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iPLA2β, ALTERNATIVE SPLICING AND APOPTOSIS OF PANCREATIC ISLETS

Bhargavi Emani
Virginia Commonwealth University

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iPLA$_2$β, ALTERNATIVE SPlicing AND APOPTOSIS OF PANCREATIC ISLETS

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biochemistry and Molecular Biology at Virginia Commonwealth University.

by

BHARGAVI EMANI
Master of Science, Microbiology, Osmania University, India, 2007

Director: SUZANNE E. BARBOUR, PH.D

PROFESSOR

DEPARTMENT OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

Virginia Commonwealth University
Richmond, Virginia
August, 2010
Acknowledgement

I would like to thank my thesis adviser Dr. Suzanne Barbour for giving me the opportunity to be a part of her laboratory. I especially thank her for the constant support, encouragement and most of all the friendship she has given me through my learning years here at VCU. She has not only been a terrific guide to me but also a great source of inspiration. Dr. Barbour’s commitment, dedication and passion for science have kept me motivated to be a better scientist. I will be forever grateful for all the valuable knowledge I have received by being a part of Dr. Barbour’s lab.

I would also like to thank my committee members Dr. Charles Chalfant and Dr. Daniel Conrad for their constructive criticism and for helping me with my project. I would also like to give my thanks to our collaborator Dr. Sasanka Ramanadham for all his words of encouragement and also providing me with the wonderful opportunity of presenting my work at an international conference. It has truly been an enriching experience. A special thank you to Dr. Xiaoyong Lei as well for providing me with many of the samples I worked with for this project.

I would especially like to give a shout out to all the members of Dr. Barbour’s lab that I have worked with. Dr. Rachael Griffiths and Ms. Andria Chambers have been a tremendous source of support, knowledge and guidance throughout my interaction with them. My co-workers, Minkyeong Son, Latonya Marshall, Dr. Palmer Wilkins III,
Vasudha Surampudi and Marianna Sukholutsky have made lab an enjoyable experience. I would like to show my sincere appreciation for Marianna’s friendship and thank her for being “my partner in crime.”

I would finally like to thank my entire family; my parents, Lakshmi R. and Suryanarayana S. Emani, my sister, Sarvani Emani and my husband Rajasekhar Challa for their undying love and support. I want to give a special mention to my husband, whose relentless and unflaunting encouragement has kept me going in some of my most difficult times as a student. I will always be indebted to you all for everything you have given me. You help me strive to be a better person every day.
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Ceramides are bioactive lipids that can promote splicing of apoptosis-related genes, including caspase 9 and BCL-x. A recent study demonstrated that expression of neutral sphingomyelinase (NSMase), an enzyme that hydrolyzes sphingomyelins to generate ceramide, is regulated by Group VIA phospholipase A2 (iPLA2β)-dependent mechanism during β-cell apoptosis. This prompted us to hypothesize that iPLA2 is upstream of ceramide generation in the process regulating splicing of apoptotic genes. To test this, Jurkat T cells were treated with the selective inhibitor of iPLA2β, bromoenol lactone (BEL), RNA was isolated and converted to cDNA, and caspase 9 and BCL-x mRNA
species were amplified using RT-PCR. Inhibition of iPLA$_2$β activity with BEL caused a significant shift in splicing favoring variants encoding the anti-apoptotic forms of caspase 9 (caspase 9b) and BCL-x (BCL-x(L)). This shift was consistent with previously reported effects of ceramide and suggested that iPLA$_2$β regulates splicing of these pre-mRNAs. We next determined whether iPLA$_2$β regulates splicing events during a biological response. Caspase-9 and BCL-x splice variants were compared in human and mouse islets, mouse islet cell lines, and in rat insulinoma (INS1) cells.

INS-1 insulinoma cells were treated with thapsigargin to induce ER stress, which can eventually lead to apoptosis. Thapsigargin-treated INS-1 cells exhibited an increase in the ratio of BCL-x(s) (pro-apoptotic) to BCL-x(L) (anti-apoptotic) but BEL prevented this shift in splicing. Splicing data obtained from genetically modified rodent mice (iPLA$_2$β knockouts and transgenics) also demonstrated the involvement of iPLA$_2$β in alternative splicing.

Together, these observations indicate that iPLA$_2$β plays an important role in the regulation of pre-mRNA splicing of key apoptotic factors. Our findings therefore suggest a novel role for iPLA$_2$β in determining whether cells survive or undergo apoptosis.
CHAPTER 1: INTRODUCTION

1.1 PHOSPHOLIPASES

Phospholipases are enzymes that hydrolyze phospholipids into fatty acids and other lipophilic substances (FIGURE 1A). There are four major classes, termed A, B, C and D distinguished by what type of reaction they catalyze (FIGURE 1B). Phospholipase A includes two groups of enzymes Phospholipase A₁ (PLA₁) and phospholipase A₂ (PLA₂). Phospholipase A₁ cleaves the phospholipids at the SN-1 acyl position and phospholipase A₂ cleaves the SN-2 acyl position. Phospholipase B cleaves both SN-1 and SN-2 acyl chains and Phospholipase C cleaves before the phosphate, releasing diacylglycerol and a phosphate-containing head group. Phospholipase Cs play a central role in signal transduction, releasing the second messenger inositol triphosphate. Phospholipase D cleaves after the phosphate, releasing phosphatidic acid and an alcohol.

Phospholipase A₂ (PLA₂) enzymes are commonly found in mammalian tissues as well as insect and snake venom. This family of enzymes, as described earlier, cleaves the phospholipids at the SN-2 acyl chain to release unsaturated fatty acids and lysophospholipids. The common fatty acids released by this hydrolysis reaction are arachidonic acid (AA) and oleic acid (OA). Arachidonic acid can function as a messenger...
and precursor of various bioactive pro-inflammatory compounds called eicosanoids (including prostaglandins, thromboxanes, lipoxins, and leukotrienes). These are known to mediate inflammation and signal transduction. Lysophospholipids, such as lysophosphatidic acid (LPA) and lysophosphatidylcholine (LPC), play important roles in cell signaling, phospholipid remodeling, and membrane perturbation (Six and Dennis., 2000 and Kudo and Murakami., 2002).

The PLA₂ family can further be categorized into three classes based on their location and dependence on calcium levels.

Secreted phospholipase A₂ (sPLA₂) is the extracellular form of PLA₂ that requires the presence of large (mM) amounts of calcium for its function. There have been 11 isoforms identified so far and they have low molecular masses ranging from 14-17KDa. These enzymes play critical roles in several biological processes like eicosanoid generation, inflammation and host defense (Six et al 2000).

Cytosolic phospholipase A₂ (cPLA₂) is an intracellular form of the enzyme that is also calcium dependent for its function. This category of phospholipases is involved in cell signaling processes, such as inflammatory responses through the initiation of arachidonic acid metabolism. This family of enzymes shows a preference towards arachidonic acids compared to other fatty acids for hydrolysis at the SN-2 acyl positions. These are also known as Group IV PLA₂s and there are six identified isoforms (cPLA₂α,
cPLA$_2$,$ \beta$, cPLA$_2$$\gamma$, cPLA$_2$$\delta$, cPLA$_2$$\varepsilon$, cPLA$_2$$\zeta$). They are large proteins ranging from 61-114KDa but are usually found at 85KDa. (Fuentes et al., 2002)

Calcium independent phospholipase A$_2$ (iPLA$_2$) is a Group VI, intracellular phospholipase A$_2$ that is not dependent on calcium for its activity. It is a key player in glycerophospholipid and energy metabolism. There are six different isoforms all of which have masses ranging from 84 to 88KDa (Wilkins and Barbour, 2008).

Our focus is mainly going to be on iPLA$_2$ and is discussed in further detail below.
FIGURE 1A:

Phospholipase Cleavage Sites

FIGURE 1B:

REACTION CATALYZED BY PLA$_2$
1.2 CALCIUM INDEPENDENT PHOSPHOLIPASE A2 (iPLA2)

Group VI phospholipase A2 (iPLA2) is a calcium independent, intracellular enzyme that breaks down phospholipids to lysophospholipids and unsaturated fatty acids. It is the least characterized of all the PLA2s. It was first isolated and characterized from murine P388D1 macrophage like cells (Ackermann et al., 1994).

This enzyme has the ability to hydrolyze both SN 1 and SN 2 fatty acids, but it shows a preference toward SN 2 fatty acids. (Ackermann et al.1994). In addition to its phospholipase activity, it also exhibits lysophospholipase and transacylase activities (Lio et al., 1998) and an acyl-CoA thioesterase activity (Carper et al., 2008).

According to the current classification, iPLA2 products from different genes have been designated as the following: VIA or iPLA2β, VIB or iPLA2γ, VIC or iPLA2δ, VID or iPLA2ε, VIE or iPLA2ζ, and VIF or iPLA2η.

Several structural elements of the enzyme have been identified that help us gain a better understanding about the functional aspects of iPLA2 (FIGURE 2). The active site of the iPLA2 consists of the consensus lipase motif GXS\textsuperscript{465}XG (Ser\textsuperscript{519} in the human isoform) (Tang et al.,1997). iPLA2 also contains a string of eight successive ankyrin repeats that are thought to be involved in protein-protein interactions (Larson et al.,1998) These repeats have also shown to be important for the enzymatic activity (Tang et al.,1997). These observations have led several groups to hypothesize that the ankyrin repeats play a role in the oligomerization of the protein. iPLA2 also consists of an ATP binding domain
(the enzyme requires the presence of ATP for optimal activity), a caspase 3 cleavage site, proline rich region, and a bipartite nuclear localization domain

iPLA₂ pre-mRNA undergoes extensive alternative splicing that produces several mRNA transcripts that encode multiple isoforms with several distinct properties. There are two iPLA₂ human isoforms-long and short. The human iPLA₂ gene extends across a total of about 70 kilobases of DNA and contains 17 exons. (Ma et al., 1999).

In 1998, two novel splice variants of iPLA₂ were identified (Larsson et al., 1998) which were named ankyrin iPLA₂-1 and ankyrin iPLA₂-2. These sequences contain an additional exon which was then considered to be exon 10, which encodes an in-frame premature stop codon, as a result of which there is translational termination and expression of a truncated protein.

Recent studies have demonstrated that the truncated iPLA₂ protein is as a result of inclusion of exon 14 and not exon 10 (FIGURE 3). This protein has the N terminus ankyrin repeats but lacks the C- terminus lipase motif and hence is catalytically inactive. The protein has been identified to be about 45KD in size. The truncated protein product of ankyrin iPLA₂ has been shown to act as a dominant negative of the full-length iPLA₂ (Larsson et al. 1998). Studies from the same group have shown that the ankyrin iPLA₂ protein can negatively regulate the activity of the full length iPLA₂ (Larsson et al. 1998).

Previous studies have shown that iPLA₂ activity is cell cycle dependent (Manguikian et al., 2004). It was shown that the iPLA₂ splice variant mRNA accumulates at the G2/M phase of the cell cycle where the iPLA₂ activity is the highest. This poses the question of
the possibility of the downstream products of the iPLA$_2$ reaction regulating the alternative splicing of the iPLA$_2$ pre-mRNA and thereby its activity.

There are several biological functions of iPLA$_2$. The first is phospholipid remodeling (Balsnide 2002) which is a process, other than de novo synthesis, through which fatty acids can be incorporated into phospholipids. Regulation of phosphotidylcholine (PC) biosynthesis is also mediated by iPLA$_2$ (Anthony et al., 1999). iPLA$_2$ leads to the release of arachidonic acid that triggers certain mitogen-activated protein kinases which activate proteins essential for cell division, by phosphorylating them. It is therefore involved in cell proliferation (Balboa et al., 2008, Roshak et al., 2000, Sanchez et al., 2002, Song et al., 2007). Several other studies have shown that iPLA$_2$ is a key player in signal transduction (Maggi Jr. et al. 2002, Atsumi et al. 1998, Jenkins et al. 2009).

A key instrument used to perform all of these studies and gain a better understanding about the function of the enzyme is the iPLA$_2$ selective inhibitor bromoenol lactone (BEL). BEL is a suicide (irreversible) inhibitor that selectively targets iPLA$_2$. It shows a thousand fold more selectivity for iPLA$_2$ than cPLA$_2$ and sPLA$_2$. The S-enantiomer inhibits iPLA$_2$$\beta$, while the R-enantiomer inhibits iPLA$_2$$\gamma$ (Jenkins et al., 2002). The mode of action of BEL is thought to be via the acylation of the Serine residue present on the active site of the enzyme (Daniels et al., 1983 and Baek et al., 1990).
BEL has been shown to inhibit the PLA$_2$, transacylase and other catalytic activities associated with iPLA$_2$ (Lio et al 1998, Jenkins et al 2006, Hazen et al 1991). This suggests that all these activities are likely mediated through the same active site. BEL has more recently been shown to inhibit serine proteases, magnesium dependent phosphotidate phosphohydrolase 1 and serine lipases. Despite this, BEL continues to remain one of the most widely used selective inhibitors of iPLA$_2$ (Song et al., 2006).
FIGURE 2:

STRUCTURAL ELEMENTS OF iPLA$_2$

- Caspase-3 Cleavage site
  - DVTD$^{41}$Y
- Lipase consensus motif
  - GTS$^{54}$YG

- Ankyrin Repeat Domain
- ATP Binding Domain
- Bipartite Nuclear Localization Domain
The iPLA₂ pre-mRNA is alternatively spliced and generates the splice variant Ankyrin iPLA₂. The splice variants are produced when alternative splicing incorporates exon 14 (bold lines) which encodes an in-frame stop codon that generates an iPLA₂ protein that retains the ankyrin repeats but not the lipase motif. This protein is catalytically inactive and functions as a dominant negative of the catalytically active full-length protein.
1.3 INVOLVEMENT OF iPLA₂β IN APOPTOSIS OF PANCREATIC β-CELLS

The term apoptosis or programmed cell death was first coined in 1972 by John Kerr, Andrew Wilie and Alashair Currie. They observed different cell types undergo distinct morphological changes that eventually led to death differing significantly from necrosis. Apoptosis is characterized by a series of typical morphological features, such as shrinkage of the cell, membrane blebbing, fragmentation into membrane-bound apoptotic bodies and rapid phagocytosis by neighboring cells. Factors such as heat, radiation, nutrient deprivation, viral infection, hypoxia and increased intracellular calcium concentration drive cells toward apoptosis (Savil et al 1997).

Two important pathways through which apoptosis is mediated have been identified (Danial and Korsmeyer., 2004). They include intrinsic apoptotic pathway and the extrinsic pathway. The intrinsic pathway is mediated via the mitochondria and is characterized by permeabilisation of the mitochondria and release of cytochrome c into the cytoplasm. Cytochrome c then forms a multi-protein complex known as the ‘apoptosome’ and initiates activation of the caspase cascade through caspase 9 (Kroemer, Galluzzi, and Brenner., 2007)

The extrinsic apoptotic pathway takes place via the activation of cell death receptors on the plasma membrane such as tumor necrosis factor receptor 1 (TNFR1) and Fas/CD95. As ligands bind to these receptors, the death inducing signaling complex (DISC) is formed
leading to initiation of the caspase cascade through caspase 8 (Taylor, Cullen, and Martin., 2008)

Recently a third mechanism through which apoptosis might take place has been identified; the ER stress pathway. The Endoplasmic Reticulum is critical for most cellular activities and cell survival. Conditions that interfere with ER function lead to the accumulation and aggregation of unfolded proteins. ER transmembrane receptors detect the onset of ER stress and initiate the unfolded protein response (UPR) to restore normal ER function. If the stress is prolonged, or the adaptive response fails, apoptosis takes place (Szegezdi et al., 2006). Several diseases including Alzheimer’s and Parkinson’s diseases, cancer, and obesity have been associated with ER stress induced apoptosis. (Hosoi and Ozawa, 2009). ER stress has also been shown to cause β-cell apoptosis eventually resulting in diabetes mellitus. Causes of both Type I and Type II diabetes have been attributed to loss of β-cell mass due to apoptosis. By nature of their secretory function, β-cells have very greatly developed ER which makes them highly sensitive to ER stress.

One of the tools used to study ER stress is the SERCA inhibitor Thapsigargin. Thapsigargin is non-competitive inhibitor of a class of enzymes known by the acronym SERCA, which stands for sarco / endoplasmic reticulum Ca\(^{2+}\) ATPase (Rogers et al 1995). Thapsigargin blocks the ability of the cell to pump calcium into the sarcoplasmic and endoplasmic reticula which causes these stores to become depleted. These conditions lead to ER stress.
One of the first studies that provided evidence for the involvement of iPLA$_2$$\beta$ in ER stress induced apoptosis was performed by Polonsky (Zhou et al, 1998) and Kudo (Atsumi et al, 2000). They showed that there was an induction of apoptosis in MIN-6 insulinoma cell when they were treated with thapsigargin and other SERCA inhibitors. This took place via a mechanism that involved the generation of arachadonic acid metabolites. They also noted that this mechanism was independent of intracellular calcium levels. This gave them reason to suspect the involvement of a PLA$_2$ that has activity even in the absence of calcium.

This was later confirmed in studies performed using U937 cell lines (human monocytes), where over expression of iPLA$_2$$\beta$ and not cPLA$_2$ increased the occurrence of apoptosis in response to TNF$\alpha$/cycloheximide. Immunoblot analyses revealed the presence of the full length iPLA$_2$$\beta$ band. (Atsumi et al, 2000).

Subsequent studies from Ramanadham group have shown that INS-1 (rat insulinoma) cells over-expressing iPLA$_2$$\beta$ have higher incidence of apoptosis upon treatment with thapsigargin. This apoptosis was suppressed with BEL (selective inhibitor for iPLA$_2$), providing further evidence that iPLA$_2$$\beta$ plays a role in ER stress induced apoptosis in yet another cell line (Ramanadham et al., 2004).
1.4. CASPASE 9 AND BCL-x

Among the key players in apoptosis are caspase 9 and BCL-x, both of which are under the influence of the biologically active lipid, ceramide.

Caspases, or cysteine-aspartic proteases, are a family of cysteine proteases, which play essential roles in apoptosis (programmed cell death), necrosis and inflammation. Caspase-9 is an initiator caspase, encoded by the CASP9 gene. The human caspase 9 gene undergoes alternative splicing to produce two dominant-negative splice-variants designated caspase 9a and caspase 9b. These splice-variants arise as an effect of inclusion or exclusion of the 3, 4, 5, and 6 cassettes from the caspase 9 gene (Figure 4). The larger splice-variant (9a, with 3,4,5,6 cassette inclusion) is pro-apoptotic, while the smaller splice-variant (9b with 3,4,5,6 cassette exclusion) is anti-apoptotic (Seol et al 1990).

BCL-x, a member of the BCL2 family, also plays a role in apoptosis. The proteins of the BCL 2 family govern mitochondrial outer membrane permeabilization (MOMP) and can be either pro-apoptotic (Bax, BAD, Bak and Bok among others) or anti-apoptotic (including BCL-2 proper, BCL-xL, and BCL-w) (Zamzami et al 1998).

The anti-apoptotic factor, BCL-x(L) was shown to have a dominant negative splice-variant, called BCL-x(s) (Minn et al., 1996). These splice variants arise as a consequence of alternative splicing of the BCL-x gene (FIGURE 5). There is a difference in the preference for the 5’ splice site in Exon two of this gene due to which pro-apoptotic variant (BCL-x(s)) lacks an exon in the coding region when compared to the anti-apoptotic variant (BCL-x(L)).
The human caspase 9 gene undergoes cassette splicing where inclusion of the 3.4.5.6 cassette gives rise to the larger, pro-apoptotic splice variant, caspase 9a while its exclusion produces the smaller, anti-apoptotic splice variant, caspase 9b.
Based on the difference in the preference for the 5’ splice site, two BCL-x splice variants arise; BCL-x(L) is the larger anti-apoptotic splice variant and BCL-x(s) is the smaller pro-apoptotic splice variant.
In 2002, Chalfant group showed that caspase 9 and BCL-x genes were under the influence of ceramide. They demonstrated that A 549 lung adenocarcinoma cells, when treated with short chain ceramide underwent alternative splicing to produce the pro-apoptotic splice variants of both genes and also went on to delineate the mechanism by which ceramide regulates alternative-splicing of these genes (Chalfant et al., 2002). According to this mechanism, ceramides activate protein phosphotases (like PP1) which dephosphorylate splicing proteins (such as SR proteins) and eventually leading to alternative splicing (Chalfant et al 2002).

Ceramides are a family of lipid molecules. A ceramide is composed of sphingosine and a fatty acid. Ceramides are found in high concentrations within the cell membrane of cells. They are one of the component lipids that make up sphingomyelin, one of the major lipids in the lipid bilayer. Perhaps one of the most fascinating aspects of ceramide is that it can act as a signaling molecule.

There are three major pathways of ceramide generation. The sphingomyelinase pathway uses an enzyme to breakdown sphingomyelin in the cell membrane and release ceramide. The de novo pathway creates ceramide from less complex molecules like palmitate. Ceramide generation can also occur through breakdown of complex sphingolipids that are ultimately broken down into sphingosine, which is then reused by reacylation to form ceramide. This latter pathway is termed the Salvage pathway (Kitatani et al 2008).

Dr. Ramanadham’s group in 2004 demonstrated that iPLA₂β leads to the accumulation of ceramides. They showed that INS-1 cells over expressing iPLA₂β had greater levels of
ceramides upon treatment with ER stress inducer thapsigargin, when compared to the cells transfected with vector alone. More importantly, they showed that these elevated levels can be dramatically suppressed with the iPLA$_2$$\beta$ inhibitor BEL. (Ramanadham et al 2004). They also pointed out that the generation of ceramides during ER stress takes place via the induction of Neutral Sphingomyelinase (NSMase) which breaks down sphingomyelins to release ceramides. Interestingly enough, they observed that BEL treatment not only suppressed ceramide levels, but also NSMase levels (Lei et al., 2008).

This suggests that iPLA$_2$$\beta$ is upstream of NSMase and that the induction of NSMase during ER stress is iPLA$_2$$\beta$ dependent.
CHAPTER 2: HYPOTHESIS

Evidence from previous studies gives us reason to believe that iPLA$_2\beta$ plays a role in apoptosis and is upstream of ceramides. We hypothesize that iPLA$_2\beta$ is involved in cell apoptosis by modulating alternative splicing of BCL-x and Caspase 9 genes in a ceramide mediated manner. The ceramides generated trigger the alternative splicing events of BCL-x and Caspase 9 to favor the production of pro-apoptotic splice variants that play a significant role in cell death.
CHAPTER 3: MATERIALS AND METHODS

3.1 CELL CULTURE AND TREATMENTS: Jurkat cells were cultured in modified RPMI-1640 medium (Cellgro), supplemented with 10% heat inactivated fetal calf serum (FCS), 2mM L-glutamine (Gln), 100µg/mL penicillin and 100µg/mL streptomycin (P/S), at 5% CO₂, 100% humidity and 37°C. Jurkat cells were treated with 10µM BEL overnight.

INS1 cells (rat insulinoma cell line) were cultured in RPMI 1640 media with 10% heat inactivated FCS, 2mM L-Gln, 100 µg/mL each P/S, 10mM HEPES, 1mM sodium pyruvate, 11mM glucose and 50µM β-mercaptoethanol.

Cells were treated with 10µM bromoenol lactone (BEL, selective iPLA2 inhibitor), 20µM C6 ceramide (16 hrs) and 10µM Thapsigargin (ER stress inducer) as indicated.

Human Embryonic Kidney (HEK) cells used for the mini-gene experiments were maintained in modified DMEM (Cellgro) supplemented with 10% heat-inactivated FCS, 2mM L-Gln, 100µg/mL each P/S, at 5% CO₂, 100% humidity and 37°C.

3.2 MOUSE ISLETS: RNA samples from mouse islets were obtained from Dr. Ramanadham, Washington University, St. Louis, MO.

All animal experiments were performed in accordance with IACUC regulations of WUSTL. Pancreas were harvested from 4-5 week old male mice and islets prepared as
described (Lei, 2010). Islets were cultured in RPMI 1640 media with 10% FCS and P/S. Where indicated, islets were treated with 0.2mM thapsigargin.

3.3 RT-PCR ANALYSIS: RNA was isolated with Trizol-LS and was converted to cDNA with oligo dT primer using the Thermoscript cDNA kit. The cDNA was then amplified using the Accuprime Taq PCR kit and gene-specific primers (Table 1) at their respective annealing temperatures.

The amplified samples were analyzed using 6% acrylamide gel electrophoresis at a 100 volts with 0.5x TBE as the running buffer, followed by staining with sybr gold (a 1:10 dilution of the solution made with the running buffer). Images were collected with a Typhoon scanner and signals were quantified with Image Quant software.

3.4 BCL-x MINIGENE: A Bcl-x minigene construct was obtained from C. Chalfant (VCU). The minigene consists of exon 1, intron 1, exon 2, and portions of intron 2 and exon 3 (Massiello, 2003). The minigene was transfected into HEK cells using Fugene 6 reagent. Transfected cells were treated as indicated and RNA was isolated and converted to cDNA with oligo dT primers. The resulting cDNA was then amplified by PCR with the Bcl-x minigene primers whose sequences are 5’ aaacttaagcttggtaccgag 3’ (forward) and 5’ ggagctggtggtgacctttc 3’ (reverse) (Massiello, 2003). The amplified samples were analyzed by acrylamide gel electrophoresis. The amplified samples were analyzed by acrylamide gel electrophoresis, as described earlier. Images were collected with a Typhoon scanner and signals were quantified with Image Quant software.
3.5 IMMUNOBLOT ANALYSES: Cells were harvested, sonicated, and an aliquot (30-90 μg) of lysate protein was analyzed by SDS-PAGE (10 or 15%), transferred onto nitrocellulose membrane and analyzed for the desired protein. The primary antibody used for Caspase 9 (1:800) was purchased from Assay designs. An HRP tagged anti-mouse secondary was used. The proteins were detected using Femto.
TABLE 1:
PRIMER SEQUENCES USED

<table>
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<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing temperature</th>
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<tbody>
<tr>
<td>Human BCL-x</td>
<td>Forward: 5’gaggccagcgcagcagttgaa 3’</td>
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</tr>
<tr>
<td></td>
<td>Reverse: 5’tgaggaggtaggtgtgtg 3’</td>
<td></td>
</tr>
<tr>
<td>Rat BCL-x</td>
<td>Forward: 5’ggagacactgatgctg 3’</td>
<td>48 °C</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’ cagtgatgtaaagaga 3’</td>
<td></td>
</tr>
<tr>
<td>Mouse BCL-x</td>
<td>Forward: 5’atccacaaagtcacctgc 3’</td>
<td>54 °C</td>
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<tr>
<td></td>
<td>Reverse: 5’cctcagagatcaccacaaagtctc 3’</td>
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<tr>
<td>Human Caspase 9</td>
<td>Forward: 5’gctcttcttcttcatcc 3’</td>
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<tr>
<td></td>
<td>Reverse: 5’catctgctctcggagtttctgc 3’</td>
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CHAPTER 4: RESULTS

Previous studies have shown that ceramides regulate alternative splicing of caspase 9 and BCL-x (Chalfant et al 2002). Studies have also correlated iPLA₂ activity with ceramide production via the generation of unsaturated fatty acids that induce sphingomyelinase (Ramanadham et al 2004 and Lei et al 2008). These evidences raise a question about the potential involvement of iPLA₂ in regulating alternative splicing of caspase 9 and BCLx.

One of the first indications we received about the role of iPLA₂β in alternative splicing, was from Jurkat T cells. These cells were treated with the iPLA₂β inhibitor BEL overnight, RNA was isolated and converted to cDNA. The cDNA was then subjected to a PCR analysis with caspase 9 and BCL-x primers to amplify for their pro-apoptotic and anti-apoptotic splice variants. We observed that there was a dramatic shift toward the anti-apoptotic splice variants in both Caspase 9 and BCL-x upon treatment with BEL, when compared to those treated with vehicle (DMSO) alone. That is, inhibition of iPLA₂β in these cells produced a shift in splicing of the Caspase 9 and BCL-x genes to their anti-apoptotic forms. This gave us the reason to believe that iPLA₂β is involved in the alternative splicing of Caspase 9 and BCL-x. (FIGURE 6)
4.1 REGULATION OF BCL-x SPLICING:

We next wanted to apply our newly acquired knowledge to a system of a greater physiological relevance: ER stress induced apoptosis of the β-cell. As previously mentioned, it has been shown that thapsigargin-induced apoptosis in INS-1 cells, can be significantly suppressed by the iPLA₂β inhibitor BEL (Ramanadham et al 2004). It was also shown that there is an increased accumulation of ceramides with thapsigargin treatment in cells that are over-expressing iPLA₂β when compared to the cells transfected with vector alone (Ramanadham et al 2004). The Ramanadham group also demonstrated that this accumulation of ceramides could be blocked by inhibiting iPLA₂β with BEL.

Putting two and two together, we asked ourselves if iPLA₂β is bringing about apoptosis in these cells by regulating alternative splicing of the apoptotic genes caspase 9 and BCL-x.

To test our hypothesis, we treated INS-1 cells with BEL and/or thapsigargin, isolated RNA and subjected it to RT-PCR analysis of the BCL-x splice variants. We observed that the ER stress inducer, thapsigargin shifts splicing toward the pro-apoptotic splice variant (BCL-x(s)), more importantly, this shift was prevented by the iPLA₂β inhibitor BEL. We also noticed that BEL by itself had the ability to shift splicing to the anti-apoptotic form of BCL-x. (BCL-x(L)). Similar shifts in BCL-x splicing were observed in the human systems as well. While thapsigargin produced more of the pro-apoptotic BCL-x(s), inhibition of iPLA₂β with BEL prior to thapsigargin treatment shifted splicing in the opposite direction; that is toward the anti-apoptotic BCL-x(L).
(FIGURE 7). These data strengthen our hypothesis about the involvement of iPLA₂β in alternative splicing of BCL-x. While ER stress drives splicing toward pro-apoptotic BCL-x(s), inhibition of iPLA₂β drives splicing in the opposite direction; toward the anti-apoptotic BCL-x(L).

Our study thus far has been focused on the chemical inhibition of iPLA₂β with BEL. But, as mentioned previously, BEL also shows certain non-specific effects. It was hence essential for us to look at certain genetically modified rodent models to confirm the proposed role for iPLA₂β in alternative splicing.

RNA from iPLA₂β knockout and transgenic mice islets was obtained from Dr. Ramanadham at Washington University, St.Louis MO. The islets were cultured in the presence or absence of thapsigargin.

We converted the RNA to cDNA and subjected it to PCR analysis of mouse BCL-x and compared the ratios of the splice-variants with that of the wild type islets. In the wild type islets, thapsigargin shifts splicing toward the pro-apoptotic splice variant, BCL-x(s) as compared to the untreated cells that had very low levels of BCL-x(s). In the transgenic islets, there was a significant level of the pro-apoptotic signal even in the untreated samples. Conversely, in the iPLA₂β knockouts, there was negligible amount of BCL-x(s) present even after treatment with thapsigargin (FIGURE 8).

These observations illustrate that iPLA₂β regulates alternative splicing of the BCL-x gene, toward the pro-apoptotic splice variant (BCL-x(s)) and knockdown of iPLA₂β shifts splicing in the opposite direction.
Up until now, ER stress in cells was artificially induced using thapsigargin. This is not a fool proof phenomenon as there could be other non-specific targets of the drug. We hence decided to see if our observations hold true in systems that are naturally undergoing ER stress. One such system is the Akita mouse. Akita mice develop diabetes as a consequence of a single base pair substitution in the Ins2 gene, resulting in improper folding of pro-insulin which leads to protein aggregate-induced ER stress in the pancreatic islets (Koizumi et al, 1997).

It was recently shown that thapsigargin accelerates apoptosis of Akita β-cells and that this is associated with increased iPLA2β expression (Lei et al, 2010). We thus wanted to see if BCL-x splicing correlates with the above data. RT-PCR analysis of RNA from Akita mice showed an increase in ratio of BCL-x(s) (pro-) to BCL-x(L) (anti-apoptotic) in the Akita islets when compared to the wild type (FIGURE 9). This observation may be attributed to the fact that there are elevated levels of iPLA2β expression in the Akita islets, compared to the wild type (Lei et al 2009), which subsequently leads to a shift toward the pro-apoptotic splice variant of BCL-x thereby increasing the ratio of BCL-x(s) (pro) to BCL-x(L) (anti).

We also developed a transfection protocol in the HEK cells for the BCL-x mini-gene that was obtained from Dr. Chalfant. This mini-gene is constructed such that it mimics the endogenous BCL-x. The mini-gene like the endogenous BCL-x also shows a
difference in the 5’ splice site so that two splice variants that are comparable to the larger BCL-x(L) which is anti-apoptotic and the smaller BCL-x(s) which is pro-apoptotic.

The PCR products of the mini-gene splice variants are 751 bp (x(L)) and 565 bp (x(s)) (Massiello et al, 2004).

On performing an RT PCR analysis on the RNA isolated from HEK cells transfected with the mini-gene, we detected a shift in splicing toward the larger (anti-apoptotic) splice variant in the BEL (iPLA2β inhibitor) treated sample (FIGURE 10). This observation now gives us the tool to direct our future studies toward the mechanistic aspects of the splicing events of BCL-x.

4.2 REGULATION OF CASPASE 9 SPlicing:
iPLA2β also seems to be regulating the splicing events of caspase 9. In the human system, a simple RT PCR analysis of caspase 9 message from RNA isolated from human islets revealed a shift in splicing toward caspase 9a with thapsigargin treatment. This shift was blocked by the iPLA2β inhibitor BEL (FIGURE 11). Therefore, there is an increase in the pro-apoptotic message of Caspase 9 in human islets under ER stress conditions and inhibition of iPLA2β in such conditions blocks this effect and brings down the ratio of pro-apoptotic (9a) to anti-apoptotic (9b).

Although we do not have RT PCR tools to quantify for caspase 9 messages in the rat system, we were able to perform western analysis of the caspase 9 protein in INS-1 cells. The caspase 9a protein is expected to be around 47kDa and the 9b around 37KDa. We
observed a dramatic increase in the pro-apoptotic caspase 9a protein in iPLA$_2$β over-expressing cells when treated with the ER stress inducer thapsigargin compared to the cells that were transfected with vector alone. The ratio of caspase 9a/9b protein was about four fold higher than in the cells over expressing iPLA$_2$β (FIGURE 9).

Hence far our data suggest that iPLA$_2$β is playing a vital role in the alternative splicing of caspase 9 and BCL-x. Over expression of iPLA$_2$β augments the pro-apoptotic splice variants of both caspase 9 (9a) and BCL-x (xL) in both the human and rodent models and its inhibition shifts the splicing events in the opposite direction. Inhibition of iPLA$_2$ in ER stress conditions protects the cells by causing a shift in splicing of caspase 9 and BCL-x toward their anti-apoptotic forms. This and evidences from previously published work (Ramanadham et al, 2004) together suggest that cell death during ER stress conditions may be mediated via iPLA$_2$β.

4.3. ALTERNATIVE SPlicing IS MEDIATED THROUGH CERAMIDE:
One of the important aspects of our hypothesis is that iPLA$_2$β is influencing alternative splicing through the induction of ceramides. As mentioned above, the Ramanadham lab has shown that there are elevated levels of ceramide in cells over-expressing iPLA$_2$β and that these levels can be significantly suppressed using the iPLA$_2$β inhibitor, BEL (Ramanadham et al 2004). Ceramides have also been implicated to shift splicing events of
caspase 9 and BCL-x in A549 lung carcinoma cells by the Chalfant group (Chalfant et al., 2002).

Our goal was to see if ceramide treatment of INS-1 cells can produce splicing results similar to what was observed by the Chalfant lab. RT PCR analysis of RNA extracted from INS-1 cells treated with ceramide produced a increase in ratio of BCL-x(s)/BCL-x(L) when compared to the vehicle control (FIGURE 12). That is, there was more of the pro-apoptotic BCL-x(s) signal upon treatment with ceramide.

Both thapsigargin and ceramide seem to be causing a similar effect on the splicing of BCL-x; while one is upstream of iPLA$_2$β, the other is downstream, respectively.
FIGURE 6:

BEL MODULATES SPlicing OF CASpASE 9 AND BCL-x IN JURKAT T CELLS

Electrophoretic analysis of amplified m-RNA from cells incubated with and without the iPLA₂β inhibitor BEL. Inhibition of iPLA₂β shifts splicing of caspase 9 and BCL-x towards their anti-apoptotic forms.
FIGURE 7:

iPLA$_2$$\beta$ INHIBITOR SHIFTS SPLICING TOWARD ANTI-APOPTOTIC BCL-x(L) IN INS-1 CELLS AND HUMAN ISLETS

Thapsigargin (ER stress inducer) shifts splicing towards the pro-apoptotic BCL-x(s), but BEL (selective inhibitor of iPLA$_2$$\beta$) prevents this effect and shifts splicing in favor of anti-apoptotic BCL-x(L). Left: INS-1 cells; Right: Human islets.
FIGURE 8:

MODULATION OF BCL-x SPlicing IN GENETICALLY ALTERED MICE

Islets were cultured in the presence or absence of thapsigargin (ER stress inducer). In wild type (WT) islets, and very little BCL-x(s) is present in untreated cells but the thapsigargin shifts splicing in favor of this pro-apoptotic variant. In contrast, BCL-x(s) is present in transgenic (TG, over expressing iPLA$_2$$\beta$) islets, even in the absence of thapsigargin treatment. Conversely, very little BCL-x(s) is present in iPLA$_2$$\beta$-/- (KO)islets even after thapsigargin treatment.
Akita mice develop spontaneous ER stress and hyperglycemia/diabetes due to β-cell apoptosis. It was recently shown that thapsigargin accelerates apoptosis of Akita β-cells and that this is associated with increased iPLA$_2$β expression. Correspondingly, the ratio of BCL-x(s) to BCL-x(L) is elevated in islets from Akita mice.
FIGURE 10:

iPLA3β INHIBITOR SHIFTS SPLICING OF BCL-x MINIGENE

*BCl*-x minigene in HEK cells. BEL (selective iPLA3β inhibitor) shifts splicing to favor the anti-apoptotic BCL-x(L).
FIGURE 11:

A.

iPLA$_2$B INHIBITOR, BEL, SHIFTS SPLICING TOWARD ANTI-APOPTOTIC CASPASE 9b IN HUMAN ISLETS

Thapsigargin (ER stress inducer) shifts splicing towards pro-apoptotic caspase 9a, but BEL (iPLA$_2$B selective inhibitor) prevents this effect.
B.

OVER-EXPRESSION OF iPLA₂β INCREASES THE ABUNDANCE OF PRO-APOPTOTIC CASPASE 9a PROTEIN IN INS-1 CELLS

<table>
<thead>
<tr>
<th>iPLA₂β</th>
<th>Thapsigargin</th>
<th>Ratio 9a/9b</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>+</td>
<td>7.8</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>2.2</td>
</tr>
</tbody>
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*Induction of ER stress in iPLA₂β over-expressing INS-1 cells produces an increase in amount of caspase 9a protein when compared to induction of ER stress in INS-1 cells transfected with the vector alone.*
FIGURE 12:

CERAMIDE BRINGS ABOUT A SHIFT IN SPLICING TO FAVOR THE PRO-APOPTOTIC BCL-x(s)

RT-PCR analysis showing that treatment of INS-1 cells with C-6 ceramide increases the pro-apoptotic BCL-x(s) message when compare to cells treated with the vector alone.
CHAPTER 5: DISCUSSION

Over the years many groups have put forward evidences that point toward the involvement of iPLA₂β in apoptosis. We hypothesized that iPLA₂β induces pre-mRNA splicing events of caspase 9 and BCLx in favor of their pro-apoptotic forms thus playing a critical role in apoptosis. Our hypothesis was based on studies that have illustrated that over-expression of iPLA₂β increases the incidence of apoptosis accompanied with a marked increase in the endogenous levels of ceramides. The Ramanadham lab has previously shown (Lei et al 2008) that accumulation of ceramides during ER stress takes place through the activation of iPLA₂β that drives the expression of neutral sphingomyelinase (NSMase); the enzyme that hydrolyses sphingomyelins to ceramides. Ceramides in turn have been shown to modulate the splicing events of caspase9 and BCL-x by the Chalfant group (Chalfant et al 2002).

These observations have made it important for us to focus our attention toward the possibility of iPLA₂β playing a direct role in the alternative splicing of caspase 9 and BCL-x.

To test our hypothesis we made use of both pharmacological and genetic tools to alter the activity of iPLA₂β, followed by a comparison of the relative expression of the pro-apoptotic to anti-apoptotic splice variants of caspase 9 and BCL-x in INS-1 cells, mouse and human islets.
On comparing the BCL-x splice variants, we were able to see that inhibition of iPLA₂β chemically (with its specific inhibitor BEL) or genetically (by a complete knockout of the enzyme) produce a shift in splicing of the BCL-x gene toward the anti-apoptotic splice variant (figures 7 and 8 respectively). Conversely, we observed more of the pro-apoptotic splice variant in the iPLA₂β over expressers and in the iPLA₂β transgenic mice islets (figure 7 and 8 respectively).

We made similar observations for caspase 9 at the RNA level (figure 11A) in human islets and at the protein level (figure 11B) in the INS-1 cells.

Taken together, our data point toward the prospect of iPLA₂β regulating the alternative splicing of caspase 9 and BCL-x. Increased iPLA₂β levels in the system drive splicing toward the pro-apoptotic splice variants while inhibition of the enzyme produce a shift to favor the anti-apoptotic forms.

We have also acquired data that suggest the involvement of iPLA₂β in ER stress induced apoptosis. Thapsigargin (ER stress inducer) treated INS-1 cells produced more of the pro-apoptotic splice variant of BCL-x (BCL-x(s)) (figure 7). This observation was consistent with the one made by the Ramanadham lab where thapsigargin treatment induces apoptosis in INS-1 cells (Ramanadham et al 2004). But a more intriguing observation was that we could shift this splicing toward the anti-apoptotic form by treatment with BEL prior to thapsigargin treatment, which again can be correlated to the...
inhibition of apoptosis of INS-1 cells under ER stress with BEL treatment, previously observed by Ramanadham group.

Another piece of the puzzle is the information obtained from the Akita mice (figure 9). Previous studies have shown that these mice develop diabetes as a result of ER stress mediated apoptosis. The islets from these mice show elevated levels of iPLA₂β expression (Lei et al 2009). Consistent with these data was an increase in ratio of the BCL-x(s)/BCL-x(L) (pro/anti) in the Akita islets. Thus, data from the Akita mice also suggest that under ER stress conditions, iPLA₂β is regulating the alternative splicing of BCL-x and caspase 9.

Our latest addition to these studies is the BCL-x mini-gene transfection which we have currently developed in the HEK cells (figure 10). The fact that inhibition of iPLA₂β shifts splicing of this exogenously expressed gene also, gives us the ability in the future to pinpoint the molecular mechanism by which this alternative splicing is taking place.

Since we postulated that iPLA₂β was bringing about alternative splicing through ceramide, it was imminent for us to see what happens to splicing upon treatment with ceramide. We have most recently performed experiments that provide evidence for the involvement of ceramide in the alternative splicing of BCL-x. Cells treated with C-6 ceramide clearly showed a marked increase in the pro-apoptotic splice variant of BCL-x (figure 12).
We hence believe that iPLA₂β participates in the regulation of both caspase 9 and BCLx pre-mRNA splicing. This seems to be true even in the cases of ER stress mediated apoptosis. The most probable means by which this is taking place is through the increase in the endogenous ceramide levels mediated via the induction of NSMase by iPLA₂β. Based on these inferences, we put forward a model for a possible pathway through which alternative splicing takes place. According to this model, an initial insult (pro-inflammatory cytokines, ER stress, etc) activates the mitochondrial apoptotic pathway resulting in caspase-3 activation and processing of iPLA₂β. Caspase-3-cleaved iPLA₂β translocates to the nucleus where it increases neutral sphingomyelinase (NSMase) message. A nuclear pool of NSMase protein generates ceramides, which activate PP1, resulting in dephosphorylation of splicing proteins and hence altering the splicing events of caspase-9 and BCL-x pre-mRNAs to increase expression of pro-apoptotic caspase-9a and reduce expression of anti-apoptotic Bcl-x(L) (Figure 13).

Our efforts in the future will be directed towards the investigation of this theory.

These findings are of significant importance in respect of addressing diabetes which has been shown to be a result of loss of β-cell mass due to apoptosis (Butler et al 2003). ER stress mediated apoptosis is gaining a lot of attention in the β-cell world and iPLA₂β, as shown above, seems to be an integral part of this mechanism via the regulation of pre-mRNA splicing. Our studies will therefore add to the ongoing efforts toward the discovery of novel therapeutic targets for diabetes.
Taken together, our work suggests a novel function for iPLA$_2$ that is independent of its effects on lipid metabolism; that is regulation of alternative splicing and involvement in cell apoptosis.

Our future experiments to strengthen the already existing evidence are mechanistic studies using the mini gene and siRNA studies against NSMase and iPLA$_2$β. Additionally, we would like to focus our attention on acquiring better tools to look at caspase 9 in rodent models. We also intend on delineating the events that are leading to the generation of ceramides downstream of iPLA$_2$β.
The initial insult (pro-inflammatory cytokines, ER stress, etc) activates the mitochondrial apoptotic pathway resulting in caspase 3 activation and processing of iPLA_2β. Caspase-3-cleaved iPLA_2β translocates to the nucleus where it increases neutral sphingo myelinase (NSMase) message. A nuclear pool of NSMase protein generates ceramides, which activate PP1, resulting in dephosphorylation of splicing proteins and altering splicing of caspase 9 and BCL-x pre-mRNAs to an increase in pro-apoptotic caspase 9a and a decrease in anti-apoptotic BCL-x(L).
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VITA

Bhargavi Emani was born on the 30th of January, 1985 in Hyderabad, India. She graduated high school in 2002 and then attended St. Francis College, Osmania University in India from where she graduated with a BSc. in Microbiology. In 2005, she went on to do her MSc. in Microbiology from Osmania University, India which she completed in May 2007. In Fall 2008, she began to work on her second Master of Science degree in Biochemistry and Molecular Biology at Virginia Commonwealth University. She joined the laboratory of Dr. Suzanne E Barbour in the spring of 2008. Bhargavi Emani completed her Masters degree in August 2010.