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Group VIA Ca^{2+}-Independent Phospholipase $A_2\beta$ ($iPLA_2\beta$) and its role in β-cell Programmed Cell Death

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Abstract

Activation of phospholipases A_2 (PLA_2s) leads to the generation of biologically active lipid mediators that can affect numerous cellular events. The Group VIA Ca^{2+}-independent PLA_2, designated $iPLA_2$®, is active in the absence of Ca^{2+}, activated by ATP, and inhibited by the bromoenol lactone suicide inhibitor (BEL). Over the past 10–15 years, studies using BEL have demonstrated that $iPLA_2\beta$ participates in various biological processes and the recent availability of mice in which $iPLA_2\beta$ expression levels have been genetically-modified are extending these findings. Work in our laboratory suggests that $iPLA_2$® activates a unique signaling cascade that promotes β-cell apoptosis. This pathway involves $iPLA_2$® dependent induction of neutral sphingomyelinase, production of ceramide, and activation of the intrinsic pathway of apoptosis. There is a growing body of literature supporting β-cell apoptosis as a major contributor to the loss of β-cell mass associated with the onset and progression of Type 1 and Type 2 diabetes mellitus. This underscores a need to gain a better understanding of the molecular mechanisms underlying β-cell apoptosis so that improved treatments can be developed to prevent or delay the onset and progression of diabetes mellitus. Herein, we offer a general review of Group VIA Ca^{2+}-independent PLA_2 ($iPLA_2\beta$) followed by a more focused discussion of its participation in β-cell apoptosis. We suggest that $iPLA_2\beta$-derived products trigger pathways which can lead to β-cell apoptosis during the development of diabetes.

Keywords

$iPLA_2\beta$; apoptosis; β-cell; ceramides; mitochondria

A. The Biology of Group VIA Phospholipase A_2 ($iPLA_2\beta$)

1. Classification of phospholipases A_2 (PLA_2s)

In pancreatic islet subcellular organelles, similar to brain tissue [1], arachidonic acid is a major *sn*-2 substituent of membrane phospholipids [2,3]. Arachidonic acid and its oxygenated metabolites and the lysophospholipids are potent bioactive mediators that regulate a myriad of physiological and pathophysiological processes [4,5]. Phospholipases A_2 (PLA_2s) are a diverse

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group of enzymes that catalyze hydrolysis of arachidonic acid (and other *sn*-2 substituents) from glycerophospholipid substrates. [6]. For more detailed descriptions of the biology of the PLA_2 enzymes, the reader is referred to a number of comprehensive reviews [7–17].

To date, 15 distinct groups of PLA_2s are recognized [7,8] and among them, the Ca^{2+}-independent PLA_2s ($iPLA_2$s) are the most recently described and the least well characterized. The $iPLA_2$ was first purified from macrophages in 1994 [18] and was subsequently cloned from multiple sources between 1997 and 1999 [19–21]. A brief review of the biology and properties of $iPLA_2$ is provided here and the reader is referred to other, more comprehensive reviews for more detailed discussions of the $iPLA_2$ enzymes [15–17].

2. Sub-classification, activity, and localization of Group VI PLA_2s ($iPLA_2$s)

The $iPLA_2$s have a conserved C-terminal lipase consensus motif (GXSXG) and manifest catalytic activity in the absence of Ca^{2+}. According to the current classification, $iPLA_2$ products of different genes are designated as follows: VIA or $iPLA_2\beta$ (Table 1), VIB or $iPLA_2\gamma$ [22–26], VIC or $iPLA_2\delta$ [27,28], and VID or $iPLA_2\varepsilon$, VIE or $iPLA_2\zeta$, and VIF or $iPLA_2\eta$ [29].

A unique characteristic of the group VIA $iPLA_2$s is the presence of 7–8 ankyrin N-terminal repeats that are not found in the other $iPLA_2$s. The VIA-1 and VIA-2 are designated as the short and long form $iPLA_2\beta$, where the long form is a product of alternatively spliced exon 8 that generates a protein containing a 54 amino acid insertion in the eighth ankyrin repeat. The Ank-1 and Ank-2 are truncated $iPLA_2\beta$ proteins that interact with full-length $iPLA_2\beta$ and suppress catalytic activity in a dominant negative fashion [31,35,36]. The VIA isoforms arising from post-translational modification (PTM), designated here as groups VIA-4 and VIA-5, are generated via cleavage of $iPLA_2\beta$ at the N-terminal by caspase-3 (VIA-4) [32,33] and at the C-terminal by an unknown mechanism (VIA-5) [34] and are catalytically active.

In addition to a PLA_2 activity, the $iPLA_2$s exhibit lysophopholipase and transacylase activities [37] and the $iPLA_2\beta$ also expresses an acyl-CoA thioesterase activity [38,39]. The group VIA $iPLA_2\beta$ is the most extensively studied $iPLA_2$ and under basal conditions is predominantly localized in the cytosol but upon certain stimulation translocates to the nucleus [33,40], ER [41–43], Golgi [42,43], and mitochondria [44].

3. Structural Features of $iPLA_2\beta$

The $iPLA_2\beta$ is encoded by mRNA species that yield a protein with an expected molecular mass of 84–88 kDa. Full-length $iPLA_2\beta$ protein consists of the lipase motif preceded by the eight N-terminal ankyrin-repeats [20,21,30]. Other salient features of the $iPLA_2\beta$ amino acid sequence include a caspase-3 cleavage site (DVTD*), an ATP-binding domain (GGGVKG), a serine lipase consensus sequence (GTSGT), a putative bipartite nuclear localization sequence (KREFGEHTKMTDVKKPK), a C- terminal 1-9-14 calmodulin-binding motif (IRKGQGNKVKKLSI), and a calmodulin-binding peptide (AWSEMVGIQYFR) [45–47]. The recent findings that certain stimuli promote translocation of $iPLA_2\beta$ to the ER and mitochondria [41,44] suggest that additional targeting sequences, not yet identified, also reside within the $iPLA_2\beta$.

4. Alternative splicing and post-translational modifications of $iPLA_2\beta$

The $iPLA_2\beta$ gene undergoes a variety of alternative splicing events, generating variants that differ in their subcellular localization, catalytic activity, and likely cellular function [31,36, 48]. Group VIA-1 $iPLA_2\beta$ is the “classic” 84–85 kDa isoform. The 88 kDa $iPLA_2\beta$ isoform is a product of an mRNA species that arises from an exon-skipping mechanism of alternate splicing [31] and contains a 54-amino acid sequence that interrupts the eighth ankyrin repeat. Two additional splice variants (Ank-1 and Ank-2) encode premature stop codons due to

alternatively spliced exon 10a. The proteins encoded by these splice variants, group VIA Ank-1 and group VIA Ank-2, terminate after the ankyrin repeat domain but before the active site.

In addition to the 70 kDa isoforms that result from proteolytic cleavage of full-length iPLA$_2$β, mass spectrometry analyses reveal that iPLA$_2$β is a candidate for N-terminal modification and truncation [43,49] *(Song et al, under review)*. Though the mechanisms responsible for generating the iPLA$_2$β protein variants or their role in biological processes have yet to be determined, these observations indicate that iPLA$_2$β is a candidate for post-translational modification by NH_2-terminal processing and that this might represent a means to regulate its activity, subcellular location, or interaction with other proteins.

5. Chemical and biological modulation of iPLA$_2$β Activity

Chemical Inhibitors—Hazen et al. (1991) reported the synthesis of (E)-6-(bromomethylene) tetrahydro-3-(1-naphthalenyl)- 2H-pyran-2-one, designated initially as haloenol lactone suicide substrate (HELSS) [50] but referred to now as bromoenol lactone (BEL). This suicide inhibitor selectively targets iPLA$_2$β and other group VI PLA$_2$ enzymes and has little or no effect on cPLA$_2$ or sPLA$_2$ activity [29,50,51]. Although BEL treatment results in covalent modification of iPLA$_2$β [50,52], the modified residues are cysteines, and not the active site, likely due to a diffusible bromoketomethyl acid that is generated when iPLA$_2$β acts on the inhibitor [53].

iPLA$_2$β is also targeted by arachidonyl trifluoromethyl ketone (AACOCF$_3$), methyl arachidonyl fluorophosphonate (MAFP), and palmitoyl trifluoromethyl ketone (PACOCF$_3$), inhibitors that are sometimes used for "selective" inhibition of cPLA$_2$ [52,54]. This underscores the importance of pairing AACOCF$_3$, PACOCF$_3$, and MAFP experiments with BEL treatments to accurately assess involvement of specific PLA$_2$s a given system.

Over the years, BEL has been used to discern the involvement of iPLA$_2$ in biological processes and is still considered the only available specific irreversible inhibitor of iPLA$_2$. Recently, the *S*- and *R*-enantiomers of BEL have been demonstrated to exhibit specific inhibition of iPLA$_2$β and iPLA$_2$γ, respectively [55]. However, several examples of inhibition of non-iPLA$_2$ enzymes by BEL have been described [28,29,56–58] and the mechanism of inhibition does not appear to involve the active site of iPLA$_2$ [53,59]. In view of this, the now available mice genetically-modified to be deficient in or overexpress iPLA$_2$β [60–62] are expected to significantly promote studies leading to further understanding of iPLA$_2$ participation in various biological processes.

Biological regulators

ATP: The iPLA$_2$β contains a consensus nucleotide binding motif (GXGXXG) that is homologous to those of the protein kinases [22,46]. This feature likely mediates the well-established regulation and stabilization of iPLA$_2$ activity by ATP [18,37,63,64], which is independent of phosphorylation of the enzyme [18,63,64]. Although the ATP binding domain is not disrupted by the 54 amino acid insertion that distinguishes group VIA-1 from group VIA-2 iPLA$_2$β, only VIA-2 is activated by ATP [21]. This is likely because the insertion is highly enriched in proline residues that can change conformation of the adjacent ATP binding site.

Phosphorylation: Several investigators have proposed that iPLA$_2$ is activated downstream of serine-threonine protein kinases, including PKC and p38 kinases [26,65–70]. However, there is little direct evidence for iPLA$_2$β activation in response to phosphorylation. Although iPLA$_2$ activity increases when the enzyme binds Ca^{2+}/calmodulin dependent protein kinase

βII (CaMKII) in pancreatic β-cells [71], there is no evidence for CaMKII-dependent phosphorylation of the enzyme.

Ca^{2+}-Calmodulin: The $iPLA_2\beta$ is bound by calmodulin affinity columns [72], suggesting that the enzyme may be regulated by calcium-calmodulin complexes. Indeed, $iPLA_2$ activity increases in Ca^{2+}-depleted cells [73–75], consistent with the observation that calmodulin suppresses catalytic activity [76]. Negative regulation of $iPLA_2\beta$ by Ca^{2+}/calmodulin complexes has important implications for activation of store-operated Ca^{2+} channels [74] and neutrophil activation in response to stress [77].

Caspase-mediated proteolysis—The $iPLA_2\beta$ has been implicated in apoptosis induced through both the intrinsic and extrinsic pathways [32,33,41,44,78,79]. The executioner caspase, caspase-3, is common to both pathways and can cleave $iPLA_2\beta$ at Asp^{183}, producing a 62–70 kDa-truncated enzyme that has enhanced $iPLA_2\beta$ catalytic activity [32,33,78]. Another group has suggested that the highly active $iPLA_2\beta$ is a 26 kDa protein, generated after processing at an additional caspase-3 cleavage site at Asp^{513}, just upstream of the active site [80]. The truncated $iPLA_2\beta$ is responsible for the arachidonic acid release that occurs during apoptosis of U937 monocytes, in response to TNFα or anti-Fas [32,78]. Caspase-processed $iPLA_2$ also generates lysophosphatidylcholine (LPC), a chemoattractant that recruits monocytes and thereby promotes phagocytosis and clearance of apoptotic cells and lysophosphatidic acid (LPA), a survival factor that protects the cells against apoptosis [79, 80].

Oligomerization—Radiation inactivation studies indicate that active $iPLA_2\beta$ is a homotetramer [18], which indicates oligomerization is another mechanism for the regulation of $iPLA_2$ activity. This is most likely due to the N-terminal ankyrin repeats, which can facilitate protein-protein interactions [81] and mediate oligomerization of $iPLA_2\beta$ into active tetramers [31,48]. The truncated $iPLA_2\beta$ proteins encoded by VIA Ank-1 and group VIA Ank-2, terminate after the ankyrin repeat domain but before the active site. These proteins retain the ability to oligomerize with full-length monomers but are catalytically inactive. As a result, the proteins encoded by VIA Ank-1 and group VIA Ank-2 are endogenous dominant-negative proteins that oligomerize with full-length monomers and prevent the monomers from assembling into active oligomers [31,36,48].

It was reported earlier that oxidants inactivate $iPLA_2$ by a mechanism involving oxidation of sulfhydryl groups within the $iPLA_2$ [82]. Subsequently, Song et al. identified oligomerization of $iPLA_2\beta$ in INS-1 cells in response to oxidative stress [59]. Oxidant-induced oligomerization alters the subcellular localization of $iPLA_2\beta$ and results in reduced release of arachidonic acid, suggesting inhibition of $iPLA_2\beta$ catalytic activity. These non-productive oligomers are DTT-sensitive and therefore likely generated through intermolecular disulfide bonds. Like $iPLA_2\beta$, $iPLA_2\gamma$ activity is also suppressed by oxidants, but restored when oxidant-inhibited enzyme is treated with reducing agent [82,83]. Together, these studies indicate that $iPLA_2\beta$ monomers are capable of assembling into both productive and non-productive monomers. The productive oligomerization is mediated through the N-terminal ankyrin repeat domain while inactive oligomers are formed through intramolecular disulfide bonds.

Regulation of $iPLA_2\beta$ expression—While $iPLA_2\beta$ was once thought to be constitutively expressed, there is mounting evidence that its expression is regulated by a variety of stimuli. For example, $iPLA_2\beta$ expression is regulated by lipids and in response to changes in systemic lipid metabolism. The human $iPLA_2\beta$ gene contains a sterol response element. Sterol response element binding protein-2 (SREBP-2) binds this element and is likely the mechanism for $iPLA_2\beta$ induction in lipid-depleted cells [84]. Both $iPLA_2\beta$ and $iPLA_2\gamma$ expression increase during adipocyte differentiation, and these responses are required for adipocyte development

[85]. Although the molecular mechanism for iPLA$_2$β induction during adipogenesis is not yet certain, it may be mediated through PPARγ or FOXO4 transcription factors which have been linked to adipogenesis [86,87] and have putative binding sites in the 5′ flanking region of the iPLA$_2$β gene (*unpublished observation*). In addition, iPLA$_2$β expression is regulated in the retina [88,89], in the cerebral cortex and hippocampus [90], and in the myocardium of rats undergoing congestive heart failure [91] through mechanisms that have not yet been elucidated.

6. iPLA$_2$β in cell survival versus apoptosis

A significant number of reports over the years from various laboratories, including ours [42, 43,71,92–99] indicate that iPLA$_2$β has a prominent role in phospholipid remodeling, the maintenance of phosphatidylcholine (PC) mass, and signal transduction [78,100–107]. Studies describing this function have escalated in the past five years and are now greatly facilitated by the availability of iPLA$_2$β knockout [61,108] and iPLA$_2$β transgenic mice [60]. A list of biological processes in which iPLA$_2$β participation has been described is provided in Table 2. For more details regarding the role of iPLA$_2$β in these and other biological processes, the reader is referred to other recent reviews [15–17]. As the focus of this review is the role of iPLA$_2$β in ®-cell apoptosis, we will focus on only one biological process in this section: the role of iPLA$_2$β in cell survival vs. apoptosis.

iPLA$_2$β involvement in cell proliferation—Although iPLA$_2$ has important roles in a variety of apoptotic responses in some systems [26,32,40,41,78,79,137–140], studies utilizing BEL have led to identification of a role for iPLA$_2$β in proliferation in other systems. Addition of BEL to culture media decreases cell proliferation [141–144] and this is reversed by addition of arachidonic acid [143,145]. Consistent with these observations, knock-down of iPLA$_2$β suppresses and overexpression of iPLA$_2$β accelerates proliferation of insulinoma cells [94, 99] and proliferation of vascular smooth muscle cells from iPLA$_2$β-null mice is severely impaired but is reversed by addition of arachidonic acid or PGE2 [146]. Other studies suggest that iPLA$_2$β is required for cell cycle progression [109,140,144,145,147], through both p53-dependent and independent mechanisms. The molecular mechanism whereby iPLA$_2$β promotes cell cycle progression and proliferation remains unclear, but is likely to be related to bioactive lipid mediators that are generated by the enzyme. For example, the products of iPLA$_2$β activity may activate genes involved in cell division [30,144,148–151]. Arachidonic acid and eicosanoids have been connected to iPLA$_2$-dependent proliferation [143,145]. Ovarian cancer cells produce lysophosphatidic acid (LPA) in an iPLA$_2$-dependent manner and this potent mitogen acts in an autocrine fashion to induce proliferation and migration [144, 148]. The effect of iPLA$_2$β on cell survival vs. apoptosis and its mechanism of action are likely to be cell type-specific and dependent on the spectrum of bioactive lipids that are generated by the enzyme.

iPLA$_2$β involvement in apoptosis—Another recognized role of iPLA$_2$β is its participation in programmed cell death (i.e. apoptosis; recently reviewed in [152]. An early indication for this was provided by Dr. Kenneth Polonsky's group [153] who reported that apoptosis of mouse insulinoma cell line MIN6 due to ER stress induced by sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) inhibitors occurred via a mechanism that does not require an increase in the cytosolic Ca^{2+} concentration but that does require the generation of arachidonic acid 12-lipoxygenase products. These observations were soon followed by a report from the group of the late Dr. Ichiro Kudo that Fas-induced death of U937 cells was unaffected by inhibition of cPLA$_2$ or sPLA$_2$ but was delayed by inhibition of iPLA$_2$β [78]. The same group subsequently demonstrated that induction of U937 cell apoptosis was associated with caspase-3-mediated cleavage of iPLA$_2$β in the N-terminal region (DVTD^{183}) that generated a more active truncated iPLA$_2$β product [32]. These latter reports arising from Dr. Kudo's group are the first demonstrations of a link between iPLA$_2$β activation during the apoptotic process.

Subsequently, several laboratories demonstrated that iPLA$_2$β activation contributes to apoptosis of various cell system and their studies are summarized in Table 3.

In contrast to these observations, other studies suggest that iPLA$_2$β involvement is not crucial for the execution of apoptosis. For instance, while arachidonic release or iPLA$_2$β activation occurred during apoptosis of S49 caused by thapsigargin [154], of human macrophages by Mycobacterium tuberculosis [166], or of cultured epithelial cells and fibroblasts by *Pseudomonas* aeruginosa [167], inhibition of iPLA$_2$β with BEL did not suppress the apoptosis. In fact, some studies suggest that BEL treatment can actually induce apoptosis [57,154]. However, in these studies the cells were exposed to BEL for up to 24h, which may allow its inhibition of non-iPLA$_2$ proteins to come into play. Further, a recent study reported that androgen receptor activation of iPLA$_2$ upregulates prostate specific antigen (PSA) expression and secretion and PSA via activation of the PI3K/Akt pathway provides a survival signal in prostate cancer cells [168]. It has also been reported that mitochondrial abnormalities promoted by increased generation of ROS and subsequent apoptosis are prevented by expression of iPLA$_2$β, which facilitates repair of membrane phospholipids, in particular cardiolipins, which are susceptible to damage by ROS-mediated peroxidation [169].

Though a more active truncated iPLA$_2$β generated by caspase-3-mediated cleavage of iPLA$_2$β at the N-terminal region is proposed to amplify apoptosis [32], it has been reported that nuclear shrinkage and PC12 cell death due to hypoxia requires activation of iPLA$_2$β but occurs via a caspase-independent pathway [40]. As noted above, caspase-cleaved iPLA$_2$ generates LPC, arachidonic acid, and LPA [79,80]. These bioactive lipids not only promote safe clearance of dying cells but are also potent mitogens that may protect against apoptosis [79,80,134,170]. It is suggested that a 32 kDa product generated by caspase-mediated cleavage of iPLA$_2$β at a site proximal to the lipase site (DLFD^{513}) or 25/26 kDa fragments generated by truncation of the 32 kDa product at other putative caspase-consensus sequences in the C-terminal region (MVVD^{733}, DCTD^{737}, or RAVD^{744}) facilitate generation of the “attraction signals” [79,80,134].

B. iPLA$_2$β role in ®-cell apoptosis

1. ER Stress and β-cell apoptosis

The work by Polonsky and co-workers [153] demonstrated that insulinoma cells were sensitive to SERCA inhibitors. These agents deplete ER Ca^{2+} stores and this can lead to ER stress. Being a site for Ca^{2+} storage, the ER responds to various stimuli to release Ca^{2+} and is therefore extremely sensitive to changes in cellular Ca^{2+} homeostasis. In addition to being a storage site for cellular Ca^{2+}, the ER is also the site where secretory proteins are synthesized, assembled, folded, and post-translationally modified. Interruption of these functions can lead to production of malfolded proteins that require rapid degradation. ER stress ensues when an imbalance occurs between the load of client proteins on the ER and the ER’s ability to process the load occurs, as when ER Ca^{2+} is depleted [171,172]. Prolonged ER stress promotes induction of stress factors and activation of caspase-12, localized in ER [173–176], and can subsequently lead to downstream activation of caspase-3, a protease that is central to the execution of apoptosis [177].

The secretory function of β-cells endows them with a highly developed ER and heightens their susceptibility to ER stress. Thapsigargin, a widely used SERCA inhibitor [178] induces ER stress and promotes caspase-12 cleavage [175,179] and apoptosis of neurons and insulin-releasing BRIN-BID11 cells [175] and Apaf-1 null cells [176]. While SERCA inhibitors promote loss of ER Ca^{2+} stores, induction of MIN-6 insulinoma cell apoptosis by these agents occurs by a pathway that does not require an increase in $[Ca^{2+}]_i$ but instead requires the

generation of arachidonic acid metabolites [153]. These findings were an early indication that ER stress-induced apoptosis may involve Ca^{2+}-independent generation of arachidonic acid.

The likelihood that this process occurs in β-cells is enhanced by the fact that glucose-responsive insulinoma cells, pancreatic islets, and β-cells express $iPLA_2\beta$ and also contain an abundance of arachidonate-containing membrane phospholipids [2,3,20,21,34,98]. Consistent with these features, thapsigargin-induced ER stress in pancreatic islets leads to hydrolysis of arachidonic acid from membrane phospholipids by a Ca^{2+}-independent mechanism that is suppressed by BEL [73], supporting the possibility that ER stress in β-cells promotes $iPLA_2\beta$ activation.

2. $iPLA_2\beta$ involvement in β-cell apoptosis

Our lab has used the INS-1 insulinoma cell line, which behaves similarly to pancreatic islet β-cells [180], to address this possibility and elucidate the mechanism by which $iPLA_2\beta$ participates in β-cell apoptosis [33,41,44]. We found that thapsigargin induces ER stress in INS-1 cells, as evidenced by increases in ER stress factors GRP78/BiP, pPERK, and peIF2a. Prolonged ER stress activated the apoptotic process and this was associated with induction of ER stress apoptotic factor CHOP, activation of ER caspase-12, activation of apoptosis executioner caspase-3, and cleavage of PARP, which facilitates cellular disassembly. These events led to apoptotic INS-1 cell death, as reflected by DNA laddering and increased TUNEL staining. Pre-treatment of the cells with BEL suppressed apoptosis, suggesting that $iPLA_2\beta$ activation was involved in ER stress-induced apoptosis. A direct role of $iPLA_2\beta$ was examined next by overexpressing in INS-1 cells. Exposure of the $iPLA_2\beta$-overexpressing INS-1 cells to thapsigargin significantly amplified the various outcomes described above, including apoptosis, and DNA laddering and TUNEL-positivity in these cells were inhibited by BEL.

To verify that the development of ER stress and subsequent apoptosis was not a unique property of insulinoma cells, we exposed human islets to thapsigargin and found that they also exhibit ER stress-induced apoptosis and that pretreatment of the human islets with BEL suppresses islet β-cell death (*Lei et al. in review*). These findings are taken to indicate that native pancreatic islets are susceptible to ER stress and that the process in islets also involves $iPLA_2\beta$ activation. Consistent with this, islets prepared from $iPLA_2\beta$-KO mice were resistant and islets prepared from $iPLA_2\beta$-Tg mice were more susceptible to ER stress-induced apoptosis (*Lei et al., in preparation*). That this was a specific effect in β-cells is suggested by the fact that the $iPLA_2\beta$-Tg mice were generated using the Rat Insulin I Promoter (RIP) to drive $iPLA_2\beta$ overexpression and as such, $iPLA_2\beta$ expression was only increased in the β-cells [60].

The above-described findings were made in insulinoma cell and islet preparations that were treated with a chemical agent to induce ER stress or in which $iPLA_2\beta$ expression was genetically-modified. We therefore considered whether $iPLA_2\beta$ participation was necessary under conditions where ER stress developed in the β-cell in the absence of chemical intervention. To address this, we compared β-cell lines generated from wild type (WT) and Akita mice. The Akita mouse contains a spontaneous mutation in the insulin-2 gene that results in insulin misfolding and leads to development of diabetes due to ER stress-induced β-cell apoptosis [181,182]. Consistent with their pre-disposition to developing ER stress, basal pPERK and activated caspase-3 are higher in the Akita cells [183]. Interestingly, basal $iPLA_2\beta$ is higher in the Akita cells, relative to WT cells and exposure to thapsigargin induces expression in both the Akita and WT cells. These findings are taken as further proof of $iPLA_2\beta$ involvement during the development and progression of ER stress in the β-cell. In support of these observations in the cultured Akita cell lines, expression of $iPLA_2\beta$ is markedly increased in islets from Akita mice, relative to WT mouse islets. Collectively, these findings suggest that increases in $iPLA_2\beta$ expression and activity during ER stress are not unique to INS-1 cells or artifacts of overexpressing $iPLA_2\beta$ in INS-1 cells or native pancreatic islet β-cells but that they are indeed evident in spontaneous models of ER stress.

3. Evidence for iPLA$_2$β-induced ceramide generation in β-cell apoptosis

An unexpected finding associated with induction of ER stress in INS-1 cell apoptosis was an increase in ceramide generation that was significantly amplified in iPLA$_2$β-overexpressing INS-1 cells [33]. Ceramides are lipid messengers that can suppress cell growth and induce apoptosis [184–186] and they can be generated via multiple pathways. Interestingly, ceramide accumulation in INS-1 cells during ER stress was not associated with changes in mRNA levels of serine palmitoyl-transferase, the rate limiting enzyme in *de novo* synthesis of ceramides [41]. However, both message and protein levels of neutral sphingomyelinase (NSMase), which hydrolyzes sphingomyelins to generate ceramides, were temporally increased in the INS-1 cells. This was reflected by increased hydrolysis of sphingomyelins and increased generation of ceramides in the INS-1 cells undergoing prolonged ER stress. The increases in NSMase expression in the ER-stressed INS-1 cells were associated with corresponding temporal elevations in ER-associated iPLA$_2$β protein and catalytic activity and pretreatment with BEL prevented induction of NSMase message and protein.

Relative to control INS-1 cells, the effects of ER stress were accelerated and/or amplified in iPLA$_2$β overexpressing INS-1 cells [41]. However, inhibition of iPLA$_2$β or NSMase (chemically or with siRNA) suppressed induction of NSMase message, ceramide generation, sphingomyelin hydrolysis, and apoptosis in both control and iPLA$_2$β-overexpressing INS-1 cells during ER stress. In contrast, inhibition of serine palmitoyltransferase did not suppress ceramide generation or apoptosis in either control or iPLA$_2$β overexpressing INS-1 cells. These findings indicate that iPLA$_2$β activation participates in ER stress-induced INS-1 cell apoptosis by promoting ceramide generation via NSMase-catalyzed hydrolysis of sphingomyelins, raising the possibility that this pathway contributes to β-cell apoptosis due to ER stress. This is in contrast to the contribution of the *de novo* pathway to lipoapoptosis of β-cells in ZDF rats [187] or of pancreatic islets exposed to free fatty acids [188,189]. Ongoing studies indicate that NSMase expression is increased in the Akita islet β-cells and thapsigargin-treated iPLA$_2$β-Tg mouse or native human pancreatic islets, relative to corresponding controls.

To determine if the same ceramide-generating mechanism is expressed and is activated in the ER or mitochondria during ER stress, ER and mitochondrial fractions prepared from INS-1 cells were analyzed by mass spectrometry. Such analyses revealed that ER stress induces ceramide generation and sphingomyelin hydrolysis in both the ER and mitochondrial fractions of INS-1 cells. Although the ER and mitochondrial fractions were not completely free of plasma membrane contamination, it is not totally unexpected that both the ER and the mitochondria are capable of generating ceramides via sphingomyelin hydrolysis. The membranes of the nucleus and ER are contiguous [190,191] and they both express NSMase [192,193]. Purified mitochondria from cells exposed to various agents have increased ceramide levels [194] and the contribution of mitochondrial sphingomyelin hydrolysis to ceramide generation and apoptosis has been demonstrated in other studies [195,196]. These observations suggest that the ER and mitochondria express components of sphingolipid metabolism and raise the possibility that these organelles may also contribute to the generation of ceramides via sphingomyelin hydrolysis during apoptosis.

4. Evidence for iPLA$_2$β-induced ceramide generation leading to the mitochondrial pathway of apoptosis

Although ER stress alone can induce the necessary factors to cause apoptosis, it is becoming increasingly apparent that the mitochondria, as an organelle that sequesters Ca^{2+} released from the ER, plays an important role in supporting the apoptosis process initiated by ER stress [197,198]. Recent studies in our lab [44] reveal that both caspase-12 and caspase-3 are activated in INS-1 cells following induction of ER stress with thapsigargin, but only caspase-3 cleavage is amplified in iPLA$_2$β overexpressing INS-1 cells, relative to control cells, and is suppressed

by iPLA$_2$β inhibition. Unexpectedly, ER stress also promoted the release of cytochrome *c* and Smac and their accumulation in the cytosol is amplified in iPLA$_2$β-overexpressing cells. These findings raise the likelihood that iPLA$_2$β participates in ER stress-induced apoptosis by activating the intrinsic apoptotic pathway.

Several lines of study support a link between iPLA$_2$β and mitochondria during apoptosis. The work of Brustovetsky et al. [158] raised the possibility that truncated BID and BAX activate ROS generation, leading to iPLA$_2$β activation in the mitochondria, which promotes changes in the outer mitochondrial membrane (OMM) and release of mitochondrial apoptotic factors into the cytosol [158]. Similarly, mitochondrial-associated iPLA$_2$β was suggested to be activated during energy-dependent Ca^{2+} accumulation leading to opening of the permeability transition pore with sustained activation of iPLA$_2$β leading to rupture of the OMM and release of cytochorome *c* into the cytosol [160]. While a precise mechanism connecting iPLA$_2$β activation with mitochondrial abnormalities was not elaborated in these studies, it has been suggested that iPLA$_2$β-mediated generation of AA causes disruption of membrane integrity [157].

Consistent with this possibility, we find that ER stress promotes iPLA$_2$β accumulation in the mitochondria, opening of mitochondrial permeability transition pore, and loss in mitochondrial membrane potential in INS-1 cells and that these changes are amplified in iPLA$_2$β overexpressing cells. These ER stress-induced mitochondrial abnormalities and apoptosis are suppressed by inactivation of iPLA$_2$β or NSMase. These data suggest that iPLA$_2$β triggers mitochondrial abnormalities through the generation of ceramides via sphingomyelin hydrolysis during ER stress. In support, inhibition of iPLA$_2$β or NSMase prevents cytochrome *c* release. Taken together, our findings indicate that the iPLA$_2$β-ceramide axis plays a critical role in activating the mitochondrial apoptotic pathway in insulin-secreting cells during ER stress (summarized in Fig. 1). Interestingly, in contrast to our findings and those of the others [157, 158,160], Seleznev et al. reported that staurosporine-induced generation of ROS in the mitochondria and apoptosis of INS-1 cells is suppressed by overexpression of iPLA$_2$β. They suggest that staurosporine-mediated down-regulation of iPLA$_2$β results in the loss of mitochondrial membrane repair and that this leads to mitochondrial failure and apoptosis. These contrasting findings suggest that different stimuli may activate different apoptotic pathways in the same or different cell systems.

C. Summary and conclusions

Diabetes mellitus is the most prevalent human metabolic disease, and it results from loss and/or dysfunction of β-cells in pancreatic islets. Type 2 diabetes mellitus (T2DM) results from a progressive decline of β-cell function and chronic insulin resistance. Autopsy studies indicate that β-cell mass in obese T2DM patients is smaller than in obese non-diabetic subjects and that the decrease is not due to reduced β-cell proliferation or neogenesis but to increased β-cell apoptosis [199]. Type 1 diabetes mellitus (T1DM) is caused by autoimmune β-cell destruction and apoptosis plays a prominent role in β-cells loss during its development and cytokine-mediated β-cell apoptosis is a recognized contributor to β-cell death during the development of T1DM [200]. It is therefore important to understand the mechanisms underlying β-cell apoptosis if this process is to be prevented or delayed.

β-cell apoptosis can be mediated not only via death receptors residing in the plasma membrane and/or mitochondrial signaling but as a consequence of prolonged ER stress. A third organelle gaining recognition as a participant in apoptosis is the endoplasmic reticulum (ER) [179]. A number of factors can induce ER stress and this process is thought to cause various diseases, including Alzheimer's and Parkinson's [201]. β-cell death in the Akita [202] and NOD.k iHEL nonimmune [203] diabetic mouse models are reported to be due to ER stress. Further, mutations

in genes encoding the ER-stress transducer pancreatic ER kinase (PERK) [204] and the ER resident protein involved in degradation of malfolded ER proteins have been linked to diminished β-cell health clinically [205,206]. As the secretory function of β-cells endows them with a highly developed ER and the β-cell is one of the most sensitive cells to nitric oxide (NO) [207], it is not unexpected that β-cells exhibit a heightened susceptibility to autoimmune-mediated ER stress. In support of this, Wolfram syndrome, which is associated with juvenile-onset diabetes mellitus, is proposed to be a consequence of chronic ER stress in pancreatic β-cells [208].

Phospholipases A_2 serve an important function in cells by providing lipid mediators (i.e. arachidonic acid) that subsequently participate in a variety of biological processes, including influencing cell survival. Among the PLA_2s is the $iPLA_2\beta$, and our work has revealed that prolonged activation of this enzyme triggers mitochondrial abnormalities that subsequently cause β-cell apoptosis and that this occurs by a novel mechanism involving $iPLA_2\beta$-dependent ceramide generation via sphingomyelin hydrolysis. Continued studies to understand the role of $iPLA_2\beta$ in β-cell apoptosis will enable us to more precisely define its contribution to the onset and progression of diabetes. Findings from such studies will further our knowledge of factors that influence β-cell health in diabetes mellitus and identify potential targets for future therapeutic interventions to prevent β-cell death.

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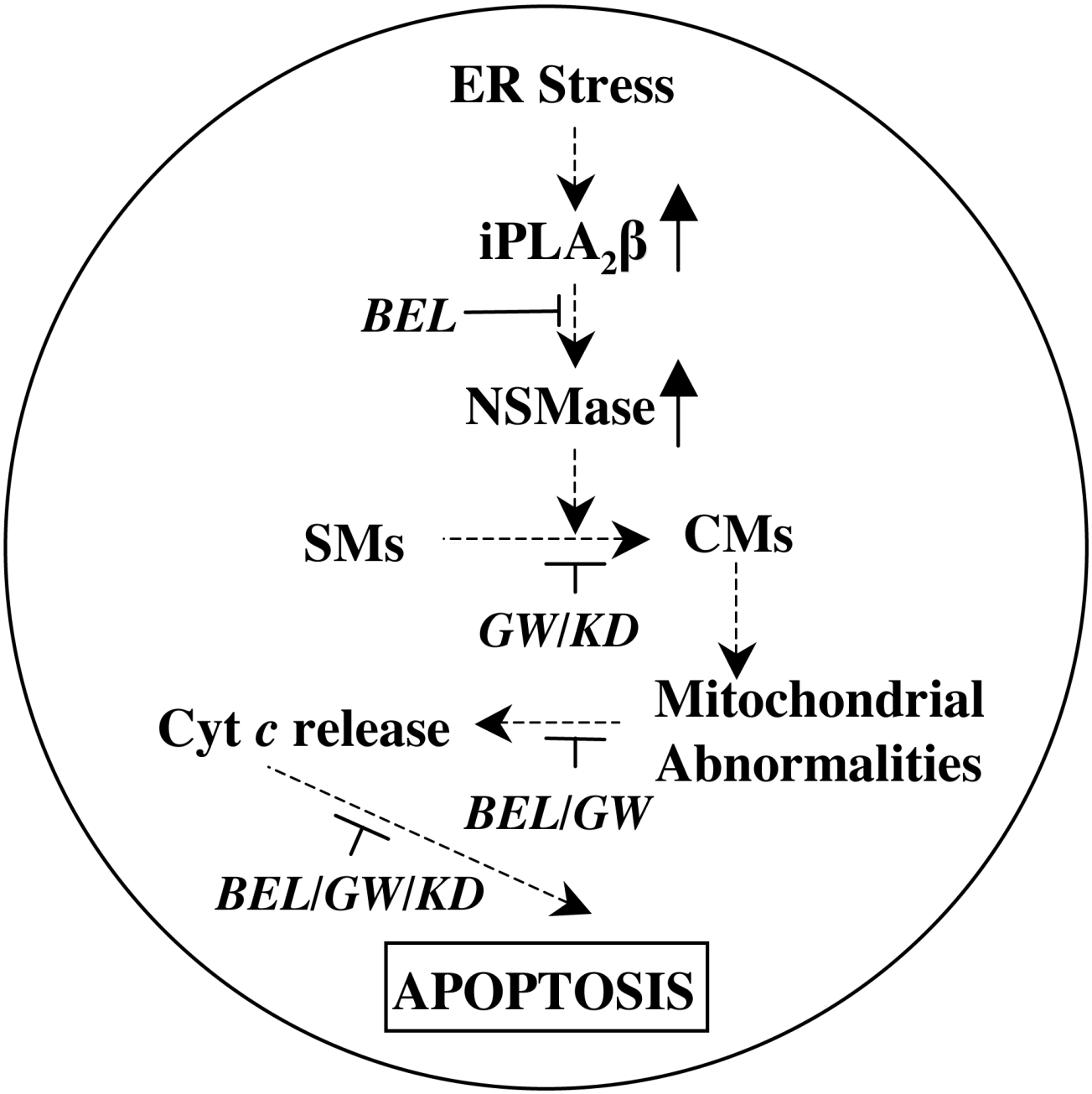

Figure 1. Role of iPLA$_2$β and ceramides in ER stress-induced β-cell apoptosis

Proposed mechanism of iPLA$_2$β and ceramide involvement in ER stress-induced β-cell apoptosis. ER stress in β-cells leads to activation of iPLA$_2$β and this induces neutral sphingomyelinase (NSMase), which promotes generation of ceramides (CMs) via hydrolysis of sphingomyelins (SMs). The ceramides cause mitochondrial membrane depolarization, opening of mitochondrial permeability transition pore, and release of cytochrome *c*. Accumulation of cytochrome *c* in the cytosol leads to activation of caspases and causes apoptosis of the β-cell. (BEL, bromoenol lactone suicide inhibitor of iPLA$_2$β; GW, GW4869 inhibitor of NSMase; KD, knock-down of NSMase with siRNA.)

Table 1

Isoforms of Group VIA PLA_2

VIA	aa	kDa	Ankyrin Repeats	ATP Binding (GXGXXG)	Lipase Activity (GXSXG)	Origin	Refs.
1	752	84–85	8	Yes	Yes	Alt Spl	[18,20,21,30]
2	806	88–90	7	Yes	Yes	Alt Spl	[21,31]
3	640	~70	7–8	Yes	Yes	Alt Spl	[31]
Ank-1	479	~53	7	No	No	Alt Spl	[31]
Ank-2	427	~47	7	No	No	Alt Spl	[31]
“4”	623	~70	7	Yes	Yes	PTM	[32,33]
“5”	~640	~70	8	Yes	Yes	PTM	[34]

Table 2

Biological Processes in Which iPLA$_2$β Participates

Biological Process	Citation
Nerve Degeneration	[109,110]
Insulin Secretion	[60,111,112]
Onset of Acute Pleurisy	[113,114]
Neurotransmission in Hippocampus	[114,115]
Diabetes- and Ischemia-Induced Arrhythmias	[116]
Impairment in Memory Acquisition	[54,117]
Schizophrenia	[118,119]
Muscle Degeneration	[120]
Skeletal Muscle Contractility	[121,122]
HIV-Induced Cardiomyopathy	[122]
Drip Formation in Muscle	[123]
Photoreceptor Cell Renewal	[89]
Exfoliation Glaucoma	[124]
Bipolar Disorder and Neuroinflammation	[90,125]
Infantile Neuroaxonal Dystrophy	[126–128]
Bone Formation	[129]
Skeletal Muscle Fatty Acid Oxidation	[38]
Chemotaxis	[130–132]
Store-Operated Ca^{2+} Entry	[133,134]
Neurodegeneration Associated with Brain Iron Accumulation	[135]
Vascular smooth muscle cell contraction	[136]

Table 3

Evidence for iPLA$_2$β involvement in apoptosis

Year	Citation	Stimulus	System
1998	[78]	Fas	Human leukemic monocyte lymphoma U937 cells
2000	[32]	Fas & TNF/CHX	Human leukemic monocyte lymphoma U937 cells
2000	[154]	Thapsigargin	T cell lymphoma S49 cells
2002	[155]	Polychlorinated biphenyls	Rat pheochromocytoma PC12 cells
2002	[139]	Polycyclic aromatic hydrocarbons	Human coronary artery endothelial cells
2003	[40]	Hypoxia	Rat pheochromocytoma PC12 cells & Mouse cerebellar granule neurons
2004	[138]	H_2O_2	Human leukemic monocyte lymphoma U937 cells
2005	[156]	Chemotherapeutic drugs	Human renal cell models
2005	[157]	Cancer	Human cancer cells
2005	[158]	ROS generation	Non-synaptosomal brain mitochondria
2006	[159]	iPLA$_2$ siRNA	Human epithelial cells (HEK 293 and Caki-1)
2006	[160]	Depolarization and Ca^{2+} accumulation	Rat liver mitochondria
2006	[161]	iPLA$_2$β overexpression	Human leukemic monocyte lymphoma U937 cells
2006–2008	[140,144,162–164]	p53/p21 mediated cell cycle arrest and cell growth	Cancer cell lines
2007	[163]	Virus	Human hepatoma (Huh7) and breast adenocarcinoma (MCF7)
2008	[137]	Free cholesterol loading	Murine macrophages
2009	[165]	Extracellular ATP	Murine macrophages