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The Role of Peroxidation of Mitochondrial Membrane Phospholipids in Pancreatic β -Cell Failure

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Abstract

Type 2 diabetes (T2D) 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 pathogenesis of T2D. This review focuses on mechanisms whereby reactive oxygen species (ROS) produced by β -cell in response to metabolic stress affect mitochondrial structure and function and lead to β -cell failure. Specifically, ROS oxidize mitochondrial membrane phospholipids such as cardiolipin, which impairs membrane integrity and leads to cytochrome c release and apoptosis. In addition, ROS activate UCP2 *via* peroxidation of the mitochondrial membrane phospholipids, which results in proton leak leading to reduced ATP synthesis and content in β -cells — critical parameters in the regulation of glucose-stimulated insulin secretion. Group VIA Phospholipase A₂ (iPLA₂ β) appears to be a component of a mechanism for repairing mitochondrial phospholipids that contain oxidized fatty acid substituents, and genetic or acquired iPLA₂ β -deficiency increases β -cell mitochondrial susceptibility to injury from ROS and predisposes to development of T2D. Interventions that attenuate the adverse effects of ROS on β -cell mitochondrial phospholipids may prevent or retard the development of T2D.

Keywords

β -cell failure; Group VIA phospholipase A₂; metabolic stress; mitochondrial phospholipid peroxidation; Reactive Oxygen Species (ROS); repair of mitochondrial membranes

1. INTRODUCTION

Type 2 diabetes (T2D) is the most common human endocrine disease and it is reaching pandemic proportions [1, 2]. Predisposition to T2D is affected by both genetic and environmental factors, many of which are incompletely understood [2, 3]. It is becoming clear, however, that the progressive failure of pancreatic islet β -cells, which normally respond to increased metabolic demands by increasing their mass as well as their synthesis

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and secretion of insulin, is a central component of the development and progression of T2D [4].

The mechanisms that underlie the progressive development of β -cell failure during the evolution of T2D are not fully understood, but likely involve both genetic and acquired factors [4, 5]. Acquired factors suggested to contribute to β -cell injury include glucotoxicity, lipotoxicity, altered islet amyloid polypeptide (IAPP) processing, advanced glycation end-products (AGEs), and increased levels of inflammatory cytokines [6-13]. Although many mechanisms are proposed to underlie effects of these contributing factors, a unifying theme is that reactive oxygen species (ROS) induced by metabolic stress causes a cascade of events that ultimately results in β -cell failure [10, 14-19]. Indeed, islets from T2D patients appear to produce more ROS than those from non-diabetic subjects do [15, 20-22].

ROS, including superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($HO^{\bullet}$), can be generated in non-mitochondrial sources including cytochrome P450 enzyme, NADPH oxidase, and 5-lipoxygenase [23, 24], but the mitochondria are the major source of ROS in pancreatic β -cells under metabolic stress [14, 22, 23, 25]. In β -cells, glucose or fatty acids are metabolized through the tricarboxylic acid (TCA) cycle and generate electron donors NADH and $FADH_2$, which transfer their electrons to the mitochondrial electron-transport chain (ETC). Electron flow through the ETC is carried out by four inner-membrane-associated enzyme complexes (I-IV), cytochrome *c*, and the mobile carrier coenzyme Q. The ETC continually generates small amounts of superoxide anion radicals, principally through complexes I and III [26]. These superoxide radicals are normally removed by Mn^{2+} -superoxide dismutase (MnSOD), which dismutates $O_2^{\bullet-}$ to produce H_2O_2 . The H_2O_2 is then reduced to water by glutathione peroxidase (GPx) at the expense of glutathione. Under metabolic stress, however, elevated glucose or fatty acids produce excessive NADH and $FADH_2$, which accelerates the ROS production (Fig. 1). When the rates of ROS generation exceed those of its removal, the subsequent accumulation of H_2O_2 in the presence of Fe^{2+} can result in the production of the highly reactive hydroxyl radical *via* the Fenton and Haber-Weiss reactions.

Activation of a series of stress-response pathways by ROS has been reviewed elsewhere [16, 27, 28]. The purpose of this review is to provide a brief overview as to how mitochondrial ROS affects mitochondrial membrane phospholipids, including cardiolipin, and leads to β -cell mitochondrial failure and, ultimately, T2D. Recent advances in complex lipid analyses by mass spectrometry have permitted the detailed molecular characterization of the effects of various pathophysiologic states on mitochondrial cardiolipin species [29-32]. This provides a powerful tool with which to increase our understanding of these processes and to identify potential targets for therapeutic intervention.

2. ROS TRIGGER APOPTOSIS VIA THE OXIDATION OF PHOSPHOLIPIDS IN THE INNER MEMBRANE OF β -CELL MITOCHONDRIA

The onset of T2D is accompanied by a progressive decrease in β -cell mass due to a marked increase in β -cell apoptosis [11, 33, 34]. Mitochondria are known to play a pivotal role in regulating apoptotic cell death [35], and the release of cytochrome *c* from the mitochondrial

membrane into the cytoplasm is a key step in the initiation of apoptosis [36]. Specifically, cytoplasmic cytochrome *c* participates in the formation of the apoptosome, which then recruits and activates caspase-9. Caspase-9, in turn, activates the executioner caspases-3, -6 and -7 that dismantle the cell during apoptosis [35].

Cytochrome *c* release appears to result from the direct action of ROS on the mitochondrial phospholipid cardiolipin [37, 38]. Cardiolipin is a structurally unique dimeric phospholipid localized exclusively in the inner mitochondrial membrane (IMM) in mammalian cells. It maintains mitochondrial architecture and membrane potential and supports proteins involved in mitochondrial bioenergetics [39, 40]. Cytochrome *c* is anchored to the outer surface of the inner mitochondrial membrane by electrostatic and hydrophobic interactions with cardiolipin [41]. During early apoptosis, mitochondrial ROS production is stimulated and cardiolipin is oxidized. This oxidation destabilizes its interaction with cytochrome *c*, which then detaches from the membrane and is released into the cytoplasm through pores in the outer membrane [37, 41].

Cardiolipin is particularly susceptible to oxidation because it is enriched in polyunsaturated fatty acid (PUFA) residues. These residues contain a bisallylic methylene group from which hydrogen is easily abstracted to permit the formation of a hydroperoxy radical *via* an interaction with molecular oxygen. In most mammalian tissues, linoleic acid (C18:2) is the most abundant fatty acid in cardiolipin [42]. For example, rat pancreatic islet cardiolipin is 89.5% PUFA, of which 71% is linoleate [43]. Mitochondrial cardiolipin is also a target of the pro-apoptotic protein tBid, which is a Bcl-2 family member produced from Bid by the activation of caspase-8. The targeting of cardiolipin by tBid results in activation of the mitochondrial death pathway upon induction of apoptosis via the engagement of death receptors [44].

Cardiolipin is thus a central participant in regulating mitochondrial- and death-receptor-triggered apoptosis, and alterations of mitochondrial cardiolipin are now recognized to be involved in the development of diabetes as well as several other pathologic conditions [28, 31, 32, 39, 40, 45-51]. We have observed that the generation of ROS by mitochondria, and the subsequent oxidation of mitochondrial phospholipids and release of cytochrome *c*, triggers apoptosis in both INS-1 insulinoma cells and mouse pancreatic islet β -cells [47, 52].

3. ROS ACTIVATE UNCOUPLING PROTEIN 2 (UCP2) VIA THE PHOSPHOLIPID PEROXIDATION IN THE INNER MEMBRANE OF β -CELL MITOCHONDRIA

Several studies have demonstrated that glucose-stimulated insulin secretion is lower in islets from T2D patients than in control islets [53, 54]. Glucose-stimulated insulin secretion by β -cells is achieved by coupling glycolysis to oxidative phosphorylation and ATP production in mitochondria [27]. In response to rising glucose levels, respiratory chain complexes pump protons out of the mitochondrial matrix, generating an electrochemical proton gradient that provides the energy for ATP synthase to produce ATP from ADP. As a result, the ratio of cytoplasmic ATP to ADP rises, which induces a series of events including the closure of

ATP-sensitive potassium channels (K_{ATP}), the depolarization of plasma membranes, the opening of voltage-gated calcium channels, the influx of Ca^{2+} , the activation of Ca^{2+} -sensitive effector elements including the Ca^{2+} /calmodulin-dependent protein kinase II β and others, and, finally, insulin exocytosis [55].

It appears that, in pancreatic islet β -cells, mitochondrial membrane potential is 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 attenuate ATP synthesis. It has been shown to negatively regulate insulin secretion and is a major link between obesity, β -cell dysfunction, and T2D [56, 57]. Specifically, obesity and chronic hyperglycemia increase mitochondrial superoxide ($O_2^{\bullet-}$) production [58], which activates UCP2 and causes pancreatic islet β -cell dysfunction [14, 59-61]. Inhibition by genipin of UCP2-mediated proton leak has been found to reverse β -cell dysfunction induced by obesity and high glucose levels in isolated pancreatic islets *in vitro* and in animals with diet-induced T2D *in vivo* [62, 63]. Together these observations suggest that UCP2 activation by mitochondrially produced superoxides could contribute to the development of β -cell dysfunction during T2D evolution.

The mechanism by which superoxides activate UCP2 is, however, not well understood. Experiments with targeted antioxidants suggest that superoxides or their products activate UCPs on the matrix side of the mitochondrial inner membrane [60], and a study with a mitochondrion-targeted spin trap derived from α -phenyl-N-tert-butyl nitron indicated that superoxides activate UCPs via the oxidation of unsaturated side chains of fatty acid substituents of mitochondrial phospholipids such as cardiolipin associated with UCPs [64]. In this model, superoxide generated by mitochondria is dismutated by matrix Mn-SOD to hydrogen peroxide (H_2O_2), which reacts with Fe^{2+} in the Fenton reaction to generate a hydroxyl radical ($\bullet OH$). The hydroxyl radical then extracts a hydrogen atom ($H\bullet$) from a bisallylic methylene moiety of PUFA substituent of a phospholipid, *e.g.*, 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 generates a complex mixture of products, including 4-hydroxynonenal (*HNE*) and 4-hydroxyhexenal, that activate UCPs [64, 65].

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 [43]. The electron transport chain complexes that generate superoxides reside in the inner mitochondrial membrane, and superoxide production is rate-limiting for the generation of all ROS. Cardiolipin PUFA substituents are especially susceptible to ROS due to 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 scenario in which high rates of mitochondrial superoxide production are associated with correspondingly high rates of cardiolipin oxidation. This oxidation contributes to the superoxide-mediated activation of UCPs, perhaps *via* the generation of *HNE* and other products of lipid peroxidation.

We propose that cardiolipin oxidation directly links ROS generation to UCP2 activation and thereby contributes to the acceleration of the proton leak that ultimately results in β -cell dysfunction. Indeed, it was recently reported that oxidation of the mitochondria-specific phospholipid tetralinoleoyl cardiolipin (L4CL) leads to the formation of 4-HNE *via* a novel chemical mechanism involving cross-chain peroxy radical addition and decomposition [66]. This proposal points to potentially important processes that may be targeted to prevent or retard the development of T2D and, perhaps, obesity [65].

4. GROUP VIA PLA₂ (iPLA₂ β) IS INVOLVED IN THE REMODELING AND REPAIR OF MITOCHONDRIAL MEMBRANES

As described above, pancreatic islet cardiolipin is enriched in PUFA, particularly linoleate [43], and the bisallylic methylene moieties of PUFA side chains are especially vulnerable to oxidation by ROS. Cardiolipin resides in the inner mitochondrial membrane, which is also the locus of ROS generation, and cardiolipin oxidation is favored under conditions of accelerated ROS production. Because pancreatic islets express lower levels of antioxidant enzymes than other tissues, it is likely that islet cardiolipin is particularly susceptible to oxidation [67, 68] and that β -cells have evolved 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 member of the phospholipase A₂ (PLA₂) family are required to eliminate oxidized fatty acids from mitochondrial phospholipids under physiological conditions [69]. Gpx4 is a selenoprotein in the glutathione peroxidase (Gpx) family that protects biomembranes, particularly in mitochondria [70]. The PLA₂ family comprises a diverse group of enzymes that catalyze the hydrolysis of the *sn*-2 fatty acyl bond of phospholipids to generate a free fatty acid and a 2-lysophospholipid [71, 72]. Therefore, PLA₂ family members are thought to be involved in the repair of oxidized membrane phospholipids [73-75].

Among PLA₂ family members, Group VIA PLA₂ (iPLA₂ β) is attracting increasing interest as a potentially critical participant in the maintenance of mitochondrial cardiolipin homeostasis [47, 52, 76, 77]. In eukaryotes, cardiolipin is synthesized *de novo* on the inner face of the inner mitochondrial membrane from phosphatidylglycerol (PG) and cytidinediphosphate-diacylglycerol (CDP-DAG) by cardiolipin synthase [78]. Nascent cardiolipin does not contain PUFAs in its four acyl chains, and the enrichment of PUFA in cardiolipin is thought to be achieved through remodeling mediated by either Tafazzin (*TAZ*) or iPLA₂ β /MLCLAT pathway [78, 79].

In the *TAZ* pathway, newly synthesized cardiolipin is proposed to be deacylated and reacylated by *TAZ*. *TAZ* encodes a putative mitochondrial phospholipid acyltransferase with both deacylation and reacylation activities [80, 81]. *TAZ* activity is essential for optimal mitochondrial function in heart muscle, and a mutation in the gene is responsible for Barth Syndrome, which is characterized by a severe cardiomyopathy [80, 82]. In the iPLA₂ β /MLCLAT-mediated pathway, it is proposed that newly synthesized cardiolipin is deacylated by iPLA₂ β to form MLCL, which is then reacylated to cardiolipin by a MLCL

acyltransferase (MLCLAT) (Fig. 1). It has recently been recognized that mutations in the PLA2G6 gene, which encodes iPLA₂β, are responsible for the neurodegenerative disease infantile neuroaxonal dystrophy (INAD) [83] and that a similar disorder develops in mice with a disrupted iPLA₂β gene [84]. It has also been suggested that iPLA₂β plays a role in cardiolipin remodeling in both a *Drosophila* model of Barth Syndrome [77] and a rat model of spontaneously hypertensive heart failure [76].

In addition to the previous findings, we also have reported observations consistent with a role for iPLA₂β in β-cell mitochondrial function. Specifically, we have shown that iPLA₂β resides in mitochondria in INS-1 insulinoma cells and that its activity protects against the ability of staurosporine — an inhibitor of various isoforms of Protein Kinase C that strongly stimulates ROS production in the mitochondria — to induce the loss of mitochondrial membrane potential, the release of cytochrome *c* and Smac/DIABLO into the cytosol, the peroxidation of mitochondrial membranes, and apoptosis [52, 85].

Both Barth Syndrome and INAD are human genetic disorders that are often fatal in childhood [80, 83] 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 [86]. Thus, the mice with administration of iPLA₂β specific inhibitor bromoenol lactone (BEL) [87] and iPLA₂β-null (iPLA₂β^{-/-}) that have a late onset of INAD [84, 88] provide valuable animal models to study the role of iPLA₂β in β-cell failure *in vivo* [47, 89-91].

We have observed that acute pharmacologic inhibition of iPLA₂β in mice impairs glucose tolerance by suppressing insulin secretion without affecting insulin sensitivity [87]. Consistent with this finding, iPLA₂β^{-/-} mice exhibit a 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, consumption of a high-fat diet, or staurosporine administration [47, 90, 91]. Islets from iPLA₂β^{-/-} mice also exhibit diminished secretory responses as compared to wild-type islets [47, 90, 91], and incubation with elevated concentrations of glucose and free fatty acids results in higher levels of β-cell apoptosis and of mitochondrial membrane phospholipid peroxidation than observed in wild-type islets [47]. These findings suggest that iPLA₂β plays an important role in the maintenance of β-cell mitochondrial membrane integrity. In this scenario, iPLA₂β deficiency or inhibition by BEL increases β-cell susceptibility to injury by ROS generated by mitochondria in response to metabolic stress [47, 91] and leads to a cascade of β-cell failure, apoptosis and eventual development of T2D [47, 91].

It has been suggested that oxidation of PUFA in cardiolipin and other mitochondrial phospholipids serves to trap ROS and protect mitochondrial proteins or DNA from oxidative injury. The reaction of PUFA with ROS may also generate signals to respiratory chain proteins to mitigate ROS generation [40, 65, 92-94]. A repair mechanism in which iPLA₂β excises oxidized fatty acid substituents from mitochondrial cardiolipin and other phospholipids for reacylation with an unoxidized subunit could allow the cell to modulate the levels and effects of ROS during stress responses.

However, when the rates of ROS generation and PUFA oxidation exceed the capacity of the repair system, the accumulation of oxidized phospholipids could impair the integrity of mitochondrial membranes and result in release of cytochrome *c* into cytosol, the induction of β -cell apoptosis, and, ultimately, T2D (Fig. 1). This situation could occur when iPLA₂ β activity is low because of pharmacologic inhibition, genetic deficiency, or other still-to-be-defined regulatory influences.

Interestingly, islets from the *db/db* mouse model of obesity, dyslipidemia, and diabetes express lower levels of iPLA₂ β than do islets from control mice [47]. In the scenario described above, this discrepancy would impair cardiolipin remodeling and repair and increase the susceptibility of *db/db* β -cells to oxidative injury, accelerating obesity-associated β -cell loss and the development of T2D.

5. CONCLUSION AND IMPLICATION FOR TREATMENT

Mitochondrial cardiolipin alteration and oxidation has been reported to be associated with many human diseases, including diabetes [45, 48, 50]. Cardiolipin is a key mitochondrial structural component in the mediation of ATP synthesis and apoptosis [40, 95]. Metabolic stress, such as that caused by obesity and hyperglycemia, is typically accompanied by increased mitochondrial production of ROS [58], and cardiolipin's composition (it is high in PUFAs) and location (on the inner mitochondrial membrane) suggest that some type of repair mechanism must be functioning to prevent apoptosis in response to ROS-induced damage of the molecule.

Evidence from our lab and others [47, 52, 76, 77, 87, 90, 91, 96] suggests that iPLA₂ β -mediated deacylation is a critical step in the repair of oxidized cardiolipin in β -cell mitochondria. iPLA₂ β dysfunction, such as that which occurs in INAD patients and when iPLA₂ β expression is downregulated in *db/db* mice, leads to an accumulation of oxidized cardiolipin that sets cytochrome *c* free and triggers apoptosis. The resulting cell death may contribute to neurodegeneration in INAD and β -cell-mass reduction in type 2 diabetes. Further study of cardiolipin remodeling and repair is vital to increase our understanding of the mechanisms of pathogenesis of diabetes and neurodegeneration. It may also provide novel insights into the development of therapeutic protocols capable of attenuating these disease processes in humans.

For example, we have shown that the antioxidant NtBHA (which accumulates in mitochondria) prevents peroxidation of mitochondrial phospholipids and blunts staurosporine-induced apoptosis in islets from iPLA₂ $\beta^{-/-}$ mice [47]. Because mitochondrial ROS are essential to glucose-stimulated insulin secretion, and also enhance insulin sensitivity [97, 98], manipulating ROS production *in vivo* will be difficult and may have unexpected negative effects. Our finding suggests that the specific inhibition of mitochondrial phospholipid oxidation, particularly that of cardiolipin, will be an important component of any successful therapy. Interestingly, the specific delivery directly to mitochondria of antioxidants such as mitoquinone (Mito-Q) and mitovitamin E (mitoVit-E) that have been shown to reduce oxidative stress and improve cardiac function [96, 99] may be useful for preventing β -cell apoptosis. In addition, melatonin, which specifically inhibits

cardiolipin oxidation in mitochondria, has been shown to prevent the induction of the mitochondrial permeability transition (MPT) and the release of cytochrome c and to protect against heart ischemia-reperfusion injury [100, 101].

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Fig. (1). Schematic summary of the proposed role of mitochondrial cardiolipin oxidation in β-cell failure in type 2 diabetes

In the presence of metabolic stress, there is more glucose (Glc) or fatty acids (FAs) being oxidized in the TCA cycle in β-cell mitochondria, which pushes more electron donors (NADH and FADH₂) into the electron transport chain (ETC) resulting in more ROS production. Under moderate stress, ROS causes cardiolipin oxidation, which may mitigate ROS generation through its interaction with respiratory chain proteins or UCP2. After ROS damage, iPLA₂β/MLCLAT-mediated deacylation and reacylation promptly repair the oxidized cardiolipin. Under conditions of high oxidative stress or a defect in repair system, the accumulation of oxidized cardiolipin may activate UCP2, impairing the glucose-stimulated insulin secretion and free cytochrome *c*, triggering apoptosis, which lead to β-cell failure and, ultimately, the onset of type 2 diabetes. Block arrows indicate the iPLA₂β-mediated deacylation. CL, cardiolipin; MLCLAT, monolysocardiolipin acyltransferase. Black arrows show the stimulatory pathway; dashed line, the inhibitory pathway.