves activation of FOXO1 [46, 47]. In the latter, advanced glycation endproducts activate FOXO1 in pericytes through the MAP kinase pathway, and the loss of pericytes is countered by activation of Akt and NF-kappaB [47].

5. FOXO in Diabetes-Induced Inflammation

Inflammation has long been considered as a major risk factor in diabetes and associated with development and progression of diabetic complications. Hyperglycemia-induced oxidative stress promotes inflammation through increased endothelial cell damage, microvascular permeability, and increased release of proinflammatory cytokines, including TNF-α, interlukin-1β (IL-1β), and interlukin-6 (IL-6), ultimately leading to decreased insulin sensitivity and diabetic complications. Hyperglycemia-induced FOXO plays an important role in the induction of proinflammatory cytokines. It was shown that FOXO1 directly binds to IL-1β promoter and increases its expression in macrophages [48]. FOXO1 is induced by inflammatory cytokines and may be involved in a forward amplification loop. For example, in microvascular endothelial cells, FOXO1 is induced *in vivo* by diabetes-enhanced TNF-α and also induces expression of TNF-α levels in these cells [46]. Increased IL-1β and TNF-α production has been implicated in pathogenesis of obesity and diabetes. Hyperglycemia in diabetes also stimulates toll-like receptor (TLR) signaling, which results in prolonged inflammation and tissue damage. Recent studies show that FOXO1 promotes inflammation during diabetes by enhancing TLR4-mediated signaling, suggesting FOXO1 as a key mediator of inflammatory responses during obesity and diabetes [49]. In diabetic fracture healing, there is enhanced upregulation of proinflammatory and proapoptotic factors [50, 51]. It has been shown that FOXO1 induces expression of both proinflammatory and proapoptotic factors in chondrocytes and that FOXO1 directly binds to the TNF-α promoter. Moreover, diabetes-enhanced TNF-α activates FOXO1 in chondrocytes *in vivo* by enhancing its nuclear localization [50].

Another transcription factor that plays an important role in stimulating inflammation during hyperglycemia and oxidative stress is NF-κB [52]. Activation of NF-κB pathway has been implicated in the development of diabetic complications, including retinopathy, and has been shown to regulate expression of various proinflammatory cytokines, including TNF-α and IL-1β [53]. Chronically elevated ROS levels associated with diabetes may induce both NF-κB and FOXO leading to increased inflammation and cellular damage. In most cell types, NF-κB is directly antiapoptotic, while FOXO1 is directly proapoptotic. Thus, in inflammatory conditions when both NF-κB and FOXO1 are activated, their relative balance may determine whether a cell ultimately survives or undergoes apoptosis [42, 47].

5.1. Mitochondria, ROS, and Diabetes. A mechanism through which diabetes can increase oxidative stress involves electron transport in mitochondria. It has been proposed that high intracellular glucose levels increase the follow of electrons through the electron transport chain in mitochondria during oxidative respiration [6]. This can result in the transfer of electrons to O_2 leading to formation of O_2^- and the generation of various reactive oxygen species in the mitochondria. Furthermore, changes caused by diabetes alter the redox balance and affect redox-sensitive proteins such as protein kinase C-epsilon, which can result in enhanced mitochondrial ROS production. Advanced glycation end products (AGEs) generated under conditions of hyperglycemia stimulate NADPH oxidase that in turn can induce production of ROS. In a surprising development, increased Wnt signaling stimulates mitochondrial biogenesis that can lead to enhanced ROS levels in mitochondria and greater oxidative damage [54]. The increased ROS in mitochondria is thought to be problematic due to a number of different mechanisms. One is that ROS damages mitochondrial components such as DNA, membrane proteins, and lipids. ROS can also induce the opening of the mitochondrial permeability transition pore (MPTP) [55]. When this pore is opened, proapoptotic proteins are released from the mitochondria such as cytochrome c that stimulate cell death. ROS generated in the mitochondrial respiratory chain have been proposed as secondary messengers for activation of NF-κB by TNF-α and IL-1 [6].

ROS may affect insulin signalling. Insulin signalling is reduced under conditions of oxidative stress, which may contribute to insulin resistance. This may occur through

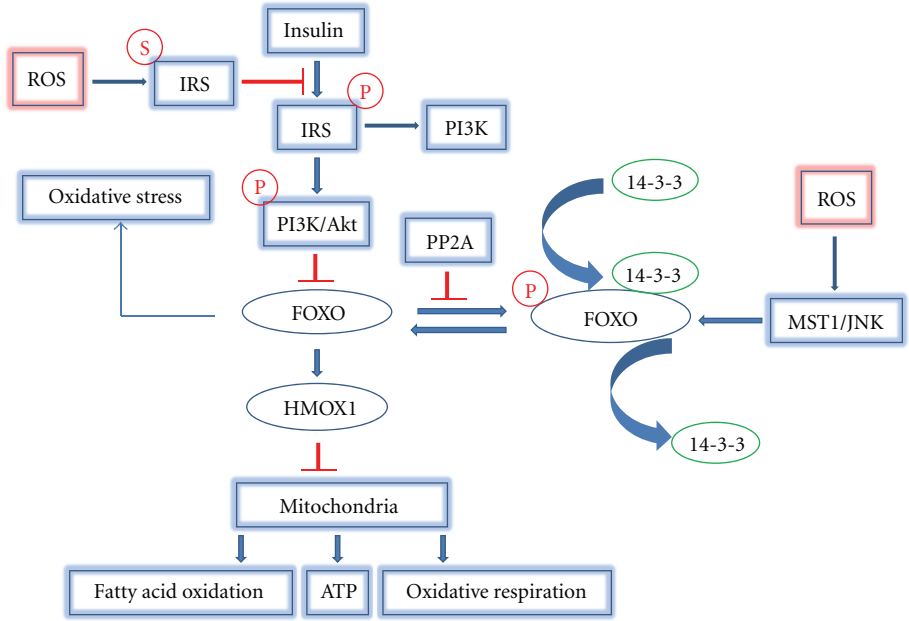

Figure 2: Oxidative stress and insulin signaling affect mitochondrial function via FOXO. ROS induce IRS serine phosphorylation which inhibits IRS activation by insulin signalling. As a result of reduced IRS activity, Akt activity is reduced. Reduced Akt reduces negative signalling of FOXO so that FOXO1 is left in an activated state since it is not exported out of the nucleus by 14-3-3. Meanwhile, ROS activate MST1 and JNK which induce FOXO nuclear translocation by disrupting the complex of FOXO and 14-3-3. PP2A activates FOXO by dephosphorylation of FOXO and by Akt dephosphorylation. FOXO nuclear translocation will induce HMOX1 gene expression which inhibits mitochondrial function by affecting like fatty acid oxidation, ATP, and oxidative respiration (arrow indicates stimulatory event; bar indicates inhibitory event).

several mechanisms. In one scenario, ROS induces serine phosphorylation of insulin receptor substrate, decreasing tyrosine phosphorylation thereby interfering with insulin signaling [56]. Similarly, ROS have been shown to partially mediate the effect of Angiotensin II inhibition of insulin signalling [57]. Methylglyoxal, a biologically active AGE precursor formed under conditions of hyperglycemia, inhibits phosphorylation of insulin receptor substrate and activation of the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (PKB) pathway [58].

Insulin signalling inactivates FOXO1, which is mediated by insulin receptor substrates-1 and -2 through AKT. A characteristic feature of insulin resistance is the elevated production of glucose that contributes to hyperglycemia. FOXO1 regulates glucose production in the liver through the expression of genes that promote gluconeogenesis [59]. Thus, a pathway exists whereby insulin resistance leads to elevated FOXO1 activation, upregulation of genes that promote glucose production, and greater serum glucose levels. Disruption of the insulin-Akt-FOXO1 balance also affects the mitochondria. Activated FOXO1 induces heme oxygenase-1 (HMOX1), which cleaves heme and disrupts the mitochondrial electron transport chain [60]. Thus, when FOXO1 activity is elevated by insulin resistance, greater expression of heme oxygenase-1 ensues. Greater heme oxygenase-1 levels interfere with mitochondria leading to impaired oxidative respiration, negatively affecting fatty acid oxidation and the production of ATP. Furthermore, enhanced activation of FOXO1 affects the expression mitochondrial fusion and fission thereby affecting mitochondrial biogenesis. Under conditions of insulin resistance, there are insufficient mitochondria and abnormal mitochondrial morphology, which is reversed when FOXO1 is deleted [60]. Figure 2 demonstrates the complex signalling pathways through which oxidative stress and insulin can modulate FOXO activity to affect mitochondria.

6. Conclusion and Perspective

Hyperglycemia-induced ROS and subsequent oxidative stress are major contributors to the development and progression of diabetes and related complications. However, effective therapeutic strategies to prevent the generation of these free radicals remain limited. It is now well established that FOXO transcription factors are the critical regulators of cell fate and play a major role in diabetes-induced oxidative stress resistance and in diabetes-enhanced apoptosis. It seems that FOXO transcription factors might function as molecular switches that determine cell fate in response to various levels of oxidative stress by either promoting antioxidants (prosurvival) responses or alternatively enhancing proapoptotic gene expression and cell death. However, the precise mechanism by which FOXO mediates prosurvival/proapoptotic response remains unclear and elucidation of molecular mechanisms involved may provide new targets for therapy. Furthermore, multiple signaling pathways, including JNK

and MAPK, regulate the activity of FOXO transcription factors in response to hyperglycemia in diabetes. Because our understanding of how these diverse signaling pathways coordinate their effects to regulate FOXO activity during diabetes remains limited, detailed understanding of these pathways may provide insights into development of new therapeutic strategies for treatment of diabetes.

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Review Article

Cellular Dysfunction in Diabetes as Maladaptive Response to Mitochondrial Oxidative Stress

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Oxidative stress has been implicated in diabetes long-term complications. In this paper, we summarize the growing evidence suggesting that hyperglycemia-induced overproduction of superoxide by mitochondrial electron transport chain triggers a maladaptive response by affecting several metabolic and signaling pathways involved in the pathophysiology of cellular dysfunction and diabetic complications. In particular, it is our goal to describe physiological mechanisms underlying the mitochondrial free radical production and regulation to explain the oxidative stress derived from a high intracellular glucose concentration and the resulting maladaptive response that leads to a cellular dysfunction and pathological state. Finally, we outline potential therapies for diabetes focused to the prevention of mitochondrial oxidative damage.

1. Introduction

The Diabetes Control and Complications Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) established that hyperglycemia is the initiating cause of the diabetic tissue damage which is verified clinically [1, 2]. Even though this process is modified by both genetic determinants of individual susceptibility and by independent accelerating factors such as hypertension, both the repeated acute changes in cellular metabolism and cumulative long-term changes in cellular constituents appear to be the mechanisms that mediate the cell-damaging effects of hyperglycemia.

The cell-damaging effects of hyperglycemia comprise the damage to a selective subset of cell types directly involved in diabetic complications: endothelial cells in the vascular system, mesangial cells in the kidney, neurons and neuroglia in the nervous system, and pancreatic β cells. Why are these cells especially vulnerable to hyperglycemic conditions? In the organism, most cells are able to downregulate the transport of glucose inside the cell when they are exposed to a hyperglycemic status, so that their intracellular glucose concentration stays constant. In contrast, the cells injured by hyperglycemia are those that cannot do this efficiently [3, 4], leading to high glucose levels inside the cell. In this scenario, available evidences demonstrate that a hyperglycemia-induced cellular oxidative stress is the basic mechanism underlying the physiopathology of the diabetic complications. Indeed it has been suggested that increased mitochondrial free radicals production during hyperglycemia may be central of the pathology of diabetes [5, 6]. Therefore, mitochondrial free radical production and oxidation-derived molecular damage may contribute to the onset, progression, and pathological consequences of diabetes. Here, we discuss how mitochondrial oxidative damage occurs, consider the maladaptive mechanisms by which it may contribute to the pathophysiology of diabetes, and outline potential therapeutic strategies to prevent it.

2. Physiology of the Mitochondrial Oxidative Damage

Inside mitochondria, electrons from reduced substrates move from complexes I and II of the electron transport chain through complexes III and IV to oxygen, forming water and causing protons to be pumped across the mitochondrial inner membrane. When glucose is metabolized through the tricarboxylic acid (TCA) cycle (or fatty acids through

β-oxidation), it generates electron donors. The main electron donor is NADH, which gives electrons to complex I. The other electron donor generated by the TCA cycle is $FADH_2$, formed by succinate dehydrogenase, which donates electrons to complex II. The proton motive force set up by proton pumping [7] drives protons back through the ATP synthase in the inner membrane, forming ATP from their precursors ADP (adenosine diphosphate) and phosphate [8]. The electron transport system is organized in this way so that the level of ATP can be precisely regulated.

In this context, a major side reaction is that electrons may leak from the respiratory chain and react with oxygen to form the free radical superoxide. Superoxide anion, the product of a one-electron reduction of oxygen, is the precursor of most reactive oxygen species (ROS) and a mediator in oxidative chain reactions [71–75]. So, oxygen reduction, needed for aerobic life, generates three main ROS, superoxide radical, hydrogen peroxide (H_2O_2), and hydroxyl radical. The hydroxyl radical can be generated by the combination of superoxide radical and H_2O_2 in the presence of traces of iron or copper during the Fenton-Haber-Weiss reaction. Thus H_2O_2, although it is not a free radical, can work as a Trojan horse, diffusing away from sites of ROS production to generate the hydroxyl and other reactive radicals at other cellular locations, hereby propagating oxidative damage. Other ROS of probable relevance for endothelial cells are the perhydroxyl radical, particularly near membranes where local pH is lower than in the bulk solution [76], singlet oxygen, and nitric oxide. In the case of mitochondria, nitric oxide production is much smaller than superoxide production. However, nitric oxide can still be important due to interaction with superoxide and other radicals to produce reactive nitrogen species like peroxynitrite [77], which can modify many kinds of macromolecules and possibly contribute to diabetes vascular complications [78].

Despite ROS can be generated at various sites and under various conditions (including, ischaemia-reperfusion, enzymatic reactions (e.g., the membrane NADPH oxidase, lipoxygenases, cyclooxygenases, peroxidases, and other heme proteins), the enzyme xanthine oxidase, peroxisomes, or the hepatic P-450 microsomal detoxifying system), in healthy cells under physiological conditions, most ROS are originated in mitochondria [79]. Currently, it is well known that mitochondrial ROS generation occurs at complex I [79–86] and at complex III [87, 88]. Concerning the electron transport component responsible for mtROS generation within complex I, flavin mononucleotide, ubisemiquinone species, or iron-sulphur clusters have been proposed [89–97].

The finding that the percentage of total electron flow directed to free radical generation in mitochondria is not constant in different tissues and different conditions inside a given tissue suggests that ROS generation is not a simple byproduct of mitochondrial respiration as is frequently assumed. Indeed there is a lack of stoichiometric coupling of ROS production to oxygen consumption [98]. Therefore, it should be better viewed as a homeostatically controlled variable.

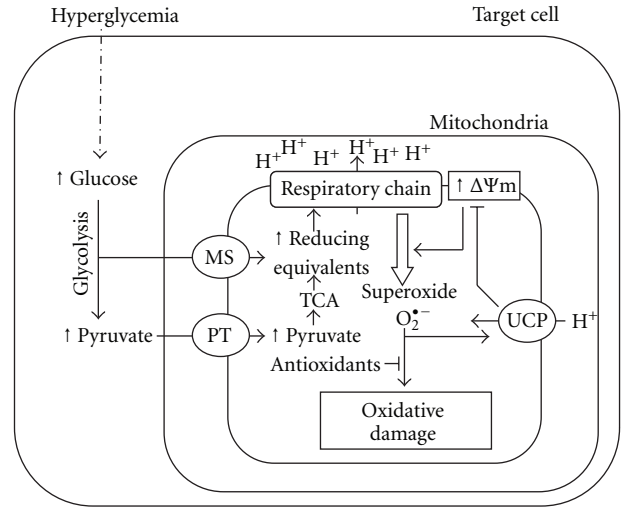

Figure 1: Uncoupling proteins (UCPs) respond to hyperglycemia-induced overproduction of mitochondrial superoxide by catalyzing mild uncoupling, which lowers membrane potential ($\Delta\Psi m$) and decreases superoxide production by mitochondrial complex I and III of the electron transport chain. Antioxidants limit the impact of superoxide production on molecular oxidative damage (for more details, see text). MS: mitochondrial redox shuttles; $O_2^{\bullet -}$: superoxide radical; PT: pyruvate transporter; TCA: tricarboxylic acid cycle.

Are there physiological adaptation mechanisms with ability to modulate the rate of mitochondrial free radical generation? Available evidence seems to suggest that this is the case [99]. Among these adaptations, two negative feedback loops protect cells from ROS-induced damage. The first mechanism is characterized by regulation of uncoupling proteins (UCPs). During oxidation of substrates, the complexes of the mitochondrial electron transport chain reduce oxygen to water and pump protons into the intermembrane space, forming a proton motive force (Δp). However, some electrons in the reduced complexes also react with oxygen to produce superoxide. Superoxide can peroxidize membrane phospholipids, forming hydroxynonenal, which induces proton transport through the UCPs and the adenine nucleotide translocase. The mild uncoupling caused by proton transport lowers Δp and slightly stimulates electron transport, causing the complexes to become more oxidized and lowering the local concentration of oxygen; both these effects decrease superoxide production. Thus, the induction of proton leak by hydroxynonenal limits mitochondrial ROS production as a feedback response to overproduction of superoxide by the respiratory chain [89, 100, 101]. So, a possible antioxidant physiological function for UCPs has been proposed [100]. In this model, UCPs respond to overproduction of matrix superoxide by catalyzing mild uncoupling, which lowers proton motive force and would decrease superoxide production by the electron transport chain (Figure 1).

The second feedback loop consists of a regulation of the flux of metabolites to mitochondria. So, a transient overproduction of ROS by the mitochondrial electron

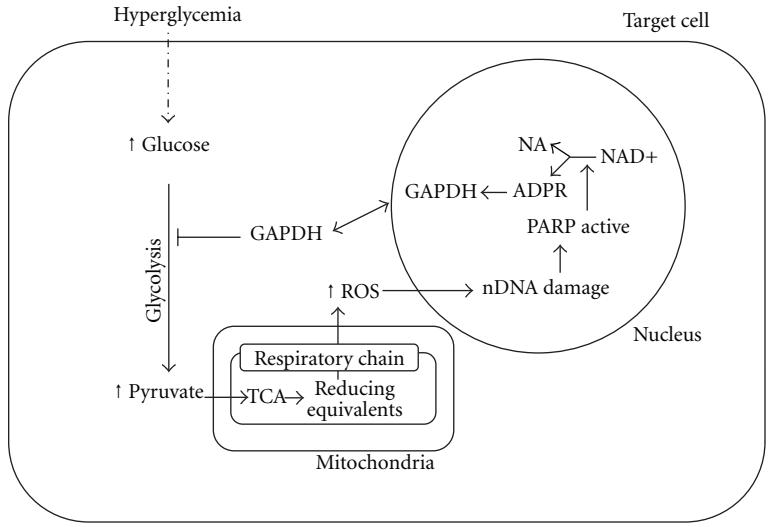

Figure 2: Hyperglycemia-induced mitochondrial free radical production induces DNA damage that activates PARP and modifies GADPH leading to a block of glycolysis (for more details, see text).

transport chain can decrease the activity of the key glycolytic enzyme glyceraldehyde-3 phosphate dehydrogenase (GAPDH) by modifying the enzyme by ADP-ribosylation [102]. Poly(ADP-ribosyl)ation represents an immediate cellular response to DNA damage induced by oxidants [103–105]. In the absence of DNA single and double-strand breaks, poly(ADP-ribosyl)ation is a very rare event, but it can increase over 100-fold upon DNA damage. Under these conditions, about 90% of poly(ADP-ribose) is synthesized by poly(ADP-ribose) polymerase 1 (PARP-1). PARP-1 is constitutively expressed but enzymatically activated by DNA strand breaks. So, PARP-1 functions as a DNA damage sensor and signaling molecule binding to both single- and double-stranded DNA breaks. It catalyses the formation of ADP-ribose from the oxidized form of nicotinamide adenine dinucleotide (NAD+) by cleavage of the glycosidic bond between nicotinamide and ribose. Glutamate, aspartate, and carboxyterminal lysine residues of target ("acceptor") proteins are then covalently modified by the addition of an ADP-ribose subunit, via formation of an ester bond between the protein and the ADP-ribose residue. So, poly(ADP-ribosyl)ation is a covalent posttranslational protein modification linked with genome protection [103, 106]. In this scenario, it is plausible to suggest that the inhibitory effect of ADP-ribosylation on GAPDH probably represents a feedback loop in order to reduce levels of glycolysis and transiently block the subsequent flux of metabolites to mitochondria allowing a decrease in the levels of reducing equivalents and the subsequent mitochondrial ROS production and oxidative cellular molecular damage (Figure 2).

3. Mitochondrial Antioxidant Defenses

Oxidative stress homeostasis (e.g., balance between ROS production and elimination) relies on endogenous cellular antioxidants [99, 107–109]. Mitochondria, from an intracellular organelle comparative approach, are endowed with the best antioxidants, detoxifying and repair systems against oxidative damage. So, the antioxidant enzyme MnSOD (manganese superoxide dismutase) converts superoxide to H_2O_2. The mitochondrial isoform of glutathione peroxidase (GPx) and the thioredoxin-dependent enzyme peroxiredoxin III both detoxify H_2O_2; alternatively, H_2O_2 can diffuse from the mitochondria into the cytoplasm. The mitochondrial glutathione (GSH) pool is different from that in the cytosol and is maintained in its reduced state by a mitochondrial isoform of glutathione reductase (GR). This enzyme requires NADPH, which is produced within mitochondria by the NADP-dependent isocitrate dehydrogenase and through a proton electrochemical potential gradient-dependent transhydrogenase. Within the mitochondrial phospholipid bilayer, the fat-soluble antioxidants vitamin E and coenzyme Q (CoQ) both prevent lipid peroxidation, while CoQ also recycles vitamin E and is itself regenerated by the respiratory chain. The mitochondrial isoform of phospholipid hydroperoxide glutathione peroxidase [110] degrades lipid peroxides within the mitochondrial inner membrane. There are also a variety of specific mitochondrial mechanisms to repair or degrade oxidatively damaged lipids [108, 110], proteins [111], and mtDNA [112].

4. Hyperglycemia Induces Permanent Overproduction of Superoxide by Mitochondrial Electron Transport Chain

As mentioned above, the major sites of ROS generation are the complexs I and III of the mitochondrial electron transport chain. In cells under sustained high glucose concentrations, there is more glucose being oxidized in the TCA cycle. This situation drives to pushing more electron donors (NADH and $FADH_2$) into the electron transport chain thus leading to an increase in ROS generation [5, 6]. This is so

because in this situation, there is a higher degree of reduction of complexes I and III increasing their rate of ROS production. The rate of mitochondrial ROS generation strongly increases with a sigmoidal kinetics when the NADH/NAD+ ratio is increased, because this dramatically increases the degree of reduction of the complex I ROS generator [84, 98]. In an identical way, in the insulin resistance syndrome, there is an increased free fatty acid (FFA) flux from adipocytes into arterial endothelial cells that might result in increased FFA oxidation by the mitochondria. Since both β-oxidation of fatty acids and oxidation of FFA-derived acetyl CoA by the TCA cycle generate the same electron donors (NADH and $FADH_2$) generated by glucose oxidation, increased FFA oxidation may cause mitochondrial overproduction of ROS [113] by exactly the same mechanism described above for hyperglycemia, and in both cases can be reversed upon exposure to agents that act as mitochondrial uncouplers or electron transport chain inhibitors.

Concomitantly with the hyperglycemia-induced mitochondrial free radical overproduction, it has been described that in hyperglycemia Ucp2 gene transcription is activated by key regulatory proteins such as peroxisome proliferator-activated receptors (PPARs), forkhead transcription factors, sterol regulatory element-binding protein-1c (SREBP-1c) [114], and AMP-activated protein kinase [115]. Additionally, the pathological and persistent overproduction of ROS by the mitochondrial electron transport chain decreases the activity of the key glycolytic enzyme GAPDH. The inhibition of GAPDH activity by "hyperglycemia" does not occur when mitochondrial overproduction of superoxide is prevented by either UCP1 or MnSOD [116]. In addition, subsequent studies demonstrate that persistent high intracellular glucose concentration-induced superoxide inhibits GAPDH activity in vivo by modifying the enzyme by ADP-ribosylation [102]. By inhibiting mitochondrial superoxide production with either UCP-1 or MnSOD, it prevented the modification of GAPDH by ADP-ribose and the reduction of its activity. Most importantly, the modification of GAPDH is prevented by a specific inhibitor of poly(ADP-ribose) polymerase (PARP), the enzyme that makes these polymers of ADP-ribose, establishing a cause-and-effect relationship between PARP activation and the changes in GAPDH [5]. Therefore, this mechanism seems to indicate that the stress-induced block of glycolysis is not the result of a passive oxidative damage but rather an active cell adaptation programmed via ADP-ribosylation for cell self-defence.

However, the chronic increase in target cells of the intracellular glucose concentration and permanent block of glycolysis leads to a maladaptive response derived from the upstream accumulation of glycolytic metabolites which are substrates for the activation of metabolic pathways involved in the development of diabetic complications. In addition to this maladaptive response, the block of glycolysis leads to a fall of mitochondrial substrates that originates a re-duced mitochondrial energy production and subsequent cell exhaustion that can be a determinant element in the endothelial cell dysfunction. In this scenario, other cellular sources of free radical generation could take the relief to mitochondria assuming a relevant role in a potential second round of cellular oxidative molecular damage.

5. Hyperglycemia-Induced Mitochondrial Free Radical Generation Activates Damaging Downstream Cellular Pathways

From the scenario described above, it was proposed that different pathogenic mechanisms leading to the development of diabetic complications do reflect a single hyperglycemia-induced process [5]. This process is based on that hyperglycemia, through the overproduction of free radicals by the mitochondrial electron transport chain, decreases the activity of the key glycolytic enzyme GAPDH. So, when GAPDH activity is inhibited, the level of all the glycolytic intermediates located upstream of GAPDH increases. Increased levels of the upstream glycolytic metabolite glyceraldehyde-3-phosphate activate two pathogenic pathways: (a) it activates the glycation pathway because methylglyoxal, a glycation precursor, is formed from glyceraldehyde-3 phosphate [117–119], and (b) it also activates the protein-kinase C pathway because diacylglycerol, one of its activators, is also formed from glyceraldehyde-3 phosphate [102, 120]. Further upstream, levels of the glycolytic metabolite fructose-6 phosphate increase, which increases flux through the hexosamine pathway, where fructose-6 phosphate is converted by the enzyme GFAT to UDP-*N*-acetylglucosamine (UDP-GlcNAc) increasing the chances for hexosamine modification of proteins [116]. Finally, inhibition of GAPDH increases intracellular levels of the first glycolytic metabolite, glucose. This increases flux through the polyol pathway, where the enzyme aldose reductase reduces it, consuming NADPH in the process and reducing available GSH [120–123].

Besides these maladaptive damaging cellular pathways, it must be considered the cellular responses derived from the PPAR overactivation as important mechanism of tissue damage also leading to an endothelial dysfunction in diabetic blood vessels, which importantly contributes to the development of various diabetic complications. Thus, PPAR activation, in addition to the mitochondrial bioenergetic depletion due to the block of glycolysis, potentiates in a maladaptive process the expression of various proteins at the transcriptional level [124]. Of special importance is the regulation by PARP-1 of the production of inflammatory mediators such as inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), and major histocompatibility complex class II. NF-κB is a key transcription factor in the regulation of this set of proteins, and PARP has been shown to act as a coactivator in the NF-κB-mediated transcription. Poly(ADP-ribosyl)ation can loosen up the chromatin structure, thereby making genes more accessible for the transcriptional machinery [118]. Therefore, all these metabolic pathways originate alterations in gene expression, inflammatory responses, and structural and functional changes in cellular constituents that also participate in the molecular basis of the vascular diabetic process (Figure 3).

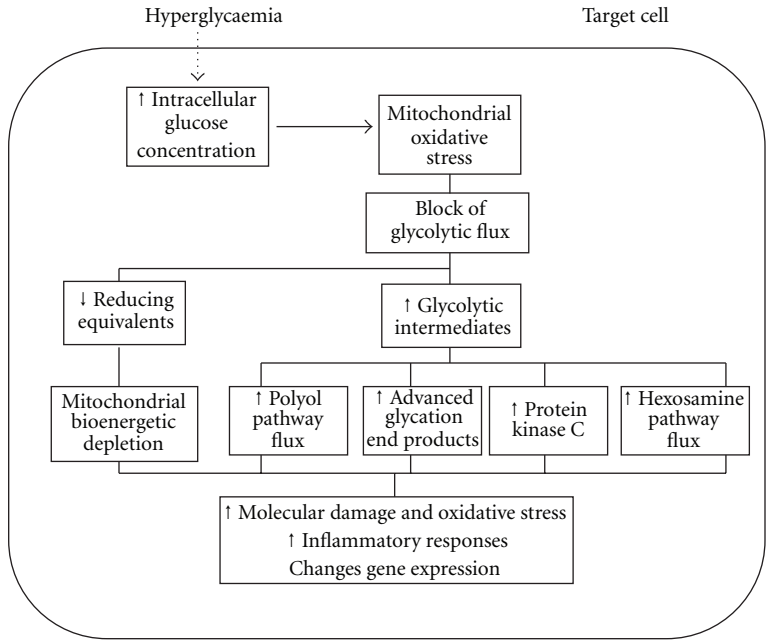

Figure 3: Intracellular high-glucose metabolism and oxidative stress. When intracellular glucose concentration increases in target cells of diabetes complications, it causes increased mitochondrial production of ROS and activates negative feedback loops to protect target cells from ROS-induced damage. The maladaptive response, however, leads to the activation of metabolic pathways that are involved in the diabetes vascular disfunction.

6. Protein Oxidative Damage: Protein Carbonyl Content in Diabetes

Oxidative damage occurs whenever the ROS produced by mitochondria evade detoxification, and the steady-state level of molecular oxidative damage depends on the relative rates of damage accumulation, repair, and degradation. ROS can damage all types of biomolecules, and oxidative damage to DNA, lipids and proteins can be deleterious and concomitant [107]. The primary cellular target of oxidative stress depends upon the cell type, the nature of the stress imposed, the susceptibility to oxidation of the target molecule, the site of generation, the proximity of ROS to a specific target, and the severity of the stress. In this context, protein oxidation demands an especial mention because proteins constitute the major "working force" for all forms of biological work. Furthermore, their exact conformation and pattern of folding are tightly related to their activity and function. So, the consequent loss of function and structural integrity of modified proteins can have a wide range of downstream functional consequences and may be the cause of subsequent cellular dysfunctions and tissue damage (Table 1). The products of oxidation of amino acids are indicators of modification to proteins in biological systems [125–129]. They include oxidized amino acids, modified amino acids by reactive nitrogen species and chlorination reactions, and crosslinks formed by a combination of enzymatic and nonenzymatic mechanisms.

Amino acid residues in proteins are highly susceptible to oxidation by one or more reactive species. Many different types of protein oxidative modification can be induced directly by ROS or indirectly by reactions of secondary byproducts of oxidative stress (basically derived from the oxidation of both carbohydrates and polyunsaturated fatty acids that lead to the formation of the named reactive carbonyl species, RCOs [130]). Cysteine and methionine are particularly prone to oxidative attack by almost all ROS. Protein modifications are elicited by direct oxidative attack on Lys, Arg, Pro, or Thr, or by secondary reaction of Cys, His or Lys residues with reactive carbonyl compounds can lead to the formation of protein carbonyl (PCO) derivatives (aldehydes and ketones) [125, 130, 131] (Table 2).

Table 1: Effects of oxidative damage in protein structure and function.

(i) Cleavage of peptide bonds
(ii) Direct reaction of proteins with ROS can lead to formation of protein derivatives or peptide fragments possessing highly reactive carbonyl groups (ketones, aldehydes)
(iii) Formation of intra- or interprotein cross-linked derivatives that can lead to the formation of aggregates by (a) direct interaction of two carbon-centered radicals; (b) interaction of two tyrosine radicals; (c) oxidation of cysteine sulfhydryl groups; (d) interactions of the carbonyl groups of oxidized proteins with the primary amino groups of lysine residues in the same or a different protein; (e) by noncovalent interactions such as hydrophobic and electrostatic interactions between oxidized residues
(iv) Partial unfolding or denaturation with a concomitant increase in surface hydrophobicity
(v) Loss of function (e.g., enzyme activity)

Table 2: Markers of oxidative damage in proteins.

Amino acid	Product
(i) Arginine	Glutamic-semialdehyde
(ii) Cysteine	Cysteine disulfides, Sulfenic acid
(iii) Histidine	Aspartate Asparagine 2-Oxoimidazoline 2-Oxohistidine
(iv) Leucine	3-,4-,5-Monohydroxyleucine
(v) Leucine, valine, isoleucine, proline, and others	Protein carbonyls
(vi) Lysine	2-Amino-adipic-semialdehyde
(vii) Methionine	Methionine sulfoxide
(viii) Phenylalanine	ortho- and meta-tyrosine
(ix) Proline	Glutamate Glutamic-semialdehyde 2-Pyrrolidone 4-,5-Hydroxyproline Pyroglutamic acid
(x) Threonine	2-Amino-3-ketobutyric acid
(xi) Tryptophan	2-, 4-, 5-, 6-, or 7-OH tryptophan N-formylkynurenine Kynurenine
(xii) Tyrosine	Di-tyrosine (Tyr-Tyr cross-links) Dihydroxyphenylalanine (DOPA) 3-Nitrotirosine 3-Chlorotyrosine

Glutamic semialdehyde is a product of oxidation of arginine and proline, and aminoadipic semialdehyde, of oxidation of lysine. They account for 55–100% of the total carbonyl value in several metal ion-catalyzed oxidation (MCO) systems [128, 132]. Sensitive gas chromatography-mass spectrometry based analytical methods has allow their quantitation in a variety of biological samples providing specific information on the oxidative status of proteins that is complementary to that afforded by protein carbonyls, and will be useful tools in the ongoing effort to define and assess the role of protein oxidation in diabetes complications [95, 132].

Other oxidation-derived protein damage markers include protein modifications derived from reactive nitrogen species (RNS). Nitric oxide generated from nitric oxide synthetases plays an important role in the regulation of various physiological parameters (very especially at the vascular level) but due to its free radical nature, it could also react with superoxide radical to form highly reactive peroxynitrite functions [133]. It has been established that aromatic amino acids, cysteine, and methionine residues of proteins are particularly sensitive to modification by RNS. These reactions lead to nitration of tyrosine residues of proteins [134, 135], the oxidation of methionine residues to methionine sulfoxide, and the nitrosation of protein sulfhydryl groups to RSNO derivatives [136–138].

Studies of the formation of PCOs cannot differentiate between those produced through direct protein oxidation and those formed by the addition of previously oxidized molecules, and hence protein carbonyl content (PCC) must be considered as a broad and unspecific marker of oxidation. Because carbonyls are relatively difficult to induce compared with, for example, methionine sulphoxide and cysteinyl derivatives, they might indicate a more rigorous oxidative stress. Indeed, elevated levels of PCC are generally a sign not only of oxidative stress, but also of disease-derived protein dysfunction. PCC can have an advantage over both carbohydrate and lipid oxidation products as markers of oxidative stress; oxidized proteins are generally more stable. PCCs form early and circulate in the blood for longer periods (their elevation in serum is stable for at least four hours), compared with other parameters of oxidative stress, such as glutathione disulphide and malondialdehyde [131]. The PCC seems to be a common phenomenon during oxidation-derived protein damage, and their quantification can be used to measure the extent of chemical and nonenzymatic oxidative modification. This has driven the development of various sensitive but unspecific biochemical (spectrophotometric and fluorometric) and immunological (western blot, enzyme-linked immunosorbent assay (ELISA), and proteomics) methods for the detection and measurement of the PCC in tissues and body fluids; in all of them 2,4-dinitrophenylhydrazine is allowed to react with the PCOs to form the corresponding hydrazone, which can be analyzed by the above mentioned methods. Currently, PCC is the most general indicator and by far the most commonly used marker of protein oxidation. Because the mechanisms of PCC generation are nonspecific, it has been argued that other protein modifications, such as the conversion of tyrosine residues to 3-chlorotyrosine, 3-nitrotyrosine or dityrosine, arginine and proline to glutamic semialdehyde, or lysine to aminoadipic semialdehyde, are better markers of oxidative stress. However, the tissue levels of such markers are orders of magnitude lower than the overall PCC and, hence, their measurement often requires highly sensitive and expensive methods such as mass spectrometry [109, 130, 132].

Tables 3 and 4 summarize available studies where PCC was analyzed by different methods in the diabetic status. From this summary of the effects of diabetes on PCC, it is possible to propose some general ideas: (1) Mouse, rabbit, and especially rat are the animal species used as reference for the study of the effects of experimental diabetes, being the STZ-induced diabetes the experimental model predominantly, but not exclusively, used. (2) PCC levels are consistently increased in all the analyzed tissues independently of the analytical method used. Of particular interest are the increased PCC levels showed by the organs containing the selective subset of cell types directly involved in diabetic complications: vascular system, kidney, brain, and pancreas. (3) In humans, most studies are focused to Type 2 diabetes and the measurement of PCC in plasma proteins. (4) In humans, elevated PCC levels have been detected in both Type 1 and Type 2 diabetes. (5) Plasma PCC levels are significantly higher in diabetic children and adolescents without complications compared with control

Table 3: Effects of experimental diabetes in levels of protein carbonyls.

Tissue	Model	Effect	Reference
	Mouse		
Aorta	*BKS.cg-m +/+ Lepr db/J mice versus wild type*	↑	[9]
Hippocampus and cerebral cortex	*Streptozotocin*	↑	[10]
Kidney	*Type 2 diabetic db/db versus normoglycemic wild type mouse*	↑	[11]
Lenses	*Streptozotocin*	↑	[12]
	Rat		
Aorta	*Goto-Kakizaki rats*	↑	[13]
Bone	*Goto-Kakizaki rats*	↑	[14]
Brain	*Galactose-induced hyperglycemia*	↑	[15]
Brain	*Goto-Kakizaki rats*	↑	[16]
Brain	*Streptozotocin*	=	[17]
Heart	*Streptozotocin*	↑	[18]
Heart	*Streptozotocin*	↑	[19]
Heart	*Streptozotocin*	↑	[20]
Heart	*Streptozotocin*	↑	[21]
Heart	*Streptozotocin*	↑	[17]
Hemoglobin	*Streptozotocin*	↑	[22]
Intestinal tissue	*Streptozotocin*	↑	[23]
Kidney	*Streptozotocin*	↑	[24]
Kidney	*Streptozotocin*	↑	[18]
Kidney	*Zucker obese hyperglycemic rats (ZDFn Gm-fa/fa)*	↑	[25]
Kidney	*Streptozotocin*	↑	[26]
Kidney	*Streptozotocin*	↑	[17]
Lens proteins	*Streptozotocin*	↑	[27]
Liver	*Streptozotocin*	↓	[24]
Liver	*Pregnant diabetic rats versus control rats*	=	[28]
Liver	*Galactose-induced hyperglycemia*	↑	[15]
Liver	*Streptozotocin*	↑	[18]
Liver	*Streptozotocin*	↑	[29]
Liver	*Streptozotocin*	↑	[23]
Liver	*Streptozotocin*	↑	[30]
Liver	*Streptozotocin*	↑	[17]
Lung	*Streptozotocin*	↑	[31]
Pancreas	*Streptozotocin*	↑	[18]
Pancreas	*Alloxan*	↑	[32]
Pancreas	*Streptozotocin*	↑	[17]
Pancreas	*Streptozotocin*	↑	[33]
Plasma proteins	*Streptozotocin*	↑	[34]
Plasma proteins	*Streptozotocin*	↑	[35]
Plasma proteins	*Streptozotocin*	↑	[36]
Red blood cells	*Streptozotocin*	↑	[18]
Retinal Müller cells	*Streptozotocin*	↑	[37]
Skeletal muscle	*Glupreclamp infusion versus control*	↑	[38]
Skeletal muscle	*Otsuka Long Evans Tokushima Fatty (OLETF) rats versus LETO rats*	↑	[39]
Skeletal muscle (Soleus muscles)	*Goto-Kakizaki rats*	↑	[13]
Skeletal muscle (Plantaris muscle)	*Obese Zucker rats versus lean Zucker rats*	↑	[40]
Skeletal muscle	*Streptozotocin*	↑	[41]
Testis and epididymal sperm	*Streptozotocin*	↑	[42]
Vascular smooth muscle cells	*Glucose incubation*	↑	[43]
	Rabbit		
Heart	*Alloxan*	↑	[44]
Lens proteins and cells	*In vitro incubation*	↑	[45]

subjects, suggesting that oxidative protein damage occurs at the onset of disease and tends to increase in the later stages. (6) The presence of a diabetic complication is associated with higher PCC levels. (7) There is a lack of studies specifically driven to the vascular system.

7. Current Antioxidant Therapeutic Strategies

Hyperglycemia-induced overproduction of superoxide by mitochondrial electron transport chain induces a cellular maladaptive response that triggers several metabolic pathways of injury involved in the endothelial dysfunction and contributes to the progressive development of micro- and macrovascular complications and multiorgan damage. Consequently, inhibition of mitochondrial oxidant generation and/or oxidative-derived molecular damage might provide a potential approach for the prevention of diabetic vascular complications.

Even though it is well established that good (but strict) glycemic control is the basis for the prevention of diabetic complications, there is no doubt that preventive measures targeting other risk factors should be also achieved. Therapeutic strategies for diabetic vascular complications should consist in the modulation of afflicted pathways. Thus, therapeutic strategies to limit mitochondrial radical production during hyperglycemia and to counteract their damaging effects could be useful complements to conventional therapies designed to normalize blood glucose. As our understanding of molecular mechanisms evolves, it is becoming clear that a more comprehensive approach is needed. Based

Table 4: Effect of diabetes in protein carbonyl content (PCC) levels from human tissues.

Tissue	Model/condition	Effect	Reference
Erythrocytes	*Obese type 2 diabetic patients*	↑	[46]
Erythrocytes	*Type 2 diabetic patients versus healthy subjects*	↑	[47]
Erythrocyte membrane	*Type 2 diabetic patients versus healthy subjects*	↑	[48]
Lymphocytes	*Type 2 diabetic patients versus age-matched controls*	↑	[49]
Lymphocytes	*DM patients versus healthy subjects*	↑	[50]
Placenta	*Women with gestational diabetes versus healthy pregnant women*	↑	[51]
Plasma proteins	*Type 2 diabetic patients versus healthy subjects*	=	[52]
Plasma proteins	*Dialysis patients versus control subjects*	↑	[53]
Plasma proteins	*Diabetic type 2 patients versus healthy subjects*	↑	[54]
Plasma proteins	*Type 1 diabetes without complications*	↑	[55]
Plasma proteins	*Type 1 diabetes with complications*	↑	[55]
Plasma proteins	*Chronic kidney disease patients versus healthy subjects*	↑	[56]
Plasma proteins	*Diabetes type 2 versus healthy subjects*	↑	[57]
Plasma proteins	*Diabetes type 2 associated with CVD versus healthy subjects*	↑	[57]
Plasma proteins	*Good glycemic control versus poor glycemic control*	↑	[58]
Plasma proteins	*Type 1 diabetic patients*	=	[59]
Plasma proteins	*End-stage renal disease*	↑	[59]
Plasma proteins	*Heart failure + diabetes versus healthy subjects*	↑	[60]
Plasma proteins	*Type 2 diabetes without microangiopathy versus healthy subjects*	↑	[61]
Plasma proteins	*Type 2 diabetes with microangiopathy versus healthy subjects*	↑	[61]
Plasma proteins	*Type 2 diabetic patients versus age-matched controls*	↑	[49]
Plasma proteins	*Childhood type 1 diabetes*	↑	[62]
Plasma proteins	*Diabetic patients without ulcer versus healthy subjects*	↑	[63]
Plasma proteins	*Diabetic patients with foot ulcer grade 1 versus healthy subjects*	↑	[63]
Plasma proteins	*Diabetic patients with foot ulcer grade 2 versus healthy subjects*	↑	[63]
Plasma proteins	*Diabetic patients versus healthy subjects*	↑	[64]
Plasma proteins	*IGT subjects versus healthy subjects*	↑	[64]
Plasma proteins	*Diabetic type 2 patients versus healthy subjects*	↑	[65]
Platelets	*Type 2 diabetes (young versus elderly)*	↑	[66]
Serum	*Type 1 diabetic patients versus healthy subjects*	↑	[67]
Serum	*Diabetic patients versus healthy subjects*	↑	[68]
Serum	*Diabetic nephropathy patients versus healthy subjects*	↑	[68]
Skin collagen	*Type 2 diabetes*	↑	[69]
Subretinal fluid	*Diabetic patients versus control subjects*	↑	[70]

on the numerous evidence of a role of oxidative stress in the pathogenesis of vascular complications, the use of for example, antioxidants, uncouplers, or PARP inhibitors should represent an appealing approach. Candidate "drugs" include: vitamins A, C, and E, alpha-lipoic acid, SOD and catalase mimetics, L-propionyl carnitine, taurine, acetyl-L-carnitine, U83836E (a ROS scavenger), M40403 (a manganese superoxide dismutase mimetic), PKC-b inhibitors, peroxynitrite catalyst FP15, mitochondrial uncoupler DNP, PARP inhibitors, transketolase inhibitors, melatonin, statins, angiotensin converting enzyme inhibitors, angiotensin II receptor blockers, thiazolidinediones, synthetic pyridoindole antioxidant stobadine (STB), extracts from different natural sources (e.g., Artemisia campestris, Centaurium erythraea), the metal chelator pyrrolidine dithiocarbamate (PDTC), and plant polyphenols (e.g., myricetin), among others.

PARP inhibition may emerge as a novel approach for the prevention or reversal of diabetic complications. The benefits and potential risks associated with chronic administration of PARP inhibitors are discussed in a recent review [139]. The comparative therapeutic utility of PARP inhibition for the experimental therapy of diabetic complications should be explored by additional preclinical and subsequent clinical investigations. The development of uncoupling strategies is not forthcoming [93]. So, the time is upon us to test antioxidant therapies in diabetes [78, 93].

8. Conclusions

Hyperglycaemia is the first trigger in the pathogenesis of diabetic vascular complications and it activates many metabolic pathways and their downstream mediators. Several mitochondrial and other intracellular pathways are implicated in the increased production of oxidants. In subjects with diabetes, oxidative damage is enhanced and contributes to the development of endothelial dysfunction and vascular complications. Nevertheless, there still is a considerable wealth of knowledge to be acquired, concerning oxidative stress and diabetes. Assuming that oxidative stress has also a signalling role (exceeding the role of NO), how the signaling role of oxidative stress is modified by diabetic status is still an open question. It needs to be elucidated how the general increase of protein oxidative damage has an impact on the signalling modules of oxidative stress. Furthermore, with a wide knowledge on protein oxidative modification chemistry, there is still lacking a comprehensive study dissecting the potential pathways of protein oxidative modifications in diabetes and diabetes complications. Numerous antioxidant agents are being investigated and there is growing interest in developing new compounds specifically targeting oxidative stress. However, up to now, there is a lack of supporting evidence for an extensive use of antioxidants for preventing or treating diabetic vascular complications. A better and more precise knowledge of the molecular mechanisms underlying hyperglycaemia-related damage will help in developing better therapies. When the answer of these and other relevant questions will be available, then a rationale intervention on ROS homeostasis, more directed than the mere supplementation with antioxidants, will be granted for therapy of diabetes vascular complications.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Mitochondrial Dysregulation in the Pathogenesis of Diabetes: Potential for Mitochondrial Biogenesis-Mediated Interventions

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Muscle mitochondrial metabolism is a tightly controlled process that involves the coordination of signaling pathways and factors from both the nuclear and mitochondrial genomes. Perhaps the most important pathway regulating metabolism in muscle is mitochondrial biogenesis. In response to physiological stimuli such as exercise, retrograde signaling pathways are activated that allow crosstalk between the nucleus and mitochondria, upregulating hundreds of genes and leading to higher mitochondrial content and increased oxidation of substrates. With type 2 diabetes, these processes can become dysregulated and the ability of the cell to respond to nutrient and energy fluctuations is diminished. This, coupled with reduced mitochondrial content and altered mitochondrial morphology, has been directly linked to the pathogenesis of this disease. In this paper, we will discuss our current understanding of mitochondrial dysregulation in skeletal muscle as it relates to type 2 diabetes, placing particular emphasis on the pathways of mitochondrial biogenesis and mitochondrial dynamics, and the therapeutic value of exercise and other interventions.

1. Introduction

Type 2 diabetes is the most common form of diabetes accounting for ~90% of diabetic cases and ~8% of the total population [1]. Type 2 diabetes is characterized by insulin resistance and is commonly associated with several clinical complications such as hypertension, atherosclerosis, and cardiovascular disease, and these are often collectively referred to as the metabolic syndrome [1]. Although the specific molecular mechanisms underlying type 2 diabetes are not well understood, insulin resistance is believed to result from reductions in glucose transport and phosphorylation and impaired fatty acid metabolism in a number of tissues, notably skeletal muscle [1, 2]. Specifically, defects in this series of reactions are directly associated with increased levels of plasma and intracellular free fatty acids and alterations in insulin signaling pathways [3, 4].

Mitochondria have several functions but are most known for their role as key regulators of metabolic activity within the cell by converting energy from the oxidation of macronutrients to ATP. Mitochondrial activity and function in skeletal muscle is a highly controlled process, under the influence of a variety of nuclear and mitochondrial factors that act as metabolic sensors and can adapt to perturbations in cellular nutrient and energy status. The renewal of mitochondria through the process of biogenesis is vital for maintaining mitochondrial integrity, and a diminished capacity for organelle biogenesis has been implicated in the pathogenesis of several diseases such as aging, neurodegeneration, as well as type 2 diabetes [1, 5]. Additionally, muscle mitochondrial metabolism is regulated by a group of morphogenesis machinery proteins which are important for mitochondrial fusion and fission events and also for their independent effects on bioenergetics, programmed

cell death, and autophagy [6]. Defects in mitochondrial biogenesis and morphogenesis factors can impair enzyme activity and reduce the oxidative capacity of the cell leading to insufficient oxidation of lipids and increased intramyocellular lipid (IMCL) levels. The inability of mitochondria to utilize these substrates along with their accumulation within muscle has been associated with impaired insulin signaling pathways and reduced glucose uptake [7]. Elevated IMCLs, in association with the increased production of lipid metabolites such as acyl coenzyme A (CoA), diacylglycerol (DAG), ceramides, and reactive oxygen species (ROS) [2, 8], can affect insulin signaling and contribute to insulin resistance associated with type 2 diabetes. Additionally, skeletal muscle from individuals with type 2 diabetes have a higher percentage of type II fibers and a lower percentage of type I fibers when compared to control individuals [9, 10]. Type II fibers have a reduced capacity to oxidize fat [11] and possess unique properties that have been shown to potentiate mitochondrial hydrogen peroxide production and oxidative stress [12]. Therefore, there are likely multiple factors that contribute to the stress environment that intensify the mitochondrial dysregulation observed in type 2 diabetes.

The multiplicity of mitochondrial functions has made it a logical target for the study of metabolic diseases, and, given that skeletal muscle represents the major site of insulin-stimulated glucose utilization in the body [13, 14], dysregulation of mitochondria is closely associated with insulin resistance and the pathogenesis of type 2 diabetes in muscle. In this paper, we will first discuss key pathways involved in the regulation of mitochondria, with specific attention given to organelle biogenesis, as well as mitochondrial fusion and fission events and their contribution to metabolic perturbations in muscle. In the second part, current therapeutic interventions will be described, with the focus on those related to stimulating mitochondrial biogenesis.

2. Mitochondrial Biogenesis

Skeletal muscle is a malleable tissue and can adapt to alterations in energy status and substrate supply in part via its ability to increase the number of mitochondria. Mitochondrial biogenesis is induced by numerous physiological, environmental, and pharmacological stimuli and results from the transcription and translation of genes both in the nuclear and the mitochondrial genomes [15, 16]. The biogenesis of mitochondria is mediated by changes in many key intracellular events, including transcriptional activation, mRNA stability, posttranslational modification of proteins, and/or alterations in the import and processing of proteins in mitochondria (Figure 1) [17, 18]. These gene products are assembled into functional multisubunit complexes within mitochondria and enhance oxidative capacity and ATP production within the cell. Thus, mitochondria are key regulators of metabolic activity within the cell, and it is these attributes that have made mitochondria a primary focus in the study of metabolic disorders such as type 2 diabetes.

Some of the early studies examining mitochondrial function and insulin resistance reported reduced mitochondrial content and impaired lipid oxidation in skeletal muscle of obese and type 2 diabetic individuals [19, 20]. The role of mitochondria in obesity and type 2 diabetes has been confirmed by studies examining insulin resistant but otherwise, healthy individuals, with non-insulin-resistant subjects. The insulin-resistant group did not only have higher intramyocellular lipid (IMCL) levels than the control group, but this was associated with a 40% decrease in both oxidative capacity and ATP levels [21]. Impaired mitochondrial oxidative capacity is also an early feature observed in insulin-resistant offspring of individuals with type 2 diabetes [22]. The altered mitochondrial phenotype observed in human skeletal muscle tissue is retained in myocyte cultures obtained from individuals with type 2 diabetes [23]. Furthermore, an A3243G mutation in the $tRNA^{Leu}$ gene in mitochondrial DNA (mtDNA) is the cause of maternally inherited diabetes and deafness (MIDD), and individuals with this mutation will likely develop diabetes as they age [24]. These data support the hypothesis that mitochondrial dysfunction may be one of the early events in the pathogenesis of this disease that predisposes an individual to elevated levels of IMCL, lipid metabolites, and insulin resistance.

In order to elucidate the molecular mechanisms that are responsible for the reduction in mitochondrial content and enzyme activity, earlier studies used DNA microarray techniques to examine the gene expression profile of mitochondrial proteins within skeletal muscle of obese and type 2 diabetic patients. These studies found significant alterations in a wide array of genes responsible for glucose uptake, fatty acid oxidation, and oxidative phosphorylation (OXPHOS) [25, 26]. These include changes in the expression of various nuclear regulatory proteins. Peroxisome proliferator-activated receptor γ (PPARγ) coactivator-1 (PGC-1) and their related family members (PGC-1α, PGC-1β, PRC, and PERC) [27–30] are perhaps among the most well-known regulators of mitochondrial biogenesis. In particular, PGC-1α binds and coactivates transcription factors such as the estrogen-related receptor alpha (ERRα) and the nuclear respiratory factors 1 and 2 (NRF-1 and NRF-2) to cause the induction of a broad spectrum of genes involved in substrate metabolism and mitochondrial biogenesis. NRF-1 and NRF-2 have been shown to transactivate target genes involved in several mitochondrial processes, including OXPHOS subunits, heme biosynthesis, mitochondrial import machinery, and mtDNA transcription [31–33]. In addition, it has been shown that a dominant negative allele of NRF-1 prevents the ability of PGC-1α to induce mitochondrial proliferation, confirming the importance of NRF-1 in PGC-1α-driven mitochondrial biogenesis [29].

In skeletal muscle, the importance of PGC-1α has been reinforced with data from studies in both cell culture, as well as transgenic mouse models, where PGC-1α levels were experimentally altered. Forced expression of PGC-1α in cultured muscle cells and cardiac myocytes results in an increase in nuclear and mitochondrial gene expression and mtDNA content [29, 34]. Animals with increased muscle PGC-1α have a longer lifespan that is associated with enhanced mitochondrial function, improved insulin sensitivity, and

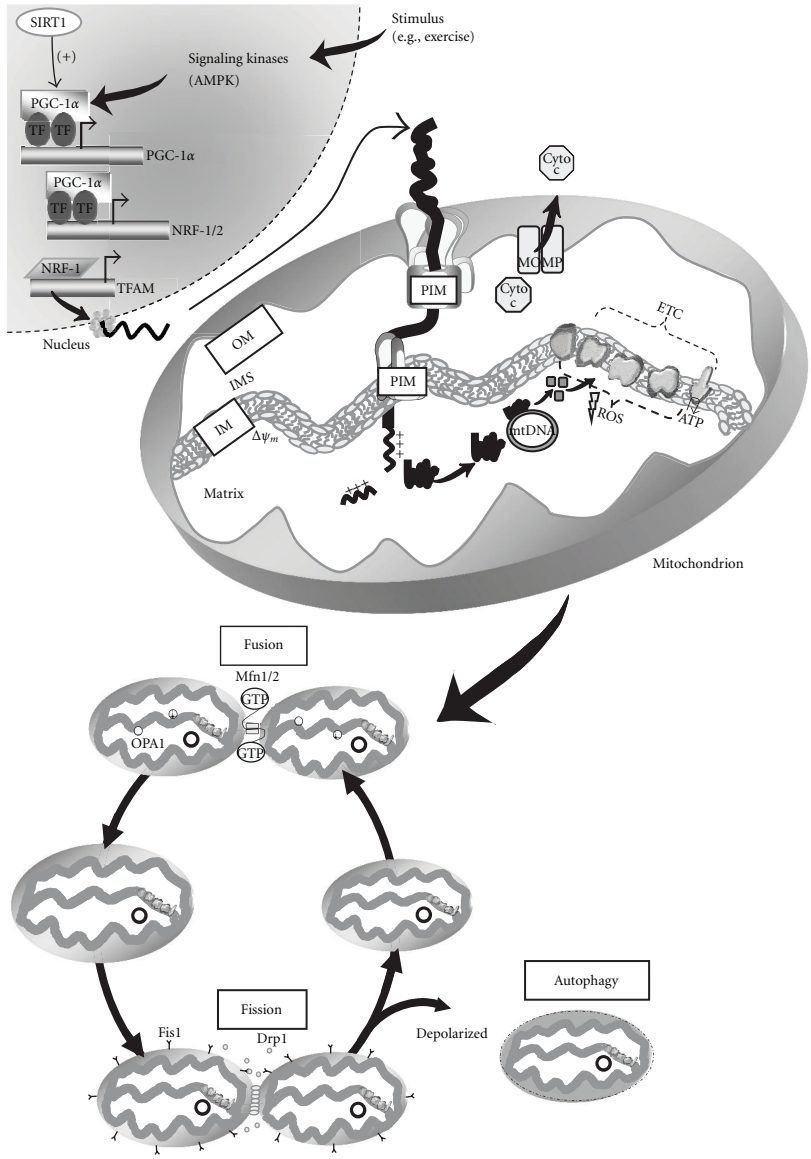

Figure 1: Proposed model of mitochondrial biogenesis. In response to a stimulus such as skeletal muscle contractile activity or exercise, intracellular Ca^{2+} levels, as well as AMP levels, increase leading to the activation of signaling molecules including AMP-activated protein kinase (AMPK). These signaling pathways converge and interact primarily with the transcriptional coactivator peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) which is a master regulator of mitochondrial biogenesis. PGC-1α activates its own expression, as well as the expression of the nuclear respiratory factor-1 and 2 (NRF-1/2). Additionally, PGC-1α has recently been shown to be deacetylated and activated by the longevity protein sirtuin 1 (SIRT1). NRF-1 and NRF-2 bind and upregulate the expression of nuclear genes encoding mitochondrial proteins (NUGEMPs), as well as the expression of mitochondrial transcription factor A (Tfam). Tfam along with other newly transcribed NUGEMPS are targeted and imported into mitochondrial subcompartments via the protein import machinery (PIM). Within the matrix, Tfam binds to mtDNA and regulates the expression of the 13 mitochondrial DNA (mtDNA) gene products. These proteins are assembled into multisubunit enzyme complexes within the electron transport chain (ETC) and mediate oxidative phosphorylation (OXPHOS) and the production of ATP. Thus, coordinated expression regulated by the two genomes allows for the proper assembly and expansion of the mitochondrial reticulum leading to mitochondrial proliferation and increased mitochondrial number/content. Another important product of the ETC is reactive oxygen species (ROS) that are associated with the mitochondrial membrane potential ($\Delta\psi_m$). Elevated levels of ROS have been shown to activate mitochondrial outer membrane permeabilization (MOMP) and the release of proapoptotic factors such as cytochrome c (Cyt c) into the cytosol that can subsequently activate caspase-dependent signaling cascades leading to mitochondrially-mediated apoptosis. Furthermore, organelle biogenesis requires a continuous cycle of fusion and fission events. Mitochondrial fusion of the outer and inner mitochondrial membranes is mediated by the GTPase proteins, mitofusin 1 and 2 (Mfn1 and Mfn2) and OPA1, respectively. Conversely, mitochondrial fission requires Drp1 and Fis1 which assemble at fission sites on the mitochondrial membrane and induce membrane division. It has been proposed that fission can lead to mitochondria with different $\Delta\psi_m$ and that damaged or depolarized organelles will exit the fusion and fission cycle and will be removed through autophagy.

reduced oxidative damage and also show resistance to age-related weight gain [35]. Furthermore, overexpression of PGC-1α in mice results in a partial fiber-type transition from white muscle with mostly glycolytic fibers to muscle that appears red and has a high oxidative capacity [36]. This fiber-type conversion coincides with the activation of calcineurin signaling cascades, the coactivation of myocyte-enhancer factor 2 (MEF2) by PGC-1α, and the induction of slow gene expression pathways. Calcium- (Ca^{2+}-) dependent PGC-1α activation was further confirmed in skeletal muscle from transgenic mice overexpressing a constitutively active form of the calcium/calmodulin-dependent protein kinase IV (CaMKIV). These mice displayed increased mtDNA copy number and an upregulation of several enzymes that are involved in fatty acid oxidation and OXPHOS [37]. Additionally, upregulation of PGC-1α mRNA and protein with acute and chronic exercise in both animals and humans leads to an increased mitochondrial content through the induction of NRF proteins and mitochondrial transcription factor A (Tfam) [38–40].

Regarding metabolic disorders, PGC-1α mRNA levels are reduced in certain cohorts of obese and type 2 diabetic individuals [25, 26], and, in some populations, polymorphisms in the PGC-1α gene have been linked to a predisposition for type 2 diabetes [41, 42]. PGC-1α induces the expression of the insulin-sensitive glucose transporter (GLUT4) by interacting and coactivating the MEF2 transcription regulator [43]. Furthermore, the tissue-specific knockout of Tfam in pancreatic β cells leads to the development of diabetes that is associated with a loss of mtDNA and impaired oxidative capacity [44]. Despite these findings, the importance of PGC-1α and other mitochondrial regulators of biogenesis in insulin resistance and type 2 diabetes has remained controversial. This is because several studies have shown elevated IMCL levels and reduced mtDNA content in the absence of changes in PGC-1α expression (mRNA or protein) or other PGC-1α-related target genes [22, 45, 46]. In addition, several studies of muscle-specific PGC-1α and/or PGC-1β null mice have demonstrated normal glucose tolerance and insulin sensitivity [47, 48]. These studies suggest that alternate mechanisms may also regulate mitochondrial content in metabolic diseases. Clearly more work is required in this area to obtain a better understanding of the molecular pathways mediating insulin sensitivity in both healthy muscle, as well as muscle with metabolic dysfunction.

Another clue into the molecular function of PGC-1α comes with the recent finding that PGC-1α is present within mitochondria and specifically localized in a complex with Tfam in mtDNA nucleoids [49]. This surprising finding is also confirmed in animals where, following an acute bout of exercise, PGC-1α protein was increased in both the nuclear and mitochondrial subfractions [50]. These preliminary studies suggest that PGC-1α coactivates mitochondrial transcription in both the nucleus and mitochondria and indicates the potential of PGC-1α as being a central messenger of nuclear-mitochondrial crosstalk during cellular stress.

Recently, another family of proteins has emerged as crucial regulators of mitochondrial activity and cellular energy metabolism. Sirtuins are a group of class III histone/protein deacetylases that are primarily known for their involvement in promoting lifespan in a number of organisms including yeast, flies, and mice, and they accomplish this by the acetylation and deacetylation of target genes [51]. To date, seven sirtuin mammalian homologs have been identified (SIRT1-7), three of which are mainly localized to mitochondria (SIRT3, SIRT4, SIRT5) [51, 52]. SIRT1 is a NAD-dependent deacetylase that is widely expressed in mammalian cells and activated in response to cellular stress conditions such as with exercise, caloric restriction (CR), and starvation [53–57]. Transgenic mice overexpressing SIRT1 have similar physiological and behavioral phenotypes as calorie restricted mice. These animals have less body fat, are more metabolically active, and display improved insulin sensitivity and glucose tolerance [58]. The CR-mediated phenotype is dependent on SIRT1, since knockout of this gene diminishes these adaptations [59]. Studies investigating the role of SIRT1 in diabetes and skeletal muscle are limited. In cardiac muscle of Otsuka Long-Evans Tokushima fatty (OLETF) rats, SIRT1 levels were lower in these animals than in control rats. Treatment with pioglitazone, a PPARγ agonist, enhanced SIRT1 expression [60]. Similar attenuations in SIRT1 levels were reported in adipocytes from ob/ob mice [61] and adipose tissue from obese women [62]. SIRT1 gain-of-function studies in various models of insulin resistance and diabetes have revealed improved glucose tolerance and decreased energy expenditure that is due to lower hepatic glucose production and increased adiponectin levels [63] suggesting that SIRT1-mediated longevity may be related to improvements in insulin sensitivity.

SIRT1 regulates AMP activated-protein kinase (AMPK), a key energy-sensing molecule that is activated during conditions of low ATP and high AMP (increased AMP/ATP ratio) such as with exercise [64] and CR [65]. Interestingly, both SIRT1 and AMPK together or independently activate PGC-1α to induce the expression of genes involved in glucose and fatty acid metabolism and restore ATP levels [66–69]. AMPK activity levels decline with aging, and this is associated with reduced insulin sensitivity and diminished fatty acid oxidation, suggesting that AMPK is an important regulator of mitochondrial metabolism in muscle [70, 71].

More recently, another histone/protein deacetylase has gained increasing attention as a key regulator of metabolic activity in muscle. SIRT3 contains a cleavable N-terminal mitochondrial targeting signal that permits its import into mitochondrial subcompartments [51]. SIRT3 levels were diminished with aging and a high fat diet [65, 72] and increased in response to exercise, CR, and fasting [65, 72, 73]. It is likely that, similar to SIRT1, SIRT3 is a key regulator of muscle adaptation since it also targets PGC-1α and influences mitochondrial transcriptional regulation in muscle [65, 74].

It is important to remember that skeletal muscle contains two subpopulations of mitochondria, subsarcolemmal (SS) and intermyofibrillar (IMF) mitochondria, that display key differences in biochemical and functional properties [75]. These differences are crucial because it has been proposed

that, due to their close proximity to myonuclei, SS mitochondria may be important for driving processes at the cell surface, including the propagation of insulin signaling pathways, fatty acid oxidation, and glucose transport [75, 76]. In contrast, IMF mitochondria are thought to provide energy for muscle contractions [75]. In both SS and IMF subfractions, electron transport chain (ETC) activity was reduced in obese and type 2 diabetic subjects when compared to lean subjects [77]. Interestingly, the decrement in enzyme activity was more pronounced in the SS, compared to the IMF subfraction, suggesting that SS mitochondria may be more readily affected in states of altered glucose homeostasis. These data are consistent with previous findings showing that SS mitochondria are more labile in response to metabolic changes [78, 79].

3. Mitochondrial Fusion and Fission

The adaptability of skeletal muscle is also associated with mitochondrial morphological plasticity. Mitochondria are dynamic and readily adapt to changes in cellular energy demands through network remodeling and continuous fusion and fission [5, 80]. Under normal conditions, mitochondrial fusion results in the formation of an interconnected mitochondrial network that allows the mixing and redistribution of proteins and mtDNA and which has been hypothesized to prevent the accumulation of mutated or damaged mtDNA in a cell [5, 81, 82]. In contrast, mitochondrial fission leads to mitochondria with a fragmented morphology facilitating the segregation of damaged mitochondria that can then be targeted for degradation via autophagy [5]. Although the molecular mechanisms mediating these morphological changes remain largely unknown, recent studies have identified distinct fusion and fission machinery that appear to regulate these processes [5, 81, 82].

The fusion of mitochondria in mammalian cells is mediated by several proteins, the most well known being the nuclear-encoded dynamin-related guanosine triphosphatases (GTPase), mitofusin 1 (Mfn1), and mitofusin 2 (Mfn2). Although these mitofusins share greater than 70% homology, they have different GTPase activity levels and display distinct expression patterns, with Mfn2 present in higher amounts in tissues such as heart and skeletal muscle [83, 84]. Additionally, mutations in Mfn2 cause Charcot-Marie-Tooth (CMT) disease-type 2A, the most common form of CMT disease and an inherited neuropathy leading to progressive weakness and sensory loss [85, 86]. While mitofusins are responsible for tethering and fusion of the outer mitochondrial membrane, another dynamin family GTPase, Optic Atrophy 1 (OPA1), is required for inner membrane fusion. OPA1 was first identified through its involvement in the neurodegenerative disease known as autosomal dominant optic atrophy (ADOA) [87]. Alternative splicing of OPA1 produces multiple variants that are distinctly present in different species and tissues [88]. Furthermore, posttranslational modification of OPA1 by mitochondrial processing peptidase (MPP) results in different length isoforms that vary based on their localization and function [89, 90]. Muscle-specific Mfn1 and Mfn2 knockout mice with a diminished capacity for mitochondrial fusion have impaired mitochondrial function and a loss of muscle mass that are associated with increased mtDNA point mutations and deletions and severe mtDNA depletion [91]. Similarly, silencing of OPA1 in mammalian cells blocked mitochondrial fusion and resulted in mitochondrial fragmentation, decreased OXPHOS, poor cell growth, and reduced mitochondrial membrane potential ($\Delta\psi_m$) [92]. These data indicate that mitochondrial fusion is important for maintaining the integrity of the organelle by allowing the intramitochondrial exchange of damaged mitochondria, preventing their localization within specific organelles and their accumulation within the cell.

Mitochondrial homeostasis is also regulated by fission machinery such as dynamin-related protein 1 (DLP1/Drp1) and fission protein 1 (Fis1). Drp1 is a dynamin-related GTPase that is found in the cytosol and recruited by Fis1 to scission sites on the mitochondrial outer membrane to induce mitochondrial fission. Downregulation of Drp1 in HeLa cells leads to a loss in mtDNA, reduced mitochondrial respiration, and higher levels of ROS, all of which are associated with mitochondrial dysfunction [93]. Also, blocking fission by reducing the levels of Drp1 and Fis1 genes in a human cell line with a mtDNA mutation exacerbates the abundance of mutant mtDNA compared with wild-type mtDNA [94]. These studies imply that mitochondrial fusion and fission are involved in mtDNA quantity and quality control and are required for the maintenance of healthy organelles.

It is also becoming more and more apparent that mitochondrial morphology is directly linked to mitochondrial function and substrate utilization. Cells with a high mitochondrial fusion capacity display interconnected mitochondria associated with increased OXPHOS, while cells with high mitochondrial fission have fragmented mitochondria and rely more on anaerobic metabolism pathways for energy [81]. A hallmark of mitochondrial biogenesis is an expansion of the mitochondrial reticulum, thereby allowing the propagation of signaling pathways and the mixing of metabolites. A perfect example of this adaptation in muscle occurs with exercise, whereby repeated bouts of an exercise stimulus lead to muscle adaptations. Substantial evidence in the last decade has shown the involvement of mitochondrial network remodeling in these exercise-induced adaptations. The expression levels of Mfn1, Mfn2, and Fis1 have been shown to be increased in skeletal muscle following an acute bout of exercise in both animals and humans [95, 96]. Additionally, transcript levels of mitochondrial dynamics proteins are upregulated with endurance exercise in healthy subjects, and these are closely correlated with muscle OXPHOS activity and PGC-1α mRNA content [97]. An important finding of these studies is that fusion and fission machinery respond rapidly to an exercise stimulus and that the levels of these proteins are dependent on the type of exercise [95, 96].

Given the involvement of mitochondrial dynamics in muscle metabolism, perturbations in the fusion-fission balance caused by changes in the levels of the molecular machinery have been shown to lead to abnormal mitochondrial morphology and to negatively impact mitochondrial

function. Mitochondrial gene expression profiles and function are associated with changes in overall mitochondrial morphology in skeletal muscle from diabetic rats [83], as well as in humans with type 2 diabetes [83, 98]. In particular, mitochondria in skeletal muscle from type 2 diabetics are smaller in size than mitochondria present in lean subjects, and they also contain abnormal cristae structure that would suggest defects in the inner membrane [83, 98, 99]. In our laboratory, we have found reduced levels of fusion proteins Mfn2 and Opa1, but no alterations in fission proteins Drp1 or Fis1 in skeletal muscle from type 2 diabetic individuals (Figure 2(a)). Similar reports of decreased Mfn2 expression and aberrant mitochondrial morphology have been documented by other studies in type 2 diabetics [98, 99].

Loss of function of the fusion protein Mfn2 leads to impaired mitochondrial metabolism and is associated with reduced mitochondrial oxygen consumption, membrane potential, and glucose metabolism in a variety of tissues [83, 92, 100]. In contrast, Mfn2 gain of function increases substrate oxidation and improves mitochondrial metabolism in HeLa cells [100]. Additionally, similar to other key metabolic factors, Mfn1 and Mfn2 are increased in response to weight loss and exercise in healthy, obese, and type 2 diabetic individuals [95, 101] and are increased also in mice subject to CR in mice [102]. This is dependent primarily on PGC-1α and ERRα transactivation of the Mfn2 promoter, suggesting that fusion is an important signaling event for mitochondrial biogenesis and healthy insulin signaling in muscle [95, 103]. Additionally, reduced levels of OPA1 are associated with insulin resistance in several cell types [104], as well as in human fibroblasts from patients with ADOA with impaired OXPHOS and reduced mitochondrial fusion events [105]. These findings are in keeping with the observation that mitochondrial fusion is an important signaling event for mitochondrial biogenesis in muscle. For the most part, the regulatory mechanisms and the functional importance of these protein changes in obesity and type 2 diabetes remain enigmatic, and more work is required to elucidate the regulatory factors of the other fusion and fission machineries.

3.1. Mitochondrial Dynamics and Cell Death. The finding that aberrant mitochondrial morphology is present in cells undergoing apoptosis has led to the examination of the possible link between mitochondrial fusion and fission processes and programmed cell death. A hallmark of apoptosis is mitochondrial outer membrane permeabilization (MOMP) and the release of cytochrome c that activates proapoptotic signaling cascades. This results in the fragmentation of DNA [106]. Imaging experiments have demonstrated that mitochondrial fragmentation occurs concurrently with MOMP. This is primarily due to fission, since either the inhibition of fission proteins or upregulation of fusion proteins can delay or prevent mitochondrial fragmentation, along with the induction of proapoptotic signaling events including MOMP, cytochrome c release, and cell death [89, 107].

Studies conducted to elucidate the molecular mechanisms responsible for the association between mitochondrial morphology and apoptosis revealed a physical interaction between morphogenesis proteins and the apoptosis machinery. In particular, proapoptotic proteins such as Bax and Bak interact with Mfn proteins [108, 109], and similar findings were reported between the fission machinery and the Bcl-2 family of proteins [110, 111]. It is highly likely that the physical interaction between apoptotic proteins and mitochondrial morphogenesis proteins is another means of regulating cell death. However, not all studies are consistent with this theory, and more work is required to clearly show whether mitochondrial fusion and fission are an important part of the apoptosis signaling cascade. Nonetheless, altering levels of fusion and fission events can confer protection to the cell, and this introduces a potential mechanism of decreasing apoptotic susceptibility through the modulation of the fusion and fission machinery.

Mitochondria represent the major source of ROS production within the cell and increased levels of ROS are a likely culprit in a variety of pathophysiological conditions, including type 2 diabetes. Increased ROS production was associated with altered mitochondrial morphology in myotubes cultured in high glucose conditions, as well as in diet-induced diabetic mice [112]. Furthermore, increased oxidative stress within mitochondria arising from impaired oxidative metabolism may contribute to greater lipid peroxidation and damage to cell membranes and DNA, activating a cascade of signaling events that further exacerbate the severity of the disease [113]. A number of studies have found that altering mitochondrial fusion and fission events can influence ROS production in mitochondria. For instance, increasing mitochondrial fusion or decreasing mitochondrial fission during hyperglycemia can prevent mitochondrial fragmentation and reduce ROS levels in the cell [107]. Treatment of cells with hydrogen peroxide, antimycin A, or rotenone to increase ROS production resulted in fragmented mitochondria [107, 114], while addition of an antioxidant reduced ROS levels and inhibited mitochondrial fragmentation [115]. The effect of mitochondrial fusion and fission machinery on apoptosis is likely associated with its ability to modulate ROS levels. Thus, mitochondrial dynamics is a relatively new field in mitochondrial biology, and despite growing progress, many questions still remain regarding how alterations in mitochondrial shape affect mitochondrial bioenergetics, morphology, and ROS production, as well as the sequential order of these events in skeletal muscle with obesity and type 2 diabetes.

Furthermore, a growing area of interest in the field of metabolic diseases is the study of obesity in the elderly, the prevalence of which has considerably increased in the last few years [116, 117]. As we age, there is a progressive decline in muscle mass and strength, as well as an increased fat mass. This combination of sarcopenia and obesity has recently been defined as "sarcopenic obesity" [116, 117]. While the molecular mechanisms mediating a loss of muscle mass in obesity and type 2 diabetes are unknown, several theories, including increased apoptosis, have been brought forward. Recently,

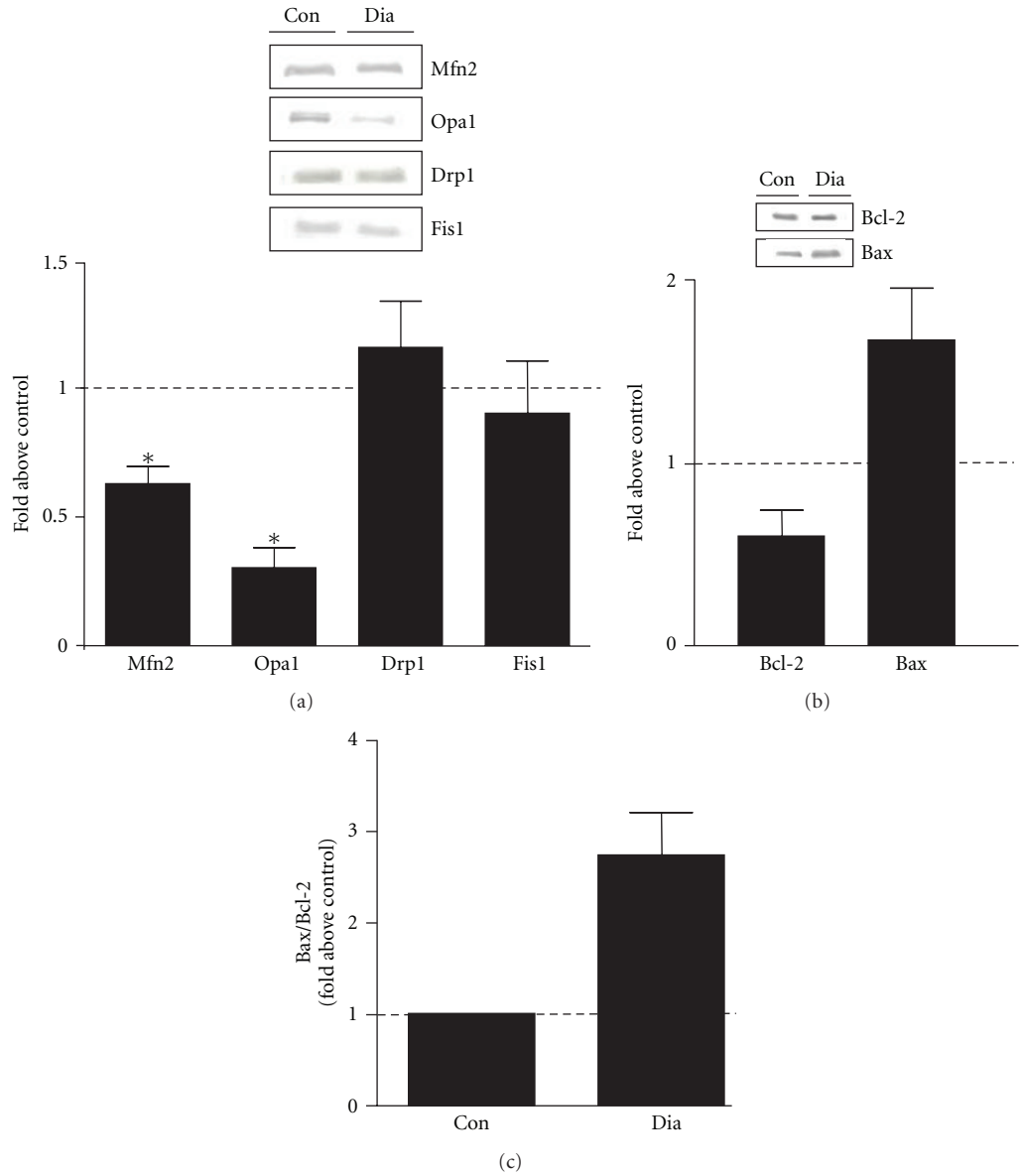

Figure 2: (a) Mitochondrial morphology proteins in type 2 diabetes. The research volunteers that participated in this study were obese subjects with type 2 diabetes undergoing coronary bypass surgery and were all male between 48 and 75 years of age. Biopsies from the vastus medialis muscle were removed from both control and type 2 diabetic subjects from within incisions of the inner thigh and protein analyses performed. The protocol was approved by the Medical Ethics Committees of Laval University and Laval Hospital, and all subjects provided informed written consent. Representative western blots of fusion proteins Mfn2 and OPA1 and fission proteins Drp1 and Fis1 from the vastus medialis muscle of control (Con) and type 2 diabetic subjects (Dia). A summary of repeated experiments is shown below with values expressed as a fold over control. Values are means ± SE; $n = 4$–9; $^*P < 0.05$ versus Con. (b) Indicators of apoptotic susceptibility in type 2 diabetes. Western blots of the antiapoptotic protein Bcl-2 and the proapoptotic protein Bax in vastus medialis muscle of Con and Dia individuals and the graphical representation of the data is shown below. Values are means ± SE; $n = 4$–9. (c) The ratio of Bax/Bcl-2 in Dia subjects when compared to Con.

Sishi et al. [118] have demonstrated that, as a result of diet-induced obesity, skeletal muscle of adult rats displayed muscle atrophy along with increased apoptosis. This was evident by increased levels of caspase-3 and poly(ADP-ribose) polymerase (PARP), two hallmark features of cell death. Additionally, electron micrographs of muscle fibers from obese and type 2 diabetic individuals display an altered mitochondrial structure and fragmented mitochondria, indicating the potential involvement of apoptosis [99]. We have obtained similar results of increased apoptotic signaling, with a trend toward higher levels of the proapoptotic protein Bax and lower levels of the antiapoptotic protein Bcl-2 observed in

muscle from type 2 diabetics (Figure 2(b)). This indicates a greater Bax/Bcl-2 ratio (Figure 2(c)) and suggests that muscle from type 2 diabetics has a greater susceptibility to apoptosis when compared to healthy individuals. Although these studies point to the involvement of apoptosis in sarcopenic obesity, much more research is required to characterize apoptotic signaling pathways in obesity and type 2 diabetes.

3.2. Mitochondrial Dynamics and Autophagy. Mitochondrial dynamics are also involved in another form of cell death known as autophagy. Autophagy protects the cell through the selective degradation and recycling of organelles such as mitochondria by lysosomal machinery [119, 120]. Under normal conditions, autophagy prevents the accumulation of damaged mitochondria within the cell that can trigger apoptotic pathways and irreversible cell death. Autophagy is particularly important for postmitotic tissues such as skeletal muscles that are exposed to high levels of oxidative stress and that do not have an inherently high capacity for regeneration [119, 120].

Recent studies have shed light on the function and regulation of autophagic pathways in skeletal muscle and have implicated the involvement of mitochondrial fission. The monitoring of individual mitochondria in cultured cells has revealed that daughter mitochondria generated by mitochondrial fission display different mitochondrial membrane potentials ($\Delta\psi_m$), with one mitochondrion possessing a high $\Delta\psi_m$ and the other a low $\Delta\psi_m$. Interestingly enough, depolarized mitochondria with a lower $\Delta\psi_m$ were selectively degraded by autophagy and displayed a lower fusion capacity when compared to mitochondria with a high $\Delta\psi_m$. The reduced capacity for fusion in this subpopulation of mitochondria was attributed to low levels of the OPA1 protein [104]. Increased autophagy levels have also been reported in Fis1-overexpressing cells, indicating the potential for both fusion and fission events in regulating autophagy signaling pathways and mitochondrial turnover [121].

Reduced autophagy has been reported in several tissues with age [122–124], and this is associated with the presence of enlarged mitochondria. In a recent study conducted by Masiero and Sandri [125], muscle-specific knockout of the autophagy-related protein 7 (Atg7) resulted in muscle loss and weakness associated with abnormal mitochondria and the accumulation of protein aggregates and vacuoles. Impaired glucose homeostasis and abnormal mitochondrial structure were also observed in Atg7-deficient pancreatic β cells [126]. Furthermore, electron micrographs of muscle fibers from obese and type 2 diabetic subjects revealed that mitochondria are smaller and contain abnormal inner membrane structure when compared to muscle form lean subjects [83, 98]. Additionally, the presence of vacuole-like structures which, when stained with a membrane marker, contain fragmented mitochondria could be detected [99]. Whether these vacuoles represent autophagosomes engulfing damaged or energy-deficient mitochondria still requires con-firmation. These studies suggest that the clearance of damaged proteins and organelles such as mitochondria is vital for maintaining cellular integrity. Whether the dysregulation of autophagy contributes to the pathogenesis of type 2 diabetes remains to be investigated.

4. Mitochondrial-Mediated Therapeutic Interventions

As mentioned above, factors involved in mitochondrial biogenesis are vital for tissue-specific metabolic control, and recent research has focused on modalities that can improve mitochondrial function and substrate oxidation by stimulating mitochondrial biogenesis and nutrient-sensing pathways. These include physiological and macronutrient interventions, as well as pharmacological interventions.

4.1. Physiological and Macronutrient Interventions

4.1.1. Exercise and Weight Loss. Skeletal muscle from obese and type 2 diabetic individuals is characterized by an impaired oxidative capacity and increased IMCL content [127, 128]. Recent studies to identify potential therapeutic modalities in obese/type 2 diabetic individuals have shown the effectiveness of both acute and chronic exercise to increase muscle glucose disposal, fatty acid oxidation, and mitochondrial biogenesis [129–131]. These changes are mediated by key metabolic factors such as AMPK and SIRT1 that are stimulated with reduced energy states and directly activate the PGC-1α-mediated induction of target genes, including NRF-1, Tfam, and Mfn2, as well as genes involved in glucose and fatty acid oxidation [69, 95, 96, 130]. Obese and type 2 diabetic individuals have impaired activation of these signaling pathways, and their responses are attenuated when compared to healthy subjects suggesting that a higher intensity and/or duration of exercise is required to achieve the same adaptations in these patients [130, 132].

Weight loss alone is associated with a reduction in IMCL content [20, 133]. However, the beneficial effects of exercise on insulin resistance occur in the absence of a change in lipid content and active lipid intermediates (e.g., ceramides, DAGs, CoAs) and in some cases even elevated IMCL levels [134, 135]. In a study conducted by He et al. [136] using a combination of weight loss and moderate-intensity exercise, increased insulin sensitivity was associated with a reduction in the size of the lipid droplets within skeletal muscle and not the total amount of lipid. The reduced size of the lipid droplets was linked to an improved aerobic capacity, suggesting that increased oxidative enzyme activity resulting from physical activity allowed for the more efficient oxidation of lipids. This is consistent with studies of trained athletes that have been reported to have higher IMCL levels, coinciding with greater oxidative enzyme capacity and improved insulin sensitivity [137, 138]. However, the adaptations that occur in patients with obesity and type 2 diabetes following these interventions do not result in an increased mtDNA content that is typical of mitochondrial biogenesis in healthy muscle. In addition, mitochondria from these individuals display abnormal morphology with altered inner membrane cristae structure [45]. These results are in keeping with the observation that the induction of mitochondrial biogenesis in patients with this metabolic

condition differs from the molecular processes typically observed in healthy individuals.

4.1.2. Macronutrient Modulation. The beneficial effects of nutritional interventions on health and lifespan have been known for decades. Nutrient deprivation by way of CR (20–40% reduction) increases lifespan in a number of species ranging from insects to mammals [139, 140]. The efficacy of CR in muscle is mediated by a variety of cellular and molecular changes including increased mitochondrial biogenesis and reduced metabolic rate and ROS levels, as well as increased mitochondrial autophagy [141–144]. Additionally, CR alters substrate utilization in muscle from carbohydrate metabolism towards a greater fatty acid oxidation [145]. The effects of CR have also been reported in humans albeit to a lesser extent than in animals. Civitarese et al. showed that 6 months of 25% CR in healthy young individuals resulted in increased mitochondrial biogenesis that was associated with higher levels of SIRT1, PGC-1α, and Tfam protein. Skeletal muscle from these subjects displayed increased mtDNA content along with decreased levels of DNA damage [146]. CR (25%) has also been shown to improve insulin sensitivity and increase mitochondrial density and oxidative enzyme activity in skeletal muscle from type 2 diabetic individuals [131]. Furthermore, prolonged CR in obese individuals with type 2 diabetes decreased myocardial triglyceride content that was associated with improved myocardial function [147]. Thus, CR may be an effective way to increase mitochondrial biogenesis in a wide range of species and tissues; however, more studies are required to characterize its role in insulin signaling in muscle from type 2 diabetics.

4.1.3. Pharmacological Stimulation of Mitochondrial Biogenesis. In an effort to reduce the severity of insulin resistance and the clinical phenotype associated with type 2 diabetes, different classes of drugs have been developed that stimulate/induce mitochondrial biogenesis and morphogenesis. Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors that mediate the expression of a wide array of genes involved in glucose and fat metabolism. Modulation of the PPAR-activated pathway may have therapeutic potential for metabolic disorders. For example, thiazolidinediones (TZDs), such as rosiglitazone, troglitazone, and pioglitazone, are classified as PPARγ agonists that improve insulin sensitivity by increasing substrate metabolism and reducing FFA levels and ROS production [148, 149]. The PPAR agonist bezafibrate has been shown to increase mitochondrial enzyme activity, as well as the levels of several respiratory chain subunit proteins in human fibroblasts with respiratory chain deficiency [150].

Another class of drugs gaining wide acceptance in the treatment of diabetes are sirtuin 1 (SIRT1) activators. Transgenic mice overexpressing SIRT1 display lower whole-body energy requirements and have decreased rates of oxygen consumption resulting in higher metabolic efficiency when compared to their wild-type littermates. Additionally, these animals display decreased susceptibility to the development of diabetes [63]. The therapeutic potential of resveratrol, a polyphenolic extract from grape skins and red wine, has been gaining increasing popularity as a CR mimetic due to its ability to induce mitochondrial biogenesis via SIRT1-mediated activation of PGC-1α in mice. Furthermore, these mice have improved insulin sensitivity and are refractory to high-fat diet-induced obesity [151, 152]. Small SIRT1 activator molecules appear to mimic the effect of CR by altering the expression of target genes involved in substrate metabolism and antioxidant defenses thereby improving metabolic function [151, 152]. In a recent study by Milne et al. [153], obese animals treated with small SIRT1 activator molecules were shown to display enhanced mitochondrial function and improved insulin sensitivity, indicating the potential use of these drugs in the treatment of type 2 diabetes.

Another group of mitochondrial biogenesis-stimulating agents showing therapeutic promise are AMPK activators such as 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) and metformin. The addition of AICAR to C_2C_{12} myoblasts upregulated PGC-1α gene expression through SIRT1 [154], at least in part via transcriptional activation [155]. The administration of AICAR or metformin improved insulin resistance and delayed the onset of diabetes [156, 157]. Furthermore, metformin has been shown to improve insulin sensitivity and has been consistently used as an antidiabetic agent [158]. Thus, AMPK activators have shown great promise as a pharmacological intervention for type 2 diabetes, and future studies in this area will further our understanding of AMPK signaling pathways and promote the development of additional potentially effective antidiabetic agents.

5. Concluding Remarks

It is well established that mitochondria are intricately involved in the pathogenesis of type 2 diabetes. This is because of the instrumental role that mitochondria play in lipid and carbohydrate metabolism, their morphological and functional plasticity in response to inactivity and disease, and their involvement in apoptosis and autophagy. Whether the alterations observed in mitochondrial function are a cause, or a consequence, of insulin resistance and type 2 diabetes remains under debate. Despite the fact that there is conflicting evidence regarding the extent of mitochondrial dysfunction in type 2 diabetics, there is a consensus among studies that skeletal muscle mitochondria from diabetic animals or humans exhibit impairments in key transcriptional regulators. In addition, alterations in genes involved in mitochondrial fusion and fission, as well as aberrant mitochondrial morphology, are commonly associated with the increased production of ROS and the accumulation of damaged DNA, proteins, and lipids. Collectively, these transcriptional alterations can impair insulin signaling pathways leading to insulin resistance and the development of type 2 diabetes (Figure 3). The potential to stimulate mitochondrial biogenesis and morphogenesis through physiological interventions such as exercise, CR, or pharmacological mimetics of mitochondrial biogenesis has shown great promise in improving insulin sensitivity and

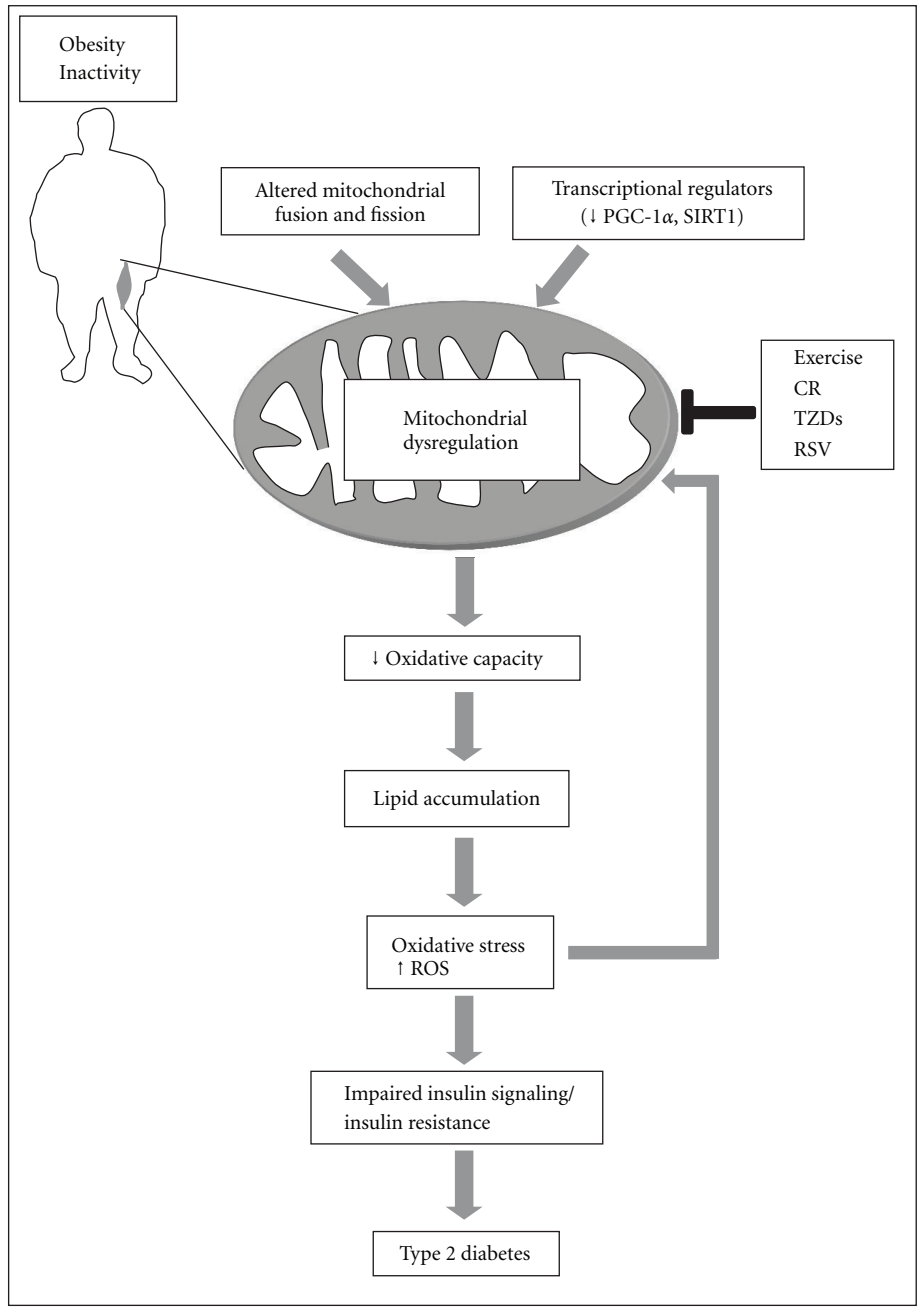

Figure 3: Simplified scheme that illustrates the role of mitochondrial dysregulation in the pathogenesis of type 2 diabetes in skeletal muscle. Obesity and physical inactivity can result in mitochondrial dysregulation through alterations in crucial transcriptional activators (e.g., PGC-1α and SIRT1), as well as impaired fusion and fission leading to aberrant mitochondrial morphology. These changes can subsequently lead to reduced oxidative capacity and cause lipid metabolite accumulation, increased oxidative stress, and the production of reactive oxygen species (ROS). Over time, the accumulation of ROS can damage DNA, proteins, and lipids, further exacerbating mitochondrial dysfunction. Collectively, these factors contribute to impaired insulin signaling pathways and increase the risk of type 2 diabetes. On the other hand, physiological interventions, including exercise and caloric restriction (CR), as well as pharmacological agents such as thiazolidinediones (TZDs) and resveratrol (RSV) have been shown to stimulate mitochondrial biogenesis and reduce mitochondrial dysfunction that is observed with type 2 diabetes in muscle.

attenuating the clinical phenotype associated with these metabolic disorders. Thus, the closer we come to understanding the molecular mechanisms governing these pathways during normal physiological conditions, the better chance we have to develop new therapeutic strategies that may one day prevent or treat type 2 diabetes.

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Review Article

Endoplasmic Reticulum Stress and Diabetic Cardiomyopathy

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The endoplasmic reticulum (ER) is an organelle entrusted with lipid synthesis, calcium homeostasis, protein folding, and maturation. Perturbation of ER-associated functions results in an evolutionarily conserved cell stress response, the unfolded protein response (UPR) that is also called ER stress. ER stress is aimed initially at compensating for damage but can eventually trigger cell death if ER stress is excessive or prolonged. Now the ER stress has been associated with numerous diseases. For instance, our recent studies have demonstrated the important role of ER stress in diabetes-induced cardiac cell death. It is known that apoptosis has been considered to play a critical role in diabetic cardiomyopathy. Therefore, this paper will summarize the information from the literature and our own studies to focus on the pathological role of ER stress in the development of diabetic cardiomyopathy. Improved understanding of the molecular mechanisms underlying UPR activation and ER-initiated apoptosis in diabetic cardiomyopathy will provide us with new targets for drug discovery and therapeutic intervention.

1. Introduction

The endoplasmic reticulum (ER) is a central organelle entrusted with lipid synthesis, calcium homeostasis, protein folding, and maturation [1]. Most secreted and integral membrane proteins of eukaryotic cells are translocated into the lumen of ER. The ER lumen provides a specialized environment for posttranslational modification and folding of secreted, transmembrane, and resident proteins of the various compartments of the endomembrane system [2]. Various factors that interfere with ER function lead to accumulation of unfolded proteins, including oxidative stress, ischemia, disturbance of calcium homeostasis, and overexpression of normal and/or incorrectly folded proteins. This process is called ER stress and further activates the unfolding protein response (UPR). There are two aims of the UPR: the UPR initially tries to restore normal function of the cell by halting protein translation and activating the signaling pathways that lead to increasing the production of molecular chaperones involved in protein folding; if these objectives are not achieved within a certain time lapse or the disruption is prolonged, the UPR tries to turn on apoptotic pathway [3–6]. Therefore, the UPR can be considered as a safeguard for protein synthesis, posttranslational modifications, folding and secretion, calcium storage and signaling, and lipid biosynthesis [7–11]. Under normal conditions the ER maintains high concentrations of resident calcium-dependent chaperone proteins, such as glucose-regulated protein-78 (GRP78, also known as BiP) and glucose-regulated protein-94 (GRP94) [12], along with a high level of calcium and an oxidized environment. Only properly folded proteins are allowed to reach their final destination, whereas unfolded and misfolded proteins are exported or dislocated from the ER and degraded by cytoplasmic proteasomes. The stresses that can cause the UPR include a variety of conditions such as elevated secretory protein synthesis, overexpression and/or accumulation of mutant proteins, aberrant Ca^{2+} regulation, hypoxia, glucose deprivation, altered glycosylation, ischemia, ER calcium depletion, viral infections, shifting of redox status to a more reduced state, exposure to natural and experimental toxins that perturb ER function, and overloading of cholesterol [3, 10, 13–17].

In human and animal models of diabetes, a heart muscle-specific disease in the absence of other vascular pathology has been described, termed diabetic cardiomyopathy [18, 19].

The pathogenesis of diabetic cardiomyopathy is a chronic and complex process that is attributed to abnormal cellular metabolism and defects in organelles such as myofibrils, mitochondria, and sarcolemma [20–24]. Probable candidates to explain this heart disease include autonomic abnormalities, metabolic disorders, abnormal enzyme function, and interstitial fibrosis [25–27]. For instance, apoptosis, as a regulated, energy-dependent, cell suicide mechanism has also been reported to play a critical role in the development of diabetic cardiomyopathy [28–31].

Recently the involvement of ER stress in the development of diabetic cardiomyopathy was also reported [32, 33]. However, the mechanisms of diabetic cardiomyopathy are not fully known, and appropriate approaches to minimize these risks are still being explored. This paper focuses on ER stress and its involvement in the development of diabetic cardiomyopathy in order to provide certain new insights into understanding of its mechanisms.

2. Adaptation to ER Stress: Mechanisms to Restore Homeostasis

The ability to adapt to physiological levels of ER stress is important to cells. The initial intent of the UPR is adaptation and restoration of the normal ER function. When unfolded proteins accumulate in the ER, resident chaperones become occupied, releasing transmembrane ER proteins involved in inducing the UPR. These proteins straddle ER membranes, with their N-terminus in the lumen of the ER and their C-terminus in the cytosol, providing a bridge that connects these 2 compartments. The N-termini of these transmembrane ER proteins are usually associated with or bound to intralumenal GRP78 in the absence of ER stress, preventing their aggregation. In some physiological or pathological conditions, the large excess of unfolded proteins in the ER lumen necessitates GRP78 dissociation and launches the UPR. The UPR initially activates intracellular signaling pathways mediated by three ER-resident proteins in mammalian cells: the inositol-requiring kinase-1 (IRE-1) [34, 35], the activating transcription factor 6 (ATF6), and the protein kinase R-like ER kinase (PERK) [36, 37].

2.1. PERK. The UPR induces an early and transient attenuation of protein biosynthesis which is mediated by PERK, an ER-resident protein whose effector the eukaryotic initiation factor 2α kinase (eIF2α) domain lies on the cytoplasmic side of the ER membrane and whose stress-sensing domain lies on the opposite side of the membrane in the ER lumen [38], as illustrated in Figure 1. The luminal domain of monomeric PERK binds with the ER chaperone GRP78 in an inactive complex under unstressed conditions. However, as client proteins accumulate in the ER lumen, in efforts to assist folding, GRP78 relocates from PERK to misfolded ER proteins. GRP78 relocalization allows PERK to dimerize, which facilitates transautophosphorylation [39, 40]. PERK is then activated, and it phosphorylates α-subunit of eIF2α on serine-51. This phosphorylation event decreases cap- or eIF2α-dependent translation, which shuts off global mRNA translation and reduces the protein load on the ER [37, 39]. Global translational inhibition acutely reduces the protein-folding load on the ER and allows the cell to focus resources on resolving the ER stress, thus facilitating survival. However, certain mRNAs encoded by ER stress response (ERSR) genes gains structural features and a selective advantage for translation that allow them to escape PERK-mediated translational inhibition [41]. For example, eIF2α phosphorylation induces expression of GRP78 and ATF4 under stress [42, 43]. During the prosurvival phase of ER stress, ATF4 induces numerous genes involved in resolution of the ER stress, such as genes that encode amino acid transporters and ER-resident chaperones [43]. However, after prolonged ER stress, continued ATF4 expression mediates the upregulation of genes that contribute to programmed cell death (Figure 1).

2.2. IRE-1. Chaperone induction and ER-associated degradation (ERAD), in response to ER stress, are regulated by the IRE-1 pathway. IRE-1 contains both a Ser/Thr kinase domain and an endoribonuclease domain, which thus functions as a kinase and an endoribonuclease. Like PERK, under normal conditions, the luminal domain of IRE-1 monomers associates with GRP78. Also in comparison with PERK, during the prosurvival phase of the ERSR, GRP78 relocates to misfolded proteins, which allows IRE-1 to dimerize, thus facilitating transautophosphorylation [44]. However, in contrast to PERK, transautophosphorylation of IRE-1 activates a novel endoribonuclease activity [45]. In mammalian cells, the substrate for the IRE-1 endoribonuclease is the X-box-binding protein-1 (XBP1) mRNA. After activation, the IRE-1 endoribonuclease splices the XBP1 mRNA with an altered reading frame. This XBP1 splice variant binds to the promoters containing ER stress response elements (ERSEs) [46]. These findings suggest that ER stress, acting through the IRE-1- and XBP1-dependent signaling pathway, upregulates the secretory apparatus in cells. Defective signaling in this pathway would affect professional secretory cells (Figure 1).

2.3. ATF6. ATF6 is an ER transmembrane protein [47, 48]. Two similar transcription factors, ATF6α and ATF6β, exist in mammals. In comparison to PERK and IRE-1, in normal cells, the luminal domain of ATF6 is associated with GRP78 in an inactive form. It should be mentioned that even though ER stress releases GRP78 from ATF6, in contrast to PERK and IRE-1, this is not thought to be attributable to competitive binding of GRP78 to other proteins [49]. Moreover, ATF6 exists in the ER as a dimer linked by intermolecular disulfide bonds in the luminal domain. GRP78 dissociation and disulfide bond cleavage on ER stress facilitate the translocation of ATF6 to the Golgi [50], where it undergoes cleavage by site-1 and site-2 proteases. This yields N-ATF6 that translocates to the nucleus and induces target ER genes [36, 48, 51–58]. Therefore, activation of ATF6, IRE-1, and the downstream XBP1 (IRE-1-XBP1) increases the expression of ER-resident chaperones. The genes induced by ATF6 during the prosurvival phase of ER stress foster resolution of the stress and, thus, survival, whereas those genes induced on the

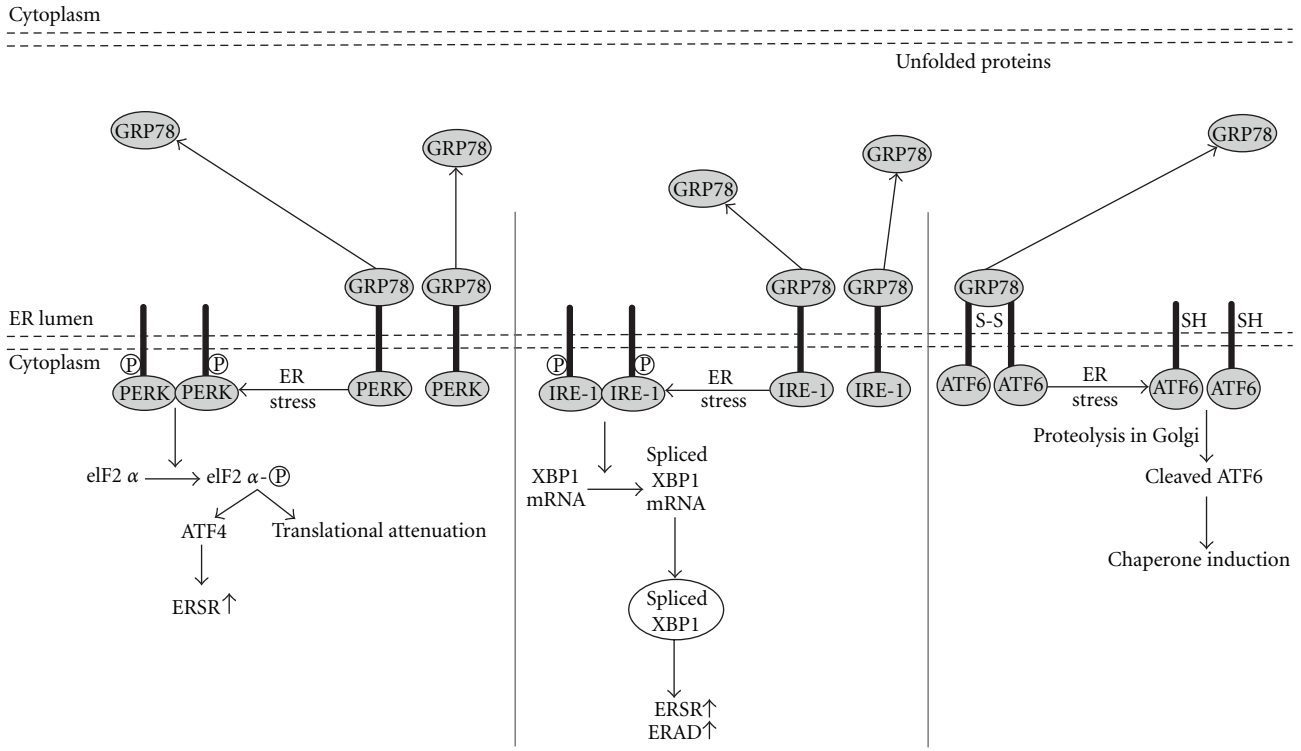

Figure 1: ER stress signaling pathways.

proapoptotic phase of ER stress contribute to programmed cell death [59] (Figure 1).

2.4. Apoptosis Induced by ER Stress. The UPR deals with adverse effects of ER stress in a timely and efficient manner at the early stage and thus enhances cell survival. However, when protein misfolding is persistent or excessive, prolonged ER stress has severe consequences, including apoptosis. 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 [1]. Although the induction of apoptosis is the least well understood among the responses to ER stress, the apoptotic mechanisms induced by ER stress remain able to be broadly divided into a few categories, as summarized in Figure 2.

The first apoptotic pathway involves activation of the c-Jun N-terminal kinase (JNK) pathway. During ER stress, activated IRE-1 recruits tumor necrosis factor receptor-associated factor 2 (TRAF2) and apoptosis signal-regulating kinase 1 (ASK-1) to form IRE-1-TRAF2-ASK1 complex which then lead to activation of JNK and downstream mitochondria/Apaf-1-dependent caspase activation [60, 61]. Also, the c-Jun-N-terminal inhibitory kinase (JIK) interacts with activated IRE-1 and promotes phosphorylation and association of TRAF2 with IRE-1 (Figure 2).

The second apoptotic pathway depends on the activation of ER-localized cysteine protease, caspase-12 in rodents [62]. Several processes have been suggested as contributing factors to caspase-12 activation in ER-stressed cells. *m*-Calpain, a cysteine protease activated by disturbed calcium homeostasis in ER-stressed cells, may directly cleave and activate caspase-12 [63]. Caspase-7, which is translocated from cytosol to the ER surface in stressed cells, can cleave and activate caspase-12 [64]. ER stress-activated IRE-1 and PERK may lead to clustering caspase-12 at the ER membranes by recruitment of TRAF2 proteins [60]. Upon activation, in rodents but not in humans, caspase-12 translocates from the ER to the cytosol, where it directly cleaves procaspase-9, which, in turn, activates the downstream effector caspase, caspase-3 without the need for mitochondrial amplification [62, 65]. Therefore, caspase-12-mediated apoptosis was a specific apoptosis pathway of ER, which is independent on mitochondria or death receptor activation. Caspase-4, one of the closest paralogs of rodent caspase-12, has been suggested to fulfill this role normally ascribed to rodent caspase-12 in the context of ER stress in human [66] (Figure 2). In a recent study, the enhanced expression of the cleaved caspase-12 as an indicator of ER stress-associated apoptosis was also observed in the diabetic heart [32].

The third apoptotic pathway activated by ER stress is mediated by transcriptional activation of CHOP/GADD153, a member of the C/EBP family of b-ZIP transcription factor that potentiates apoptosis, possibly through repressing expression of antiapoptotic Bcl2 and Bcl-X_L and induction of ER oxidase 1α which generates reactive oxygen species (ROS) and depletes glutathione (GSH) [67]. While CHOP is barely detected under physiological conditions,

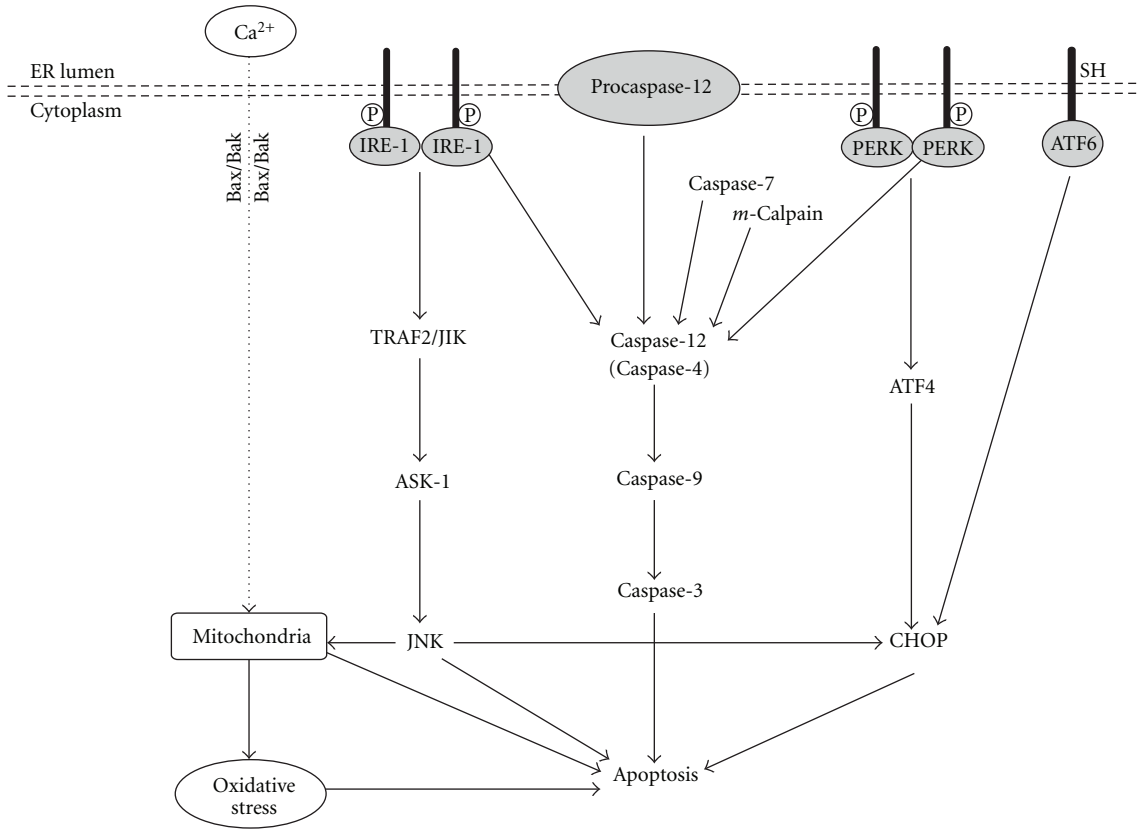

Figure 2: Apoptosis mechanisms induced by ER stress.

it is strongly induced in response to ER stress [68]. Although both the IRE-1 and ATF6 pathways can upregulate CHOP, the PERK pathway predominates through selective upregulation of translation of ATF4, which subsequently induces transcription of CHOP and other genes involved in amino acid metabolism and transport, and oxidation-reduction reactions [69, 70]. The downstream targets of CHOP leading to apoptosis are still unclear (Figure 2).

In addition, prolonged ER stress is associated with release of ER Ca^{2+} stores which can perturb mitochondria, triggering oxidative stress. Ca^{2+}-induced oxidative stress can induce cell death. Increased cytosol Ca^{2+} also activates calpains, a family of Ca^{2+}-dependent cysteine proteases which proteolytically cleave caspase-12 (activated), Bcl2, and Bcl-X_L (inhibited). Apoptosis is rapidly initiated after ER-Ca^{2+} depletion in photodynamic therapy and strictly requires Bax/Bak at the mitochondria [71] (Figure 2).

3. ER Stress in the Heart

3.1. Requirement of the ER Stress for the Heart. Several studies have indicated that the ER stress is required for the proper cardiac differentiation and development. It is reported that many genes encoding ER-resident proteins are activated during the early stages of cardiogenesis. For instance, GRP78 can be activated to promote early heart organogenesis through cooperation between the cell type-specific transcription factors and ERSE-binding factors [72]. GRP78 deficiency is lethal at a very early stage of embryogenesis [73]. GRP94 knockout cells fail to develop mesoderm, resulting in a prevention of cardiac development from beginning [74]. Targeted disruption of the XBP1 gene in mice is embryonic lethal because of cardiac development defects [75]. All these studies clearly confirm the requirement of ERSR for the proper cardiac development.

ERSR also involves in the cardiac protection against certain challenges. For instance, GRP78 antisense oligodeoxynucleotide partially abrogated the protective effect of endothelin-1 pretreatment on hypoxic cardiomyocyte injury [76]. Overexpression or pharmacological induction of GRP78 can attenuate cardiomyocyte death induced by proteasome inhibition [77]. Similarly, overexpression of GRP94 also reduces cardiac cell death caused by calcium overload or ischemia [78]. In cardiac ischemia/reperfusion injuries model, ATF6 transgenic hearts exhibited a better recovery of ventricular pressure and lower incidence of cardiac cell death [79]. A recent study showed that induction of autophagy by ER stress before ischemia (similar to ischemic preconditioning) could reduce ischemia/reperfusion-induced lethal injury [80]. Therefore, the ER stress is not only required for the cardiac development, but also provide certain protective mechanisms for the heart against damage caused by various stresses.

3.2. The Deleterious Effect of the ER Stress on the Heart. However, ER stress is demonstrated to be pathologically involved in cardiac diseases and damages under numerous conditions, including myocardial infarction, ischemia/reperfusion, and pressure overload. For example, the ERSR is activated in the hearts of transgenic mice that overexpress monocyte chemoattractant protein-1 (MCP-1) and develop heart failure [81]. They found that the heart failure is mainly due to the activated proapoptotic phase of ERSR that led to massive loss of cardiomyocytes [81]. A further support of a role for ER stress in heart failure is the finding that transgenic overexpression of a mutant KDEL receptor, a retrieval receptor for ER chaperones in the early secretory pathway, induced the ERSR in the hearts, in parallel with a consequence of dilated cardiomyopathy [82].

Hypoxia is an insult that activates cardiac ER stress. During ischemia that could result from the reduced availability of molecular oxygen, significant changes in cardiac energy metabolism happen [83]. Under severe hypoxia, anaerobic metabolism has to occur and results in a massive increase in ROS, leading to cardiac oxidative damage, cell death, and, ultimately, cardiac dysfunction [84]. In addition, reperfusion after ischemia generates increased oxidative stress as the heart converts back to aerobic respiration, thereby generating lethal levels of ROS. Ischemia/reperfusion injury in the heart results in numerous cellular and molecular events that lead to loss of cardiac damage and dysfunction, such as disrupting ER oxidative state or Ca^{2+} homeostasis, triggering cellular damage, and eventually causing cardiac apoptotic death [84, 85].

Apoptotic cell death as an early cellular event in response to diabetes has been reported to play a critical role in the development of diabetic cardiomyopathy [28–30]. Because myocytes rarely proliferate in adult cardiac muscles, the loss of cardiomyocytes would eventually lead to compromised cardiac function. Loss of endothelial cells will lead the vascular system to dysfunction and aggravate the ischemia of the heart. Apoptosis of cardiomyocytes and endothelial cells has been observed in the heart of patient with diabetes and in streptozotocin- (STZ-) induced diabetic mice and rats [27, 29, 86]. Pieces of evidence have demonstrated that apoptosis induced by ER stress was involved in pathogenesis of diabetic and nondiabetic heart failure [87–89]. UPR and ER-initiated apoptosis coexist in failing hearts and the CHOP-dependent cell death pathway may be involved in the transition from cardiac hypertrophy to heart failure in mice [90]. Recent studies have also demonstrated the UPR and activation of ER-initiated apoptotic signaling in models of autoimmune cardiomyopathy [91] and alcoholic cardiomyopathy [92]. Overexpression of the ERSR gene product, p53-upregulated modulator of apoptosis (PUMA), contributes to ER stress-mediated apoptosis in cultured cardiomyocytes [93] and targeted deletion of PUMA in mouse hearts attenuates cardiomyocyte death during Langendorff ex vivo ischemia/reperfusion [94]. All these studies demonstrated that apoptotic death of cardiac cells plays a critical role of the development of diabetic cardiomyopathy, whether the cardiac cell death at diabetic early stage is mediated by diabetic induction of the ER stress still needs to be further studied.

4. Contribution of the ER Stress to Diabetic Cardiomyopathy

As early as 1985, whether the ER stress may play an important role in the development of diabetic cardiomyopathy has been questioned since in the diabetic hearts the ERs become swollen under ultrastructural examination [95, 96], suggesting the disorder of the ER under diabetic condition. In 2007, Li et al. provided the experimental evidence for the involvement of the ER stress in the cardiac apoptosis in a STZ-induced type 1 diabetic rat model [32]. They examined the heart function with echocardiography, morphological changes with hematoxylin-eosin staining, and cardiac cell death with TUNEL staining. Immunohistochemistry, Western blot, and real-time PCR methods were used to examine two ER stress hallmarks, GRP78 and caspase-12. They found that GRP78 and caspase-12 were upregulated at both protein and mRNA levels in the diabetic hearts compared to normal hearts. Since apoptosis plays critical role in diabetic cardiomyopathy [28, 30], those results suggested that the ER stress was induced in the diabetic hearts, and the ER stress-associated apoptosis took part in the pathogenesis of diabetic cardiomyopathy. We have also demonstrated the cardiac up-regulation of several ERSR proteins, including PERK- and ATF6-mediated pathways in diabetic hearts of mice model induced by multiple low doses of STZ (MLD-STZ). However, these ERSR were not observed in the diabetic mice with cardiac-specific overexpression of metallothionein (MT) gene (MT-TG) [86]. Since we have demonstrated that diabetes-induced cardiac cell death was prevented by MT at the diabetic early stage, resulting in a significant prevention of cardiac remodeling and dysfunction at the 5 or 6 months, that is, a significant prevention of diabetic cardiomyopathy. Based on that study we further assumed that MT prevented diabetic cardiomyopathy via suppression of diabetic induction of ER stress and associated cell death. To define the direct protection by MT from ER stress-mediated apoptotic cell death, both MT-TG mice and the age-matched wild-type (WT) mice were administrated with chemical ER stress inducer, tunicamycin. We found that cardiac ERSR such as *p*-eIF2, cleaved ATF6 and GRP78 and cardiac cell death all were significantly upregulated in tunicamycin-treated WT mice, but not in tunicamycin-treated MT-TG mice [86].

We have approved that Ang II plays a critical role in the development of diabetic cardiomyopathy [97]; therefore, we have explored and demonstrated that the exposure of embryonic rat heart-derived cells (H9c2) to induced a significant ERSR, effect abolished by MT in Ang II-treated MT-TG mice [86]. The direct role of Ang II in the induction of cardiac ERSR was also approved by a recent study that olmesartan treatment downregulated the cardiac expressions of GRP78 and caspase-12, along with oxidative and nitrosative damage [98]. To support this study, Wu et al. reported that valsartan, another selective AT1 receptor antagonist, could relieve the ER stress along with cardiomyocyte apoptosis,

resulting in a significant prevention of cardiac remodeling [99].

It is well appreciated now that in addition to hyperglycemia and Ang II, diabetic heart experiences many other conditions that can invoke ER stress, such as increased oxidative stress, hypoxia, homocysteine, lipid deposition, and increased synthesis of secretory proteins [100–102]. A recent study reported the role of homocystein- (Hcy-) induced ER stress in diabetic cardiomyopathy [103]. Since significant increase in Hcy has been indicated in the development of diabetic cardiomyopathy, whether its pathogenic effect on diabetic heart is also related to ER stress has been addressed using a rat model of Hcy. These rats were given overloaded methionine to induce high-plasma Hcy. In the heart of these rats, there were significant increases in GRP78, CHOP, and caspase-12, suggesting the existence of cardiac ER stress induced by Hcy that also plays a critical role in the development of diabetic cardiomyopathy [103].

5. Potential Mechanisms by Which the ER Stress Causes the Development of Diabetic Cardiomyopathy

The molecular mechanisms by which hyperglycemia causes cell death are probably related to ROS production. ROS is mainly produced by mitochondria and NADPH oxidase in cardiomyocytes [29, 104]. We have used MT transgenic model to indicate the importance of oxidative stress in the induction of ER stress in the diabetic heart. Since MT is a potent, nonspecific antioxidant that can scavenge multiple ROS and/or RNS [104–106], we have examined whether diabetes induces cardiac ER stress and, if so, whether MT can prevent diabetic induction of the ER stress, resulting in a prevention of cardiomyopathy as we observed in previous studies [30, 107, 108]. Therefore we used STZ to induce diabetes in both MT-TG and WT mice. Two weeks, and 2 and 5 months after diabetes onset, cardiac ER stress was detected by expression of ER chaperones, and apoptosis was detected by CHOP, cleaved caspase-3 and caspase-12. Cardiac apoptosis in the WT diabetic mice, but not in MT-TG diabetic mice, was significantly increased 2 weeks after diabetes onset [86]. In parallel with apoptotic effect, significant up-regulation of the ER chaperones, including GRP78, GRP94, cleaved ATF6 and phosphorylated eIF2α, in the hearts of WT, but not MT-TG diabetic mice. Pretreatment with antioxidants completely prevented Ang II-induced ER stress and apoptosis in the cultured cardiac cells. Therefore, our results suggested that ER stress exists in the diabetic heart, which may cause the cardiac cell death. MT prevents both diabetes-induced cardiac ER stress and associated cell death most likely via its antioxidant action, which may be responsible for MT prevention of diabetic cardiomyopathy [86].

In consistence with our finding, recently, Younce et al. [109] also reported that high glucose induces cardiomyocyte death via production of MCP-1 and induction of MCP-1-induced protein (MCPIP) that results in ROS production, leading to ER stress to cause autophagy and eventual cell death. Selective inhibition of Rac1 or NADPH oxidase prevents ER stress by blocking ROS production in high-glucose-stimulated cardiomyocytes [110].

Diabetes may impair ERSR so that certain functions that can be observed in nondiabetic condition may not be observed in diabetic condition. For instance, under nondiabetic condition, pretreatment with erythropoietin (EPO) can prevent ischemia/reperfusion-induced cardiac damage, but the ER stress in diabetic hearts abolished EPO-induced cardiac protection by impairment of phospho-glycogen synthase kinase-3β- (GSK-3β-) mediated suppression of mitochondrial permeability transition [111]. In this study, the authors used type 2 diabetic (OLETF) rats and its control (LETO) to be treated with tauroursodeoxycholic acid (TUDCA) to induce ER stress. Infarction was induced by 20 min coronary occlusion and 2 h reperfusion. Levels of ER chaperones (GRP78 and GRP94) in the heart and level of non-phoshopho-GSK-3β in the mitochondria were significantly higher in OLETF than in LETO rats. TUDCA normalized levels of GRP78 and GRP94 and mitochondrial GSK-3β in OLETF rats. Administration of EPO induced phosphorylation of Akt and GSK-3β and reduced infarct size in LETO hearts. However, neither phosphorylation of Akt and GSK-3β nor infarct size limitation was induced by EPO in OLETF rats. The threshold for mitochondrial permeability transition pore (mPTP) opening was significantly lower in mitochondria from EPO-treated OLETF rats than in those from EPO-treated LETO rats. Therefore, disruption of protective signals leading to GSK-3β phosphorylation is due to the increased ER stress that inhibited EPO-induced suppression of mPTP opening and cardioprotection in diabetic hearts [111].

There was a recent study that has addressed how glucose induces ER stress in cardiac cells [109]. They found that glucose-induced cardiomyocyte death is mediated via MCP-1 production and MCPIP, which causes sequential events—ROS production, ER stress, autophagy, and cell death [109]. This is an elegant study, in which H9c2 cardiomyoblasts treated with 28 mmol/L glucose were evaluated for MCP-1 production and induction of MCPIP. They disrupted MCP-1 to interact with its receptor, CCR2, and knocked down MCPIP with siRNA to determine if MCP-1 and MCPIP mediate glucose-induced cell death glucose treatment of H9c2 cardiomyoblasts and isolated cardiomyocytes caused MCP-1 production, MCPIP induction, ROS production, ER stress, autophagy, and cell death. Treatment with CCR2 antagonists and knockdown of MCPIP attenuated glucose-induced ROS production, ER stress, autophagy, and cell death. Inhibition of ROS with antioxidants attenuated ER stress, autophagy, and cell death. Specific inhibitors of ER stress and knockdown of IRE-1 attenuated glucose-induced autophagy and cell death. Inhibitors of autophagy and knockdown of beclin-1 attenuated glucose-induced death. Therefore, they have demonstrated a novel mechanism: hyperglycemia-induced cardiomyocyte ER stress and cell death are mediated via MCP-1 production and induction of a novel zinc-finger protein MCPIP [109].

In summary, various pathogenic factors of diabetes most likely induce ROS that in turns causes the ER stress and associated cell death, as illustrated in Figure 3.

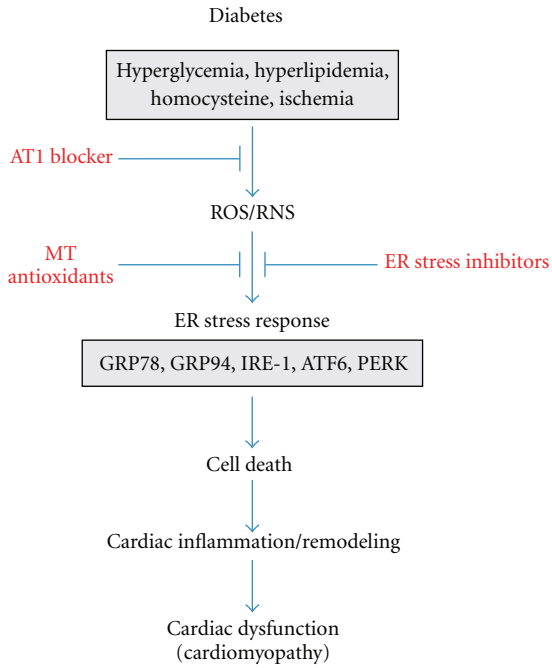

Figure 3: Outlining the involvement of ER stress in the development of diabetic cardiomyopathy.

Cardiac cell death will initiate the cardiac inflammation and remodeling and eventually cardiac dysfunction, that is, diabetic cardiomyopathy. The pathogenic factors of diabetes include hyperglycemia, hyperlipidemia, and Ang II and so on (Figure 3).

6. Potential Prevention of Diabetic Cardiomyopathy by Inhibition of ER Stress

Since the above studies have strongly demonstrated the critical role of ER stress in the development of diabetic cardiomyopathy, ER stress inhibitors would be the new targets for drug discovery and therapeutic intervention in diabetic cardiomyopathy. Chemical or pharmaceutical chaperones, such as 4-phenyl butyric acid (PBA), TUDCA are a group of low-molecular-weight compounds known to stabilize protein conformation to improve ER folding capacity and facilitate the trafficking of mutant proteins. Studies have shown that these chemical ER chaperones can reduce ER stress in a mouse model of type 2 diabetes [112]. TUDCA normalized levels of GRP78 and GRP94 and mitochondrial GSK-3β in the rat model of type 2 diabetes [111]. Inhibition of ER stress with tauroursodeoxycholate (TUDC) and PBA resulted in the attenuation of cardiomyoblast death [109]. Our recent studies have shown cardiac overexpression of MT rescued diabetes-, Ang II-, and even chemical ER stressor-induced cardiac cell death via suppression of cardiac ER stress. MT protection against Ang II-induced ER stress and associated apoptotic effects are mediated by its antioxidant action; this suggests that MT inducers such as zinc may also play the same role as the ER stress direct inhibitors [109, 112]. We have treated diabetic mice for the first three months after the establishment of STZ-induced hyperglycemia, resulting in a significant prevention of the development of diabetic cardiomyopathy [113]. Although we did not examine the status of ER stress in the heart for that study, zinc treatment significantly induced cardiac MT expression that might prevent diabetic induction of the ER stress and associated cell death as we observed in other studies [86].

There were also some studies to indirectly inhibit ER stress for the prevention of diabetic cardiomyopathy. Wu et al. have explored the possibility to use valsartan to prevent diabetic ER stress and consequently prevented diabetic cardiomyopathy [99]. They demonstrated that valsartan can ameliorate ER stress-induced cardiac remodeling and myocardial apoptosis in diabetic cardiomyopathy. Similarly, hydrogen sulfide was also explored for the potential to attenuate hyperhomocysteinemia-induced cardiomyocytic ER stress in the rat model [103], with a finding that hydrogen sulfide attenuated cardiomyocyte ER stress in Hcy-induced cardiac injury.

14-3-3 protein was also found to protect against cardiac ER stress and ER stress-initiated apoptosis in experimental diabetes [114]. STZ was used to induce transgenic mice that showed cardiac-specific expression of a dominant negative (DN) 14-3-3eta protein mutant. The expression levels of cardiac GRP78, IRE-1α, and TRAF 2 protein were significantly increased in the diabetic DN 14-3-3eta mice compared with the diabetic wild-type control. Moreover, cardiac apoptosis and the expression of CHOP, caspase-12, and cleaved caspase-12 protein were significantly increased in the diabetic DN 14-3-3eta mice. Therefore, they found that partial depletion of 14-3-3 protein in the diabetic heart exacerbates cardiac ER stress and activates ER stress-induced apoptosis pathways, at least in part, through the regulation of CHOP and caspase-12 via the IRE-1α/TRAF2 pathway. The enhancement of 14-3-3 protein expression can be used as a novel protective therapy against ER stress and ER stress-initiated apoptosis in the diabetic heart [114].

It is reported that low levels of adiponectin, a fat-derived hormone, were found to be correlated with coronary heart disease, type 2 diabetes, obesity, and insulin resistance. Conversely, high adiponectin levels are predictive of reduced coronary risk in long-term epidemiologic studies. Whether adiponectin has certain cardiac protection and, if so, whether the cardiac protection of adiponectin is related to ER stress have been questioned by Dong and Ren [115]. They have examined the role of adiponectin in cardiac contractile function in the db/db model of diabetic obesity and demonstrated that adiponectin improved cardiomyocyte contractile function in db/db diabetic obese mice, which was not associated with improvement of the ER stress [115]. They demonstrated that cardiomyocytes from db/db mice exhibited greater cross-sectional area, depressed peak shortening, and maximal velocity of shortening/relengthening as well as prolonged duration of relengthening. Consistently, myocytes from db/db mice displayed a reduced electrically stimulated rise in intracellular Ca^{2+} and prolonged intracellular Ca^{2+} decay. These functional and Ca^{2+} changes were abrogated by adiponectin treatment. Levels of the phosphorylated ER

stress makers PERK (Thr980), IRE-1, and eIF2α were significantly elevated in db/db mice compared with lean controls, but the effect was unaffected by adiponectin. Collectively, they concluded that adiponectin improves cardiomyocyte dysfunction in db/db diabetic obese mice through an ER stress-independent mechanism. However, this study was in contrast to another study that showed that TUDCA normalized levels of GRP78 and GRP94 and mitochondrial GSK-3β in the rat model of type 2 diabetes resulted in significant prevention of diabetic cardiac damage [111]. Therefore more studies with different types of diabetes and different conditions are needed to address whether inhibition of ER stress can prevent diabetic cardiomyopathy in T2D model.

As illustrated in Figure 3, these studies have indicated the potential application of various direct and indirect ER stress inhibitors to prevent diabetic ER stress-mediated cell death, and eventually the development of the cardiac remodeling and dysfunction, as a potential approach applied clinically in the future.

7. Conclusions

In conclusion, although our understanding of the pathophysiological role of ER stress in diabetic cardiomyopathy has progressed in recent years, many important issues are still unresolved. There are several fundamental questions. One of these important issues is how the cell decides between life and death after the onset of ER stress. Improved understanding of the molecular mechanisms underlying UPR activation and ER-initiated apoptosis in diabetic cardiomyopathy will provide us with new targets for drug discovery and therapeutic intervention.

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Review Article

Mitochondrial Dysfunction and β-Cell Failure in Type 2 Diabetes Mellitus

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Type 2 diabetes mellitus (T2DM) is the most common human endocrine disease and is characterized by peripheral insulin resistance and pancreatic islet β-cell failure. Accumulating evidence indicates that mitochondrial dysfunction is a central contributor to β-cell failure in the evolution of T2DM. As reviewed elsewhere, reactive oxygen species (ROS) produced by β-cell mitochondria as a result of metabolic stress activate several stress-response pathways. This paper focuses on mechanisms whereby ROS affect mitochondrial structure and function and lead to β-cell failure. ROS activate UCP2, which results in proton leak across the mitochondrial inner membrane, and this leads to reduced β-cell ATP synthesis and content, which is a critical parameter in regulating glucose-stimulated insulin secretion. In addition, ROS oxidize polyunsaturated fatty acids in mitochondrial cardiolipin and other phospholipids, and this impairs membrane integrity and leads to cytochrome *c* release into cytosol and apoptosis. Group VIA phospholipase A_2 (iPLA$_2\beta$) appears to be a component of a mechanism for repairing mitochondrial phospholipids that contain oxidized fatty acid substituents, and genetic or acquired iPLA$_2\beta$-deficiency increases β-cell mitochondrial susceptibility to injury from ROS and predisposes to developing T2DM. Interventions that attenuate ROS effects on β-cell mitochondrial phospholipids might prevent or retard development of T2DM.

1. Introduction

Type 2 diabetes mellitus (T2DM) is the most common human endocrine disease and is reaching pandemic proportions [1]. Predisposition to T2DM is affected both by genetic and acquired factors, and there are contributions from many genes and environmental influences that are incompletely understood [1, 2]. It is becoming clear that the progressive failure of pancreatic islet β-cells is a central component of the development and progression of T2DM [3]. Normally, pancreatic islet β-cells respond to increased metabolic demands by increasing their mass and insulin synthetic and secretory activity, as demonstrated both in rodent models of obesity without diabetes and in nondiabetic obese humans.

Most humans who are obese do not develop diabetes, and T2DM develops only in those who are unable to sustain compensatory β-cell responses to increasing metabolic stress [4]. The United Kingdom Prospective Diabetes Study (UKPDS) has clearly demonstrated that the progressive nature of T2DM reflects an ongoing decline in β-cell function without a change in insulin sensitivity [5]. Longitudinal studies of subjects who eventually develop T2DM reveal a progressive rise in serum insulin levels in the prediabetic phase that is followed by a decline in serum insulin levels upon development of fasting hyperglycemia [1]. Many T2DM patients ultimately require therapy with exogenous insulin in the later stages of the disease because endogenous insulin production becomes insufficient to maintain acceptable levels of glycemia despite ongoing therapy with other antidiabetic agents, including sulfonylureas and metformin, *inter alia* [3].

Reductions in both β-cell mass and function contribute to the pathogenesis of β-cell failure in human T2DM [6, 7]. Several studies have demonstrated that glucose-stimulated

insulin secretion is lower in islets from T2DM patients compared to control islets [8, 9]. In addition, islets from T2DM subjects exhibit both structural and functional abnormalities and fail to reverse hyperglycemia when transplanted into diabetic mice under conditions in which equivalent numbers of control human islets do so [9]. Interestingly, T2DM human islets secrete significantly higher amounts of insulin in response to arginine and glibenclamide than in response to D-glucose, suggesting that T2DM β-cell insulin secretory defects reflect a relatively selective loss of responsivity to glucose compared to other insulin secretagogues [10].

Moreover, it has been demonstrated that the ATP content of islets from T2DM subjects fails to increase normally upon acute stimulation with glucose. Consequently, their ATP/ADP ratio rises to values only about 60% of that in control islets, and this is likely to account for or contribute to the blunted or absent glucose-stimulated insulin secretory responses of T2DM islets [11]. Mitochondria in T2DM β-cells exhibit both morphologic and functional abnormalities that are not observed in control β-cells [11]. Together, these findings indicate that human T2DM β-cells exhibit abnormalities in glucose metabolism and in mitochondrial structure and function that result in impaired ATP production and glucose-stimulated insulin secretion [7].

Accumulating evidence indicates that progressive reduction in β-cell mass also contributes to the overall decline in β-cell functional capacity in the pathogenesis of T2DM. Early observations indicated that β-cell volume is significantly reduced in T2DM islets [12–14]. More recent studies with postmortem and surgical specimens of human pancreata have characterized changes in β-cell mass that occur during the evolution of T2DM [6, 15]. One such study based on specimens from 124 autopsies revealed a 63% lower β-cell volume in obese T2DM subjects compared to nondiabetic, weight-matched control subjects and a 41% lower β-cell volume in lean T2DM subjects compared to nondiabetic lean control subjects. Another study revealed a 40% lower β-cell mass in subjects with elevated fasting blood glucose levels compared to weight-matched control subjects with fasting euglycemia, which suggests that reductions in β-cell mass may not be confined to late-stage T2DM but may rather occur progressively throughout the prediabetic phase and continue after the onset of impaired glucose tolerance and then hyperglycemia [6]. Moreover, the decreased β-cell volume observed in subjects with fasting hyperglycemia is associated with increased β-cell death by apoptosis [6]. Evidence also indicates that the loss of β-cells is selective among islet cell types in the evolution of T2DM and that comparable losses of islet α-cells do not occur [15]. Together, these findings demonstrate that progressive structural and functional abnormalities occur in islets during the development of T2DM.

The mechanisms that underlie the progressive development of β-cell failure during the evolution of T2DM are not fully understood at present [3]. Identifying the factors involved and characterizing the mechanisms by which they lead to β-cell failure would be important steps in elucidating the pathogenesis of T2DM and identifying potential targets for therapeutic interventions designed to retard or prevent these processes. Both genetic and acquired factors contribute to β-cell failure in T2DM [16], and, among the acquired factors, glucotoxicity, lipotoxicity, altered islet amyloid polypeptide (IAPP) processing, advanced glycation end-products (AGEs), and increased inflammatory cytokines have been suggested to contribute to β-cell injury [1, 7, 17–20].

Although many mechanisms are proposed to underlie effects of these factors, a unifying theme is that production of reactive oxygen species (ROS) induced by metabolic stress represents a common pathway of injury in the cascade of events that ultimately results in β-cell failure [3, 21–27]. Activation of a series of stress-response pathways by ROS has been reviewed elsewhere [28–30]. The purpose of our paper is to provide a brief overview of how mitochondrial ROS affect mitochondrial membrane phospholipids, including cardiolipin, and how this might lead to β-cell mitochondrial failure and ultimately result in T2DM. Recent advances in complex lipid analyses by mass spectrometry permit detailed molecular characterization of the effects of pathophysiologic states on mitochondrial cardiolipin species [31–34], and this provides a powerful tool with which to increase our understanding of these processes and to identify potential targets for therapeutic intervention.

2. Mitochondria Are the Most Important Cellular Source of ROS in β-Cells

Oxidative stress can arise from various sources [35], and ROS appear to be produced in larger amounts by islets from T2DM patients than by those from nondiabetic subjects [23, 36–38]. Accumulating evidence indicates that obesity and hyperglycemia are associated with increased ROS production [22, 39]. Although ROS are generated in peroxisomes, for example, by cytochrome P450- and NADPH oxidase-catalyzed reactions, and in other nonmitochondrial loci, the major source of ROS production in cells is the mitochondrion [40].

Electron flow through the mitochondrial electron-transport chain is carried out by four inner membrane-associated enzyme complexes (I–IV), cytochrome *c*, and the mobile carrier coenzyme Q. Molecular species of ROS include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\bullet$HO), *inter alia*. The electron-transport chain continually generates small amounts of superoxide anion radicals, principally through complexes I and III [41]. Superoxide production increases substantially in the settings of obesity and hyperglycemia [22, 39]. Superoxide radicals are normally removed by Mn^{2+}-superoxide dismutase (MnSOD), which dismutates $O_2^{\bullet -}$ to produce H_2O_2 that is then reduced to water by catalase or glutathione peroxidase (GPx) at the expense of glutathione. When rates of H_2O_2 generation exceed those of its removal, H_2O_2 accumulation can result in production of the highly reactive hydroxyl radical in the presence of Fe^{2+} via the Fenton reaction and via the Haber-Weiss reaction of $O_2^{\bullet -}$ and $\bullet$HO (Figure 1).

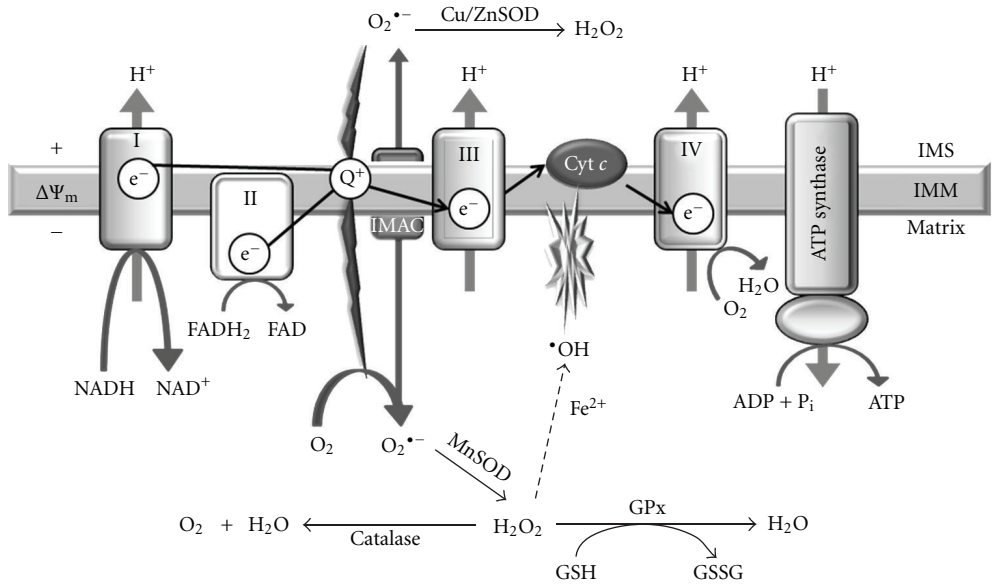

Figure 1: Mitochondrial ROS production and defense. The electron transport chain consists of four protein complexes (I–IV) and the ATP synthase located in the inner mitochondrial membrane (IMM). The activity of complex I converts NADH to NAD^+, and the activity of complex II converts succinate to fumarate. Complexes I, III, and IV transport protons (H^+) across the membrane, and complexes I and III generate superoxide anion radical ($O_2^{\bullet -}$) during the electron transfer process. $O_2^{\bullet -}$ can naturally dismutate to hydrogen peroxide (H_2O_2) or is enzymatically dismutated by matrix manganese superoxide dismutase (MnSOD). $O_2^{\bullet -}$ is not membrane permeable but can pass through inner membrane ion channel (IMAC) and is dismutated to H_2O_2 by Cu/ZnSOD in the intermembrane space (IMS)/cytoplasm. H_2O_2 is detoxified in the matrix by catalase and the glutathione peroxidase (GPx). Alternately, H_2O_2 can react with metal ions to generate via Fenton chemistry (dash line) the highly reactive hydroxyl radical (•OH) that can initiate the peroxidation of the inner mitochondrial membrane phospholipids, such as cardiolipin. Cyt. *c*: cytochrome *c*; IMS: intermembrane space; GSH: glutathione; GSSG: glutathione disulfide; $\Delta\Psi_m$: membrane potential.

If they are not rapidly eliminated, ROS can injure mitochondria by promoting DNA fragmentation, protein crosslinking, and peroxidation of membrane phospholipids and by activating a series of stress pathways [29]. Indeed, β-cell mitochondria in islets from T2DM subjects have been found to exhibit morphologic abnormalities that include hypertrophy, a rounded rather than elliptical shape, and higher density compared to β-cell mitochondria in islets from control subjects [11, 42]

3. ROS Trigger Apoptosis via Oxidation of Mitochondrial Inner Membrane Phospholipids in β-Cells

The onset of T2DM is accompanied by a progressive decrease in β-cell mass that results from a marked increase in β-cell apoptosis [6, 7, 43], and mitochondria are known to play a pivotal role in regulating apoptotic cell death [44]. Proapoptotic stimuli induce release of cytochrome *c* from mitochondria into the cytoplasm, where cytochrome *c* participates in apoptosome formation that results in caspase-9 activation and subsequent activation of the executioner caspases 3, 6, and 7 that dismantle the cell during apoptosis [44].

Cytochrome *c* release from mitochondria is a key step in the initiation of apoptosis [45] and appears to result from direct action of ROS on the mitochondrial phospholipid cardiolipin [46, 47]. Cardiolipin is a structurally unique dimeric phospholipid exclusively localized in the inner mitochondrial membrane (IMM) in mammalian cells and is essential for maintaining mitochondrial architecture and membrane potential and for providing support to proteins involved in mitochondrial bioenergetics [48, 49]. Cytochrome *c* is anchored to the outer surface of the inner mitochondrial membrane by electrostatic and hydrophobic interactions with cardiolipin [50]. During the early phase of apoptosis, mitochondrial ROS production is stimulated, and cardiolipin is oxidized. This destabilizes the interaction with cytochrome *c*, which then detaches from the membrane and is released into the cytoplasm through pores in the outer membrane [46, 50].

Cardiolipin is particularly susceptible to oxidation because it is enriched in polyunsaturated fatty acid (PUFA) residues, especially linoleate (C18:2), which contain a bisallylic methylene group from which hydrogen is easily abstracted to provide a center for formation of a hydroperoxy radical via interaction with molecular oxygen. Linoleic acid (C18:2) is the most abundant fatty acid substituent of cardiolipin in most mammalian tissues [51], and rat pancreatic islet cardiolipin, for example, contains 89.5% PUFA and 71% linoleate [52]. Mitochondrial cardiolipin is also a target of the proapoptotic protein tBid, which is a Bcl-2-family member produced from Bid by the

activation of caspase-8. This results in activation of the mitochondrial death pathway upon induction of apoptosis via engagement of death receptors [53]. Cardiolipin serves as a mitochondrial target of tBid, which promotes pore formation in the outer mitochondrial membrane by Bax or Bak in a process that is inhibited by Bcl-2 or Bcl-XL [54].

The mitochondrial phospholipid cardiolipin is thus a central participant in regulating apoptosis triggered by both the mitochondrial- and death receptor-mediated pathways, and alterations of mitochondrial cardiolipin are now recognized to be involved in the development of diabetes and several other pathologic conditions [29, 33, 34, 48, 49, 55–61]. We have observed that generation of ROS by mitochondria triggers apoptosis in INS-1 insulinoma cells and in mouse pancreatic islet β-cells in a process that involves mitochondrial phospholipid oxidation and cytochrome *c* release [57, 62].

4. ROS Activate Uncoupling Protein 2 (UCP2) through Initiation of Phospholipid Peroxidation in β-Cells

Glucose-stimulated insulin secretion by residual β-cells is impaired in subjects with T2DM [7]. Glucose sensing in β-cells requires the coupling of glycolysis to oxidative phosphorylation in mitochondria to produce ATP [28]. The respiratory chain complexes pump protons out of the mitochondrial matrix to generate an electrochemical proton gradient that provides the energy required by ATP synthase to produce ATP from ADP. This glucose-stimulated ATP production at the expense of ADP causes the cytoplasmic ATP/ADP ratio to rise, which induces closure of ATP-sensitive potassium channels (K_{ATP}), depolarization of the plasma membrane, opening of voltage-gated calcium channels, influx of Ca^{2+}, a rise in $[Ca^{2+}]$ in cytosol and other cellular compartments, activation of Ca^{2+}-sensitive effector elements including the Ca^{2+}/calmodulin-dependent protein kinase IIβ and others, and triggering of insulin exocytosis [63]. That oxidative phosphorylation is essential to glucose-stimulated insulin secretion is reflected by the observations, *inter alia*, that specific inhibition of mitochondrial respiratory chain complexes by various means invariably results in blockade of insulin secretion [64]. Moreover, mitochondrial mutations that cause defects in insulin secretion underlie maternally inherited T2DM [65–67].

It appears that pancreatic islet β-cell mitochondrial membrane potential can be regulated by uncoupling protein-2 (UCP2), which is a member of the mitochondrial anion carrier protein (MACP) family. UCP2 facilitates proton leak to reduce the mitochondrial membrane potential and thus attenuates ATP synthesis. It has been reported that UCP2 negatively regulates insulin secretion and is a major link between obesity, β-cell dysfunction, and T2DM [21, 68]. Obesity and chronic hyperglycemia increase mitochondrial superoxide ($O_2^{\bullet -}$) production [69], and this causes activation of UCP2 and results in pancreatic islet β-cell dysfunction [70–73]. Inhibition of UCP2-mediated proton leak by Genipin has been found acutely to reverse obesity- and high-glucose-induced β-cell dysfunction in isolated pancreatic islets *in vitro* and in animals with diet-induced T2DM *in vivo* [74, 75]. Together, these observations suggest that activation of UCP2 by superoxide produced by mitochondria could contribute to the development of β-cell dysfunction during the evolution of T2DM.

The mechanism by which superoxide activates UCP2 is nonetheless not well understood at present, although studies with probes targeted to subcellular compartments have provided an outline of some possibly contributory processes. Experiments with targeted antioxidants suggest that superoxide or its products activate UCPs on the matrix side of the mitochondrial inner membrane [71]. A study with a mitochondrion-targeted spin trap derived from α-phenyl-N-tert-butylnitrone indicated that superoxide activates UCPs via oxidation of unsaturated side chains of fatty acid substituents in mitochondrial phospholipids, for example, cardiolipin, associated with UCPs [76]. In this model, superoxide generated by mitochondria is dismutated by matrix Mn-SOD to hydrogen peroxide (H_2O_2), which reacts with Fe^{2+} by the Fenton reaction to generate hydroxyl radical ($\bullet$OH). The hydroxyl radical extracts a hydrogen atom ($H^{\bullet}$) from a *bis*-allylic methylene moiety of PUFA substituent of a phospholipid, for example, cardiolipin. The resultant carbon-centered radical reacts with molecular oxygen (O_2) to form a peroxy radical (HC-O-O$^{\bullet}$), which then initiates a chain reaction of lipid peroxidation that results in generation of a complex mixture of products, including 4-hydroxynonenal (*HNE*) and 4-hydroxyhexenal, which activate UCPs [76, 77].

Cardiolipin is a major phospholipid constituent of the mitochondrial inner membrane, and the PUFA linoleate is the major fatty acid substituent of β-cell cardiolipin [52]. The electron transport chain complexes that generate superoxide reside in the inner mitochondrial membrane, and superoxide production is rate limiting for generating all ROS. Cardiolipin PUFA substituents are especially susceptible to reaction with ROS because of their bisallylic methylene moieties. Like cardiolipin and the electron transport chain complexes, UCP2 also resides in the inner mitochondrial membrane. Together, these observations suggest a sequence in which high rates of mitochondrial superoxide production are associated with correspondingly high rates of cardiolipin oxidation and that this contributes to superoxide-mediated activation of UCPs, perhaps via the generation of HNE and other lipid peroxidation breakdown products. Thus, we propose that cardiolipin oxidation may directly link ROS generation to UCP2 activation and thereby contribute to acceleration of the proton leak that ultimately results in β-cell dysfunction. Indeed, it was recently reported that oxidation of a mitochondria-specific phospholipid tetralinoleoyl cardiolipin (L4CL) leads to the formation of 4-HNE via a novel chemical mechanism that involves cross-chain peroxyl radical addition and decomposition [78]. This proposal points to potentially important target processes for the design of interventions to prevent or retard the development of T2DM and perhaps obesity [77].

5. The Role of Group VIA PLA_2 ($iPLA_2\beta$) in Remodeling and Repairing Mitochondrial Membranes

Pancreatic islet cardiolipin is enriched in PUFA (89.5%) substituents, including linoleate (71%) [52], and PUFA side chains are especially vulnerable to oxidation because of their bisallylic methylene moieties. Cardiolipin resides in the inner mitochondrial membrane, which is the locus of ROS generation, and this spatial proximity would also favor cardiolipin oxidation under conditions of accelerated ROS production. This susceptibility would be expected to be enhanced in islets, which express low levels of antioxidant enzymes including superoxide dismutase (SOD), catalase, and glutathione peroxidase (Gpx) compared to other tissues, such as liver, kidney, brain, lung, muscles, pituitary gland, and adrenal gland [36, 79–82]. To counteract the continual oxidation of cardiolipin and the associated impairment of mitochondrial function, it thus seems likely that β-cells must have some means of repairing or replacing oxidized cardiolipin molecules in order to maintain mitochondrial function.

It has been proposed that the consecutive actions of mitochondrial phospholipid glutathione peroxidase (PHGPx or Gpx4) and a phospholipase A_2 (PLA_2) are required to eliminate oxidized fatty acids from mitochondrial phospholipids under physiological conditions [83]. Gpx4 is a selenoprotein in the glutathione peroxidase (Gpx) family that protects biomembranes, particularly in mitochondria [84]. The PLA_2 family comprises a diverse group of enzymes that catalyze hydrolysis of the *sn*-2 fatty acyl bond of phospholipids to generate a free fatty acid and a 2-lysophospholipid [85, 86]. Because the PUFAs in phospholipids tend to be located in the *sn*-2 position, it is not surprising that members of the PLA_2 family can hydrolyze oxidized *sn*-2 fatty acid substituents [85, 87] and are thought to be involved in the repair of oxidized membrane phospholipids [88–90].

Among PLA_2 family members, Group VIA PLA_2 ($iPLA_2\beta$) is attracting increasing interest as a potentially critical participant in mitochondrial cardiolipin homeostasis [57, 62, 91, 92]. In eukaryotes, cardiolipin is synthesized *de novo* from phosphatidylglycerol (PG) and cytidine diphosphate-diacylglycerol (CDP-DAG) by cardiolipin synthase on the inner face of the inner mitochondrial membrane [93]. Nascent cardiolipin does not contain PUFAs in its four acyl chains, and the enrichment of PUFA in cardiolipin is thought to be achieved by a remodeling process [94]. Currently, two potential mechanisms, Tafazzin- (*TAZ*-) and $iPLA_2\beta$/MLCLAT-mediated mechanisms, have been proposed to participate in cardiolipin remodeling [93].

In the *TAZ* pathway, newly synthesized cardiolipin is proposed to be deacylated and reacylated by *TAZ*. It appears that this mechanism is essential for optimal mitochondrial function in heart because Barth Syndrome, which is characterized by a severe cardiomyopathy [95, 96], is caused by a mutated *TAZ* gene that encodes a putative mitochondrial phospholipid acyltransferase with both deacylation and reacylation activities [95, 97]. In the $iPLA_2\beta$/MLCLAT-mediated pathway, newly synthesized cardiolipin is proposed to be deacylated by $iPLA_2\beta$ to MLCL that is reacylated to cardiolipin by a MLCL acyltransferase (MLCLAT) (Figure 2). It has recently been recognized that mutations in the PLA2G6 gene that encodes $iPLA_2\beta$ underlie the neurodegenerative disease infantile neuroaxonal dystrophy (INAD) [98] and that a similar disorder develops in mice with a disrupted *iPLA*$_2\beta$ gene (Malik et al. [99]). It has been suggested that $iPLA_2\beta$ also plays a role in cardiolipin remodeling both in a *Drosophila* model of the Barth Syndrome [92] and in the spontaneously hypertensive rat heart failure model [91].

We have also reported observations that are consistent with a role for $iPLA_2\beta$ in β-cell mitochondrial function that include that $iPLA_2\beta$ resides in mitochondria in INS-1 insulinoma cells and that its activity provides protection against the effects of staurosporine to induce loss of mitochondrial membrane potential, release of cytochrome *c* and Smac/DIABLO into cytosol, peroxidation of mitochondrial membranes, and apoptosis [62]. Staurosporine is an inhibitor of various isoforms of Protein Kinase C and strongly stimulates mitochondrial generation of ROS [100].

Both Barth Syndrome and INAD are human genetic disorders that are often fatal in childhood [95, 98] at an age before type I DM might be manifest, which requires loss of about 80–90% of the islet β-cell mass at the age of onset [101]. Animal models that have been used to evaluate the potential involvement of $iPLA_2\beta$ in disease processes include administration of a suicide substrate bromoenol lactone (BEL) inhibitor of $iPLA_2\beta$ [102] and $iPLA_2\beta$-null ($iPLA_2\beta^{-/-}$) mice generated by homologous recombination to disrupt the *iPLA*$_2\beta$ gene [103]. These $iPLA_2\beta$-null mice develop a disorder similar to INAD [99, 104], exhibit several other phenotypic abnormalities [103, 105–113], and have permitted evaluation of the role of $iPLA_2\beta$ in β-cell failure *in vivo* [57, 103, 114, 115].

We have observed that acute pharmacologic inhibition of $iPLA_2\beta$ in mice impairs glucose tolerance by suppressing insulin secretion and that insulin sensitivity is not affected under these conditions, which suggests that $iPLA_2\beta$ deficiency adversely affects glucose-induced insulin secretion by β-cells [102]. Consistent with that interpretation, studies with $iPLA_2\beta^{-/-}$ mice that are genetically deficient in $iPLA_2\beta$ expression because of homozygous disruption of the *iPLA*$_2\beta$ gene by homologous recombination [103] have revealed that they exhibit greater impairment in islet function, as reflected by fasting blood glucose levels and glucose tolerance testing responses, than do wild-type mice in response to metabolic stress imposed by low-dose streptozotocin (STZ) treatment, by consumption of a high-fat diet, or by staurosporine administration [57, 114, 115].

Moreover, findings with pancreatic islets isolated from $iPLA_2\beta^{-/-}$ mice corroborate the involvement of $iPLA_2\beta$ in glucose-stimulated insulin secretion because $iPLA_2\beta^{-/-}$ islets exhibit diminished secretory responses compared to wild-type islets [57, 114, 115]. In addition, incubation with elevated concentrations of glucose and free fatty acids *in vitro* results in higher levels of β-cell apoptosis and of peroxidation of mitochondrial membrane phospholipids with islets isolated from $iPLA_2\beta^{-/-}$ mice compared to those from wild-type mice [57]. These findings suggest that $iPLA_2\beta$

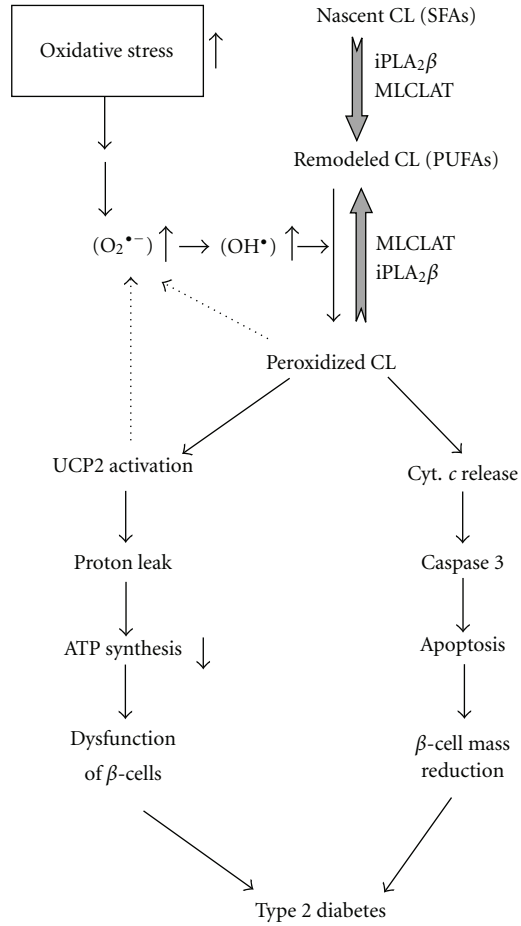

Figure 2: Schematic summary of the proposed role of mitochondrial cardiolipin oxidation in β-cell failure in type 2 diabetes mellitus. Oxidative stress results in increased mitochondrial ROS generation in β-cells. With moderate oxidative stress, ROS oxidize polyunsaturated fatty acid (PUFA) substituents in mitochondrial cardiolipin molecules, which may generate signals that mitigate ROS production via effects on respiratory electron transport chain complexes or on uncoupling protein 2 (UCP2) (dotted arrows). After delivery of the signal from the ROS-PUFA interaction, the oxidized cardiolipin molecule is repaired in a pathway in which iPLA$_2\beta$ excises the oxidized PUFA residue to yielded monolysocardiolipin (MLCL), which is then reacylated with an unoxidized PUFA substituent by MLCL acyltransferase (MLCLAT) to complete the oxidation and repair cycle. Under conditions of overwhelming oxidative stress imposed by high metabolic loads, the rate of cardiolipin oxidation exceeds the capacity of the repair mechanism and oxidized cardiolipin molecules accumulate and compromise mitochondrial membrane integrity, and this leads to cytochrome *c* (Cyt. *c*) release into the cytosol and induction of apoptosis, which eventuates in β-cell failure and the development of T2DM. Circumstances in which the capacity of the repair mechanism is overwhelmed in this way would include reductions in iPLA$_2\beta$ activity caused by genetic deficiency, pharmacologic inhibition, or yet to be defined regulatory influences on expression. Block arrows denote the iPLA$_2\beta$-mediated deacylation; line arrows denote the stimulatory pathway. SFAs: saturated fatty acids.

plays an important role in maintenance of β-cell mitochondrial membrane integrity and that iPLA$_2\beta$ deficiency increases β-cell susceptibility to injury by ROS generated by mitochondria in response to metabolic stress [57, 115]. This could lead to increased vulnerability to induction of apoptosis under conditions of metabolic stress that lead to β-cell failure and T2DM [57, 115]. β-cell mitochondrial membrane peroxidation is also more readily induced under conditions in which iPLA$_2\beta$ is inhibited pharmacologically with the suicide substrate BEL [57].

It has been suggested that oxidation of PUFA in mitochondrial cardiolipin and other phospholipids may serve to trap ROS in order to protect mitochondrial proteins or DNA from oxidative injury or that reaction of PUFA with ROS may generate signals to respiratory chain proteins and UCP2 that mitigate ROS generation and increase proton leak [49, 77, 116–118]. A repair mechanism in which iPLA$_2\beta$ excised oxidized fatty acid substituents from mitochondrial cardiolipin and other phospholipids would generate monolysocardiolipin (MLCL) that could be reacylated with an unoxidized PUFA substituent might complete a cycle that could modulate the levels and effects of ROS during stress responses.

Under conditions in which the rates of ROS generation and oxidation of PUFA in mitochondrial cardiolipin and other phospholipids exceed the capacity of the repair system, accumulation of oxidized phospholipids could eventually impair the integrity of mitochondrial membranes and result in release of cytochrome *c* into cytosol and induction of

β-cell apoptosis. One circumstance in which the capacity of this repair system would be reduced is when iPLA$_2\beta$ activity is low because of pharmacologic inhibition, genetic deficiency, or still to be defined regulatory influences. Under such conditions, accumulation of oxidized mitochondrial phospholipids and leakage of cytochrome *c* could result in accelerated induction of apoptosis that ultimately leads to β-cell failure and T2DM (Figure 2).

Of interest in this regard are findings with the *db/db* mouse, which is a model of obesity, dyslipidemia, and diabetes in which there is a defective leptin receptor. Islets isolated from *db/db* mice express lower levels of iPLA$_2\beta$ than do islets from control mice [57], and this could impair cardiolipin remodeling and repair in *db/db* β-cells and increase their susceptibility to oxidative injury, which could accelerate obesity-associated β-cell loss and the development of T2DM.

6. Conclusions and Therapeutic Implications

Modification of mitochondrial cardiolipin molecular species by oxidation and other processes is now recognized to be associated with many human diseases, including diabetes mellitus [55, 58, 60]. Cardiolipin is a critical structural component of mitochondrial membranes and plays important roles in regulating ATP synthesis and the mitochondrial pathway of apoptosis [49, 119]. Metabolic stresses imposed by obesity and hyperglycemia are often accompanied by increased rates of mitochondrial ROS production [69]. PUFAs are especially susceptible to oxidation by ROS because they contain a highly reactive bisallylic methylene moiety from which hydrogen is readily abstracted to yield a center for initiation of peroxidation chain reactions, and cardiolipin is enriched in PUFA substituents.

A repair mechanism in which iPLA$_2\beta$ excises oxidized PUFA substituents of cardiolipin to yield an MLCL intermediate that can be reacylated with an unoxidized PUFA substituent may be critical for the maintenance of mitochondrial membrane integrity, and it seems likely that some such repair mechanisms would be necessitated by the close spatial proximity of mitochondrial cardiolipin to the locus of ROS generation. Failure of this repair mechanism could compromise mitochondrial membrane integrity and facilitate release of cytochrome *c* into cytosol and induction of apoptosis. Observations from several laboratories [57, 62, 91, 92, 102, 114, 115] suggest that iPLA$_2\beta$-catalyzed deacylation participates in a cardiolipin remodeling and repair cycle that maintains an optimal mitochondrial functional status in β-cells.

Reduced iPLA$_2\beta$ activity resulting from genetic deficiency, as in INAD patients or iPLA$_2\beta^{-/-}$ mice, or downregulated expression, as in *db/db* mouse islets, could impair this cardiolipin repair mechanism and result in accumulation of oxidized cardiolipin species that compromise mitochondrial membrane integrity. The ensuing release of cytochrome *c* into cytosol and induction of apoptosis might result in the neurodegeneration in INAD and in β-cell loss during the development of T2DM. Further study of cardiolipin remodeling and repair and the role of iPLA$_2\beta$ in these processes could increase our understanding of the pathogenesis of diabetes mellitus and neurodegeneration and suggest novel strategies for design of therapeutic interventions to prevent or retard the development of T2DM and neurodegenerative diseases in humans.

An example of such a potential intervention would be administration of an agent that accumulated in mitochondria and protected them from injurious effects of ROS. The antioxidant NtBHA accumulates in mitochondria, and we have found that it attenuates staurosporine-induced apoptosis and prevents peroxidation of mitochondrial phospholipids in islets from iPLA$_2\beta^{-/-}$ mice [57]. A similar approach to protecting mitochondrial cardiolipin and other phospholipids from oxidation might represent an attractive therapeutic strategy in humans with metabolic or neurodegenerative diseases. Such approaches might be complicated by the fact that some effects of ROS are not injurious but represent essential signaling roles in physiological regulatory mechanisms. For example, mitochondrial ROS generation has been suggested to be an essential signal in the glucose-stimulated insulin secretory pathway in β-cells and also to be involved in insulin signaling and sensitivity [120].

Thus, manipulating ROS production or interaction with intracellular targets *in vivo* could have unexpected and unwanted adverse effects, and the ability to target such interventions with high selectivity to specific intracellular processes, such as inhibition of mitochondrial phospholipid oxidation, might be desirable. It is of interest in this regard that specific delivery of antioxidants to mitochondria, such as mitoquinone (Mito-Q) and mitovitamin E (mitoVit-E), has been demonstrated to reduce oxidative stress and to improve cardiac function [121, 122] and might be similarly beneficial in β-cells. In addition, melatonin specifically inhibits mitochondrial cardiolipin oxidation and has also been found to prevent induction of the mitochondrial permeability transition (MPT) and release of cytochrome into cytosol and to protect against myocardial ischemia-reperfusion injury [120, 123].

Disclosure

The authors have nothing to disclose.

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Review Article

Autophagy as a Therapeutic Target in Diabetic Nephropathy

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Diabetic nephropathy is a serious complication of diabetes mellitus, and its prevalence has been increasing worldwide. Therefore, there is an urgent need to identify a new therapeutic target to prevent diabetic nephropathy. Autophagy is a major catabolic pathway involved in degrading and recycling macromolecules and damaged organelles to maintain intracellular homeostasis. The study of autophagy in mammalian systems is advancing rapidly and has revealed that it is involved in the pathogenesis of various metabolic or age-related diseases. The functional role of autophagy in the kidneys is also currently under intense investigation although, until recently, evidence showing the involvement of autophagy in the pathogenesis of diabetic nephropathy has been limited. We provide a systematic review of autophagy and discuss the therapeutic potential of autophagy in diabetic nephropathy to help future investigations in this field.

1. Introduction

The prevalence of diabetes mellitus has been increasing worldwide during recent years, and this is estimated to continue in the future [1, 2]. Diabetic nephropathy is a serious complication of diabetes mellitus and is the most common cause of end-stage renal disease [3, 4]. The increasing prevalence of diabetes mellitus and its complications, including diabetic nephropathy, has therefore become a major health problem worldwide. There is now an urgent need to identify new therapeutic target molecules or cellular processes that underlie the pathogenesis of diabetic nephropathy to establish an additional therapeutic option.

Hyperglycemia-mediated alteration of extra- and intracellular metabolism, such as advanced glycation end products [5], increased protein kinase C activity [6], and abnormal polyol metabolism [7], has been recognized as classical pathogenesis of diabetic nephropathy. In addition, intracellular stress associated with renal hypoxia [8, 9], mitochondrial reactive oxygen species (ROS) [10–13], and endoplasmic reticulum (ER) stress [14–16] has recently been proposed and focused as new pathogenesis of diabetic nephropathy. Thus, to maintain the cellular homeostasis against stress condition derived from organelle dysfunction or hypoxia may be a new therapeutic target of diabetic nephropathy.

Autophagy, a lysosomal protein degradation pathway in cells, plays a crucial role in removing protein aggregates as well as damaged or excess organelles to maintain intracellular homeostasis and cell integrity [17]. It has recently been highlighted because it can be stimulated by multiple types of cellular stressors including starvation, hypoxia, or ER stress. The study of autophagy in mammalian systems and in disease states is advancing rapidly, and many investigators are entering this new and exciting field (Figure 1). It has been revealed that autophagy plays a crucial role in several organs, especially in metabolic organs, and that its alteration is involved in the pathogenesis of metabolic [18–21] and age-related diseases [22–27]. The functional role of autophagy in the kidneys is currently under intense investigation (Figure 1), and it has been revealed that autophagy has a renoprotective role in several animal models including those used for aging and acute kidney injury [26–31]. However, the role of autophagy in diabetic nephropathy remains unclear.

Alteration of several nutrient-sensing pathways is related to the development of metabolic diseases, such as type 2 diabetes and its vascular complications. Major nutrient-sensing

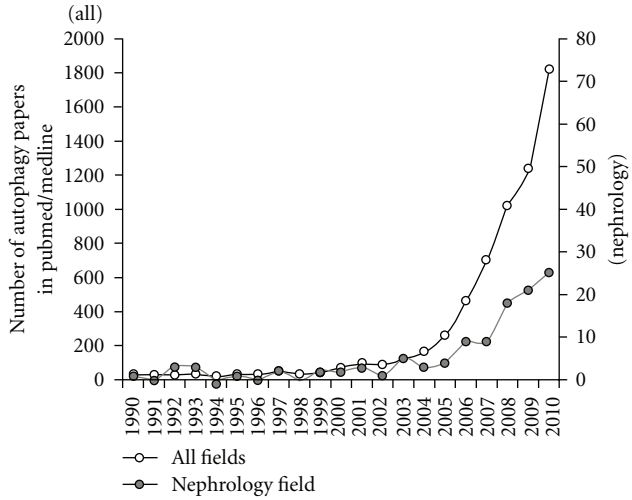

Figure 1: *Substantial increases in the number of autophagy-related papers indexed in PubMed/Medline.* The number of autophagy-related papers in all fields has increased remarkably over recent decades. Corresponding with the increase of autophagy-related papers in all fields, publication of autophagy-related papers in nephrology fields, also gradually increased.

pathway involves the mammalian target of rapamycin (mTOR) [32–35], AMP-activated protein kinase (AMPK), [36–40] and oxidized NAD- (NAD^+-) dependent histone deacetylase (Sirt1) [41–43], which are also recognized as the regulatory factors of autophagy under nutrient-depleted condition. As described above, autophagy can be induced by intracellular stresses that are involved in the pathogenesis of diabetic nephropathy [44]. Thus, alteration of these nutrient-sensing pathways under diabetic condition may impair the autophagic stress response stimulated by intracellular stress, which may lead to exacerbation of organelle dysfunction and subsequent diabetic nephropathy.

The above findings lead us to hypothesize that autophagy is involved in the pathogenesis of diabetic nephropathy and is a potential therapeutic option. Therefore, we provide a systematic review of autophagy and discuss its therapeutic potency in diabetic nephropathy to help future investigations in this field.

2. Autophagy

The term autophagy is derived from Greek and means self-eating. It is highly conserved from yeast to mammals and is a bulk degradation process that is involved in the clearance of long-lived proteins and organelles. Autophagy has two major roles in cells: to recycle intracellular energy resources in response to nutrient-depleted conditions and to remove cytotoxic proteins and organelles under stressful conditions. Autophagy works to maintain cell homeostasis under various stressful conditions. Several types of autophagy have been recognized in cells: macroautophagy, microautophagy, and chaperone-mediated autophagy; these differ in their mechanisms and functions [45, 46]. Of these three types of autophagy, macroautophagy is most prevalent and hereafter is referred to as autophagy. In this paper, we focus on the mechanisms and functions of autophagy.

3. Molecular Mechanisms of Autophagy

During macroautophagy, *de novo* isolation membranes (phagophores) elongate and fuse while engulfing a portion of the cytoplasm within double-membraned vesicles (autophagosomes) (Figure 2). Several origins of autophagosomes have been reported, including the ER [47–49], mitochondria [50], and plasma membrane [51]. Four major steps are involved in the formation of autophagosomes: initiation, nucleation, elongation, and closure. During these steps, autophagy-related proteins are involved (Figure 2). Autophagy is initiated by the unc-51-like kinase (Ulk) 1 (mammalian ortholog of the yeast autophagy-related genes (Atg)1) complex, which is composed of Ulk1 Ser/Thr protein kinase, Atg13, and FIP200 (mammalian homolog of the yeast Atg17) [52–54]. The phosphorylation of Atg13 and FIP200 by Ulk1 is essential for triggering autophagy. Phagophore nucleation is dependent on Beclin 1 (Atg6 in yeast)—an hVps34 or class III phosphatidylinositol 3-kinase (PI3K) complex, which consists of hVps34, hVps15, Beclin 1, and Atg14 [55, 56].

During autophagosome elongation/closure, two dependent ubiquitin-like conjugation systems are involved: Atg12 and LC3 (the mammalian ortholog of the yeast Atg8) [57]. The Atg12-Atg5 conjugate, which forms the Atg12-Atg5-Atg16 complex, contributes to the stimulation and localization of the LC3 conjugation reaction. The cytosolic isoform of LC3 (LC3-I) is conjugated to phosphatidylethanolamine through two consecutive ubiquitination-like reactions that are catalyzed by E1-like enzyme Atg7 and the E2-like enzyme Atg3 to form LC3-II [58]. Thus, LC3-II formation is recognized as a marker of existence of autophagosomes in cell or animal experiments [59–61]. After formation, the autophagosomes merge with the lysosomal compartment to form autolysosomes. The protein p62, also known as sequestosome 1 (SQSTM1), is known to localize to autophagosomes via LC3 interaction and to be constantly degraded by the autophagy-lysosome system [62, 63]. The accumulation of p62 is observed in autophagy-deficient cells [62, 63].

4. Methods Available for Monitoring Autophagy

It is necessary to keep in mind several important points as we monitor and assess the autophagy activity to prevent misconceptions. Some reviews about the methods for autophagy research have been published [59–61]. As briefly below summarized, some methods including electron microscopy (EM), detection of endogenous LC3 or green fluorescent protein (GFP)-LC3 by fluorescence microscopy, and detection of LC3-II by Western blotting are useful in monitoring the number of autophagosomes. However, these methods have some limitations. An accumulation of autophagosomes does not always mean increased formation of autophagosomes and may represent inhibited maturation of autolysosomes (or amphisomes). Simply counting

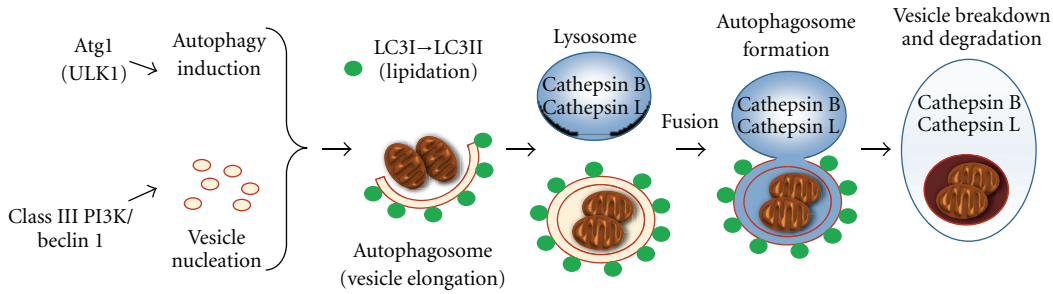

FIGURE 2: *Scheme of autophagic pathways.* Autophagic pathways consist of four steps: initiation, nucleation, elongation, and closure. Autophagy is initiated by the nucleation of an isolation membrane (phagophore). The phagophore elongates and closes on itself to form an autophagosome. Fusion of an autophagosome with a lysosome forms an autolysosome and, where the acid hydrolases in the lysosome, breaks down the inner membrane and cytoplasmic contents.

the number of autophagosomes is insufficient for assessing autophagy activity. Autophagy flux is a term that represents a serial process of autophagy, including the synthesis of autophagosomes, the delivery of cargo to lysosomes, and the degradation of autolysosomes. To distinguish whether the accumulation of autophagosomes is caused by induction of autophagy or inhibition of autophagosome maturation and/or degradation of autophagic substrates in the lysosome, and then assess autophagy activity, an autophagy flux assay is more reliable than counting the number of autophagosomes. There are some useful assays to monitor autophagy flux. These include the LC3 turnover assay, or measurement of total levels of autophagic substrates such as LC3, GFP-LC3, and p62. Furthermore, several types of autophagy inhibitors and activators have recently become available to modulate the activity of autophagy processes. Pharmacological inhibitors of autophagy are PI3-kinase inhibitors such as wortmannin, LY294002, or 3-methladenine (3-MA) and inhibitors that block autophagosome-lysosome fusion or degradation of autophagic cargo in autolysosomes, such as E64d, pepstatin A, and bafilomycin A. However, a major problem is that there are no highly specific inhibitors or activators of autophagy. Thus, it is strongly recommended that pharmacological studies should be combined with studies that investigate deficiency/reduction of autophagy-related genes by genetic knockout/knockdown of ATG genes or dominant-negative mutant autophagy proteins, including Atg3, Atg5, Atg7, and Beclin 1.

5. Role of Nutrient Stress in Autophagy and Diabetic Nephropathy

The kidney is a structurally complex organ and is essential in several functions including excretion of the waste products of metabolism, regulation of body fluid volume, maintenance of appropriate acid balance, and secretion of a variety of hormones. The basic unit of the kidney is the nephron, which consists of a glomerulus and a series of tubules lined by a continuous layer of epithelial cells (Figure 3). The glomerulus consists of mesangial cells and a capillary wall with endothelial cells, glomerular basement membrane, and visceral epithelial cells (podocytes) (Figure 3). Among them, since podocytes play essential role to maintain glomerular filtration barrier, podocyte injury leads to proteinuria and glomerulosclerosis, which are major features of diabetic nephropathy. Podocytes are terminally differentiated cells with a limited proliferative capacity. Therefore, the fate of podocyte depends on its ability to cope with stress. Excess fluid filtered through glomerulus enters urinary space and is reabsorbed by the proximal tubular cells (Figure 3). The proximal tubular cells serve as a system to degrade several molecules reabsorbed from urinary space. Thus, autophagy may be essential to maintain their homeostasis and functions in both podocytes and proximal tubular cells, which might be altered in diabetic condition. If autophagy system is altered in diabetic condition, this alteration of autophagy may be involved in the pathogenesis of diabetic nephropathy.

As expected, autophagy has been identified in both podocytes and proximal tubular cells and is regulated by a variety of stimuli including nutrient stress. Nutrient depletion is the most potent physiological inducer of autophagy, among several that have been reported to regulate autophagy. Here, we show the roles of mTOR, AMPK, and Sirt1, in the regulation of autophagy. The alteration of these pathways is involved in the pathogenesis of several kidney diseases including diabetic nephropathy.

5.1. mTOR. Several studies have shown that hyperactivation of the mTOR pathway in diabetic nephropathy plays a pivotal role in the hypertrophy of existing glomerular and tubular cells [64, 65] and is associated with podocyte injury and the progressive decline of glomerular filtration rates. Other studies have suggested that inhibition of the mTORC1 pathway with rapamycin has renoprotective effects on the progression of diabetic nephropathy in models of type 1 [33] and type 2 diabetes [32, 66–69]. Some reports have shown the additive renoprotective effects of rapamycin treatment including prevention of mesangial expansion and glomerular membrane thickness in type 1 diabetic rats [33] and attenuation of increased glomerular expression of laminin-β1 protein in type 2 diabetic mice [32, 67]. It has been reported that activation of the mTOR pathway is involved in the increased expression of profibrotic cytokines, such as TGF-β1 and connective tissue growth factor, and subsequent interstitial fibrosis in diabetic nephropathy [32–34]. Furthermore, more

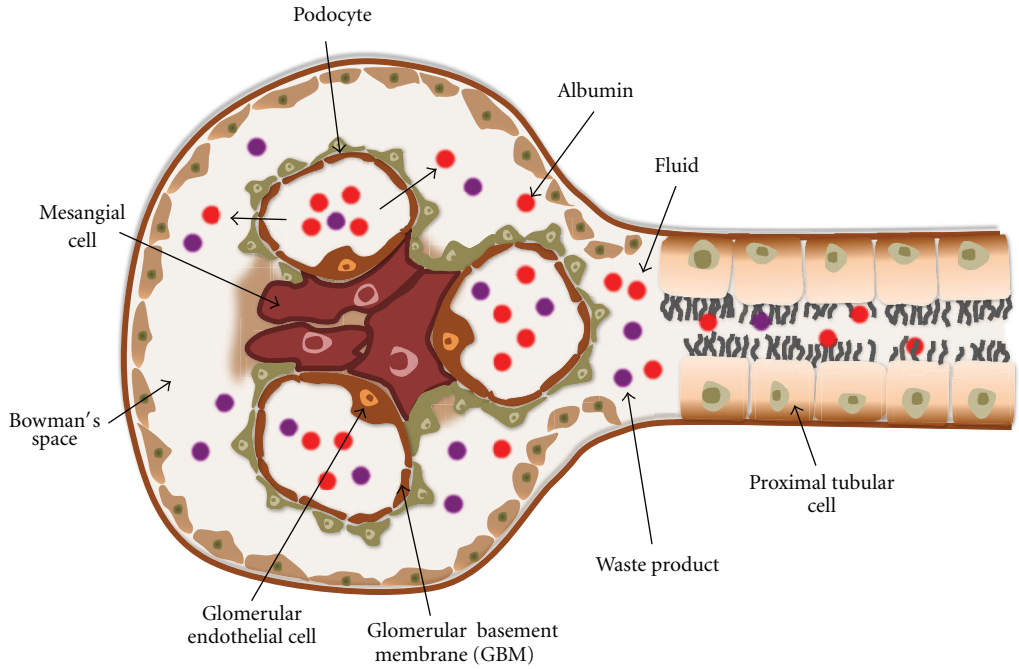

Figure 3: *Schematic representation of nephron.* The basic unit of the kidney is the nephron, which consists of a glomerulus and a series of tubules lined by a continuous layer of epithelial cells. The glomerulus consists of mesangial cells and a capillary wall with endothelial cells, glomerular basement membrane, and visceral epithelial cells (podocytes). Fluid containing albumins and waste products is filtered through glomerulus, enters urinary space, and is reabsorbed by the proximal tubular cells.

recent reports have shown that mTORC1 activity is essential to maintain podocyte homeostasis, but its hyperactivation is a causes of glomerular lesion of both type 1 and type 2 diabetic nephropathy [68, 69]. Complete deletion of podocyte mTORC1 activity in podocyte-specific Raptor-deficient mice causes podocyte injury [68]. In contrast, podocyte-specific mTORC1 hyperactivation by podocyte-specific tuberous sclerosis complex (TSC) 1-knockout mice show podocyte injury and glomerular lesion similar to diabetic nephropathy [69]. Finally, podocyte-specific Raptor-heterozygous mice show partial deletion of mTORC1 activity in podocyte and resistance to the development of diabetic nephropathy in both STZ-induced type 1 diabetic mice [68] and type 2 diabetic *db/db* mice [69].

In addition to the above-mentioned function, inhibition of autophagy is a main role of mTOR pathway [44, 58, 70]. Among the several signaling pathways that regulate autophagy in mammalian cells, the classical pathway of serine/threonine kinase, mTOR, plays a major role in the negative regulation of autophagy because it integrates signals that are emitted by growth factors, amino acids, glucose, and energy status [71]. Autophagy is inhibited by the activation of TOR under hypernutrient conditions [44, 58]. The mTOR pathway involves two functional complexes: mTOR complex 1 (mTORC1) and mTORC2. mTORC1, which consists of the mTOR catalytic subunit, regulatory associated protein of mTOR (Raptor), G protein β-subunit-like protein (GβL), proline-rich Akt substrate of 40 kDa (PRAS40), and DEP domain-containing mTOR-interacting protein (Deptor) [72], is sensitive to the immunosuppressant rapamycin [73, 74]. This complex regulates cell growth, metabolism (by integrating amino acid and growth factor signals), energy, and oxygen status [75]. The mTORC1 complex suppresses autophagy via phosphorylation and inactivation of Ulk1, an initiator of autophagosome formation [76]. Although no direct evidences have been provided, hyperactivation of mTOR pathway may suppress autophagy in podocyte and tubular cells in diabetic condition. Furthermore, enhanced activity of autophagy may be involved in the renoprotective effects of rapamycin treatment in diabetic nephropathy. The mTORC2 complex is less sensitive to rapamycin and includes mTOR, rapamycin-insensitive companion of mTOR (Rictor), GβL, stress-activated protein kinase-interacting protein 1 (Sin1), protein observed with Rictor (PROTOR), and Deptor [75, 77]. The mTORC2 complex regulates cytoskeletal organization, metabolism, and cell survival [75, 78, 79]. However, until now, the role of mTORC2 in regulation of autophagy has remained unclear.

5.2. AMPK. AMPK is activated under energy-depleted conditions and is likely to be suppressed in diabetic nephropathy. It has been reported that AMPK is inactivated (decreased phosphorylation of AMPK) in glomeruli and tubules in both type 1 and type 2 diabetic animal models [40, 81–84], which are reversed by agents such as metformin and resveratrol along with attenuation of diabetic glomerular and tubular injury [40, 85, 86]. This introduces the question of how decreases in AMPK activity can be involved in the pathogenesis of diabetic nephropathy. In type 1 and 2 diabetic kidneys, intrarenal lipid metabolism is altered, which is

characterized by enhanced renal lipogenesis and suppressed lipolysis [87–90]. AMPK-mediated phosphorylation inactivates a lipogenic enzyme, acetyl-CoA carboxylase, which results in decreased lipogenesis and enhanced lipolysis [91]. Decreases in renal AMPK activity in these mouse models may be a mechanism of altered renal lipid metabolism and subsequent lipotoxicity-associated renal damage. Since AMPK can affect various cellular metabolism as wells as lipid metabolism [92, 93], the other molecular mechanism should be involved in AMPK-mediated renoprotection.

AMPK plays a central role in the integration of several stress stimuli and is a positive regulator of autophagy in response to nutrient-depleted conditions. AMPK monitors the energy status of the cell by sensing its AMP/ATP ratio [93]. Several upstream kinases, including liver kinase B1 (LKB1), calcium/calmodulin kinase kinase (CaMKII) β, and TGF-β-activated kinase-1 (TAK1), can activate AMPK by phosphorylating a threonine residue on its catalytic α subunit [93]. AMPK can crosstalk with the mTORC1 signal during multiple steps of autophagy regulation. AMPK induces autophagy by inhibiting mTORC1 activity via phosphorylation of its regulatory-associated proteins [44, 58, 94]. Recent studies have shown that AMPK-dependent phosphorylation of Ulk1 induces autophagy [94, 95]. A balance between mTORC1 and AMPK likely directly regulates Ulk1 activity and subsequent autophagy initiation [44]. Thus, in addition to the above-mentioned mechanism, AMPK-mediated induction of autophagy may be involved in its renoprotection. AMPK activation may be linked to autophagy for the maintenance of renal homeostasis in diabetic kidney.

5.3. Sirt1. Sirtuins, the silent information regulator 2 family, were originally identified as NAD^+-dependent deacetylases in experiments in lower species and consist of seven members, Sirt1–Sirt7, in mammals [96, 97]. Sirtuins have been identified as antiaging molecules under calorie-restricted conditions and environmental stress. Some mammalian sirtuins, especially Sirt1, have been shown to play important roles in the regulation of aging, or in the pathogenesis of age-related metabolic diseases such as type 2 diabetes [41, 42, 96]. An increase in the intracellular concentration of NAD^+ by caloric restriction can activate Sirt1. Results that demonstrate the role of Sirt1 in autophagy are still lacking compared with those for mTOR and AMPK, but they have recently been increasing. Sirt1 can deacetylate essential autophagic factors such as Atg5, Atg7, and LC3 [98] and has been shown to induce autophagy. Furthermore, Sirt1 deacetylates the transcription factor Forkhead box O3a (Foxo3a), which leads to enhanced expression of proautophagic BCL2/adenovirus E1V 19-kDa interacting protein 3 (Bnip3) [26].

Renal expression of Sirt1 decreases in type 1 diabetic animal models [99, 100]. Also, reduced forms of nicotinamide adenine dinucleotide (NADH) are metabolites of glucose and fatty acids. Thus, NAD^+/NADH ratios are decreased in cells under conditions where nutrients are in excess, such as diabetes. Sirt1-deacetylase activity should decrease in diabetic nephropathy. Although direct renoprotective effects of Sirt1 in diabetic nephropathy have yet to be elucidated, Sirt1 has shown renoprotective activity in aging kidneys and fibrotic kidney diseases. The previously mentioned findings lead us to speculate that activation of Sirt1 should also have therapeutic efficacy in diabetic nephropathy. Furthermore, Sirt1-induced autophagy activation may contribute to Sirt1-mediated renoprotective effect in diabetic nephropathy.

6. Regulation of Autophagy by Intracellular Stress

Besides nutrient stress, autophagy is upregulated by several intracellular stresses, such as hypoxia, ROS, and ER stress [44]. Based on recent reports, this process is probably a compensatory response to maintain cell integrity. Furthermore, these intracellular stresses have recently been focused on as a pathogenesis of diabetic nephropathy, in addition to the classical pathogenesis of diabetic nephropathy.

6.1. Oxidative Stress. Under conditions where nutrients are in excess, such as diabetes and obesity, the production of ROS in the kidneys is enhanced by high glucose concentrations [101, 102]. Furthermore, high levels of free fatty acids, especially polysaturated fatty acids, also induce ROS production in the kidneys [88, 103]. Oxidative stress is a by-product of mitochondrial respiration and is associated with cell dysfunction. Actually, a recent report has shown abnormal mitochondrial morphology in diabetic kidney [95, 104], suggesting that diabetic kidney fails to remove damaged mitochondria. Thus, restoring the ability to control mitochondria homeostasis should be a therapeutic target of diabetic nephropathy.

Mitochondrial quality control is mediated by mitochondrial autophagy (mitophagy) [105]. Similarly, oxidative stress can induce autophagy to remove damaged mitochondria to protect cells. Thus, autophagy-mediated quality control of mitochondria and subsequent reduction of ROS should be essential to protect kidney in diabetic condition. It has been reported that exogenous hydrogen peroxide activates protein kinase RNA-like ER kinase (PERK), which subsequently phosphorylates eukaryotic initiation factor-2α, activates Atg4, and inhibits mTOR [106]. In response to cellular stress or damage, mitochondrial membranes can be permeabilized. The autophagic recognition of depolarized mitochondria is mediated by a refined voltage sensor, which involves the mitochondrial kinase, phosphatase, and tensin homolog-induced putative kinase 1.

6.2. Hypoxia. In early-stage diabetic nephropathy, hypoxia is aggravated by manifestations of chronic hyperglycemic abnormalities of red blood cells [107, 108], oxidative stress [109], and diabetes mellitus-induced tubular apoptosis; as such, tubulointerstitial hypoxia in diabetes mellitus might be an important early event.

Hypoxia is also a stimulatory factor of autophagy. Hypoxia-induced autophagy largely depends on hypoxia-inducible factor-1α (HIF-1α), which is a transcription factor that is activated and stabilized under hypoxic conditions

Table 1: Autophagy-related kidney diseases.

Species and methods to monitor autophagy	Disease model	Effects of autophagy	Reference
Sprague-Dawley rats, immunohistochemistry of LC3 and Western blotting of LC3-II	Cyclosporine A-induced nephrotoxicity	Protection against tubular cell death	[80]
C57BL/6 mice, EM, and Western blotting of LC3-II	Cisplatin injury	Protection against tubular cell death	[30]
C57BL/6 mice, EM, immunofluorescence of LC3, and Western blotting of LC3-II	Aging	Protection against aging and hypoxia-related tubular damage	[26]
GFP-LC3 mice	Cisplatin injury	Protection against tubular cell death	[31]
C57BL/6 mice, EM, and Western blotting of LC3-II with 3-MA and chloroquine	Ischemia reperfusion	Protection against tubular cell death	[28]
Proximal tubular epithelial cell-specific Atg5-deficient mice	Ischemia reperfusion	Protection against tubular cell death	[29]
Podocyte-specific Atg5-deficient mice	Aging, protein overload-, LPS-, PAN-, and adriamycin-induced glomerular injury	Protection against podocyte injury	[27]

EM: electron microscopy; GFP: green fluorescent protein; 3-MA: 3-methladenine; Atg: autophagy-related genes; LPS: lipopolysaccharide; PAN: puromycin aminonucleoside.

[110, 111]. HIF-1α activates transcription of Bnip3 and Bnip3L and subsequently induces autophagy. Normally, Beclin 1 interacts with Bcl-2 proteins. Bnip3 can disrupt this interaction, liberating Beclin 1 from Bcl-2 in cells and leading to autophagy. Thus, HIF1α-induced Bnip3 overexpression promotes autophagy [112]. The transcription of Bnip3 is also upregulated by the transcription factor FOXO3, which is deacetylated and positively regulated by Sirt1 [26]. Hypoxia causes damage to the mitochondria and intracellular accumulation of ROS [113]. Removing the damaged mitochondria under hypoxic conditions is also an important role of Bnip3-mediated autophagy. Thus, to investigate whether hypoxia-induced and Sirt1-mediated autophagy is altered in diabetic kidney is interesting. If it is altered, to restore autophagy activity even under diabetic condition should be important to protect kidney form hypoxia.

6.3. ER Stress. ER stress has recently been focused as a pathogenesis of diabetic nephropathy. The induction of ER stress and subsequent apoptosis by hyperglycemia and high levels of free fatty acids (polysaturated fatty acids) are observed in podocytes [114]. Additionally, in proteinuric kidney diseases, including diabetic nephropathy, massive proteinuria filtered from glomeruli causes ER stress responses and subsequent apoptosis in renal tubular cells [14, 115]. Thus, to suppress inadequate ER stress is thought as a therapeutic strategy of diabetic nephropathy.

It is known that ER stress as well as hypoxia and ROS also cause autophagy. The ER is not only involved in protein synthesis and maturation but may also constitute a major source/scaffold for the autophagic isolation membrane [47]. When misfolded proteins are not exported efficiently to the cytoplasm and accumulate in the ER, the unfolded protein response (UPR) is often induced [116–118]. The UPR consists of three main branches that are controlled by the ER membrane proteins: PERK; activating transcription factor-6 (ATF6); inositol requiring enzyme 1 (IRE1) [116–118]. Among these UPR-related proteins, PERK and ATF6 have been reported to induce autophagy [44]. PERK induces the transcriptional activation of LC3 and Atg5 through the action of the transcription factors ATF4 and CCAAT-enhancer-binding protein homologous protein, respectively [119]. It has been suggested that IRE1 is also involved in the induction of autophagy by phosphorylation of Beclin 1 via c-Jun NH2-terminal kinase-1 [44]. Enhanced and prolonged ER stress causes several pathogenic features such as apoptosis and inflammation [117, 118]. Thus, autophagy-mediated ER degradation (ERphagy) may be required for cell protection from prolonged cytotoxic ER stress shown in diabetic kidney.

7. Autophagy in the Kidneys

The study of autophagy has previously been undertaken in lower species. The study of autophagy in mammalian systems is advancing rapidly and has revealed that mammalian autophagy is involved in the pathogenesis of various metabolic or age-related diseases [18–27].

Recently, nephrologists have also entered this exciting field of study. In this section, we review recent studies on the pathophysiology of autophagy in the kidneys. Autophagy has been observed in various parts of the kidneys, including proximal tubules, and thick ascending limbs. In particular, in podocytes, higher levels of constitutive autophagy have been

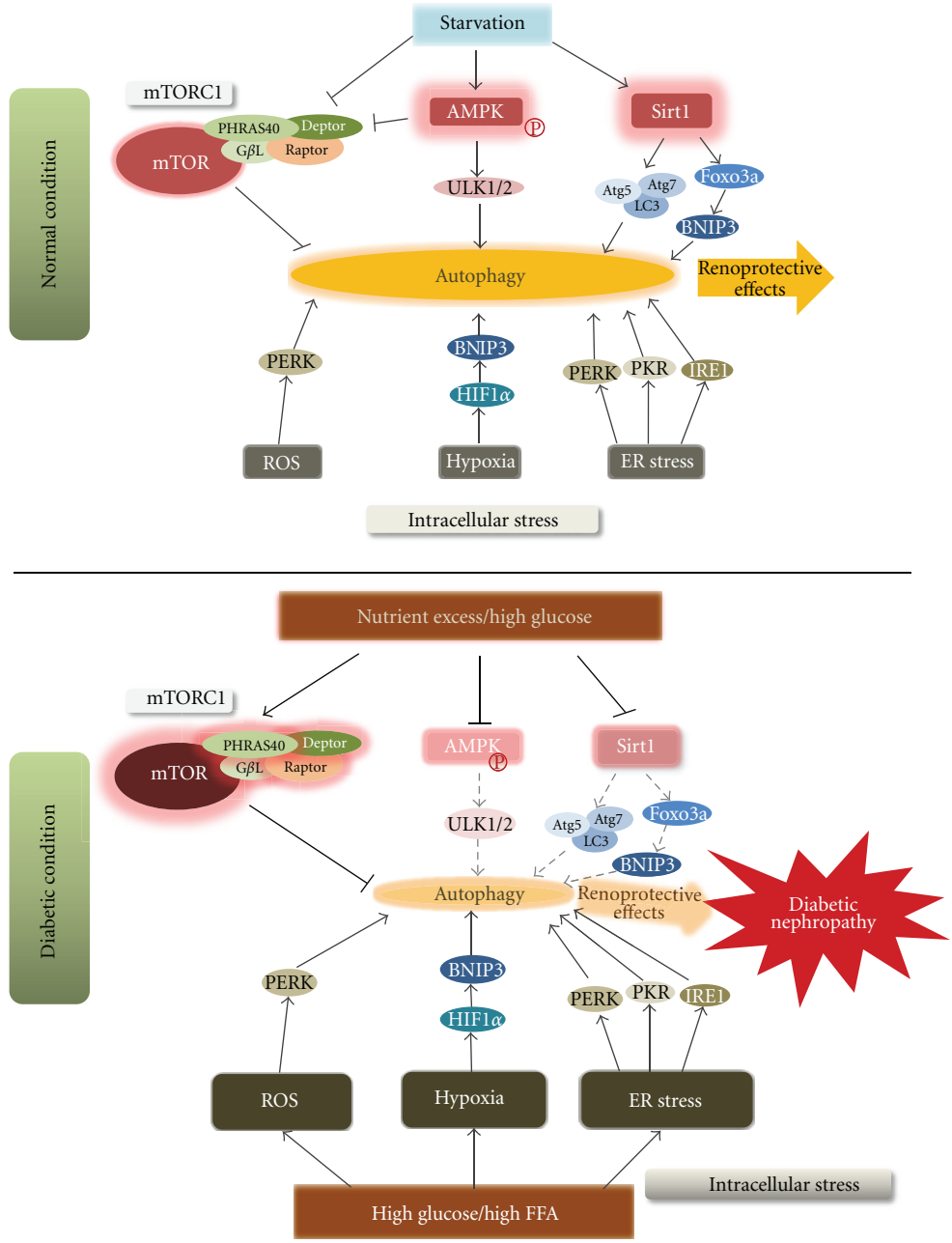

Figure 4: *Regulation of autophagy by nutrient and intracellular stresses and the relationship between autophagy and the progression of diabetic nephropathy.* Under normal conditions, intracellular stresses such as hypoxia, mitochondrial ROS, and ER stress induce autophagy. Nutrient depletion enhances autophagy by inhibiting mTORC1 and by activating AMPK and Sirt1. This activation of autophagy helps to maintain intracellular homeostasis and may have renoprotective effects. In contrast, under diabetic conditions, high glucose or FFA levels increase intracellular stresses, leading to the progression of diabetic nephropathy. Furthermore, nutrient excess and high glucose levels under diabetic conditions inhibit autophagy by inhibiting AMPK and Sirt1, and by activating mTORC1. This inactivation of autophagy may reinforce the progression of diabetic nephropathy. ROS: reactive oxygen species; ER: endoplasmic reticulum; mTORC1: mammalian target of rapamycin (mTOR) complex 1 (mTORC1); AMPK: AMP-activated protein kinase; FFA: free fatty acid.

observed using GFP-LC3 transgenic mice even under normal conditions [27]. As for the role of autophagy in renal pathophysiology, several researchers have reported the significance of autophagy in experimental renal injury models. In several experimental animal models of glomerulonephritis, including puromycin aminonucleoside and adriamycin-induced proteinuria, autophagy has been identified and shown to play renoprotective and antiproteinuric roles in podocytes through the use of podocyte-specific Atg5 knockout mice [27]. It has been recently reported that the normal aging process suppresses autophagy in podocytes, and that podocyte-specific deletion of *Atg5* leads to glomerulopathy in aging

mice that is accompanied by accumulation of oxidized and ubiquitinated proteins, ER stress, and proteinuria [27].

In renal tubules as well as in podocytes, autophagy has been reported to play a renoprotective role under several pathological conditions. In renal ischemia-reperfusion injury models, the upregulation of autophagy to protect the kidneys was observed using 3-MA, chloroquine [28], and proximal tubular epithelial cell-specific Atg5 knockout mice [29]. Additionally, in cisplatin-induced acute kidney injury models, the increase of autophagosomes was observed by EM, LC3-II Western blotting [30], and GFP-LC3 transgenic mice [31]. Hypoxia is one of the causes of renal tubular damage in aged kidney [120]. We have previously shown that hypoxia-induced autophagy activity declined with age, which led to accumulations of damaged mitochondria and mitochondrial ROS in the kidney [26]. Interestingly, long-term calorie restriction (CR) restored autophagy activity even in aged kidney [26]. As a mechanism, Sirt1-mediated autophagy was essential in CR-mediated renoprotection in aged kidney [26]. Bnip3 expression is essential to induce autophagy under hypoxic condition [121] and is positively regulated by a transcriptional factor Foxo3a [122]. This regulation was altered in aged kidney. On the other hand, CR-mediated Sirt1 activation deacetylated and activated Foxo3a transcriptional activity and subsequent Bnip3-mediated autophagy even in aged kidney [26]. Furthermore, the kidney of heterozygous Sirt1-knockout mice showed lower autophagy activity along with the decrease in Bnip3 expression, and thus they were resistant to CR-mediated antiaging effects [26]. This finding suggests that Sirt1 is essential for CR-mediated renoprotection.

Thus, accumulative evidence has demonstrated the pathophysiological importance of autophagy in the kidneys (Table 1). However, the role and existence of autophagy in other types of renal cells besides podocytes and proximal tubular cells is not known.

8. Perspective

It is evident that the above-mentioned nutrient-sensing signals exist in the kidneys. However, what are their physiological roles in this organ? The kidneys require sufficient amounts of ATP for maintenance of their functions and avidly consume oxygen to drive mitochondrial oxidative phosphorylation among major organs. A small percentage of oxygen consumed by mitochondria is incompletely reduced to ROS, and this unremitting generation of oxidants during mitochondrial respiration, albeit in small amounts, may cumulatively damage the kidneys, which are heavily dependent on mitochondrial metabolism. Regulating mitochondrial metabolism in response to nutrient conditions via regulation of autophagy that can remove damaged mitochondria and subsequent ROS may be a physiological role of renal nutrient-sensing signals.

Autophagy is regulated by nutrient conditions, and its alteration associates with various metabolic and age-associated diseases. Although studies on autophagy have methodological limitations, as outlined above, it is evident that autophagy deficiency is associated with podocyte and tubular cell injuries from the studies using Atg5-knockout mice [27, 29]. These findings lead us to hypothesize that autophagy is altered in diabetic kidneys, and autophagy deficiency should contribute to the pathogenesis of diabetic nephropathy. Altered nutrient-sensing signals in diabetic kidneys may contribute to accumulation of mitochondrial ROS via suppression of autophagy, which may be associated with initiation of the early stages of diabetic nephropathy. Both hypoxia and proteinuria-induced ER stress contribute to proximal tubular cell damage in the progressive and overt stages of diabetic nephropathy. Why do diabetic kidneys show a weakness against these stresses? How can we protect the kidneys from these stresses even under diabetic conditions? One answer may be derived from autophagy studies. Autophagy deficiency in diabetic kidneys may make tubular cells fragile under hypoxic and ER stress and possibly lead to progression of diabetic nephropathy (Figure 4). Activation of autophagy may be a therapeutic option for the advanced stages of diabetic nephropathy.

9. Concluding Comments

In recent decades, numerous investigators have been making efforts to identify the molecular mechanisms involved in the initiation and progression of diabetic nephropathy to develop new therapeutic strategies. However, end-stage renal disease due to diabetic nephropathy continues to increase worldwide. There is an urgent need to identify additional new therapeutic targets for prevention of diabetic nephropathy. We have provided a perspective on whether autophagy is involved in the pathogenesis of diabetic nephropathy and whether it is an acceptable new therapeutic target. Unfortunately, there have still not been many studies that have focused on autophagy in diabetic nephropathy. In the next few years, studies using Atg-gene knockout/knockdown mice combined with different methodologies will elucidate this possibility. Finally, these studies will ultimately give us a clearer perspective as to whether autophagy should be considered as a novel therapeutic target to halt the progression of diabetic nephropathy.

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Review Article

Guards and Culprits in the Endoplasmic Reticulum: Glucolipotoxicity and β-Cell Failure in Type II Diabetes

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The endoplasmic reticulum (ER) is a cellular organelle responsible for multiple important cellular functions including the biosynthesis and folding of newly synthesized proteins destined for secretion, such as insulin. The ER participates in all branches of metabolism, linking nutrient sensing to cellular signaling. Many pathological and physiological factors perturb ER function and induce ER stress. ER stress triggers an adaptive signaling cascade, called the unfolded protein response (UPR), to relieve the stress. The failure of the UPR to resolve ER stress leads to pathological conditions such as β-cell dysfunction and death, and type II diabetes. However, much less is known about the fine details of the control and regulation of the ER response to hyperglycemia (glucotoxicity), hyperlipidemia (lipotoxicity), and the combination of both (glucolipotoxicity). This paper considers recent insights into how the response is regulated, which may provide clues into the mechanism of ER stress-mediated β-cell dysfunction and death during the progression of glucolipotoxicity-induced type II diabetes.

1. Introduction

Type II diabetes is a heterogeneous syndrome resulting from progressive impairment of β-cell insulin secretion and insulin resistance in target tissues. Prevalence of type II diabetes, in which the body attempts to compensate for insulin resistance by augmenting insulin secretion, has increased because of the rising rate of obesity [1]. However, it is increasingly clear that pancreatic β-cell dysfunction also contributes to type II diabetes [2]. Healthy pancreatic β-cells display a dramatic response to nutrients and to obesity-associated insulin resistance through hypersecretion of insulin to maintain energy homeostasis. But, in type II diabetes, β-cells are unable to sustain a compensatory response, leading to β-cell dysfunction and death [3]. Although the cause of the metabolic deterioration is unknown, several hypotheses have been proposed, including mitochondrial dysfunction, oxidative stress, ER stress, hyperglycemia (glucotoxicity), dyslipidemia (lipotoxicity), and the combination of both (glucolipotoxicity) [4–7].

Recent studies have suggested that elevated glucose, along with circulating free fatty acids (FFAs), and particularly those originating from intra-abdominal fat stores are the major culprits in insulin resistance and β-cell dysfunction [6, 7]. The chronic hyperglycemia and hyperlipidemia cause combined, detrimental effects defined as glucolipotoxicity on β-cell function and survival [7]. However, the underlying molecular and cellular mechanisms by which glucolipotoxicity contributes to β-cell dysfunction and death in type II diabetes remain under debate. A recent observation based on experimental, clinical, and genetic evidence suggests that the endoplasmic reticulum (ER) may be responsible for the molecular mechanisms of glucolipotoxicity contributing to β-cell dysfunction in type II diabetes [8, 9]. In this paper, we discuss the involvement of ER in glucolipotoxicity-induced β-cell dysfunction and death along with the involvement of mitochondria.

2. ER Stress Response

Pancreatic β-cells display a marked response to nutrient signals through balance between the anabolic hormone insulin and the catabolic hormone glucagon, which are used

to maintain energy homeostasis. To mount an appropriate response, pancreatic β-cells require suitable sensors and signaling molecules, which integrate these signals to modulate insulin secretion to maintain homeostasis.

The ER is an integral contributor to protein synthesis, folding, maturation, trafficking, and degradation, and it may be an ideal site for nutrient sensation at the subcellular level [10]. The quality control machinery of the ER operates via specialized proteins and a specific chemical environment to ensure proper folding and processing of secretory proteins, and degradation of misfolded/unfolded proteins, including insulin, to maintain glucose homeostasis. However, overloading this machinery causes the more accumulation of misfolded/unfolded proteins in the ER and reduces the quality and quantity of ER, leading to ER stress [11]. To cope with this condition, cells activate an adaptive system linking the ER lumen with the cytoplasm and nucleus called the unfolded protein response (UPR). The UPR restores ER homeostasis, by attenuating global protein translation to decrease the protein overload and by increasing the expression of genes that induce protein folding and also promote ER-associated protein degradation (ERAD) to remove misfolded proteins [12].

The three branches of the UPR are mediated by the ER-membrane associated proteins PERK (protein kinase R-like ER kinase), IRE1α (Inositol requiring enzyme 1), and ATF6 (activating transcription factor 6). Under unstressed conditions, these three proteins are held by the abundant ER chaperone Bip/glucose-regulated protein 78 (GRP78) at the N-terminal domains of PERK and IRE1α and at the carboxyl terminal of ATF6, preventing their aggregation and rendering them inactive [13]. Under ER stress conditions, PERK is autophosphorylated and in turn phosphorylates serine 51 of eukaryotic translation initiation factor 2α (eif2α), rendering it unable to efficiently initiate translation, leading to global inhibition of protein synthesis and at the same time inducing translation of the transcription factor ATF4. ATF4 protein translocates to the nucleus and upregulates ER stress target genes, including C/EBP homologous protein (CHOP) and downstream growth arrest and DNA damage-inducible protein (GADD34) that acts as a nonenzymatic cofactor for protein phosphatase-1 (PP-1), leading to eif2α dephosphorylation for translational recovery [14, 15].

Under ER stress condition, activation of ATF6 involves the dissociation of Bip/GRP78 from its luminal domain and translocation to the Golgi for proteolytic processing where it is cleaved into its active form, which translocates into the nucleus to induce chaperon protein genes such as Bip/GRP78, GPR94, and calreticulin to enhance protein folding [16]. IRE1α, the third ER stress sensor, is a type 1 transmembrane protein with endoribonuclease activity. Similar to PERK, IRE1α is autophosphorylated in response to accumulation of misfolded/unfolded proteins in the ER. Once activated, IRE1α catalyzes the splicing of X-box-binding protein 1 (XBP-1) mRNA, leading to translation of the active transcription factor XBP-1 that induces the expression of genes required for protein folding, ER to Golgi transport, and endoplasmic-reticulum-associated protein degradation (ERAD) [17].

3. UPR under Glucolipoadaptation in β-Cells

Adaptation to metabolic changes requires regulation and coordination of many homeostatic systems, since the quality and quantity of available nutrients does not temporally match cellular needs. Acute exposure of β-cells to high glucose induces mild UPR signaling accompanied by phosphorylation and activation of IRE1α, leading to glucose-induced insulin biosynthesis [18]. Conversely, inactivation of IRE1α signaling by siRNA or inhibition of IRE1α phosphorylation hinders glucose-induced insulin biosynthesis, indicating that acute IRE1α activation is required for proinsulin biosynthesis. Surprisingly, however, acute high glucose-induced IRE1α was found not to splice the downstream target XBP-1, implying that IREα-mediated XBP-1 splicing is not essential for proinsulin biosynthesis.

Another study suggested that transient high glucose upregulates the ER resident protein oxidoreductase 1α (ERO1α), an activator of protein disulfide isomerase (PDI), which plays an important role in disulfide bond formation. Thus, ERO1α may activate insulin biosynthesis by enhancing disulfide bond formation in proinsulin in the ER [19]. Rutkowski and Kaufman suggested that eif2α phosphorylation limits proinsulin mRNA translation under low-glucose condition [10]. However, paradoxically, eif2a phosphorylation seems to be needed to upregulate proinsulin mRNA translation to compromise the uncontrolled insulin translation in response to physiologic intermittent high glucose levels [11]. Steady-state eif2α phosphorylation in glucose-induced protein translation is short, and it can be rapidly dephosphorylated by physiological stimuli via a signaling pathway that activates GADD34 and PPI [20].

In parallel with glucose, saturated and unsaturated FFAs elicit quantitatively and qualitatively different ER stress signaling in β-cells. Most investigators have employed palmitate or oleate as the fatty acids of choice because they represent the major species to which β-cells might be exposed *in vivo* [21]. However, considering the specific effects of fatty acids on β-cell viability, palmitate is more potent than oleate in triggering ER stress in clonal and primary rodent β-cells, and in human islets [22–24]. Exposure to long-chain FFAs within the physiologic range can directly stimulate insulin secretion through changes in ER calcium (Ca^{2+}) handling [25]. ER is thought to be the main dynamic intracellular Ca^{2+} storage compartment in β-cells. Palmitate causes increases in phosphorylation of the PERK branch and IRE1α-activated spliced form of XBP-1, but the effects of oleate are much less significant [26]. The marked activation of PERK-eif2α phosphorylation by palmitate leads to the induction of ATF4 and CHOP expression, which results in inhibition of protein translation [27]. Thus, FFAs differentially regulate the UPR response under physiological conditions to maintain homeostasis. Growing evidence supports the notion that early activation of UPR signaling improves β-cell homeostasis in glucolipoadaptation.

4. ER Stress under Glucolipotoxicity

Accumulating evidence suggests that glucolipotoxicity contributes to β-cell dysfunction during the development of type II diabetes. Chronic exposure of β-cells to supraphysiological levels of glucose or FFAs has been shown to be cytotoxic and causes β-cell dysfunction and failure [28–30]. Briaud et al. have provided evidence that lipotoxicity occurs in the presence of concomitantly elevated levels of glucose [31]. Several mechanisms have been proposed for glucolipotoxicity-induced β-cell dysfunction and failure, such as increased ROS, ceramide, and nitric oxide levels, and mitochondrial perturbations [32–34]. Recent evidence suggests that ER stress is linked to insulin resistance in diabetes and also expansion of ER was detected in β-cells from type II diabetic patients [35, 36]. Furthermore, increased expression of ER stress markers has been demonstrated in db/db mouse islets and β-cells of type 2 diabetes patients [23, 36]. These findings suggest that ER stress may be a pathophysiological event responsible for β-cell dysfunction and failure in type II diabetes.

Endoplasmic reticulum Ca^{2+} is an important signaling molecule in the β-cell, and fluctuations in Ca^{2+} levels in the ER can affect many functions of the endoplasmic reticulum, including protein synthesis, processing, and interchaperone interactions [37]. However, experimental data suggest that saturated and, to a lesser extent, unsaturated FFAs, trigger the ER stress response through depletion of ER Ca^{2+} stores [26, 38, 39]. Several discrepancies appear in studies of FFA-induced ER Ca^{2+} depletion, but the mechanism appears to be similar to the direct effects on sarcoplasmic-endoplasmic reticulum Ca^{2+} ATPase-2b (SERCA) pump activity [39]. ER Ca^{2+} depletion affects protein folding in the ER, because high luminal Ca^{2+} is essential for proteolytic processing and folding of proinsulin [37]. In addition, palmitate causes rapid redistribution and degradation of carboxypeptidase E (CPE) by depleting ER Ca^{2+}. CPE is a soluble membrane-bound enzyme in secretory granules involved in insulin processing. Degradation of CPE by palmitate was found to cause accumulation of unprocessed proinsulin in the secretory pathway [40]. Along with depletion of ER Ca^{2+}, palmitate also hampers ER-to-Golgi trafficking, as monitored using a temperature-sensitive vesicular stomatitis virus G protein, contributing to the accumulation of misfolded proteins and impacting ER integrity and function [41].

5. Unfolded Death Response

As described above, elevated glucose and FFA act synergistically in causing pleiotropic effects leading to β-cell decompensation and apoptosis during type II diabetes. It has been shown that palmitate induces β-cell dysfunction and apoptosis via activation of ER stress. This activates the UPR to restore normal ER function; when the UPR fails to adequately restore ER function, it turns on signaling pathways leading to apoptosis [42, 43]. The ER-localized protein Bip/GRP78 is a multifunctional chaperone and sensor of protein misfolding and controls activation of the UPR response in ER stress. Bip/GRP78 is upregulated during ER stress; overexpression of Bip/GRP78 under hyperglycemic conditions improves insulin levels and β-cell function [44]. In mouse MIN6 cells, Bip/GRP78 overexpression reduces ER stress and partially protects cells against fatty acid-induced apoptosis [23]. C/EBPbeta, a CCAAT/enhancer-binding protein (C/EBP) family basic leucine zipper (bZip) transcription factor, was found to be increased in diabetic islets and to block the induction of Bip/GRP78 due to the suppressed transactivation of ATF6α, thereby increasing the vulnerability of β-cells to ER stress [45]. However, the role of Bip/GRP78 under palmitate-induced ER stress is under debate. In BRIN-BD11 cells, palmitate exposure does not induce Bip/GRP78 [46]. These discrepancies may be dependent on cell type, and a direct comparison between studies would be required to understand the exact phenomenon that happened under these experimental conditions.

As with Bip/GRP78, the effects of palmitate on the ATF6α pathway in β-cells are also controversial. Transient transfection of INS-1 cells with HA-tagged ATF6α revealed that ATF6α protein was distributed around the nucleus and in the periphery of the cell in response to palmitate without induction of Bip/GRP78, but not in control or oleate-treated cells [47]. By contrast, Kharroubi et al. showed induction of the ATF6α-GPR78 signaling pathway by palmitate in INS-1 cells [48]. Recent evidence has also shown that missense mutations and polymorphisms within ATF6α may be linked to type II diabetes [49, 50]. Therefore, further studies are in need to delineate the exact ATF6α signaling pathways induced by palmitate in β-cell failure.

By contrast, mice with a PERK deletion develop diabetes within a few weeks of birth due to progressive β-cell loss, highlighting the importance of the PERK-mediated ER stress response in the regulation of β-cell function and survival [51]. Like other ER sensors, PERK is maintained in an inactive state by binding to Bip/GRP78. Once activated, PERK autophosphorylates and catalyzes the phosphorylation of eif2α [52, 53]. This results in a general attenuation of translation. Several studies have demonstrated that palmitate and, to a lesser extent, oleate activate rapid phosphorylation of the PERK by depletion of ER calcium leading to phosphorylation of eif2α, resulting in an overall decrease in translation, but increased translation of selected proteins including ATF3, ATF4, and CHOP [23, 39, 47, 54]. Induction of ATF4 by upstream PERK-eif2α leads to the induction of CHOP via binding of ATF4 to the C/EBP-ATF binding site in the CHOP promoter [55]. Induction of ATF3, a proapoptotic protein, by palmitate leads to β-cell apoptosis [56]. In addition, it has been shown that ATF3 downregulates the expression of IRS-2 in β-cells [57]. By contrast, knockdown of ATF3 was found to increase palmitate-induced apoptosis instead of protecting against apoptosis [56]. Recently, Zmuda et al. showed that ATF3 knockout mice fed a high-fat diet for 12 weeks had significantly reduced serum insulin levels without insulin sensitivity being affected and without β-cell apoptosis being induced [58]. So far, the downstream targets of ATF3 in β-cells are not well known and further investigation is needed to clarify these unexpected findings.

Expression of CHOP induced by palmitate induces cell death through transcriptional regulation of survival and

death effectors. CHOP was found to be localized in the nucleus as opposed to the cytoplasm in pancreatic sections from diabetic patients, suggesting that CHOP nuclear translocation is a discrete and necessary step for apoptosis induction [59]. CHOP also downregulates the expression of the antiapoptotic protein Bcl-2 and increases cellular reactive oxygen species, which likely contribute to ER stress-associated cell death [60]. CHOP also upregulates expression of ERO1α, an ER oxidase, causing hyperoxidizing conditions in the ER leading to apoptosis [61, 62].

Growing evidence shows that the IRE1α-XBP-1 pathway is activated in β-cells by palmitate treatment, but that it is less sensitive to the monounsaturated fatty acid oleate [46, 47, 54]. Oleate can efficiently counteract palmitate-induced XBP-1 mRNA splicing, and knockdown of XBP-1 was shown to potentiate oleate- but not palmitate-mediated β-cell apoptosis, suggesting differential activation of pro- and antiapoptotic signals by downstream of IRE1α [63]. In this regard, IRE1α recruits the adaptor protein TNF receptor-associated factor 2 (TRAF2) to the ER membrane, leading to activation of JNK and downstream proapoptotic signaling [64]. In rodent β-cells, the association of IRE1α and TRAF2 contributes to ER-triggered apoptosis [65, 66]. Increased levels of saturated FFAs lead to JNK activation, IRS1 and IRS2 ser/thr phosphorylation, and downregulation of insulin signaling and gene expression [67]. Furthermore, inactivation of JNK in β-cells prevents palmitate-induced inhibition of insulin gene expression [68]. In addition, blockade of palmitate-induced activation of JNK using a JNK inhibitor partially protected β-cells from the effects of palmitate [64]. However, the downstream mechanism by which JNK leads to apoptosis is not clear, and this may be mediated via caspase activation, which is described in the next section (Figure 1).

6. ER is Linked to Mitochondria to Induce Apoptosis

The ER and mitochondria are capable of modifying their structure and function in response to changing environmental challenges. These two organelles form a highly dynamic interconnected network for activating apoptosis [69]. However, the regulatory mechanisms, which determine cell status leading to cell survival or cell death in response to ER stress, have not been well established. Mitochondria have a heterogeneous shape among different cell types and their ability to effectively function is influenced by their dynamic behavior. Interestingly, mitochondrial shape and morphology are determined by two dynamically opposed processes: fusion and fission. Ablation of both fusion and fission produces a profound effect on the progression of cells to apoptosis [70]. It has been shown that the mitochondria of β-cells from Zucker diabetic rats are fragmented, suggesting an imbalance in the regulation of mitochondrial fusion and fission [71]. Under normal conditions, mitochondria in β-cells continuously undergo fusion and fission and these interactions may function to negate the detrimental effects of long-term exposure of β-cells to palmitate under high-glucose conditions, causing mitochondrial fragmentation and impairing network dynamics by abolishing fusion and fission activity. However, in the absence of palmitate, high glucose does not affect mitochondrial architecture [72].

It has been clearly demonstrated that apoptosis induction leads to fragmentation of the mitochondrial network. Moreover, inhibition of mitochondrial fission by Fis1, an outer mitochondrial membrane fission protein that determines the frequency of mitochondrial fission, reduced apoptosis in β-cells [72]. This has been questioned by another study, which ruled out the possibility that fragmentation occurs early in the cell death pathway [73]. These conflicting results may be reconciled by the fact that mitochondria are able to receive different stress signals and integrate them. How, then, do high-fat glucose conditions promote mitochondrial fragmentation and apoptosis? It is assumable that crosstalk between the ER and mitochondria may determine cellular commitment to apoptosis through Ca^{2+}. Exposure of β-cells to high-fat glucose conditions causes release of Ca^{2+} from the ER to the cytoplasm, leading to a rise in cytosolic Ca^{2+} concentration that reflects increased mitochondrial Ca^{2+} uptake. Increased mitochondrial Ca^{2+} uptake enhances local buffering capacity and release of proteins capable of apoptosis activation [74]. Subsequently, Ca^{2+} activates the phosphatase calcineurin, which dephosphorylates and inactivates dynamin-related protein 1 (Drp1), a master regulator of mitochondrial fission. Interestingly, inhibition of calcineurin activation partially prevented palmitate-induced apoptosis [75]. In addition, palmitate-induced activation of mitochondrial transition pores caused depolarization of the mitochondrial inner membrane and cytochrome c release into the cytosol, which stimulates the assembly of the apoptosome leading to activation of caspase-9, which, in turn, activates caspase-3, leading to DNA fragmentation and cell death [63, 64, 76, 77]. Moreover, mitochondrial cytochrome c translocates to the ER, where it selectively binds to InsP3R, leading to a sustained rise in cytosolic Ca^{2+} [78].

In addition, the Bcl-2 family of proteins has a role in mediating ER stress-induced apoptosis. Under normal conditions, Bcl-2 can bind and sequester BH3-only proteins, preventing these proteins from triggering oligomerization and activation of Bax and Bak [79]. Upon stimulation, Bax translocates from the cytosol to the mitochondria and oligomerizes with Bak, resulting in mitochondrial outer membrane permeabilization and release of cytochrome c into the cytosol [80, 81]. Mcl-1, a member of the anti-apoptotic Bcl-2 protein family, prevents Bax activation and translocation by sequestering factors contributing to Bax translocation [81]. Allagnat et al. recently demonstrated that, under lipotoxic conditions, Mcl-1 expression was downregulated in β-cells leading to translocation of Bax into mitochondria, cytochrome c release, and caspase-3 cleavage and apoptosis [82]. However, supporting this concept, another study has suggested the involvement of translationally controlled tumor protein (TCTP) with no sequence similarity to any other known protein. TCTP binds to antiapoptotic proteins in the Bcl-2 family, such as Mcl-1 and Bcl-XL, and antagonizes apoptosis by enhancing the antiapoptotic actions of these proteins [83]. Diraison et

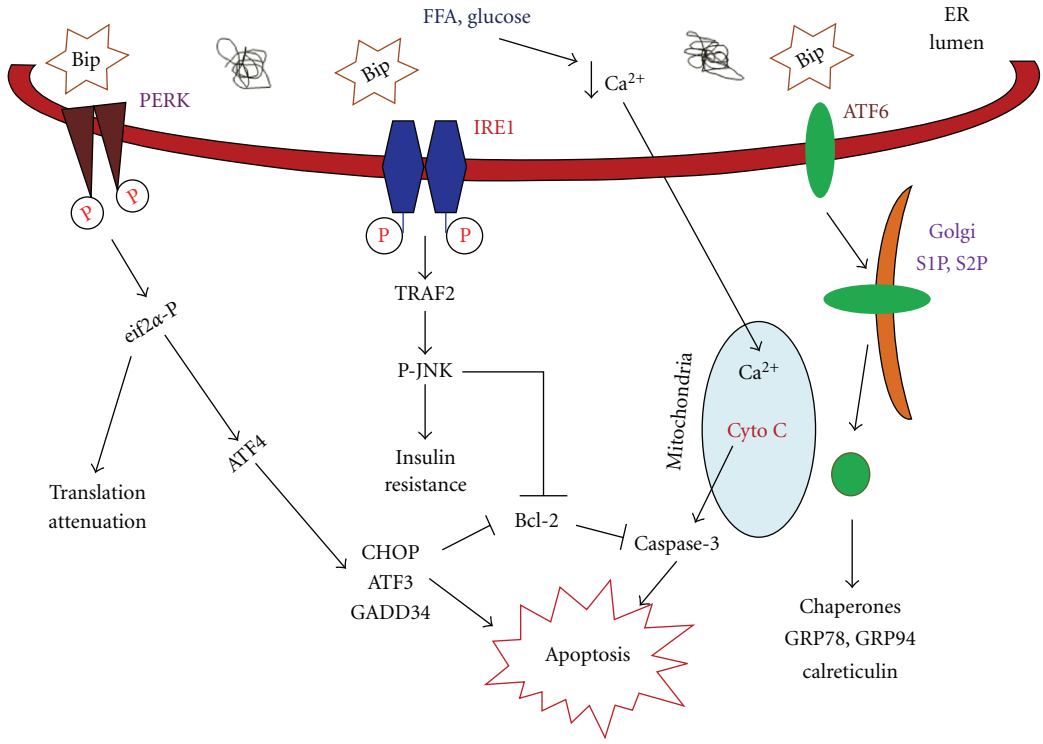

Figure 1: FFA-induced endoplasmic reticulum stress transduction and apoptosis in pancreatic β-cells, and its mechanisms; how FFA elicits β-cell apoptosis is discussed in detail in the text.

al. showed that, in β-cells, glucose regulates the expression of TCTP and its translocation from the cytosol to the nucleus by phosphorylation and glucose levels influence the sensitivity of β-cells to apoptosis. Palmitate treatment of β-cells decreased TCTP, resulting in increased β-cell death, and overexpression of TCTP prevented β-cell death. These authors also demonstrated that TCTP partially translocates to the mitochondrion in response to glucose. But it is not clear how glucose regulates TCTP translocation to various organelles and protects cells from apoptotic stimuli [84].

In addition, as described previously, FFA-induced activation of CHOP and JNK pathways decreased expression and increased the phosphorylation of Bcl-2, respectively [63, 76, 85, 86]. Phosphorylated Bcl-2 also enhances Ca^{2+} efflux from the ER and increases Ca^{2+} uptake by mitochondria [87]. Indeed, overexpression of Bcl-2 can influence the distribution of Ca^{2+} within the ER and mitochondria and can protect against apoptosis [88–90]. Together, these findings suggest the existence of a novel pathway involving the ER and mitochondria, through which these organelles orchestrate the regulation of death signals. In this complex scenario, further understanding of this link should give insights valuable for the identification of therapeutic targets to protect β-cell function and prevent type II diabetes.

7. Therapeutic Agents Targeted to the ER

ER stress has been found to be associated with obesity, insulin resistance, and type II diabetes. Therefore, agents that reduce ER stress are useful in treating obesity, peripheral insulin resistance, and hyperglycemia and type II diabetes. These agents reduce or prevent ER stress by improving the folding or processing capacity of the ER. Chemical chaperones such as PBA and TUDCA have been shown to regulate ER stress and improve insulin sensitivity *in vivo* [91]. In addition, salubrinal, a selective inhibitor of eif2α dephosphorylation, has been proposed as a novel therapy for diabetes. Salubrinal blocks eif2α dephosphorylation mediated by herpes simplex virus protein and inhibits viral replication [92]. But, in β-cells, selective inhibition of eif2α was found to potentiate lipotoxicity [27].

A growing body of evidence suggests that glucagon-like peptide 1 (GLP-1) and its analogues ameliorate experimental diabetes and preserve β-cell mass, protecting β-cells from apoptosis [93–95]. Activation of GLP-1 receptor by exendin-4 was shown to improve the survival of β-cells exposed to chemically induced ER stress via increased ATF4-CHOP expression. Interestingly, upon exposure to lipotoxic conditions, GLP-1 receptor activation prevented β-cell apoptosis by increasing cellular defense through the induction of Bip/GPR78 and the antiapoptotic protein junB [63].

Recently, in a search for novel therapeutic agents, plant-derived flavonoids were found to exhibit a broad spectrum of bioactivities. They display a remarkable array of biochemical and pharmacological actions, which may significantly affect the functions of various cellular systems [96]. Among flavonoids, methoxyflavonoids have beneficial hypolipidemic effects and suppress ER stress both *in vivo*

and *in vitro* [97, 98]. In addition, quercetin, a flavonoid found in many plants, protects β-cells from oxidative damage and preserves β-cell integrity [99, 100]. Several studies have demonstrated that the isoflavone genistein, isolated from legumes, has antidiabetic effects presumably mediated by hypolipidemic effects, thereby increasing insulin sensitivity. Administration of genistein to animal and human islets increased islet proliferation, survival, and mass, mediated by activation of cAMP/PKA/ERK1/2 phosphorylation [101–103]. To date, the beneficial activities of flavonoids have been attributed mainly to their antioxidant properties. Detailed studies are needed to reveal the mechanisms beyond antioxidant activity underlying the beneficial effects of flavonoids on the ER-mitochondrial connection in the apoptotic cascade.

8. Conclusion

It is known from experimental evidence that ER stress contributes to tissue dysfunction and damage caused by glucolipotoxicity in diabetes. However, the mechanisms underlying β-cell dysfunction and death in diabetes are complex. Understanding this complex scenario and the agents that modulate the ER stress response to β-cell resistance to lipotoxic stress could have considerable impact on the treatment of β-cell failure and type II diabetes. Thus, additional studies are required to determine the link between ER stress and β-cell failure. This may potentially lead to the development of novel therapeutics to prevent type II diabetes.

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Review Article

Endoplasmic Reticulum Stress in the β-Cell Pathogenesis of Type 2 Diabetes

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Type 2 diabetes is a complex metabolic disorder characterized by high blood glucose in the context of insulin resistance and relative insulin deficiency by β-cell failure. Even if the mechanisms underlying the pathogenesis of β-cell failure are still under investigation, recent increasing genetic, experimental, and clinical evidence indicate that hyperactivation of the unfolded protein response (UPR) to counteract metabolic stresses is closely related to β-cell dysfunction and apoptosis. Signaling pathways of the UPR are "a double-edged sword" that can promote adaptation or apoptosis depending on the nature of the ER stress condition. In this paper, we summarized our current understanding of the mechanisms and components related to ER stress in the β-cell pathogenesis of type 2 diabetes.

1. Introduction

Modern lifestyle, with overconsumption of energy-rich foods and reduced physical activity, has increased the rate of type 2 diabetes (T2D). T2D is a major cause of morbidity and mortality, decreasing both life quality and expectancy of affected individuals. Obesity is linked to insulin resistance and T2D [1]. In order to adapt to an increased metabolic load in obesity and insulin resistance, the normal pancreatic islets usually increase beta-cell mass through an increase in β-cell proliferation and neogenesis, as well as beta cell hypertrophy [2, 3] and enhancing β-cell function [4, 5]. However, failure of adaptation to the increased metabolic load results in a progressive decline in β-cell functions and cell death. As a consequence, individuals progress from normal glucose tolerance to impaired glucose tolerance and finally to established T2D [6, 7]. Accumulating evidence indicates that β-cell loss in T2D results from intertwined stress responses of gluco-/lipotoxicity, oxidative stress, and ER stress [6–14]. However, detailed molecular mechanisms underlying β-cell dysfunction and death remain to be clarified.

2. The Unfolded Protein Response in β Cells

The endoplasmic reticulum (ER) is a major subcellular compartment involved in calcium storage, lipid production, and protein biosynthesis in which a variety of extracellular signaling molecules and protein receptors critical for cellular homeostasis are properly folded, assembled, matured, and finally transported to their destination to function. These processes rely on the protein folding activity of chaperones densely populated in the ER [13]. However, folding activity can be overwhelmed with the amount of proteins imported into the ER under the instance of "ER stress", during which unfolded proteins accumulate in the ER and trigger downstream signaling pathways, which is called the unfolded protein response (UPR) [15]. The UPR is triggered by three ER stress signaling transducers—PKR-like ER kinase (PERK, EIF2AK3), inositol requiring 1α (IRE1α), and activating transcription factor 6α (ATF6α)—on the ER membrane, resulting in attenuation of protein translation and transcriptional activation of UPR genes [15]. In addition, cells activate a pathway to dispose of

misfolded proteins from the ER, termed "ER-associated degradation (ERAD)" [16]. Regulation of these processes from biosynthesis to degradation is required for protein homeostasis, and disruption in these processes can lead to terminal misfolding and/or aggregation of proteins in the ER. Then, terminally misfolded proteins which cannot be dealt by ERAD machineries need to be cleared from the ER by an additional process such as autophagy [17]. Thus, the adaptive pathways maintain cellular function and avoid apoptosis during ER stress. However, if ER stress is severe and chronic, UPR-mediated efforts to correct the protein folding defect fail, and the apoptotic pathway is preferentially activated over time [18, 19].

Increasing evidence indicates that ER stress is associated with a variety of diseases including diabetes, neurodegenerative disease, cancer, bipolar disease, liver disease, cardiac disease, muscle degeneration, autoimmune disease, and others [20, 21]. Several scientists have found evidence that T2D may be an example of an important human disease caused by ER stress [22, 23]. T2D occurs in patients who fail to compensate for insulin resistance by increasing insulin secretion. Therefore, pancreatic β-cell dysfunction and apoptosis are central to T2D pathogenesis. In this paper, we will discuss the complex ER stress responses responsible for β-cell protection as well as dysfunction and death during T2D.

2.1. Three Stress Response Pathways in the UPR. Cells have evolved an intertwined three cellular pathway termed "the unfolded protein response" to prevent accumulation of misfolded proteins in the ER lumen. ER stress such as misfolded protein accumulation is sensed by the luminal domains of three ER transmembrane proteins: PERK, IRE1α and ATF6α. Then activated stress sensors initiate the complex signaling pathways (Figure 1) [15].

2.1.1. PERK Pathway. During ER stress, PERK is dissociated from GRP78 (BiP), an abundant ER chaperone, then multimerizes and autophosphorylates [24]. Activation of PERK leads to phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which is an early response required for the attenuation of global protein translation in response to ER stress aimed to prevent further overload of the nascent polypeptides to be folded in the ER lumen [15, 25, 26]. PERK, on the other hand, induces efficient translation of several specific transcripts (such as cationic amino acid transporter 1 (cat-1), growth arrest, and DNA damage 34 (GADD34), ATF5, and ATF4) even under the condition of significant eIF2α phosphorylation [27–29]. Among them, translational increase of ATF4 induces expression of several genes involved in ER protein folding, ERAD, amino acid biosynthesis and transport function, and antioxidative stress response. Thus, translational inhibition to general mRNA transcripts but translational activation to specific mRNA transcripts by PERK is an important component of the UPR-mediated adaptation pathways to ER stress [29, 30]. Therefore, PERK activity and eIF2α phosphorylation are particularly important to maintain function of pancreatic β-cells, which continuously synthesize and secrete large amounts of insulin molecules according to physiological fluctuation of blood glucose [29, 31]. Several studies discussed below have shown that disruption of their activities increases susceptibility of β cells to death and induces β-cell failure associated with insulin resistance in T2D [23, 32, 33]. In contrast, PERK also induces expression of CHOP through transcriptional activation by ATF4 [34], an important proapoptotic gene of ER stress-mediated β-cell death [35, 36]. Therefore, it is now believed that the PERK-mediated signaling pathway may behave like a binary switch determining life and death of β cell depending on the nature of ER stress condition.

2.1.2. IRE1α Pathway. The luminal domain of PERK is functionally interchangeable in transmitting ER stress signal with IRE1α, another ER sensor, from which GRP78 is released upon exposure to ER stress [24]. IRE1α, like PERK, is autophosphorylated and dimerized by its cytoplasmic kinase domain as misfolded proteins in the ER accumulate, leading to activation of the C-terminal ribonuclease domain and specific cleavage of mRNA encoding a basic leucine zipper containing transcription factor X-box-binding protein (XBP1) [37–39]. The spliced *Xbp1* mRNA encodes a strong transcription factor (XBP1s) for many UPR genes important in protein folding, trafficking, secretion, and ER-associated degradation [40–42]. Therefore, the transcriptional function of XBP1s is important for many professional secretory cells, particularly, β cells [42, 43]. Thus, the IRE1α-XBP1 pathway contributes to restoring ER homeostasis to meet protein folding demand and protein transport [44]. Beside homeostatic function, IRE1α, under chronic ER stress, also activates proapoptotic c-Jun N-terminal kinase (JNK) signaling pathway and interacts with members of the B-cell lymphoma 2 (BCL2) family, causing cellular dysfunction and apoptosis [15, 45]. Moreover, it has been shown more recently that endonuclease activity of IRE1α cleaves ER-localized mRNAs, including proinsulin mRNA, resulting in β-cell dysfunction and apoptosis [46, 47].

2.1.3. ATF6 Pathway. The stress sensors mediating the third UPR pathway activating transcription factor α (ATF6α) and ATF6β, a structural homologue of ATF6α [48], are also associated with GRP78 and retained in the ER membrane. During ER stress, both proteins released from GRP78 traffic to the Golgi apparatus [49, 50] from which their active cytosolic fragments (p50ATF6α and p60ATF6β) are generated by S1P and S2P protease and migrate into the nucleus [51]. Although the activation mode of ATF6β during ER stress seems the same to ATF6α and biochemical studies to ATF6β suggest it has similar biological functions to ATF6α, analysis of mouse embryonic fibroblasts (MEFs) deficient in ATF6α or ATF6β revealed that ATF6α but not ATF6β is responsible for transcriptional induction of ER chaperones including GRP78 and that p50ATF6α heterodimerized with XBP1s are capable of binding both ER stress response element (ERSE) and UPR elements (UPRE) conserved in the promoters of UPR genes, resulting in significant activation of genes to restore proper ER function, protein folding,

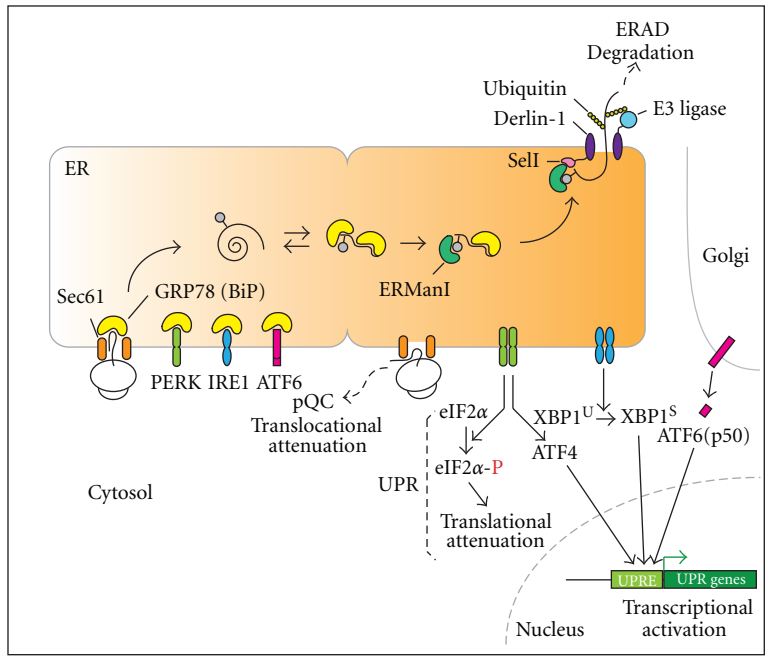

Figure 1: Activation of cellular responses during ER stress. In the resting state, newly synthesized polypeptides are cotranslationally translocated from the ribosome to the inside of the ER, in which GRP78 (BiP) plays two very important roles. First, GRP78 interacts and stabilizes polypeptides entering the ER and facilitates their proper folding, assembly, and maturation. Second, GRP78 interacts with PERK, IRE1α, and ATF6α, making them stay monomeric and functionally inactive on the membrane. However, these interactions are sensitive to protein folding status and can be easily disrupted by accumulation of misfolded proteins in the ER, resulting in activation of several pathways for protecting cells from accumulation of misfolded proteins: UPR, ERAD, and pQC. The pQC pathway is characterized by substrate-specific inhibition of protein translocation during ER stress, resulting in efficient degradation of mistranslocated proteins in the cytosol. This pathway is physiologically important in terms of controlling protein quantity in damaged ER. Following dissociation of GRP78 from ER stress sensors under ER stress, cells activate the UPR pathways to transfer signals to the nucleus and cytosol. PERK and IRE1α are autophosphorylated and modify their downstream signaling molecules, eIF2α phosphorylation, and *Xbp1* mRNA splicing, respectively. Phosphorylated eIF2α attenuates general protein translation in short. In addition, accumulation of phosphorylated eIF2α induces ATF4 expression. Together with ATF4, spliced XBP1 and cytosolic fragments of ATF6α (p50) transcriptionally activate various UPR genes involved in either adaptation or apoptosis during ER stress.

and ERAD [52, 53]. However, double knockout of ATF6α and ATF6β caused embryonic lethality whereas ATF6α-or ATF6β-deficient mice are dispensable for embryonic and postnatal development, respectively, these results suggest that ATF6α and ATF6β possess at least an overlapping function which is essential for mouse development [52, 53]. Although ATF6α-null murine model did not show pancreatic β-cell demise from functional deficiency of ATF6α [52, 53], hyperactivation of ATF6α decreases insulin gene expression via upregulation of the orphan nuclear receptor small heterodimer partner (SHP; NR0B2) which has been shown to play a role in β-cell dysfunction [54].

3. ERAD and Non-ERAD Mechanisms

The UPR involves three distinct mechanisms, namely transcriptional induction of ER-resident chaperones to facilitate protein folding, translational attenuation to decrease the demand of protein folding, and ERAD to degrade the unfolded proteins accumulated in the ER lumen. Emerging data now indicate that the function of the UPR restoring ER homeostasis is facilitated by both ERAD and non-ERAD (such as autophagy and preemptive quality control) mechanisms to remove aggregated proteins from the ER and reduce new substrates during stress. Thus, efficient removal of misfolded proteins is essential to protect cells from ER stress. Here, we will review the protein degradation pathways associated with the ER.

3.1. ER-Associated Degradation (ERAD) Pathway. Eukaryotic cells have protein quality and quantity control systems aimed to dispose of misfolded proteins from the ER [55]. Consequently, chaperones in the ER distinguish physical differences between properly folded and unfolded proteins in the ER [56, 57]. Hsp70-type (such as GRP78) and glycan-dependent chaperones (such as calreticulin and calnexin) bind unfolded proteins and contribute to maintain solubility of substrates, leading to remodeling of proteins that have incorrect conformation [57–59]. Efficient removal of misfolded proteins by the ERAD pathway seems to be very specific because it directly deals with specific substrates and, apparently, is essential to protect cells from ER stress and restore

proper ER function. Several distinct steps complete this pathway. First, although the mechanism selecting substrates is still under investigation, when cells recognize terminally misfolded proteins unable to acquire their native structures [60], ER-mannosidase I (ERManI) and mannosidase-like proteins (EDEMs) flag a target glycoprotein for degradation and activate the degradation pathway thereafter [61–63]. Second, substrates are retrotranslocated across the ER membrane via multicomplex channels, including Sec61, Derlin-1, or E3 ubiquitin ligase family members [64–66]. Third, target proteins are ubiquitylated by an E3 ligase. Finally, target proteins are then removed from the ER membrane and transported to the proteasome for degradation. Although the importance of the ERAD mechanism in pancreatic β-cells has not been studied extensively, recent studies suggest that defective protein degradation by reduction of ubiquitin carboxyl-terminal hydrolase L1 (UCH-L1) activity can compromise viability in β cells in T2D. Downregulation of UCH-L1 expression and activity in β cells induces ER stress and apoptosis [67]. In addition, E3 ubiquitin ligase HRD1 may have a protective role as an ubiquitin ligase for ATF6α [68], which inhibits hyperactivation of ATF6α in the islets of WFS1-deficient mice.

3.2. Autophagy. While ERAD controls the degradation of smaller units of unfolded and misfolded proteins, larger aggregates and long-lived proteins are detoxified via degradation in the lysosome, a process called autophagy [69]. Autophagy was originally identified as a dynamic process for degradation of cytosolic organelles [70]. Now it has also been addressed as an additional degradation pathway for proteins strongly linked to the UPR pathway [69]. For example, the phosphorylation of eIF2α is also required for the induction of autophagy [71]. Therefore, ER stress stimulates autophagy as an adaptive response to clean up terminally misfolded proteins from the ER.

3.3. Preemptive Quality Control (pQC). In addition to typical quality control pathway in mammals such as ERAD, a new degradation pathway for secretory proteins has recently been discovered. During acute ER stress, some secretory and membrane proteins are rerouted in a signal sequence-selective manner from its normal fate of being translocated into the ER to a pathway of proteasome-mediated degradation. Their cotranslational rerouting to the cytosol for degradation reduces the burden of misfolded substrates entering the ER, termed this process pre-emptive quality control (pQC) [72] For example, prion protein (PrP) is mistranslocated and rerouted to the cytosol for immediate degradation by the proteasome during ER stress. This process is largely regulated by the specific signal sequence of proteins [72, 73].

Efficient UPR pathway activated at the early stage of ER stress readily remodel misfolded proteins and restore proper ER function. As ER stress is excessive and prolonged, terminally misfolded proteins are disposed of from the ER by the ERAD pathway. At the same time, the pQC pathway reroutes misfolded proteins from the ER to the cytosol for degradation, leading to a reduction in the burden of misfolded substrates entering the ER. Therefore, when the UPR and/or ERAD pathways are compromised, the pQC pathway is apparently beneficial for cells under ER stress (Figure 1). Furthermore, to remove large aggregations, cells activate autophagy by which a large portion of aggregations can be transported directly to lysosomes for degradation without passing through the Golgi (Figure 2). However, terminally misfolded proteins often accumulate and aggregate in the ER. When the previously mentioned protein degradation mechanisms are not functionally efficient in dealing with the increasing amount of substrates, cells fail to be rescued from accumulation of misfolded proteins in the ER and cytosol. This may activate several ER stress-mediated pro-apoptotic pathways resulting in the death of stressed cells [74].

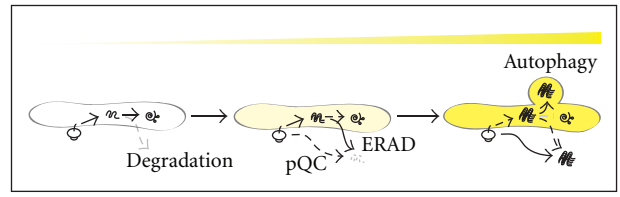

Figure 2: Potential activation mechanisms for disposing misfolded proteins from the ER. Misfolded proteins in the ER can be disposed of by serial activation of ERAD, pQC and autophagy according to the degree of protein misfolding and aggregation. In general, a small amount of protein is spontaneously misfolded and efficiently degraded in the ER even in the resting state. Under ER stress, accumulation of misfolded proteins in the ER activates pQC to reduce the burden of proteins in damaged ER as well as the ERAD pathway to dispose of misfolded proteins from the ER. However, under prolonged ER stress, ERAD does not efficiently dispose of and degrade protein aggregates from the ER, resulting in activation of an alternative way to clean them up from the ER, called "autophagy". Activation of these pathways is aimed at increasing ER capacity for protecting cells from misfolded proteins.

4. Roles of UPR-Related Genes in β Cells

4.1. PERK/eIF2α Pathway. A rare human autosomal recessive genetic disorder, the Wolcott-Rallison syndrome, is characterized by early infancy diabetes, multiple epiphyseal dysplasia, and growth retardation [75, 76]. These patients have endocrine and exocrine insufficiency and pancreatic atrophy with reduced number of β-cells [77, 78]. This syndrome was found to be associated with mutations in the *Perk* gene. In addition to that, linkage between diabetes and *Perk* gene was reported in Scandinavian families [79] and South Indian populations [80].

In addition to human studies, investigations using *PERK*$^{-/-}$ mice and mice (*Ser51Ala*) with mutation in the phosphorylation site of eIF2α showed a potential relationship between ER stress and β-cell function [29, 30, 81]. Pancreatic β cells developed normally in whole body *Perk*-null mice but showed a diabetic phenotype soon after birth mainly due to β-cell death [30, 81, 82]. In these studies, ER distention was observed in pancreatic β cells. In addition,

there was more proinsulin production in *Perk*$^{-/-}$ β cells challenged by high glucose, causing them to experience higher translational loads and higher levels of ER stress than *wild type*. These results suggested that ER overload and unresolved ER stress may cause β-cell death in those mice. Conditional deletion of *Perk* at different developmental stages showed that PERK expression in β-cells is not required postnatally in adult mice to maintain glucose homeostasis, whereas its expression is a prerequisite for fetal/neonatal β cell proliferation and differentiation [82].

Similar to *Perk*$^{-/-}$ mice, *eIF2α* homozygous mutant mice showed deficiency in pancreatic β cells in the embryonic stage, and they died within 18 hr after birth [29]. Moreover, the β cells of late embryonic stage in the mutant mice have reduced insulin contents and show severe distension of the ER [31, 33]. The results suggest that function of eIF2α phosphorylation may be required for β-cell differentiation and proliferation, along with embryonic β cells of *Perk*$^{-/-}$ mice. However, recent results showed that eIF2α phosphorylation but not PERK expression in β cells is required at the adult stage to maintain β-cell functions and glucose homeostasis. The absence of eIF2α phosphorylation in β cells caused defective intracellular trafficking of ER cargo proteins, increased oxidative damage, and reduced expression of stress response and β-cell-specific genes and apoptosis due to heightened and unregulated proinsulin translation [33]. Since Ser51Ala homozygous mutation in *eIF2α* (*A/A*) does not allow any compensatory phosphorylation by other eIF2α kinases (such as heme-regulated inhibitor kinase (HRI, EIF2AK1), general control of nitrogen metabolism kinase 2 (GCN2, EIF2AK4), and dsRNA-activated protein kinase (PKR, EIF2AK2)) [83] in adult β cells in response to physiological stimuli, whereas other eIF2α kinase in PERK-deficient adult β cells might compensate the requirements for eIF2α phosphorylation. This characteristic might contribute to phenotypic discrepancies between *Perk*$^{-/-}$ and *A/A* mice. The heterozygous mutant (S/A) mice did not spontaneously develop diabetes; however, on a 45% high fat diet, these mice showed glucose intolerance and insulin secretion defect in β cells [31]. These results suggested that translational regulation through eIF2α phosphorylation is required to maintain functional integrity of the ER. This hypothesis was further demonstrated by a study using mice with conditional A/A mutation in β cells [33]. The *A/A* mice were rescued by transgenic expression of wild-type eIF2α cDNA, which could be specifically deleted in β cells by tamoxifen-inducible Cre recombinase. As early as 3 weeks after deletion of wild-type eIF2α cDNA, β cells showed significantly distended ER and swollen mitochondria [33].

4.2. WFS1. Wolfram syndrome (WFS) is a rare autosomal recessive neurodegenerative disorder characterized by early onset diabetes, optic atrophy, and hearing impairment [84]. This syndrome is genetically linked with mutations in the *Wfs1* gene that encodes the protein wolframin [85, 86]. As in human cases, mice deficient in the *Wfs1* gene developed glucose intolerance and overt diabetes due to insufficient insulin secretion. Pancreatic β cells in mutant mice experienced ER stress shown by phosphorylation of eIF2α and spliced form of XBP1 [87–89]. Recent study suggests that WFS1 may affect maturation of plasma membrane proteins or stability of ER membrane proteins. Deficiency of WFS1 in β cells destabilizes Na^+/K^+ ATPase β1 subunit and E3 ubiquitin ligase HRD1 [68, 90]. Thus, wolframin may be involved in the ER folding and assembly of subunits of oligomeric proteins. In addition, Wolframin may suppress ATF6α hyperactivation in β cells by stabilizing HRD1, which brings ATF6α to the proteasome. Therefore, WFS may have a role as a negative regulator of chronic or unresolvable ER stress.

4.3. P58^{IPK}. Although p58$^{\text{IPK}}$ (IPK, inhibitor of protein kinase) was first known to be an inhibitor of the PKR, its function has been shown to inhibit another eIF2α kinase, PERK. However, recent evidence suggests that p58$^{\text{IPK}}$ serves as a cochaperone in the ER lumen for the Hsp70 family member BiP [91]. Mice lacking the p58$^{\text{IPK}}$ gene showed gradual onset of glucosuria and hyperglycemia mainly due to apoptosis of pancreatic β cells [92]. In addition, *p58^{IPK}* deletion in *Akita* mice (carrying a C96Y mutation in the *Ins2* gene) exacerbate the diabetic phenotype [93]. These results indicate that chaperoning ability in the ER is important to preserve ER function in β cells.

4.4. ATF6α. ATF6α was also found to be associated with β-cell function in genetic studies. In a study of Pima Indians, a native American population with a high prevalence of type II diabetes, [94] they found an association of variants in ATF6 with T2D [95]. In another study conducted in a Dutch cohort, they also found that the majority of single nucleotide polymorphisms (SNPs) in *Aft6* allele were significantly associated with impaired fasting glucose, impaired glucose tolerance, and T2D [96]. Furthermore, associated variants differed from those identified in the Pima Indians. Since ATF6α is important for protective cell response to accumulation of unfolded and misfolded proteins in the ER, disturbances of this process might contribute to β-cell apoptosis. However, there is no direct evidence of pancreatic β-cell demise in the ATF6α null murine model.

5. Causes of ER Stress in β Cell

Increasing evidence indicates that ER stress is one of the main causes of β-cell dysfunction and death [6–14]. However, it is not clear what is the main cause of ER stress in β cells, and which subpathway of UPR is responsible for this process. In this section, we will describe the potential sources and mechanisms for ER stress-mediated β-cell demise.

5.1. Lipotoxicity-Mediated ER Stress. Hyperlipidemia (elevated serum lipid levels) also results from sustained insulin resistance. It is thought that chronically elevated levels of circulating free fatty acids (FFAs) are putative mediators of progressive β-cell dysfunction and death in T2D [97]. When FFA levels are elevated twofold above the basal upon lipid infusion, obese nondiabetic individuals showed

a significant reduction in glucose-stimulated insulin release [98]. It has recently been shown that a saturated long-chain FFA palmitate induces ER stress in both clonal and primary murine and human β cells, whereas unsaturated long-chain FFAs do or do not induce ER stress to a lesser content [10, 11, 99, 100]. Palmitate preferentially activates both PERK and IRE1α pathways [101–103]. However, it is uncertain whether palmitate can specifically activate the ATF6α branch because both palmitate and oleate induce total *Xbp1* mRNA, and the expression of the known ATF6α target gene *Hspa5* (*Grp78*) is controversial [102, 104]. How saturated FFAs activate the unfolded protein response has not been answered. Several recent studies indicate that palmitate triggers ER stress in β cells through perturbation in ER Ca^{2+} handling. Calcium-specific dye assays revealed palmitate depletes ER Ca^{2+} and slows ER Ca^{2+} uptake in β cells [104]. Although palmitate-mediated ER Ca^{2+} depletion increased misfolded protein accumulation as the mechanism of the sarco-/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors thapsigargin and cyclopiazonic acid, two commonly known synthetic ER stressors, the detailed molecular mechanism of ER Ca^{2+} depletion by palmitate is not well known. Other reports indicated palmitate rapidly increases the saturated lipid content of the ER, leading to compromised ER morphology and integrity and thereby may directly or indirectly induce ER stress [100, 105]. For example, impairment of lipid content control of the ER by palmitate hampers ER-to-Golgi protein trafficking of vesicular stomatitis virus G protein (VSVG), contributing to the unfolded protein response through accumulation of misfolded proteins in the ER lumen [106]. Interestingly, increased fatty acid desaturation by stearoyl coenzyme A desaturase 1 (SCD1) reduced palmitate-induced cell death in MIN6 β cells and human embryonic kidney (HEK) cells [107]. In contrast, knockdown of SCD in INS-1 β cells decreased desaturation of palmitate to monounsaturated fatty acid (MUFA), lowered fatty acid partitioning into complex neutral lipids, and augmented palmitate-induced ER stress and apoptosis [108]. Furthermore, the importance of lipid content control of the ER by SCD in β cells was further manifested by studies of diabetic murine models. First, loss of SCD1 worsens diabetes in leptin-deficient obese mice [109]. Second, SCD1 and SCD2 mRNA expression were shown to be induced in islets from prediabetic hyperinsulinemic Zucker diabetic fatty (ZDF) rats, whereas several fatty acid desaturases including SCD1 mRNA levels were markedly reduced in diabetic ZDF rat islets [108].

5.2. Misfolded Protein-Mediated ER Stress. Since proinsulin represents up to 20% of the total mRNA and 30–50% of the total protein synthesis in the β cell [110–112], misfolded mutant insulin proteins might be a potent cause of ER stress. The Akita mutant in both mouse and human, which carries a cysteine 96 to tyrosine substitution in the *Ins2* gene, showed hyperglycemia and a reduced β cell mass [35]. This missense mutation disrupts a disulfide bond formation of mature insulin causing incorrect folding of proinsulin in the ER. Accumulation of the mutant insulin induced cell death mainly through ER stress evidenced by upregulated expression of ER stress marker genes such as BiP, spliced XBP1s, activated ATF6α, and CHOP in the Akita mutant islets and β-cell lines [113–115]. In heterozygous, but not homozygous, Akita's mutant mice, the homozygous disruption of CHOP delayed diabetes development suggested that β-cell death is partially CHOP dependent [93].

Similar to Akita diabetes due to accumulation of misfolded proinsulin, ER accumulation of islet amyloid polypeptide (IAPP, amylin) oligomers may contribute to β-cell loss in T2D [12]. Human IAPP is an 89 amino acid protein that undergoes processing to a 37-amino acid amyloidogenic peptide coexpressed and cosecreted with insulin by β cells. A study showed that islet amyloid is present at autopsy in over than 90% of patients with T2D [8]. Moreover, increased expression of ER stress marker genes such as BiP and CHOP was observed in islets of human T2D patients [22]. The IAPP-driven ER stress theory was exemplified by animal studies overexpressing human IAPP in β cells. Human IAPP but not murine IAPP forms toxic oligomers and triggers ER stress-induced apoptosis in β cells of both rat and mouse murine models [12]. Therefore, intracellular deposit of human IAPP toxic oligomer could be a link between ER stress and β-cell death in human T2D.

5.3. High Glucose-Mediated ER Stress. Insulin resistance in T2D causes blood glucose levels to remain high [116]. In a high blood glucose state, called hyperglycemia, the β-cell increases its metabolic activity, which eventually leads to cellular stress. This in turn may further impair β-cell function and survival, a process called glucotoxicity [117]. It has been shown that glucotoxicity is mediated at least in part by excess generation of reactive oxygen species (ROS) [117, 118]. When excess glucose is available to the β cell, excessive ROS can be generated in β cells by several biochemical pathways including mitochondrial oxidative phosphorylation, ER oxidative folding pathway, and other alternative metabolism pathways; overflowed glucose is shunted (such as glucosamine and hexosamine metabolism and sorbitol metabolism) [117, 118]. Elevated ROS perturbs insulin synthesis and secretion by decreasing the expression and activity of key transcription factors such as PDX-1 and MafA, which regulate proinsulin genes and other multiple genes involved in β-cell differentiation, proliferation, and survival [119].

Accumulating evidence suggests that protein folding in the ER and production of ROS are closely linked events [120]. In several reports, prolonged UPR activation leads to the accumulation of ROS via two sources: the UPR-regulated oxidative protein folding machinery in the ER and oxidative phosphorylation in mitochondria [118, 121]. In the ER lumen, oxidative protein folding is conducted by protein disulfide isomerase (PDI) and a family of ER oxidoreductases 1 (ERO1) that catalyze disulfide bond formation in folding proteins. In this reaction, an oxidant flavin adenine dinucleotide (FAD)-bound ERO1 oxidizes PDI, which then subsequently oxidizes folding proteins directly. FAD-bound ERO1 then passes two electrons to molecular oxygen, perhaps resulting in the production of ROS such as hydrogen peroxide or peroxide [120]. Overexpression

of a misfolded protein CPY (yeast vacuolar protein carboxypeptidase Y) activates the UPR, causes oxidative stress, and induces apoptosis. However, removal of all cysteine residues in CPY reduced oxidative stress and cell death [122]. Therefore, oxidative protein folding in the ER can be a source of ROS generation. It is known that ER stress increase leakage of Ca^{2+} from the ER lumen through mainly the inositol-1,4,5-trisphosphate receptor (IP_3R) [21]. Recent studies suggest that ER Ca^{2+} leakage may occur by oxidation-induced activation of the Ca^{2+} release channel IP_3R during ER stress and oxidative stress [123]. Increases in cytosolic Ca^{2+} can stimulate mitochondrial ROS production through multiple mechanisms [124]. Introduced by Ca^{2+} uniporter or mitochondrial ryanodine receptor, Ca^{2+} stimulates the TCA cycle and nitric oxide synthase (NOS), which subsequently generates nitric oxide. Nitric oxide and Ca^{2+} inhibits respiration complex I, III, or IV, which enhance ROS generation. High levels of ROS generation within the mitochondria then further increase Ca^{2+} release from the ER. In turn, Ca^{2+} also dissociates cytochrome *c* from the inner membrane cardiolipin, which triggers permeability transition pore (PTP) opening and cytochrome *c* release across the outer membrane. Now the vicious cycle of ER Ca^{2+} release and mitochondrial ROS production activates cytochrome-*c*-mediated apoptosis. The ER of diabetic pancreatic β cells synthesizing great quantities of proinsulin to maintain normoglycemia can be an important site of ROS production because correct folding of proinsulin absolutely depends on the formation of disulfide bond by oxidative protein folding. This theory is partially supported by recent reports that PERK and eIF2α phosphorylation-deficient β cells having reduced UPR responses showed increased proinsulin synthesis due to losing of translational control; thereby, large accumulation of proinsulin in the ER attributed to accumulated ROS in β cells possibly by both ER oxidative protein folding machinery and Ca^{2+} mediated-mitochondria activation [33, 82]. Thus, excess ROS and the mitochondrial cell death pathway may induce β-cell death. Therefore, the finding that increased proinsulin synthesis causes oxidative damage in β cells may reflect events in β-cell failure associated with insulin resistance in T2D [74].

Under high glucose conditions, it is possible that increased proinsulin biosynthesis may overwhelm the ER protein folding capacity leading to UPR activation. Chronic exposure (more than 24 hrs) of β cells to high-glucose caused hyperactivation of IRE1α showing *Xbp1* mRNA splicing, whereas acute expose (1–3 hrs) to high glucose activated IRE1α without *Xbp1* mRNA splicing [47, 118, 125]. In the chronic high-glucose state, activated IRE1α degrades proinsulin mRNA contributing to the reduction of proinsulin biosynthesis [47, 125]. Moreover, recent reports suggest that IRE1α's RNase causes endonucleolytic decay of many ER's localized mRNAs, including those encoding chaperones, thereby culminating in cellular dysfunction and death of several mammalian cells including β cells [46, 47, 125]. However, the studies also revealed that activated IRE1α at the physiological level may have a beneficial effect aiding in the enhancement of proinsulin biosynthesis in pancreatic β cells with the induction of a subset of downstream genes of IRE1α [47]. Thus, depending on the forms of ER stress, β cells may generate binary signaling of life and death through IRE1α.

It is well established that high blood sugar amplifies FFA-mediated toxicity in β cells [117, 118]. Although why glucose exacerbates β-cell lipotoxicity is not well known, a recent report suggests that chronic hyperglycemia may amplify fatty acid-induced ER stress in β cells [101]. The study showed that high glucose amplifies palmitate-mediated stimulation of the IRE1α and PERK pathways. Glucose stimulates the mammalian target of rapamycin complex 1 (mTORC1), an important nutrient sensor involved in the regulation of cellular stress, and the activated mTORC1 mediates amplification of fatty acid's lipotoxicity by increasing IRE1α protein levels and activating the JNK pathway, leading to increased β-cell apoptosis.

6. Concluding Remarks

Increasing evidence indicates that hyperactivation of the UPR indispensible for ER homeostasis has a role in β cell dysfunction and death during the progression of T2D and genetic forms of diabetes. Therefore, it is currently believed that ER stress-related diseases including T2D occur from adaptation to apoptosis of stressed cells. The complete understanding of this molecular mechanism responsible for life and death will shed light on future T2D prevention and treatment.

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Review Article

Interaction between Mitochondria and the Endoplasmic Reticulum: Implications for the Pathogenesis of Type 2 Diabetes Mellitus

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Mitochondrial dysfunction and endoplasmic reticulum (ER) stress are closely associated with β-cell dysfunction and peripheral insulin resistance. Thus, each of these factors contributes to the development of type 2 diabetes mellitus (DM). The accumulated evidence reveals structural and functional communications between mitochondria and the ER. It is now well established that ER stress causes apoptotic cell death by disturbing mitochondrial Ca^{2+} homeostasis. In addition, recent studies have shown that mitochondrial dysfunction causes ER stress. In this paper, we summarize the roles that mitochondrial dysfunction and ER stress play in the pathogenesis of type 2 DM. Structural and functional communications between mitochondria and the ER are also discussed. Finally, we focus on recent findings supporting the hypothesis that mitochondrial dysfunction and the subsequent induction of ER stress play important roles in the pathogenesis of type 2 DM.

1. Introduction

Type 2 diabetes mellitus (DM) is characterized by impaired insulin secretion from pancreatic β-cells. In addition, insulin-responsive tissues, such as muscle, liver, and adipose tissue, exhibit insulin resistance. A number of findings suggest that both of these major features of type 2 DM are associated with mitochondrial dysfunction and/or endoplasmic reticulum (ER) stress [1–4]. Recently, it was shown that mitochondria and the ER interact both physically and functionally [5, 6]. In this paper, we will focus on the roles that mitochondrial dysfunction and ER stress play in the pathogenesis of type 2 DM. Particular emphasis will be placed on recent findings elucidating the interaction between mitochondria and the ER.

2. Role of Mitochondrial Dysfunction in Type 2 DM

2.1. Mitochondria. The mitochondrion is an intracellular double-membraned organelle found in most eukaryotic cells [7]. Mitochondria are well known to be power stations within cells, as one of their major functions is production of ATP [8]. In addition, mitochondria play essential roles in intracellular reactive oxygen species (ROS) production [9], regulation of apoptosis [10], and Ca^{2+} storage [11].

2.2. Mitochondrial and Pancreatic β-Cell Dysfunction. Insulin-resistant patients can develop overt type 2 DM when pancreatic β-cells are unable to produce enough insulin to maintain normoglycemia. Pancreatic β-cells from patients with type 2 DM cannot sense glucose properly, and this contributes to impairment of insulin secretion. Interestingly, glucose sensing by β-cells appears to be controlled by mitochondrial metabolism. Reduced forms of nicotinamide adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$) are generated during glucose metabolism via both glycolysis and the tricarboxylic acid (TCA) cycle. Electron transfer to the mitochondrial electron-transport chain (ETC) by NADH and $FADH_2$ leads to production of ATP via the process of oxidative phosphorylation (OXPHOS). Increases in the ATP/ADP ratio in β-cells inhibit ATP-sensitive potassium channels (K_{ATP}), in turn inducing depolarization of

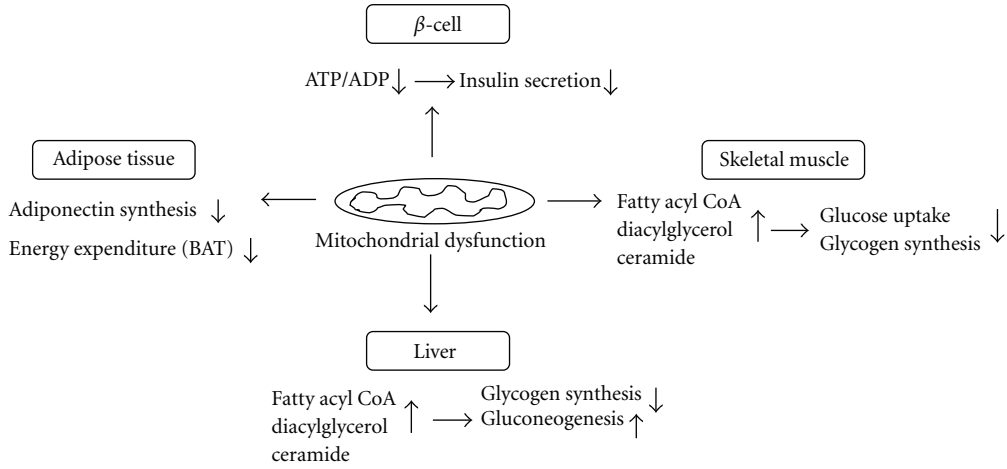

Figure 1: Roles of mitochondrial dysfunction in the pathogenesis of β-cell dysfunction and insulin resistance.

plasma membranes. The opening of voltage-sensitive Ca^{2+} channels allows Ca^{2+} uptake by β-cells, thereby contributing to secretion of insulin. Thus, mitochondrial dysfunction can impair glucose-stimulated insulin secretion by reducing the ATP/ADP ratio within β-cells (Figure 1) [12].

2.3. Mitochondrial Dysfunction and Skeletal Muscle Insulin Resistance. Defective mitochondrial fatty acid metabolism in skeletal muscle is thought to affect insulin signaling pathways, thereby leading to insulin resistance [13–15]. Impairment of mitochondrial fatty acid β-oxidation, either alone or in conjunction with increased delivery of free fatty acids (FFAs) from plasma, leads to elevated levels of intracellular fatty acid metabolites such as fatty acyl CoA, diacylglycerol, and ceramide [16–18]. Metabolites formed under such circumstances activate serine/threonine kinases including protein kinase C (PKC), leading to phosphorylation of serine sites on insulin receptor substrate-1 (IRS-1) [19, 20]. Increased serine phosphorylation of IRS-1 inhibits the tyrosine kinase activity of the insulin receptor on IRS-1 and the activity of insulin-stimulated phosphatidylinositol 3-kinase (PI 3-kinase), resulting in decreased activity of insulin-stimulated protein kinase B (PKB, also known as AKT). Reduced AKT activity leads to suppression of insulin-stimulated glucose transporter 4 (GLUT4) translocation and subsequent reduction of glycogen synthesis (Figure 1).

2.4. Mitochondrial Dysfunction and Hepatic Insulin Resistance. The liver plays a crucial role in the development of insulin resistance and type 2 DM [21]. Several lines of evidence indicate that defects in liver mitochondrial oxidative function can induce hepatic insulin resistance [14, 15, 22, 23]. For example, reduced levels of mitochondrial fatty acid β-oxidation in the liver, as in skeletal muscle, lead to accumulation of intracellular fatty acid metabolites [24, 25]. Note that similar results were observed either when de novo hepatic lipogenesis rose or when delivery of FFAs from the plasma increased. Under either circumstance, the metabolites adversely affected intracellular insulin signaling, leading to reduced insulin stimulation of glycogen synthesis and increased hepatic gluconeogenesis (Figure 1) [19].

2.5. Mitochondrial Dysfunction and Adipose Tissue. Adipose tissue has been described as an endocrine organ that plays a central role in fuel metabolism [26]. Adipocytokines such as leptin, adiponectin, resistin, and tumor necrosis factor-α (TNF-α) are released by adipose tissue, and these cytokines regulate fuel metabolism [27]. Adiponectin is known to have insulin-sensitizing effects. However, in contrast to other adipocytokines, the plasma levels of adiponectin are significantly decreased in obese subjects and in type 2 DM patients [28, 29]. Recently, we reported that the levels of adiponectin in plasma and adipose tissue were significantly lowered in obese mice; an associated reduction of mitochondrial content and function in adipose tissue was also documented [30]. Rosiglitazone, a peroxisome proliferator-activated receptor γ (PPARγ) agonist, reversed decreases in plasma adiponectin levels and adiponectin expression in obese mice, and elevated mitochondrial content and function in adipose tissue. These findings suggest that mitochondrial dysfunction in adipose tissue leads to decreased plasma adiponectin levels in obese subjects (Figure 1).

Many studies on rodents have shown that the capacity of mitochondria for oxidizing fatty acids in brown adipose tissue (BAT) plays a critical role in the regulation of adaptive thermogenesis, energy balance, and body weight [31, 32]. The presence of BAT was considered to be relevant only in human newborn and small mammals. However, recent studies using positron-emission tomography and computed tomography (PET-CT) demonstrated that adult humans possess active BAT [33, 34]. Thus, mitochondrial dysfunction in BAT appears to be linked to impaired thermogenesis and energy expenditure, contributing to the development of obesity and insulin resistance in adult humans (Figure 1) [35].

3. Role of ER Stress in Type 2 DM

3.1. ER. The ER is a complex organelle that is found in all eukaryotic cells. Structurally, the ER is formed by an interconnected network of cisternae and microtubules. From a functional viewpoint, the ER plays a central role in protein folding and in quality control of newly synthesized proteins [36]. The ER also serves as an essential site for synthesis of lipids [37] and for high-capacity buffering of intracellular Ca^{2+} [38].

3.2. ER Stress. If proteins are to be folded properly within the ER, a balance must be struck between the ER protein load and ER folding capacity. A number of conditions can disrupt ER homeostasis, leading to accumulation of misfolded proteins within the lumen of the ER [4, 39]. Such conditions include a large biosynthetic load, defects in folding machinery, and disturbances in the handling of Ca^{2+}. Accumulation of misfolded proteins in the ER causes ER stress, and this activates an elaborative adaptive process termed the unfolded protein response (UPR) [40].

The UPR is triggered by three ER transmembrane proteins: protein kinase R-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6). In unstressed conditions, ER luminal domain of these proteins are bound by the chaperone Bip, maintaining them in an inactive state until ER stress is present [41]. During ER stress, misfolded proteins sequester, Bip, leading to free PERK and IRE1 monomers to oligomerize and trans-autophosphorylate. Activated PERK mediates inhibition of protein translation via phosphorylation of eukaryotic translation initiation factor 2α (eIF2α), resulting in reduced global protein synthesis in an attempt to decrease the protein-folding load in the ER lumen [42]. PERK-mediated eIF2α phosphorylation also contributes to the activation of a subset of translational targets including activating transcription factor 4 (ATF4). ATF4 activates transcriptionally the proapoptotic transcription factor CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) [43].

Activation of IRE1, which has endoribonuclease activity, leads to splicing of X-box binding protein-1 (XBP1) mRNA and translation of the active form (XBP1s) [44]. XBP1s translocates to the nucleus and regulates expression of ER chaperones and proteins involved in ER-associated degradation (ERAD) [45]. In addition, the cytosolic domain of IRE1 can associate with TNF receptor-associated factor 2 (TRAF2) to activate the apoptosis signal-regulating kinase 1 (ASK1) and c-Jun N-terminal kinase (JNK) pathway, independently with its endoribonuclease activity [46, 47].

In response to ER stress, ATF6, released from Bip, translocates to the Golgi where it is cleaved by proteases into an active amino-terminal form [48]. N-terminal ATF6 in turn moves to the nucleus to stimulate expression of ER chaperones and proteins involved in ERAD.

3.3. ER Stress and β-Cell Dysfunction. ER stress plays an important role in the pathogenesis of type 2 DM, as such stress contributes to pancreatic β-cell dysfunction and insulin resistance [4, 49]. When the demand for insulin overwhelms the folding capacity of the ER, the UPR becomes chronically activated. Several stimuli have been shown to cause sustained accumulation of misfolded proteins within the ER lumen of β-cells [4]. These include high levels of FFA (caused by either a high-fat diet or obesity) and glucose (chronic hyperglycemia), as well as aggregation of islet amyloid polypeptide. Accumulation of misfolded proteins triggers chronic activation of the UPR, inducing β-cell dysfunction and apoptosis [50, 51].

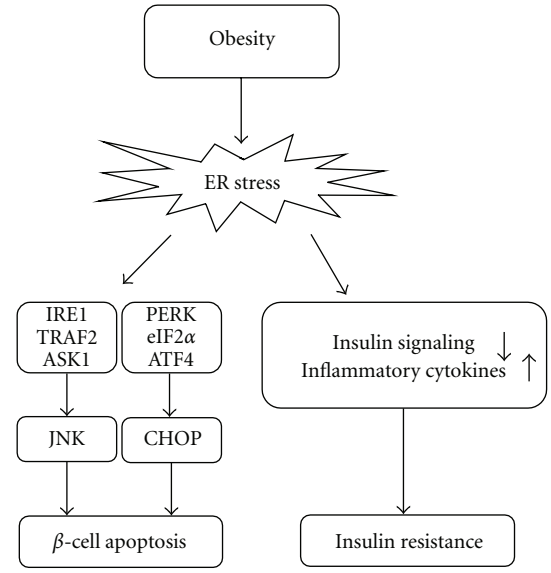

Figure 2: Roles of ER stress in the pathogenesis of β-cell apoptosis and insulin resistance.

Several components of the UPR that contribute to β-cell apoptosis have been shown (Figure 2). ER stress can induce β-cell apoptosis through prolonged activation of IRE1-TRAF2-ASK1 cascade and JNK pathway [52]. CHOP also plays a crucial role in the induction of ER stress-mediated β-cell apoptosis [53].

3.4. ER Stress and Insulin Resistance. In addition to β-cell dysfunction, ER stress is involved in peripheral insulin resistance (Figure 2). Obesity results in chronic stimulation of ER stress, leading to continuous activation of the UPR. Recent studies have suggested that this may, in fact, be the main mechanism of peripheral insulin resistance and type 2 DM [3, 54]. In obese mice, the levels of ER stress markers are increased in the liver and adipose tissue [3]. Obesity-induced ER stress inhibits insulin signaling, and this leads to insulin resistance. ER stress can also activate nuclear factor-κB (NF-κB) signaling in the liver [55], thereby increasing production of proinflammatory cytokines and causing development of insulin resistance [56]. A recent study showed that treatment of obese diabetic mice with the chemical chaperones 4-phenyl butyric acid (PBA) and taurine-conjugated ursodeoxycholic acid (TUDCA) improved peripheral insulin sensitivity by alleviating ER stress [57]. TUDCA therapy also improved insulin sensitivity in the liver and muscle of obese subjects [58].

4. Structural Communication between Mitochondria and the ER

A number of studies have shown structural communication between the mitochondria and the ER. The evidence includes cosedimentation of ER particles with mitochondria, as well as electron microscopic observation of a close physical apposition between mitochondria and the ER [59, 60]. More recently, high-resolution three-dimensional images have been obtained showing an interaction between mitochondria and the ER; specific color labels were employed to this end [61]. A recent study using electron tomography also demonstrated that the outer mitochondrial membrane (OMM) and the ER are joined by tethers, enabling ER proteins to associate directly with proteins and lipids of the OMM [62].

The structural membrane hat bridges between mitochondria and the ER is known as the mitochondria-associated membrane (MAM) [63]. The MAM plays an essential role in several cellular functions, including lipid transport, Ca^{2+} signaling, and apoptosis [64]. A number of mitochondrial or ER-bound proteins are important for maintaining structural communication between the two organelles at the MAM [64, 65]. In particular, communication between the organelles is modulated by a family of chaperone proteins. The voltage-dependent anion channel (VDAC) is physically linked to the inositol 1,4,5-triphosphate receptor (IP_3R) via the molecular chaperone grp75 [66]. Overexpression of the cytosolic form of grp75 selectively increases IP_3-induced Ca^{2+} uptake into the mitochondrial matrix, whereas overexpression of the mitochondrial form of the protein does not have this effect. Another protein that modulates interaction between mitochondria and the ER is phosphofurin acidic cluster sorting protein 2 (PACS-2), which is known to integrate ER-mitochondrial communication and apoptosis signaling [67]. Accordingly, PACS-2 depletion induces mitochondrial fragmentation, dissociates the ER from mitochondria, and blocks apoptosis signaling. More recently, Sigma-1 receptors have been shown to be located at the MAM of the ER, where they form complexes with Bip [68]. Sigma-1 receptors dissociate from Bip and bind to type-3 IP_3Rs under conditions of ER Ca^{2+} depletion. Thus, type-3 IP3Rs are not degraded by proteasomes. Ca^{2+} depletion appears to induce a prolonged Ca^{2+} signaling event from the ER to the mitochondria, via IP_3Rs. Together, the data suggest that Sigma-1 receptors are involved in maintaining normal Ca^{2+} signaling from the ER to mitochondria.

Structural communication between mitochondria and the ER is also modulated by fission and fusion of mitochondria. Fission and fusion are regulated by a family of mitochondrion-shaping proteins including dynamin-related protein 1 (DRP1), mitofusin 1, and mitofusin 2 [69]. Mitofusin-2 is a mitochondrial transmembrane GTPase that regulates mitochondrial fusion [70], and this protein is enriched at MAMs [71]. Mitofusin-2 tethers the ER to mitochondria via formation of both homotypic and heterotypic complexes. For example, ER mitofusin-2 interacts with either mitofusin-2 or mitofusin-1 on mitochondria. The tethering effect of mitofusin-2 appears to play a role in the control of Ca^{2+} flow between mitochondria and the ER [71].

5. Functional Communication between Mitochondria and the ER

5.1. Role of ER Stress in Induction of Mitochondrial Dysfunction. Mitochondrial dysfunction and ER stress have each been recognized to play crucial roles in the pathogenesis of type 2 DM. However, the individual stressors appear to act sequentially in various tissues. For example, accumulating evidence has shown that ER stress induces mitochondrial dysfunction, thereby leading to disruption of various physiological responses within cells [5, 6].

Interactions between mitochondria and the ER facilitate control of Ca^{2+} signaling and Ca^{2+}-dependent cellular processes such as apoptosis [72, 73]. Prolonged ER stress leads to release of Ca^{2+} from the ER lumen at the MAM. In contrast, such stress leads to increased Ca^{2+} uptake into the mitochondrial matrix. Elevated Ca^{2+} uptake induces an imbalance between mitochondrial Ca^{2+} load and the buffering capacity of the matrix, and such imbalance ultimately leads to a prolonged episode of massive mitochondrial Ca^{2+} accumulation. Sustained Ca^{2+} accumulation triggers opening of the mitochondrial permeability transition pore (mtPTP). Ultimately, this results in swelling of the organelle, rupture of the OMM, and release of proapoptotic proteins into the cytosol [74].

ROS are thought to act as local messengers between the ER and mitochondria [6]. Many ROS sources and targets are localized to the ER and mitochondria [75, 76]. Disulfide bond formation is a critical step in folding of newly synthesized proteins, and this is mediated by members of the ER oxidoreductin 1 (Ero1) family [77]. Importantly, ROS are concomitantly produced by Ero1. Previous studies have shown that Ero1 can be activated under conditions of ER stress [78, 79]. Thus, conditions that trigger such stress may lead to excessive production of ROS in the ER. Such elevated ROS levels inactivate the sarco-endoplasmic reticulum Ca^{2+} ATPase (SERCA) and activate IP_3R via oxidation [80, 81]. Modulation of Ca^{2+} channel activity by ROS increases the level of Ca^{2+} on the cytosolic face of the ER and also promotes Ca^{2+} uptake into the mitochondrial matrix. Therefore, ROS production mediated by Ero1 provides an additional mechanism by which ER stress can induce mitochondrial dysfunction.

5.2. Role of Mitochondrial Dysfunction in Induction of ER Stress

5.2.1. NO-Mediated Induction of the ER Stress Response via Inhibition of Mitochondrial Respiration. Protein folding processes and the handling of Ca^{2+} within the ER each require large amounts of ATP. Accordingly, ATP depletion is one of the best-known mechanisms by which ER stress may be induced [82]. Such observations have raised significant questions regarding the modes by which changes in mitochondrial function affect processes within the ER. It is widely accepted that ER stress induces mitochondrial dysfunction. However, it appears that this is not a one-way process;

Xu et al. have shown that the ER stress response can be induced following disruption of the mitochondrial respiratory chain by nitric oxide [83].

NO can bind to cytochrome c oxidase and inhibit the enzyme, in competition with oxygen [84]. Thus, the respiratory chain is disrupted in NO-generating cells [83]. Because this process is accompanied by mitochondrial Ca^{2+} flux, disruption of electron transfer by cytochrome c oxidase may result in changes in the extent of Ca^{2+} flux between the mitochondria and the ER. NO-mediated changes in Ca^{2+} flux between these organelles increase expression of ER stress-responsive genes such as glucose-regulated protein 78 (Grp78), elevated levels of which provide significant cytoprotection against thapsigargin, a selective ER Ca^{2+} ATPase inhibitor. Interestingly, chemical disruption of mitochondrial Ca^{2+} flux has been shown to reverse NO-mediated cytoprotection. In addition, the NO-mediated ER stress response was diminished in *rho*^{o} cells devoid of mitochondrial DNA [83]. Together, these results suggest that NO signals the ER stress response via inhibition of mitochondrial respiration.

5.2.2. Mitochondrial Dysfunction Induces ER Stress and Decreases Adiponectin Synthesis. Recently, we showed that impairment of mitochondrial function increases the levels of ER stress markers [30]. Adenovirus-mediated overexpression of nuclear respiratory factor-1 (NRF-1), a transcription factor that regulates the expression of nuclear-encoded mitochondrial genes, reduced the upregulation of ER stress markers associated with mitochondrial dysfunction. Previous studies showed that JNK and activating transcription factor 3 (ATF3) were activated by ER stress [3, 85]. Further, impairment of mitochondrial function sequentially activated JNK and ATF3. However, inhibition of JNK and ATF3 reversed the reduction in adiponectin transcription that was induced by mitochondrial dysfunction [30]. Together, the data suggest that mitochondrial dysfunction induces ER stress. This, in turn, activates signaling cascades involving JNK and ATF3, thereby decreasing adiponectin synthesis in adipose tissue.

5.2.3. Induction of ER Stress by Mitochondrial Dysfunction and Hepatic Insulin Resistance. Mitochondrial dysfunction induces ER stress, and this, in turn, causes hepatic insulin resistance [86]. In human liver cell lines, inhibition of mitochondrial function by oligomycin disturbs insulin signaling. In contrast, hepatic gluconeogenesis is abnormally increased. The levels of ER stress markers were elevated in cells containing functionally inactivated mitochondria. However, this rise was reversed by decreasing the level of cytosolic-free Ca^{2+}. Importantly, mitochondrial dysfunction elevated the level of cytosolic-free Ca^{2+}, which in turn promoted an increase in the concentrations of the ER Ca^{2+} channels IP_3Rs and the ryanodine receptor-2 (RyR-2). Elevated levels of these channels induced Ca^{2+} depletion within the lumen of the ER. Disturbances in Ca^{2+} homoeostasis in the ER are also known to trigger the ER stress response, leading to activation of p38 mitogen-activated protein kinase (MAPK), as well as increasing phosphoenolpyruvate carboxykinase (PEPCK) expression [87, 88]. Abnormal activation of JNK by mitochondrial dysfunction also increased PEPCK expression by affecting insulin signaling and forkhead box protein O1 (FOXO1) activity [86]. Together, the results suggest that mitochondrial dysfunction induces ER stress in a Ca^{2+}-dependent manner, leading to disturbance of insulin signaling and an abnormal rise in gluconeogenesis within hepatocytes.

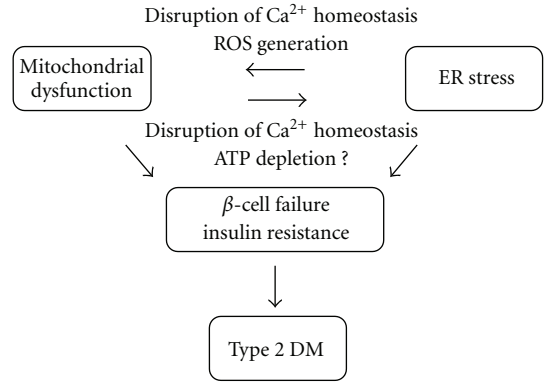

Figure 3: Bidirectional communication between dysfunctional mitochondria and the ER under stress contributes to the development of type 2 DM.

5.2.4. Induction of ER Stress by Mitochondrial Dysfunction and Local ATP Depletion. A number of events may contribute to the linking of mitochondrial dysfunction and ER stress. For example, local ATP pools in the mitochondria and the adjacent ER may be essential to supply the energy required by SERCA to import Ca^{2+} into the lumen of the ER. In agreement with this idea, inhibition of OXPHOS was shown to cause a prolonged delay in uptake of Ca^{2+} into the lumen of the ER; in addition, Ca^{2+} levels within the ER fell [89]. Inhibition of OXPHOS caused rapid local ATP depletion in mitochondria and the ER, although global cytosolic ATP levels decreased at a much later time. These results suggest that local ATP depletion in the region in which SERCA is active may reduce the uptake of Ca^{2+} into the lumen of the ER. This would cause Ca^{2+} depletion within the ER, which may trigger the ER stress response. Whether this mechanism is operative in pancreatic β-cells and/or insulin-responsive tissues remains to be determined.

6. Conclusions

We have provided a brief overview of the interaction between mitochondrial dysfunction and ER stress. In particular, we examined the role played by such interaction in the pathogenesis of type 2 DM. Mitochondrial dysfunction and ER stress are essential for β-cell dysfunction and peripheral insulin resistance. To date, substantial progress has been made in understanding structural and functional communications between mitochondria and the ER. We now know that ER stress can induce mitochondrial dysfunction. Thus, such stress plays a central role in apoptosis signaling via Ca^{2+}- and/or ROS-dependent mechanisms. Together with

recent findings linking mitochondrial dysfunction and ER stress, it appears that bidirectional communication exists between these two organelles (Figure 3). Characterization of interactions between mitochondria and the ER is a dynamic and growing area of interest; future research will carefully dissect such processes. Hopefully, the studies will help us to gain a better understanding of the pathogenesis underlying type 2 DM. Therapeutic approach aimed at restoring mitochondria function will prevent or treat insulin resistance and type 2 DM through suppression of ER stress.

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