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Characterization of Palmitic Acid Induced Lipotoxicity in Schwann Cells

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Characterization of Palmitic Acid Induced Lipotoxicity in Schwann Cells

by

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Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality, as a dissertation for the degree Doctor Philosophy.

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ABBREVIATIONS

7AAD	7-Aminoactinomycin D
AGEs	Advance Glycation End products
AIF	Apoptosis Inducing Factor
AKT	Protein Kinase B
AO	Acridine Orange
AP1	Activator Protein 1
Apaf 1	Apoptosis activating factor 1
ASCOS	Augmented State of Cellular Oxidative Stress
ASK1	Apoptosis Signal Regulating Kinase 1
ATP	Adenosine Triphosphate
BAPTA-AM	(1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester
Bak	BCl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
BMI	Body Mass Index
BMP	Bis Monoacylglycerol Phosphate
BNIP3	Bcl-2/adenovirus E1B 19kDa protein interacting protein 3
BSA	Bovine Serum Albumin
CAT	Carnitine Acetyltransferase
CACT	Carnitine Acylcarnitine translocase
CE	Cholesterol Esters
CHO	Chinese Hamster Ovarian cells
CHOP	C/EBP Homologous Protein

CD36/FAT	Cluster of Differentiation 36/ Fatty Acid Translocase
CMT-1A	Charcot Marie Tooth Disease 1A
CPT	Carnitine Palmitoyltransferase
D-AP5	D-(−)-2-amino-5-phosphonopentanoic acid
dATP	Deoxyadenosine triphosphate
DHA	Decohexodecosamine acid
DMEM/F12	Dubelcco's Modified Eagle's Media/Ham 12
DMSO	Dimethyl Sulfoxide
DPBS	Dubelcco's Phosphate Buffered Saline
DPN	Diabetic Peripheral Neuropathy
DRG	Dorsal Root Ganglion
EndoG	Endonuclease G
ER	Endoplasmic Reticulum
ERAD	ER Associated Degradation
ETC	Electron Transport Chain
ETOH	Ethanol
FA	Fatty Acid
FADH	Flavin adenine dinucleotide
FADH2	Flavin adenine dinucleotide (reduced)
Fas-R	FAS Receptor
FBS	Fetal Bovine Serum
FC	Free Cholesterol
FFA	Free Fatty Acid
FOXO 1	Forkhead box Protein 01

GRP78	Glucose Regulated Protein 78
H_2DCFDA	2', 7' dichlorodihydrofluorecein deacetate
HNPP	Hereditary Neuropathy with liability to Pressure Palsies
Hsp	Heat Shock Protein
IRE-1	Inositol Requiring protein 1
iSC	Immortalized Schwann Cells
JC-1	5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylben-zimidazol-carbocyanine iodide
JNK	c-Jun N-terminal kinase
KU32	Kansas University 32
LCSFAs	Long Chain Saturated Fatty Acids
LCSFFAs	Long Chain Saturated Free Fatty Acids
LDL	Low Density Lipoprotein
LMD	Lysosomal Membrane Destabilization
LTx	Lipotoxicity
LOX-1	Lectin like Oxidized LDL receptor 1
MCI-186	2,4-dihydro-5methyl-2-phenyl-3H-pyrazol-3-one
MBP	Myelin Basic Protein
MMD	Mitochondrial Membrane Depolarization
NAD	Nicotineamide Adenine Dinucleotide
NAD(P)H	Nicotineamide Adenine Dinucleotide Phosphate
NADH	Nicotineamide Adenine Dinucleotide (reduced)
NFkB	Nuclear Factor Kappa B
NCV	Nerve Conduction Velocity
NGFDPC12	Neuronal Growth Factor Differentiated Pheochromocytoma cells

NMDA	N-methyl D- Aspartate
NO	Nitric Oxide
Nrf2	NF-E2 related factor 2
NRG1	Neuregulin-1
oxLDLs	Oxidized LDLs
P0	Myelin Protein Zero
PA	Palmitic Acid
PA-LTx	Palmitic Acid Lipotoxicity
PARP	Poly (ADP-ribose) Polymerase
PDC	Pyruvate Dehydrogenase Complex
PMP22	Peripheral Myelin Protein 22
PN	Peripheral Neuropathy
pSC	Primary Schwann Cells
RNS	Reactive Nitritive Species
ROS	Reactive Oxidative Stress
RTPCR	Real Time Polymerase Chain Reaction
SA	Saturated Fatty Acids
SC	Schwann Cells
SCM	Schwann Cell Media
SCGS	Schwann Cell Growth Supplement
SiRNA	Small interfering RNA
STZ	Streptozotosin
TCA	Tricarboxilic acid
TG	Triglycerides

TLR4	Toll Like Receptor 4
TRAF2	TNF receptor-associated factor 2
TRB3	Tribbles Homolog 3
UPR	Unfolded Protein Response
Xbp1	X-box binding protein 1

ABSTRACT OF THE DISSERTATION

Characterization of Palmitic Acid Induced Lipotoxicity in Schwann Cells

by

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Doctor of Philosophy, Graduate Program in Pathology and Human Anatomy
Loma Linda University, December 2011
Dr. Marino De Leon, Chairperson

Lipotoxicity is a response to lipid overload that has been associated with cellular dysfunction leading to cellular death. The mechanism of lipotoxicity and its impact on the nervous system is critical as it can lead to debilitating neurological conditions. This study examines key cellular events induced by palmitic acid lipotoxicity (PA-LTx) in Schwann cells cultured in euglycemic and hyperglycemic conditions. The data show that immortalized Schwann cell (iSC), as well as primary Schwann cell (pSC) cultures exposed to elevated levels of PA induced an apoptotic cell death that is dose and time-dependent.

The earliest indication of cellular dysfunction was a decrease in Ca^{++} levels in the endoplasmic reticulum (ER, $[Ca^{++}]_{ER}$). This decrease in $[Ca^{++}]_{ER}$ was associated with a significant change in the expression of ER stress signature genes such as CHOP, Xbp1 and GRP78. In SC cultured in euglycemic conditions, lysosomal membrane destabilization preceded mitochondrial membrane depolarization, oxidative stress and caspase 3/7 activation. The release of cathepsin L and B were also observed with PA treatments. However, in hyperglycemic SCs, PA-LTx induced- ER stress followed mitochondrial injury, oxidative stress, caspase 3/7 activation with a subsequent

lysosomal release of cathepsins. Of interest is the observation that hyperglycemia was able to magnify SC injury resulting in an earlier and more severe cell death.

Treatment of iSC cultures with the calcium chelator BAPTA-AM resulted in an increase in cell viability in iSC cultures. In addition, treatment with antioxidant MCI-186 reduced oxidative stress and increased cell viability in both euglycemic and hyperglycemic SC exposed to PA. Furthermore, PA-LTx was able to disrupt mRNA and protein levels of Peripheral Myelin protein 22 within the first 12hrs. Understanding the mechanism of lipotoxicity is of importance in order to bring innovative therapies to ameliorate debilitating conditions such as diabetic neuropathy.

CHAPTER ONE

INTRODUCTION

Fatty Acids

Fatty Acids' Structure and Function

Fatty acids (FAs) are carboxylic acids with hydrocarbon chains, usually 12-24 carbons in length**.** The classification into saturated or unsaturated FAs is based on the presence of double bonds. Unsaturated FAs, such as docosahexanoic acid (DHA), have one or more double bonds in their hydrocarbon chain. On the other hand, saturated FAs, such as palmitic acid (PA), lack carbon double bonds resulting in a straight hydrocarbon tail. The double carbon-carbon bond makes the unsaturated FAs more angled and flexible, while lack of it renders the saturated FA less pliable and more rigid.

FAs are also grouped by their chain length. Short chain FAs have a carbon tail that is fewer than 6 carbons, medium chain FAs have 6-11 carbons and long chain FAs (LCFAs) have 12 or more carbons in their tail. LCSFAs are less soluble and more rigid, and their increase in chain length makes them critical factors in the development of diseases [1].

However, physiological normal levels of FAs are not deleterious to their surroundings. FAs provide fuel for tissues and cells. Cells and organs depend on the availability of adequate levels of FAs to carry out normal cellular functions and perform important and critical metabolic activities [2]. In addition to being essential components in the architecture and construction of cellular membranes, FAs are

associated with the makeup of secondary messengers needed for appropriate cellular signaling.

The transportation of FAs to their adequate locations is facilitated by albumin, a predominant protein found in plasma. Albumin binds to circulating FAs and transports them to adipocytes for proper energy storage or to tissue for energy utilization. The utilization of FAs as an energy source requires their activation before proper metabolism can take place. Fatty acyl CoA synthetase, the first enzyme in β oxidation, transforms FAs into fatty acyl CoA. Through protein carriers and other enzymes, such as carnitine palmitoyl transferase I/II and carnitine acylcarnitine translocase, acyl-CoA is taken up by the inner mitochondrial membrane and converted to acetyl-CoA in the mitochondrial matrix. Acetyl-CoA enters the Krebs cycle to produce FADH and FADH2. These two molecules reach the mitochondrial electron transport chain to produce the energy molecule, ATP (Figure 1) [3].

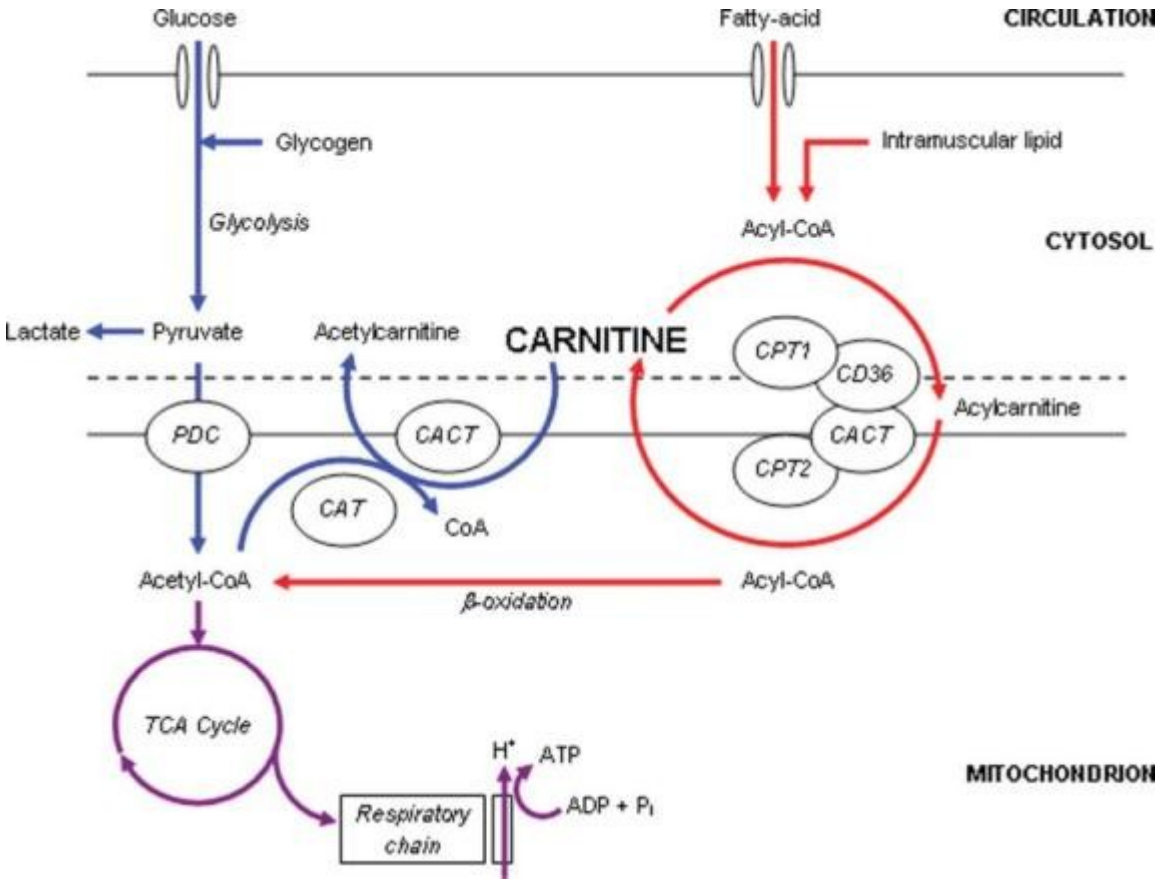
Glucose
Fatty-acid
CIRCULATION
Glycogen
Intramuscular lipid
Glycolysis
Acyl-CoA
CYTOSOL
Lactate
Pyruvate
Acetylcarnitine
CARNITINE
CPT1
CD36
Acylcarnitine
PDC
CACT
CACT
CPT2
CAT
CoA
Acetyl-CoA
Acyl-CoA
β-oxidation
TCA Cycle
H+
ATP
ADP + Pi
MITOCHONDRION
Respiratory chain

Figure 1. Glucose and FA metabolism**.** Glucose is converted to pyruvate and further modified to acetyl-CoA by pyruvate dehydrogenase complex (PDC). Similarly, FAs are activated by fatty acyl CoA synthetase to produce acyl CoA . Acyl CoA is transported into the mitochondria by carnitine palmitoyl transferase 1,2 (CPT), fatty acid translocase (CD36), and carnitine acylcarnitine translocase (CACT). β oxidation of acyl-CoA results in the production of acetyl CoA. The acetyl CoA produced by glucose and FAs advances to the tricarboxylic acid cycle (TCA) and concludes in the electron transport chain resulting in ATP production [3-4].

When excess levels of FAs are encountered by cells, the esterification process begins. Cells are able to esterify FAs into triglycerides (TG) for proper energy storage. The acceptance of two activated fatty acyl CoA molecules to form diaglycerol phosphate begins the esterification process. The removal of phosphate and the addition of a third fatty acid is the last step to TG formation. The formation of TG lipid droplets in the cytosol is suggested to be the first line of defense of non-adipose cells to prevent cellular dysfunction [5].

In addition to energy generation, FAs also play a role in cellular membrane structure and can influence the overall performance of the cell. An example of the importance of FAs in cellular membrane function is the composition of sphingomyelin, an important component of cellular membranes. Proper membrane structure is dependent on adequate levels of sphingomyelin. Ceramide, made of a long chain FA attached to a sphingosine molecule, makes part of the sphingomyelin protein. In this way, FAs also serve as vital constituents in the structure of cellular membranes.

Fatty Acids and Obesity

According to the World Health Organization, obesity has reached global epidemic proportions. It is estimated that by the year 2015, approximately 2.3 billion adults will be overweight (BMI 25-30 kg/m^2) and at least 700 million will be obese (BMI $\geq$ $30kg/m^2$) [6]. Between the years of 1980 to 2006, obesity rates among 6 to 11-year-olds have more than doubled and have tripled among 12 to 19-year-olds [7]. More alarming is that current global studies reveal that childhood obesity will continue to increase significantly [8-10]. Obesity has been strongly linked to the increase in risk for developing many

diseases such as cancers, cardiovascular disorders, neurodegenerative diseases and diabetes type 2. A common feature found in obese individuals is elevated levels of circulatory fatty acids (FAs) [11-13].

Therefore, an elevation of FAs in the diet results in unbound free fatty acids (FFAs) circulating in the blood that can be toxic to the entire biological system. Non-adipose cells, such as neurons and Schwann cells, are vulnerable to sustained elevated levels of FFAs. In addition, excessive TG deposition and FFA overload, similar to that observed in obese and diabetic individuals, can lead to cellular dysfunction and cellular death. The succession of pathological cellular responses triggered by chronic and elevated levels of FFAs is termed lipotoxicity (LTx) [14-23].

Lipotoxicity

Lipotoxicity and Saturated FAs

LTx can be trigger by LCSFAs, which are considered to be among the most damaging in the FA family. Three decisive factors that may contribute to LCSFA LTx include 1) their difficulty in undergoing esterification, 2) their ability to form diaglycerides and toxic by products such as ceramide, a key mediator of cytotoxicity and apoptosis, and 3) their ability to compact in lipid membrane rafts, thus decreasing cell membrane fluidity leading to cellular membrane dysfunction [5, 24-30].

Gelb and Kessler (1963) analyzed the influences of FA structure on esterification in the small intestine of male golden hamsters. The data demonstrated an inverse correlation between the length of the FA chain and the percentage of FA esterification. Possible explanations for the decrease in LCSFA esterification were 1) the requirement of

an extra step or additional enzymes in the esterification procedure, thus decreasing the efficiency of the cell to process LCSFAs, and 2) the possible preference in the esterification of certain FAs by the cell [25].

The difficulty in FA esterification can lead to the generation of toxic molecules. Toxic byproducts of LCSFA, such as ceramide, have been reported in many neurodegenerative disorders [31-32]. A study conducted by Blaszquez et al. (2001) demonstrated that cortical astrocytes undergo apoptosis through de novo production of ceramide by FFAs, and the inhibition of ceramide by an inducer of AMP-activated protein kinase prevented cellular death [33]. Another study found that the production of ceramide by PA induced apoptosis through ER damage and caspase 3 independent pathways in a Schwann cell (SC) model. Interestingly, this same study demonstrated that the inhibition of ceramide led to ER damage and a caspase-3 dependent cell death pathway [34].

In neuronal growth factor differentiated pheochromocytoma (NGFDPC12) cells, previous work from our lab revealed that exposure to LCSFAs, such as PA and stearic acid, activated the lipotoxic response. However, treatment with unsaturated acids, such as oleic or arachidonic acids, did not induce LTx [35].

Palmitic acid Lipotoxicity (PA-LTx)

PA (C:16) is a LCSFA predominantly found in high saturated fat diets, such as the western diet [36-38]. PA is able to induce lipotoxicity (PA-LTx) that is more severe and deleterious on tissues and cells when compared to other FAs [35, 39-40]. Studies reported from our laboratory have demonstrated that NGFDPC12 cells undergo PA-LTx

that is caspase-independent and involved a significant increase in gene expression of fas ligand/receptor and pro-apoptotic members of the Bcl2 family. The proposed mechanism of PA-LTx in a neuronal cell model included the involvement of lysosomal and mitochondrial dysfunction, caspase activation and an augmented state of cellular oxidative stress (ASCOS) (Figure 2) [41].

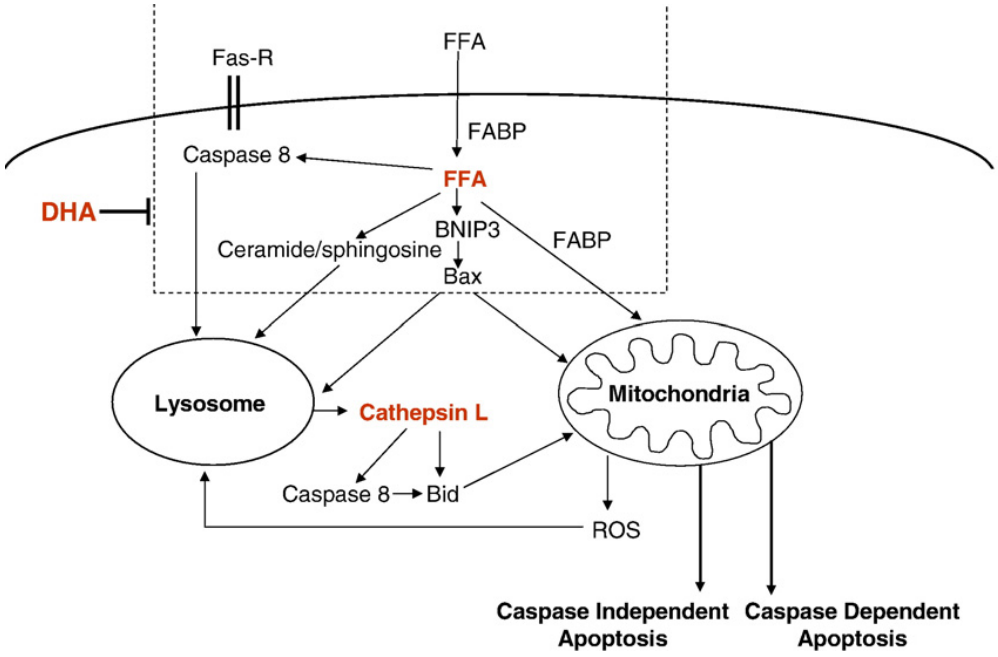
Fas-R
FFA
FABP
Caspase 8
FFA
DHA
BNIP3
Ceramide/sphingosine
FABP
Bax
Lysosome
Cathepsin L
Mitochondria
Caspase 8
Bid
ROS
Caspase Independent Apoptosis
Caspase Dependent Apoptosis

Figure 2. Mechanisms of PA-LTx in neuronal cell model. PA induces LTx in NGFDPC12 cells through mitochondrial dysfunction resulting in the increase of reactive oxidative species (ROS) and the activation of caspases. PA-LTx also results in lysosomes membrane destabilization (LMD) and the release of cathepsins into the cytosol [41].

Cellular Targets

In an attempt to discern the mechanisms of PA-LTx we focused our experiments in examining the cellular targets of PA. In specific, we examined PA's ability to target certain cellular organelles such as the ER, mitochondria, and lysosomes which may result in cellular dysfunction and cell death (Figure. 3).

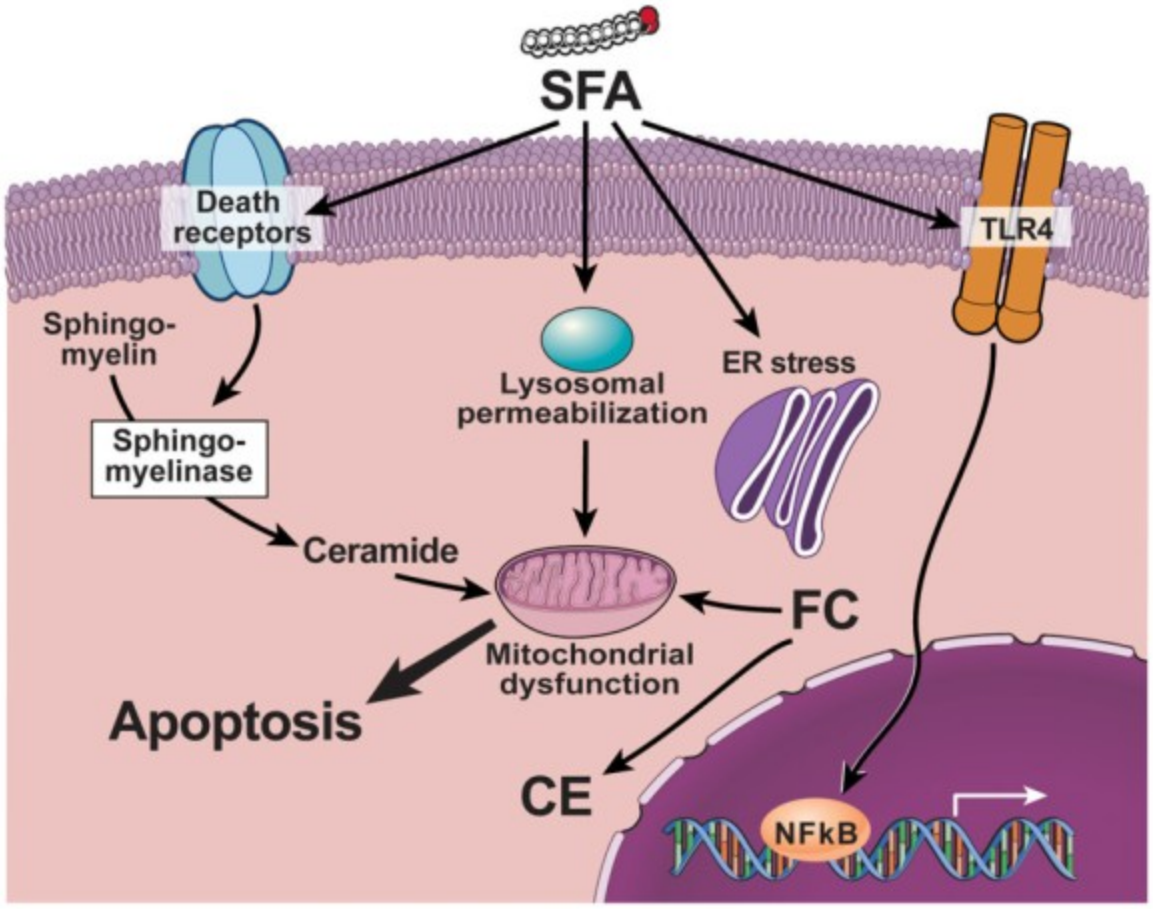
SFA
Death receptors
TLR4
Sphingo-myelin
Sphingo-myelinase
Lysosomal permeabilization
ER stress
Ceramide
FC
Mitochondrial dysfunction
Apoptosis
CE
NFkB

Figure 3. Saturated Fatty Acids' induction of cellular injury. Saturated FAs are able to induce apoptosis through the activation of death receptors, such as Fas and Toll like receptor 4 (TLR4). Death receptors damage cellular membranes and release ceramide, which can injure the mitochondria. TLR4 is able to activate the transcription of NF-kB to upregulate a variety of pro-inflammatory cytokines . SFA can also directly damage the endoplasmic reticulum (ER) and the lysosome to induce apoptosis. In addition, free cholesterol (FC), which can injure the mitochondria, elevates cholesteryl-esters (CE) inducing cell death [42-43].

Endoplasmic Reticulum (ER)

The endoplasmic reticulum (ER) has many important cellular functions such as protein assembly, Ca^{++} storage, and lipid synthesis. All proteins are assembled by the ER; however, certain proteins enter the lumen of the ER and are folded, modified and transported to the golgi apparatus for vesicular release into the cytosol. An improperly folded protein is held in the ER lumen for repair, modification or degradation. Chaperones, such as GRP78, assist in the quality control of assembled proteins by 1) facilitating protein conformation and 2) retaining and repairing misfolded proteins.

The increase in misfolded proteins in the ER can damage or overload the ER resulting in the activation of the unfolded protein response (UPR) and the ER-associated degradation pathway [44-45]. The first line of defense is through activation of the unfolded response [44], which attempts to decrease the load to the ER by 1) increasing the transcription of chaperones to aid in protein folding and 2) activate factors that will reduce protein translation. If the UPR does not decrease ER stress, the ER associated degradation pathway is activated. In this pathway the misfolded proteins in the ER are released to the cytosol to be degraded by proteosomes [45].

In addition to regulating protein folding, cellular signaling is also an important function of the ER. Studies in the literature have shown that intracellular Ca^{++} can control nuclear transport, mitochondrial oxidative metabolism, and other cell signaling activities [46-48]. However, inadequate levels of intracellular Ca^{++} are able to activate pro-apoptotic factors within the cell. Studies have demonstrated that significant ER overload induced the efflux of Ca^{++} from the ER into the cytosol and resulted in the activation of pro-apoptotic pathways [49-52]. Exposure of hypothalamic cells to PA for

4 hrs was able to phosphorylate c-Jun N-terminal kinase (JNK) and damage the ER, leading to an upregulation of pro-apoptotic genes [51, 53-54].

Other studies have gone further and suggest that PA is able to directly injure the ER, resulting in apoptosis [24]. Research by Borradaile et al. (2006) revealed that elevated levels of PA are quickly (5 hrs) incorporated into ER lipid membrane rafts of Chinese hamster ovarian cells (CHO) leading to apoptosis. Exposure of CHO to PA resulted in 1) a notable dilation of the ER membrane, 2) the increase in ER membrane rigidity and loss of ER membrane fluidity, and 3) a significant depletion of ER Ca^{++} stores that led to significant levels of intracellular Ca^{++}. This study suggested that the activation of cell death pathways and cellular damage were consequences of the increase in cytosolic Ca^{++} levels due to ER damage [24].

Mitochondria and ASCOS

The primary task of the mitochondria is to metabolize molecules, such as glucose and pyruvate, and convert them into adenosine triphosphate (ATP) for cellular energy. Adequate levels of ATP are required for cellular reactions and signal transduction pathways critical for cell survival. The process begins with the catabolism of glucose and FAs into acetyl-CoA, which enter the tricarboxilyc acid cycle in the mitochondrial matrix to produce NADH and FADH2. The electron transport chain [3], uses NADH and FADH2 to pump H+ ions out of the matrix to create a proton gradient between the mitochondrial matrix and the intramembranous space [3]. Complex V of the mitochondrial ETC, also known as ATP synthase, allows the influx of H+ ions into the matrix causing a phosphorylative reaction and, thus, generating ATP. During this

process, low levels of reactive oxidative species (ROS) are produced by the occasional escape of electrons from the ETC [55]. Normally, the free radicals formed by the electrons are neutralized by mitochondrial superoxide dismutase or cytochrome c. However, a disruption in the ETC can increase the release of numerous electrons into the intramembranous space, leading to an interaction with other cellular elements, such as oxygen, resulting in oxidative stress [56].

Mitochondrial dysfunction and the production of an augmented state of oxidative stress (ASCOS) has been suggested to be a key factor in the cellular damage produced by LCSFAs like PA [41, 57-58]. FAs induce ROS and reactive nitritive stress (RNS), stimulating lipid peroxidation. The byproducts of lipid peroxidation, lipid peroxides, are known to damage lipids, proteins, and cellular DNA [59].

Another mechanism of mitochondrial damage is through the activation of pro-apoptotic proteins, such as Bax. The pro-apoptotic protein, Bax, is known to perforate the mitochondrial membrane resulting in mitochondrial injury. The formation of pores in the mitochondrial membrane by Bax results in the release of cytochrome c into the cytosol. Cytochrome c binds to apoptosis activating factor 1 (Apaf1), caspase 9, and deoxyadenosine triphosphate (dATP) to form the apoptosome. The generation of the apoptosome is able to activate initiator caspases, inducing apoptosis [60-61]. Interestingly, a study found that PA was able to activate a "Bax- mediated mitochondrial induced" apoptotic pathway in C_2 C_{12} myotubes. This study proposed the direct activation of Bax by PA. The activation of Bax results in mitochondrial injury and the induction of the apoptotic pathway. Furthermore, Bax SiRNA experiments found that

suppression of the Bax gene decreases DNA fragmentation and caspase activation after PA exposure [62].

Studies have suggested that the mitochondrion, and not the ER, is the critical component in the apoptotic pathway. Hypothalamic neurons revealed that PA metabolism by the mitochondria was required to induce ER stress, active caspase-3 and trigger PA-LTx. The importance of the mitochondria in PA-LTx was confirmed by studies demonstrating that a non-metabolizable form of PA, methylpalmitate, had no negative effects on hypocampal neurons [53]. Along these lines, studies from our lab demonstrated that bromo-palmitate, a non metabolizable palmitate analog, was unable to induce lipotoxic effects in NGFDPC12 cells [63].

In addition, studies from our laboratory have demonstrated that PA increases the expression of BNIP3, a pro-apoptotic member of the Bcl-2 family, and Fas-R, a death receptor, that led to mitochondrial membrane destabilization (MMD) and ROS production in NGFDPC12 cells. Furthermore, caspase-3 activation and cleavage of lamin B and PARP confirmed PA-LTx to be apoptotic [64].

Lysosomes and Cathepsin Activation

Lysosomes are membrane-bound cellular organelles that contain hydrolytic enzymes, such as proteases, nucleases, and phopholipases, active in the lysosomal acidic environment (pH. of 4.5 to 5). The function of the lysosomes is to digest macromolecules and recycle cellular material. Following lysosomal injury by ASCOS or lysosomotrophic agents, proteases are released into the cytosol and initiate cellular death pathways [65-66]. Proteases, such as cathepsin B, are stable at physiologic pH

levels for a couple of minutes to a full hour following their release from lysosomes, thus supporting their ability to cause cellular injury. Lysosomal membrane destabilization (LMD) is the result of the cell's reaction to insults, such as caspase activation, Bax/Bak induction and translocation to lysosomal membranes, and mitochondria-induced generation of ASCOS [67-69].

Previous studies in our lab determined that LMD was an early event in PA-LTx. LMD led to the release of proteases into the cytosol resulting in cellular damage. In addition, the release of cathepsin L from the lysosomes was detected as early as 3 hours after PA exposure in NGFDPC12 cells and the inhibition of cathepsin L significantly eliminated MMD and apoptosis [41].

Hepatocytes exposed to PA for 3 hours resulted in the activation and translocation of Bax to the lysosome that preceded MMD. It was proposed that Bax's induction of LMD was independent of ceramide and caspase activation. Furthermore, inhibition of Bax after PA treatment 1) reduced LMD 2) inhibited the release of cathepsin B and 3) increased cell viability, suggesting a significant role of the lysosome in PA-LTx [70]. However the exact mechanism of lysosomal injury by PA continues to be unclear.

Lipotoxicity and Mechanisms of Peripheral Neuropathy

Peripheral Neuropathy

Over 20 million people suffer from PN, which is considered to be one of the most common and chronic diseases in the United States [75]. PN is a debilitating complication observed in obese and diabetic individuals [76-78]. A direct correlation between elevated

lipid levels and the occurrence of neuropathy in obese individuals has been observed in many studies [79-80].

A significant number of people who are obese, but do not demonstrate overt diabetes, have demonstrated sensory neuropathy in the median, ulnar and sural nerves. In addition, they also exhibit a decrease in action potential amplitude in the tibial and peroneal nerves [81]. The development of PN in individuals who demonstrate metabolic lipid derangement without hyperglycemia suggest the possible role of FFAs in the induction of neuronal injury.

Clinical Signs of Peripheral Nerve Injury

Individuals suffering from peripheral neuropathy (PN) frequently suffer from altered sensations (dysesthesias and parasthesias) that progress to painful perceptions (hyperalgesias) and, in later stages, to extreme responses to non-painful stimuli (allodynia). Axons will also be lost and result in muscle weakness and loss of motor function [71-72]. Studies in PN have determined that small myelinated and unmyelinated sensory fibers are affected first, followed by large sensory and motor neurons [73],[74].

Mechanisms: FAs

Recent studies are now discovering the relationship between dyslipidemia and its role in peripheral neuropathy. Studies suggest that elevated and chronic levels of FFA's, found in the pre-diabetic state, may exert detrimental and toxic effects on peripheral nerves [82]. A study performed by Andrea Vincent, found that in C57/BL6 mice that were fed a high fat diet, developed PN prior to the onset of hyperglycemia. These mice

exhibited high levels of systemic oxidative stress, plasma triglycerides, and FFAs. Plasma protein levels were found to be significantly oxidized, specifically low density lipoproteins (LDLs). In addition, lectin-like oxidized low-density lipoprotein receptor- 1 (LOX-1), a receptor for oxidized LDLs (oxLDLs) known to increase oxidative stress in endothelial cells, was also observed to be activated in the membranes of dorsal root ganglions (DRGs) [83-84]. Upon closer examination, it was found that exposure of oxLDLs to cultured DRGs from mice fed a high fat diet stimulated LOX-1 activity, increased mitochondrial superoxide levels, and activated caspase 3. Furthermore, the inhibition of nicotinamide adenine dinucleotide phosphate-oxidase (NAD(P)H) oxidase, an enzyme that produces superoxide, was able to prevent injury to DRGs. However, inhibition of NAD(P)H did not prevent DRG death in an elevated FFA and hyperglycemic environment. The findings elucidated the different death mechanisms activated by the cell when it encounters a hyperglycemic condition in combination with a high FA environment [84].

The physiological stress of a hyperglycemic and/or an LTx environment is able to injure many of the surrounding tissues. Schwann cells (SC), the glial cells of the peripheral nervous system, undergo dysfunction leading to demyelination and axonal atrophy, which are prominent abnormalities associated with peripheral neuropathies and axon degeneration [85-87]. Damage to SCs leads to dysfunctions in nerve conduction velocities, axonal atrophy, and axonal degeneration [88-90]. Suzuki et al. (2011) examined the role of ceramide in the activation of apoptosis in an immortalized SC (iSC) clone [78]. iSC clones were exposed to PA: Bovine Serum Albumin (BSA) 2:1 (500μM:250μM) for 48 hours which resulted in apoptosis, an increase in ceramide levels,

caspase 3 activation, and the production of ER stress. It was found that ceramide, through PA or de novo synthesis, was able to induce apoptosis through a caspase 3 independent pathway since inhibition of caspase 3 did not completely restore iSC viability. Interestingly, inhibition of ceramide did not prevent caspase 3 activation, ER stress, and cellular death [34]. This study suggested that PA may directly induce cellular injury without ceramide production through the activation of caspase 3 [91-92].

Diabetic Peripheral Neuropathy

Out of all cases of peripheral neuropathies, 60-70% are related to diabetes [75, 93]. Diabetes affects approximately 189 million people worldwide, and it is estimated that by the year 2025, there will be 324 million people affected with this disorder [94]. A strong link has been established between peripheral neuropathy and the development of foot ulcers [95]. Studies have shown that approximately 25% of diabetics will develop neuropathy that will result in foot ulcers, and more than half will become infected and require hospitalization. Furthermore, one in 5 cases of foot ulcers will require some form of amputation with approximately 50% of the patients requiring a second amputation after 2 years of the first one. After a limb amputation, the mortality rate of individuals with diabetes type 2 increases to approximately 50% [95-97].

Clinical Signs of Diabetic Peripheral Neuropathy

Diabetic peripheral neuropathy (DPN) commonly presents with the loss of sensation in a “stock and glove” pattern [98]. The first symptoms usually begin in the feet and ascend through the lower extremities, followed by impairment in the hands that will

progress proximately [77, 99]. The decrease in nerve conduction velocity, which is pathognomonic for DPN, is frequently asymptomatic [100]. Individuals with DPN will also develop dysesthesias and parasthesias that will progress to hyperalgesias and, in later stages, allodynia. Muscle weakness and loss of motor function will be observed at later stages [71-72].

Mechanisms of DPN: Hyperglycemia

It is difficult to suggest that DPN is caused by a single insult due to the multifactorial components found in diabetes. Hyperglycemia, elevated levels of FFA's, a decrease in neuronal blood flow, oxidative stress, and an altered growth factor level are some of the causative factors implicated in peripheral nerve injury [67-70, [101].

Hyperglycemia has been the major focus of many studies in the development of DPN. It is hypothesized that increased levels of aldose reductase and advanced glycation end products (AGEs) are important components in hyperglycemic toxicity within the SC [55, 101]. The increase in sorbitol levels can lead to the accumulation of fructose, a powerful glycator, and an increase in osmotic stress in neuronal cells [102]. Furthermore, the activation of poly (ADP-ribose) polymerase (PARP) by hyperglycemia can deplete NAD levels resulting in a dysfunction of glycolitic and mitochondrial respiration pathways [103-106].

The modification of proteins by elevated glucose levels can result in protein aggregation resulting in cellular dysfunction. Since chaperones aid in protein folding and are elevated during the unfolded protein response, recent studies have examined the role of heat shock proteins (Hsp) in the development of DPN [44]. Hsp 90, the principle

coordinator in the chaperone stress response, was examined using KU-32, an Hsp90 inhibitor. However, KU-32 is also known to increase levels of Hsp70, an anti-apoptotic chaperone. In vitro experiments using rat embryonic dorsal root ganglions (DRGs) and SC-DRG cocultures in 45mM glucose concentrations demonstrated an increase in cell death that was prevented by the addition of KU-32. Interestingly, a disturbance in sensory nerve conduction and motor nerve conduction velocities in C57BI/6 streptozotosin (STZ) induced diabetic mice were also prevented by the addition of KU-32. Since it was known that KU-32 was also able to increase Hsp70, a diabetic mouse model with the genetic deletion of Hsp70.1 and 70.3 (Hsp70.1/70.3 double KO) was generated and, after the development of diabetes, treated with KU-32. The data showed that Hsp 70.1 and 70.3 double KO mice developed DPN that was not prevented by the addition of KU-32. The results indicated that the prevention of DPN by KU-32 was dependent on levels of Hsp70 [107].

Current studies have suggested that hyperglycemia alone is not sufficient to induce significant SC death, myelin damage or reduce the total number of myelin segments associated with DPN [59, 84, 108]. Gumy et al., (2008), observed that SC rat cultures exposed to high glucose alone (24 hours) demonstrated a hindrance of neurite outgrowth, a decline in proliferation and a delay in migration without the induction of cell death. Cytochrome c release, caspase3 activation and nuclear fragmentation were not detected in hyperglycemic SC. Furthermore, co-culture of SC and axon explants and SCs alone cultured on laminin demonstrated a disorganized alignment in hyperglycemic conditions with no cellular death. It is believed that a similar situation occurs when SCs separate from the peripheral nerve when high glucose concentrations are encountered *in*

vivo. Thus, this study hypothesized that hyperglycemic changes observed in SCs is an additive but not a causative factor in the pathogenesis of DPN [108].

Vincent, et al., (2009) suggested that a possible explanation for increased resistance of SCs to hyperglycemic conditions may be due to their ability to increase cellular antioxidant concentrations, such as catalase, when they encounter an insult. The study found that under hyperglycemic conditions (20mM glucose), SCs were able to increase levels of Nrf2, a transcription factor that increases antioxidant gene expression in the brain [84]. The capacity of SCs to be resistant to a hyperglycemic environment leads to the belief that a second insult, possibly FFAs, will exceed the cell's survival threshold and results in SC cell death.

Peripheral Myelin Protein 22 and Neuropathy

Since SCs are the manufacturers of myelin in the peripheral nervous system, the death of these cells can lead to a disruption in myelin production, leading to segmental demyelination. Myelin is comprised of SC plasma membrane which is 70-80% lipid and 15 -30% protein. The plasma membrane of one SC is able to wrap around one portion of a single axon. The resulting unmyelinated axonal areas, called nodes of Ranvier, are replete with sodium channels that will depolarize the axonal membrane causing an electrical impulse to course through the myelinated portions of the axon to the next node of Ranvier. The movement of the impulse from one node of Ranvier to the next is called salutatory conduction. Due to the insulating characteristics of myelin, axonal membrane depolarization is conducted with far less energy and increased speed when compared to an unmyelintaed axon (Figure. 4).

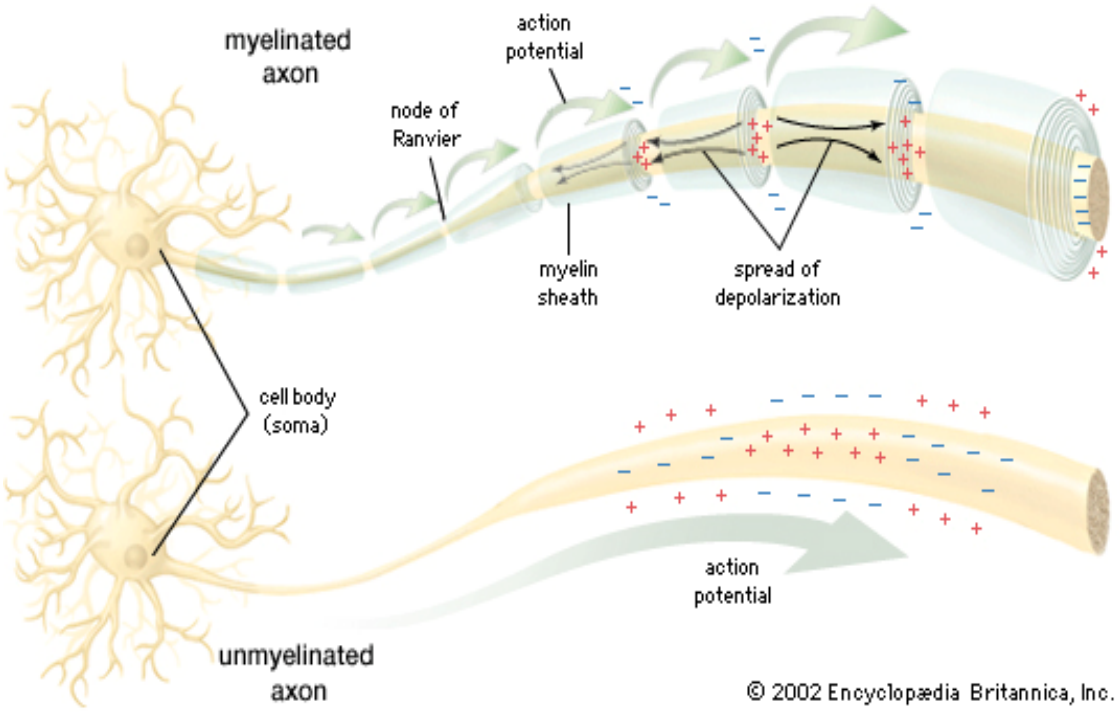
myelinated
axon
action
potential
node of
Ranvier
myelin
sheath
spread of
depolarization
cell body
(soma)
action
potential
unmyelinated
axon
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Figure 4. Anatomy and transmission of nerve conduction velocity in the peripheral nerve. Schwann cells (SC) will wrap around axons and produce myelin in small sections of the peripheral nerve. Depolarization will take place at the Nodes of Ranvier. The impulse generated will coast through the myelinated areas until the next node of Ranvier is reached where depolarization will be repeated. Depolarization occurring from one node to the next node of Ranvier is termed salutatory conduction [109].

The increase in conduction velocity is only one of the many functions of myelin. Studies have shown that axonal development and regeneration are not efficient and cannot be completed in the absence of myelin [110-111]. Recent studies found that neuregulin-1 (NRG1), a protein secreted by axons that regulate SC differentiation, proliferation, motility, axon ensheathment and myelination, is essential in the regenerative process after peripheral nerve injury. Uninjured NRG1- deficient mice showed normal myelin sheaths and undisturbed neuromuscular junctions. After a sciatic nerve crush injury, NRG1-deficient mice demonstrated 1) severe defects in remyelination or exhibited no myelin sheath production, 2) slower axonal regeneration process, and 3) a malformed neuromuscular junction. The study suggests that NRG1 is needed in the production of myelin after demyelination caused by neuronal injury and, furthermore, points to the necessity of myelin sheath in the nerve regeneration process [112].

Metabolic damage to the peripheral nerves can affect the myelin sheaths first, leading to segmental demyelination and axonal damage [113-114]. Nerve biopsies of two cats exhibiting diabetic neuropathy demonstrated a disruption and a ballooning of the myelin sheaths before axonal damage was observed. SCs exhibited "reactive, degenerative and proliferative changes" in the absence of axonal injury. These findings and others suggest that the metabolic disruptions observed in DPN may have a direct effect on SCs first before axonal damage is achieved [115-116]. Because myelin is initially disrupted, the Peripheral Nerve society has stated that myelinated fiber density may be a useful indicator of electrophysiological dysfunctions in the injured nerve [117].

Myelin is critical for the proper function of the peripheral nerve. However, in order for myelin to work properly, adequate levels of myelin components, such as

proteins, are necessary. Peripheral myelin protein 22 (PMP22) is one of the most important myelin proteins in the peripheral nervous system and has been associated with the development in peripheral neuropathies [118]. PMP22 makes up 2-5% of the total myelin protein and is incorporated into the membrane of compact myelin. It has been demonstrated that PMP22 protein decreases when myelin is disrupted and increases in axonal regeneration [119].

Schwann Cell Model

SCs are in direct contact with the axons and are responsible for many functions in the peripheral nerve such as 1) producing of myelin that will sustain the nerve conduction velocities of myelinated axons, 2) providing immunological and functional integrity to the axons through the blood brain barrier, 3) regulating axonal diameter and neurofilament spacing, and 4) most importantly, aiding in nerve regeneration and axonal guidance [120]. Unfortunately, studies have demonstrated that SCs are unable to regulate glucose entry into the cell, which makes them more vulnerable to elevated levels of glucose. In addition, SCs tend to be more sensitive to conditions that generate calcium overload and ASCOS [121-123]

Studies have shown that SC dysfunction, demyelination and axonal atrophy are three prominent abnormalities associated with peripheral neuropathies and axon degeneration [84-87, 89, 124-126]. Since it has been demonstrated that SCs undergo detrimental changes before axonal injury is detected and, furthermore, the wellbeing of the SC is required for proper peripheral nerve regeneration, it is critical to examine the mechanisms of SC damage in respect to peripheral neuropathy.

Treatments

Currently, the treatment of neuropathy continues to be ineffective. The primary target of DPN and PN treatments only focuses on symptoms. It is suggested that pain medications can only reduce neuropathic pain by 30-50% but cannot completely eliminate it [127]. Medications such as tricyclic antidepressants and anticonvulsants continue to be used to treat DPN without the understanding the mode of action. Furthermore, these medications do not address the prevention or reduction of neuronal injury in the progression of neuropathy [128].

Metformin, an oral hypoglycemic agent, has also proven to be ineffective in treating DPN. Presently, clinical studies are investigating the effects of lipid lowering therapies on DPN. The Fremantle diabetes study concluded that lipid-lowering therapy may protect against DPN. It is suggested that SC function, polyol pathway function and neuronal blood supply are improved with lipid-lowering therapy; however, the actual mechanism continues to be unclear [129-130]. Similarly, it has also been demonstrated that pancreatic transplantation can only stabilize neuropathy without offering any neuronal recovery [131].

Rationale and Hypothesis of this Study

Elevated levels of saturated long chain fatty acids are a major contributor of lipotoxic cellular injury. Obese and type 2 diabetic individuals demonstrate a serious derangement in lipid metabolism, which results in elevated free fatty acids (FFAs) circulating in the blood. One of the debilitating complications in type 2 diabetes is the development of PN. Hyperglycemia, exhibited by type 2 diabetics, has been greatly

examined in the field of PN. However, the mechanism by which PN develops continues to be unclear. In addition, studies are now demonstrating that hyperglycemia alone is not sufficient to induce significant myelin damage or damage a sufficient number of myelin segments that can lead to peripheral neuropathy [132]. **Therefore, the long term goal** of this study is to better understand the mechanism by which FFAs in combination with hyperglycemia injures the SC and results in the development of DPN. Knowledge of this mechanism could lead to the exploration of conventional therapeutic opportunities to treat DPN. Our **experimental focus** is on the injury of SC by elevated levels of PA, a saturated fatty acid which has been demonstrated to be a major contributor of LTx.

Our hypothesis is that elevated levels of PA will induce LTx in Schwann cells cultured in a euglycemic and hyperglycemic environment leading to Schwann cell death. These findings can elucidate the possible role of LTx in the development of DPN.

Approach of this Dissertation Research

The specific aims of this study were to 1) examine whether PA induces cell death in Schwann cells (SC) cultured in hypoglycemic, euglycemic and hyperglycemic conditions, 2) investigate the involvement of the endoplasmic reticulum (ER), mitochondria and lysosome in PA-LTx *in vitro*, and 3) explore the effects of increased concentrations of PA on myelin proteins and their possible contribution to the development of peripheral neuropathy.

PA-LTx was examined by culturing immortalized Schwann cells (iSC) and primary SCs (pSC) in different concentrations of glucose. Cells were cultured for 4 days in their respective glucose concentrations to mimic a chronic hyperglycemic environment

before being treated with PA. PA was dissolved in ETOH and complexed with fatty acid free Bovine Serum Albumin (BSA) in serum free medium to serve as a buffer for PA. Cells were treated with PA: BSA ratio of 1:1(150uM/150uM) and 2:1 (300uM/150uM).

To examine the apoptotic cell death pathway by PA-LTx, a time course using dual parameter flow cytometry for Annexin V-FITC (which binds to early apoptotic cells) and 7AAD (which detects cells in apoptosis) was performed, in addition to trypan blue, and Hoechst staining. Our data demonstrated that iSCs as well as pSC cultures exposed to elevated levels of PA exhibited a loss in cell viability that was dose and time-dependent. This lipotoxic effect was more dramatic in hyperglycemic cultures.

The earliest indication of cellular dysfunction was a decrease in Ca^{++} levels in the endoplasmic reticulum ($[Ca^{++}]_{ER}$) which was detected by the QuantiChromTM Calcium Assay Kit. The release of Ca^{++} into the cytosol was observed at an earlier time in hyperglycemic conditions. This decrease in $[Ca^{++}]_{ER}$ was associated with a significant change in the expression of ER stress signature genes such as CHOP, Xbp1 and GRP78. The disruptions in ER stress genes were analyzed by Real Time PCR (RTPCR). Furthermore, treatment of iSC cultures with the calcium chelator BAPTA-AM resulted in an increase in cell viability. Following the ER stress response, a strong mitochondrial membrane depolarization was also observed by flow cytometry using JC-1 dye in which an earlier MMD was observed in hyperglycemic conditions. Using acridine orange assays, our data further demonstrates that PA-LTx can trigger lysosomal membrane destabilization and release cathepsins that can intensify cellular dysfunction. Interestingly, a delay in lysosomal involvement was exhibited in hyperglycemic SCs.

Furthermore, flow cytometry using 2', 7'-dichlorodihydrofluorescein diacetate (H2DCF-DA) showed an increase in oxidative stress after PA treatment. A significant amount of oxidative stress was observed in hyperglycemic environments before it was detected in euglycemic conditions. Treatment with antioxidant MCI-186 reduced oxidative stress and increased cell viability.

Significance

The proposed study is important for various reasons. First, data generated may provide insights about the relevance of the lipotoxicity process in terms of its effects to supporting cells of the peripheral nervous system. Most of the studies of lipotoxicity have been done in non-neuronal cells, such as hepatocytes, and little is known of mechanisms on how the lipotoxic process affects neuron/glial function. Recently, there has been an increased interest in the effects of lipids on neurons and glial cells because of their potential role in neurodegenerative diseases, such as Alzheimer's disease [133].

Therefore, it is our objective to reveal the possible induction of SC death by FFAs and the potential augmentation of cellular injury by hyperglycemia in the development of DPN. This study is of great significance because it will impart insight into the possible mechanism of the development of DPN. In addition, understanding this mechanism will provide innovative and effective therapies to delay or halt the progression of DPN.

References

1. Bell, G.H., *The metabolism of long-chain fatty acids and alcohols by Candida tropicalis and Saccharomyces cerevisiae.* Antonie Van Leeuwenhoek, 1973. **39**(1): p. 137-49.

2. Bezuglov, V.V., M. Bobrov, and A.V. Archakov, *Bioactive amides of fatty acids.* Biochemistry (Mosc), 1998. **63**(1): p. 22-30.

3. Fortun, J., et al., *Impaired proteasome activity and accumulation of ubiquitinated substrates in a hereditary neuropathy model.* J Neurochem, 2005. **92**(6): p. 1531-41.

4. Stephens, F.B., D. Constantin-Teodosiu, and P.L. Greenhaff, *New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle.* J Physiol, 2007. **581**(Pt 2): p. 431-44.

5. Listenberger, L.L., et al., *Triglyceride accumulation protects against fatty acid-induced lipotoxicity.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3077-82.

6. Shah, A. *Obesity*. 2010; Available from: http://www.globalissues.org/article/558/obesity.

7. Carroll, C.O.a.M. (June 2010) *Prevalence of Obesity Among Children and Adolescents: United States, Trends 1963–1965 Through 2007–200*.

8. Ahrens, W., et al., *The IDEFICS cohort: design, characteristics and participation in the baseline survey.* Int J Obes (Lond), 2011. **35 Suppl 1**: p. S3-15.

9. Lee, A., M.M. Ho, and V.M. Keung, *Global epidemics of childhood obesity is hitting a 'less industrialized' corner in Asia: a case study in Macao.* Int J Pediatr Obes, 2011. **6**(2-2): p. e252-6.

10. Yanovski, S.Z. and J.A. Yanovski, *Obesity prevalence in the United States--up, down, or sideways?* N Engl J Med, 2011. **364**(11): p. 987-9.

11. Kusminski, C.M., et al., *Diabetes and apoptosis: lipotoxicity.* Apoptosis, 2009. **14**(12): p. 1484-95.

12. Unger, R.H., *Reinventing type 2 diabetes: pathogenesis, treatment, and prevention.* JAMA, 2008. **299**(10): p. 1185-7.

13. Ho, A.J., et al., *Obesity is linked with lower brain volume in 700 AD and MCI patients.* Neurobiol Aging, 2010. **31**(8): p. 1326-39.

14. Unger, R.H., *Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications.* Diabetes, 1995. **44**(8): p. 863-70.

15. Shimabukuro, M., et al., *Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2498-502.

16. Lupi, R., et al., *Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated.* Diabetes, 2002. **51**(5): p. 1437-42.

17. Kato, A., et al., *Protective effects of dietary chamomile tea on diabetic complications.* J Agric Food Chem, 2008. **56**(17): p. 8206-11.

18. Koshkin, V., et al., *Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic beta-cells.* J Biol Chem, 2008. **283**(12): p. 7936-48.

19. Unger, R.H., *How obesity causes diabetes in Zucker diabetic fatty rats.* Trends Endocrinol Metab, 1997. **8**(7): p. 276-82.

20. Unger, R.H. and Y.T. Zhou, *Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover.* Diabetes, 2001. **50 Suppl 1**: p. S118-21.

21. Schaffer, J.E., *Lipotoxicity: when tissues overeat.* Curr Opin Lipidol, 2003. **14**(3): p. 281-7.

22. van Herpen, N.A. and V.B. Schrauwen-Hinderling, *Lipid accumulation in non-adipose tissue and lipotoxicity.* Physiol Behav, 2008. **94**(2): p. 231-41.

23. Song, Z., et al., *Silymarin prevents palmitate-induced lipotoxicity in HepG2 cells: involvement of maintenance of Akt kinase activation.* Basic Clin Pharmacol Toxicol, 2007. **101**(4): p. 262-8.

24. Borradaile, N.M., et al., *Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death.* J Lipid Res, 2006. **47**(12): p. 2726-37.

25. Gelb, A.M., M.I. Davidson, and J.I. Kessler, *Effect of Fasting on Esterification of Fatty Acids by the Small Intestine in Vitro.* Am J Physiol, 1964. **207**: p. 1207-10.

26. Hardy, S., et al., *Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin.* J Biol Chem, 2003. **278**(34): p. 31861-70.

27. Moffitt, J.H., et al., *Adverse physicochemical properties of tripalmitin in beta cells lead to morphological changes and lipotoxicity in vitro.* Diabetologia, 2005. **48**(9): p. 1819-29.

28. Prentki, M. and S.R. Madiraju, *Glycerolipid metabolism and signaling in health and disease.* Endocr Rev, 2008. **29**(6): p. 647-76.

29. Ricchi, M., et al., *Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes.* J Gastroenterol Hepatol, 2009. **24**(5): p. 830-40.

30. Maedler, K., et al., *Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function.* Diabetes, 2003. **52**(3): p. 726-33.

31. Goswami, R., et al., *Differential regulation of ceramide in lipid-rich microdomains (rafts): antagonistic role of palmitoyl:protein thioesterase and neutral sphingomyelinase 2.* J Neurosci Res, 2005. **81**(2): p. 208-17.

32. Argia, T., R.K. Yu, and T. Miyatake, *Characterization of gangliosides by direct inlet chemical ionization mass spectrometry.* J Lipid Res, 1984. **25**(10): p. 1096-101.

33. Blazquez, C., et al., *The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes.* FEBS Lett, 2001. **489**(2-3): p. 149-53.

34. Suzuki, J., et al., *Palmitate induces apoptosis in Schwann cells via both ceramide-dependent and independent pathways.* Neuroscience, 2011. **176**: p. 188-98.

35. Ulloth, J.E., C.A. Casiano, and M. De Leon, *Palmitic and stearic fatty acids induce caspase-dependent and -independent cell death in nerve growth factor differentiated PC12 cells.* J Neurochem, 2003. **84**(4): p. 655-68.

36. Chilton, F.H., et al., *Depression and altered serum lipids in cynomolgus monkeys consuming a Western diet.* Physiol Behav, 2011.

37. Kanoski, S.E. and T.L. Davidson, *Western diet consumption and cognitive impairment: Links to hippocampal dysfunction and obesity.* Physiol Behav, 2011. **103**(1): p. 59-68.

38. Schmid, A., *The role of meat fat in the human diet.* Crit Rev Food Sci Nutr, 2011. **51**(1): p. 50-66.

39. Li, Z., et al., *Using dynamic gene module map analysis to identify targets that modulate free fatty acid induced cytotoxicity.* Biotechnol Prog, 2008. **24**(1): p. 29-37.

40. Wei, Y., et al., *Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells.* Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E275-81.

41. Almaguel, F.G., et al., *Lipotoxicity-mediated cell dysfunction and death involve lysosomal membrane permeabilization and cathepsin L activity.* Brain Res, 2010. **1318**: p. 133-43.

42. Nakhjavani, M., et al., *Serum heat shock protein 70 and oxidized LDL in patients with type 2 diabetes: does sex matter?* Cell Stress Chaperones, 2011. **16**(2): p. 195-201.

43. Alkhourl, N.D., L.J.; Feldstein, A.E., *Lipotoxicity in Nonalcoholic Fatty Liver Disease: Not All Lipids Are Created Equal.* Expert Rev Gastroenterol Hepatol., 2009. **3**(4): p. 445-451.

44. Lin, W., et al., *Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress.* Brain, 2006. **129**(Pt 5): p. 1306-18.

45. Yasuda, H., et al., *Diabetic neuropathy and nerve regeneration.* Prog Neurobiol, 2003. **69**(4): p. 229-85.

46. Greber, U.F. and L. Gerace, *Depletion of calcium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus.* J Cell Biol, 1995. **128**(1-2): p. 5-14.

47. Lam, M., G. Dubyak, and C.W. Distelhorst, *Effect of glucocorticosteroid treatment on intracellular calcium homeostasis in mouse lymphoma cells.* Mol Endocrinol, 1993. **7**(5): p. 686-93.

48. Nutt, L.K., et al., *Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores.* J Biol Chem, 2002. **277**(11): p. 9219-25.

49. Brodsky, J.L. and A.A. McCracken, *ER-associated and proteasomemediated protein degradation: how two topologically restricted events came together.* Trends Cell Biol, 1997. **7**(4): p. 151-6.

50. Kaufman, R.J., *Orchestrating the unfolded protein response in health and disease.* J Clin Invest, 2002. **110**(10): p. 1389-98.

51. Martinez, S.C., et al., *Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis.* Diabetes, 2008. **57**(4): p. 846-59.

52. Urano, F., et al., *Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1.* Science, 2000. **287**(5453): p. 664-6.

53. Mayer, C.M. and D.D. Belsham, *Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons:*

rescue of resistance and apoptosis through adenosine 5' monophosphate-activated protein kinase activation. Endocrinology, 2010. **151**(2): p. 576-85.

54. Mayer, C.M. and D.D. Belsham, *Central insulin signaling is attenuated by long-term insulin exposure via insulin receptor substrate-1 serine phosphorylation, proteasomal degradation, and lysosomal insulin receptor degradation.* Endocrinology, 2010. **151**(1): p. 75-84.

55. Sekido, H., et al., *Reduced cell replication and induction of apoptosis by advanced glycation end products in rat Schwann cells.* Biochem Biophys Res Commun, 2004. **320**(1): p. 241-8.

56. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* J Physiol, 2003. **552**(Pt 2): p. 335-44.

57. Schrauwen, P. and M.K. Hesselink, *Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes.* Diabetes, 2004. **53**(6): p. 1412-7.

58. Schrauwen, P., et al., *Etomoxir-induced increase in UCP3 supports a role of uncoupling protein 3 as a mitochondrial fatty acid anion exporter.* FASEB J, 2002. **16**(12): p. 1688-90.

59. Green, K., M.D. Brand, and M.P. Murphy, *Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes.* Diabetes, 2004. **53 Suppl 1**: p. S110-8.

60. Kirkland, R.A. and J.L. Franklin, *Bax, reactive oxygen, and cytochrome c release in neuronal apoptosis.* Antioxid Redox Signal, 2003. **5**(5): p. 589-96.

61. Kroemer, G., N. Zamzami, and S.A. Susin, *Mitochondrial control of apoptosis.* Immunol Today, 1997. **18**(1): p. 44-51.

62. Peterson, J.M., et al., *Bax signaling regulates palmitate-mediated apoptosis in C(2)C(12) myotubes.* Am J Physiol Endocrinol Metab, 2008. **295**(6): p. E1307-14.

63. Almaguel, F., *Mechanisms of DHA neuroprotection from PA indiuced lipotoxicity*. 2010. p. 223.

64. Almaguel, F.G., et al., *Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid.* J Neurosci Res, 2009. **87**(5): p. 1207-18.

65. Roberts, D.L., et al., *Molecular basis of lysosomal enzyme recognition: three-dimensional structure of the cation-dependent mannose 6-phosphate receptor.* Cell, 1998. **93**(4): p. 639-48.

66. Turk, B., D. Turk, and V. Turk, *Lysosomal cysteine proteases: more than scavengers.* Biochim Biophys Acta, 2000. **1477**(1-2): p. 98-111.

67. Bidere, N., et al., *Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis.* J Biol Chem, 2003. **278**(33): p. 31401-11.

68. Boya, P., et al., *Lysosomal membrane permeabilization induces cell death in a mitochondrion-dependent fashion.* J Exp Med, 2003. **197**(10): p. 1323-34.

69. Schotte, P., et al., *Cathepsin B-mediated activation of the proinflammatory caspase-11.* Biochem Biophys Res Commun, 1998. **251**(1): p. 379-87.

70. Feldstein, A.E., et al., *Bax inhibition protects against free fatty acid-induced lysosomal permeabilization.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(6): p. G1339-46.

71. Sugimoto, K., Y. Murakawa, and A.A. Sima, *Diabetic neuropathy--a continuing enigma.* Diabetes Metab Res Rev, 2000. **16**(6): p. 408-33.

72. McLeod, J.G., *Investigation of peripheral neuropathy.* J Neurol Neurosurg Psychiatry, 1995. **58**(3): p. 274-83.

73. Dyck, P.J., et al., *Fiber loss is primary and multifocal in sural nerves in diabetic polyneuropathy.* Ann Neurol, 1986. **19**(5): p. 425-39.

74. Said, G., D. Baudoin, and K. Toyooka, *Sensory loss, pains, motor deficit and axonal regeneration in length-dependent diabetic polyneuropathy.* J Neurol, 2008. **255**(11): p. 1693-702.

75. ; Available from: http://www.neuropathy.org/site/PageServer?pagename=About_Facts). .

76. Dumitru, D. and J.C. King, *Median/ulnar premotor potential identification and localization.* Muscle Nerve, 1995. **18**(5): p. 518-25.

77. Greene, D.A., et al., *Complications: neuropathy, pathogenetic considerations.* Diabetes Care, 1992. **15**(12): p. 1902-25.

78. Miscio, G., et al., *Obesity and peripheral neuropathy risk: a dangerous liaison.* J Peripher Nerv Syst, 2005. **10**(4): p. 354-8.

79. Green, A.Q., et al., *Altered C-fiber function as an indicator of early peripheral neuropathy in individuals with impaired glucose tolerance.* Diabetes Care, 2010. **33**(1): p. 174-6.

80. Smith, A.G., et al., *Lifestyle intervention for pre-diabetic neuropathy.* Diabetes Care, 2006. **29**(6): p. 1294-9.

81. Bland, J.D., *The relationship of obesity, age, and carpal tunnel syndrome: more complex than was thought?* Muscle Nerve, 2005. **32**(4): p. 527-32.

82. Buschbacher, R.M., *Body mass index effect on common nerve conduction study measurements.* Muscle Nerve, 1998. **21**(11): p. 1398-404.

83. Chen, X.J., et al., *Proprioceptive sensory neuropathy in mice with a mutation in the cytoplasmic Dynein heavy chain 1 gene.* J Neurosci, 2007. **27**(52): p. 14515-24.

84. Vincent, A.M., et al., *Dyslipidemia-induced neuropathy in mice: the role of oxLDL/LOX-1.* Diabetes, 2009. **58**(10): p. 2376-85.

85. Dyck, P.J. and C. Giannini, *Pathologic alterations in the diabetic neuropathies of humans: a review.* J Neuropathol Exp Neurol, 1996. **55**(12): p. 1181-93.

86. Song, Z., et al., *Transgenic mice overexpressing aldose reductase in Schwann cells show more severe nerve conduction velocity deficit and oxidative stress under hyperglycemic stress.* Mol Cell Neurosci, 2003. **23**(4): p. 638-47.

87. Wiggin, T.D., et al., *Elevated triglycerides correlate with progression of diabetic neuropathy.* Diabetes, 2009. **58**(7): p. 1634-40.

88. Ray, L., et al., *Mechanisms of association between obesity and chronic pain in the elderly.* Pain, 2011. **152**(1): p. 53-9.

89. Sango, K., et al., *High glucose-induced activation of the polyol pathway and changes of gene expression profiles in immortalized adult mouse Schwann cells IMS32.* J Neurochem, 2006. **98**(2): p. 446-58.

90. Vincent, A.M., et al., *Sensory neurons and schwann cells respond to oxidative stress by increasing antioxidant defense mechanisms.* Antioxid Redox Signal, 2009. **11**(3): p. 425-38.

91. Turpin, S.M., et al., *Apoptosis in skeletal muscle myotubes is induced by ceramides and is positively related to insulin resistance.* Am J Physiol Endocrinol Metab, 2006. **291**(6): p. E1341-50.

92. Rachek, L.I., et al., *Palmitate induced mitochondrial deoxyribonucleic acid damage and apoptosis in l6 rat skeletal muscle cells.* Endocrinology, 2007. **148**(1): p. 293-9.

93. Poncelet, A.N., *An algorithm for the evaluation of peripheral neuropathy.* Am Fam Physician, 1998. **57**(4): p. 755-64.

94. Wild, S., et al., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* Diabetes Care, 2004. **27**(5): p. 1047-53.

95. Boulton, A.J. and L. Vileikyte, *The diabetic foot: the scope of the problem.* J Fam Pract, 2000. **49**(11 Suppl): p. S3-8.

96. Amos, A.F., D.J. McCarty, and P. Zimmet, *The rising global burden of diabetes and its complications: estimates and projections to the year 2010.* Diabet Med, 1997. **14 Suppl 5**: p. S1-85.

97. Gupta, S.K., et al., *Diabetes prevalence and its risk factors in rural area of Tamil Nadu.* Indian J Community Med, 2010. **35**(3): p. 396-9.

98. Llewelyn, J.G., *Diabetic neuropathy.* Curr Opin Neurol, 1995. **8**(5): p. 364-6.

99. Said, G., G. Slama, and J. Selva, *Progressive centripetal degeneration of axons in small fibre diabetic polyneuropathy.* Brain, 1983. **106 (Pt 4)**: p. 791-807.

100. Dyck, P.J. and P.C. O'Brien, *Quantitative sensation testing in epidemiological and therapeutic studies of peripheral neuropathy.* Muscle Nerve, 1999. **22**(6): p. 659-62.

101. Obrosova, I.G., *How does glucose generate oxidative stress in peripheral nerve?* Int Rev Neurobiol, 2002. **50**: p. 3-35.

102. Obrosova, I.G., *Increased sorbitol pathway activity generates oxidative stress in tissue sites for diabetic complications.* Antioxid Redox Signal, 2005. **7**(11-12): p. 1543-52.

103. Obrosova, I.G., et al., *Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy.* Diabetes, 2004. **53**(3): p. 711-20.

104. Virag, L., et al., *Nitric oxide-peroxynitrite-poly(ADP-ribose) polymerase pathway in the skin.* Exp Dermatol, 2002. **11**(3): p. 189-202.

105. Pacher, P., et al., *Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies.* Curr Med Chem, 2005. **12**(3): p. 267-75.

106. Sharma, S.S., A. Kumar, and R.K. Kaundal, *Protective effects of 4-amino1,8-napthalimide, a poly (ADP-ribose) polymerase inhibitor in experimental diabetic neuropathy.* Life Sci, 2008. **82**(11-12): p. 570-6.

107. Urban, M.J., et al., *Inhibiting heat-shock protein 90 reverses sensory hypoalgesia in diabetic mice.* ASN Neuro, 2010. **2**(4).

108. Gumy, L.F., E.T. Bampton, and A.M. Tolkovsky, *Hyperglycaemia inhibits Schwann cell proliferation and migration and restricts regeneration of axons and Schwann cells from adult murine DRG.* Mol Cell Neurosci, 2008. **37**(2): p. 298-311.

109. Encyclopedia, B., *Action Potential Conduction.*

110. Nguyen, T., et al., *Axonal protective effects of the myelin-associated glycoprotein.* J Neurosci, 2009. **29**(3): p. 630-7.

111. Brant-Zawadzki, M. and D.R. Enzmann, *Using computed tomography of the brain to correlate low white-matter attenuation with early gestational age in neonates.* Radiology, 1981. **139**(1): p. 105-8.

112. Fricker, F.R., et al., *Axonally derived Neuregulin-1 is required for remyelination and regeneration after nerve injury in adulthood.* J Neurosci, 2011. **31**(9): p. 3225-33.

113. Sugimoto, K., M. Yasujima, and S. Yagihashi, *Role of advanced glycation end products in diabetic neuropathy.* Curr Pharm Des, 2008. **14**(10): p. 953-61.

114. Verheijen, M.H., et al., *Local regulation of fat metabolism in peripheral nerves.* Genes Dev, 2003. **17**(19): p. 2450-64.

115. Mizisin, A.P., et al., *Myelin splitting, Schwann cell injury and demyelination in feline diabetic neuropathy.* Acta Neuropathol, 1998. **95**(2): p. 171-4.

116. Yu, C.L., Z.G. Zhu, and L.S. Lei, *[Study of dynamic changes of blood sugar and body signs in streptozotocin-induced diabetic animal models].* Nan Fang Yi Ke Da Xue Xue Bao, 2008. **28**(1): p. 132-3.

117. Veves, A., et al., *The relationship between sural nerve morphometric findings and measures of peripheral nerve function in mild diabetic neuropathy.* Diabet Med, 1991. **8**(10): p. 917-21.

118. Laura, M., et al., *Rapid progression of late onset axonal Charcot-Marie-Tooth disease associated with a novel MPZ mutation in the extracellular domain.* J Neurol Neurosurg Psychiatry, 2007. **78**(11): p. 1263-6.

119. De Leon, M., et al., *SR13/PMP-22 expression in rat nervous system, in PC12 cells, and C6 glial cell lines.* J Neurosci Res, 1994. **38**(2): p. 167-81.

120. Eckersley, L., *Role of the Schwann cell in diabetic neuropathy.* Int Rev Neurobiol, 2002. **50**: p. 293-321.

121. Dugan, L.L., et al., *IL-6 mediated degeneration of forebrain GABAergic interneurons and cognitive impairment in aged mice through activation of neuronal NADPH oxidase.* PLoS One, 2009. **4**(5): p. e5518.

122. Lu, Q., et al., *Guattegaumerine protects primary cultured cortical neurons against oxidative stress injury induced by hydrogen peroxide concomitant with serum deprivation.* Cell Mol Neurobiol, 2009. **29**(3): p. 355-64.

123. Barber, S.C. and P.J. Shaw, *Oxidative stress in ALS: Key role in motor neuron injury and therapeutic target.* Free Radic Biol Med, 2010. **48**(5): p. 629-641.

124. Welcher, A.A., et al., *Molecular approaches to nerve regeneration.* Philos Trans R Soc Lond B Biol Sci, 1991. **331**(1261): p. 295-301.

125. Cameron, N.E. and M.A. Cotter, *Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway.* Curr Drug Targets, 2008. **9**(1): p. 60-7.

126. Obrosova, I.G., et al., *High-fat diet induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition.* Diabetes, 2007. **56**(10): p. 2598-608.

127. Sultan, A., et al., *Duloxetine for painful diabetic neuropathy and fibromyalgia pain: systematic review of randomised trials.* BMC Neurol, 2008. **8**: p. 29.

128. Lindsay, T.J., et al., *Treating diabetic peripheral neuropathic pain.* Am Fam Physician, 2010. **82**(2): p. 151-8.

129. Davis, T.M., et al., *Prevalence, characteristics, and prognostic significance of HFE gene mutations in type 2 diabetes: the Fremantle Diabetes Study.* Diabetes Care, 2008. **31**(9): p. 1795-801.

130. Davis, T.M., et al., *Lipid-lowering therapy and peripheral sensory neuropathy in type 2 diabetes: the Fremantle Diabetes Study.* Diabetologia, 2008. **51**(4): p. 562-6.

131. Smith, A.G. and J.R. Singleton, *Impaired glucose tolerance and neuropathy.* Neurologist, 2008. **14**(1): p. 23-9.

132. Yu, C., S. Rouen, and R.T. Dobrowsky, *Hyperglycemia and downregulation of caveolin-1 enhance neuregulin-induced demyelination.* Glia, 2008. **56**(8): p. 877-87.

133. Adibhatla, R.M. and J.F. Hatcher, *Altered Lipid Metabolism in Brain Injury and Disorders.* Subcell Biochem, 2008. **48**: p. nihpa41041.

CHAPTER TWO

HYPERGLYCEMIA MAGNIFIES SCHWANN CELL DYSFUNCTION AND CELL DEATH TRIGGERED BY PA-INDUCED LIPOTOXICITY

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Abstract

Lipid overload resulting in lipotoxicity is prominent in a number of chronic diseases and has been associated with cellular dysfunction and cell death. This study characterizes palmitic acid-induced lipotoxicity (PA-LTx) in Schwann cell cultures grown in normal and high glucose concentrations. The study shows for the first time that Schwann cell (SC) cultures exposed to elevated levels of PA exhibit a dose and time – dependent loss in cell viability. Hoescht and Annexin V/7AAD staining confirmed cell death through apoptosis and the lipotoxic effect was more dramatic in SC cultures grown under high glucose conditions. The first indication of cellular dysfunction in treated SC cultures was a decrease in Ca^{++} levels in the endoplasmic reticulum (ER, $[Ca^{++}]_{ER}$) observed five minutes following the initial challenge with PA. This decrease in $[Ca^{++}]_{ER}$ was followed by a significant increase in the expression of ER stress signature genes CHOP, Xbp1 and GRP78. The early ER stress response induced by PA-LTx was followed by a strong mitochondrial membrane depolarization. Flow cytometry using 2', 7'-dichlorodihydrofluorescein diacetate (H_2DCF-DA) showed an increase in oxidative stress within three to six hours after PA treatment. Treatment of cultures undergoing PA-LTx with the calcium chelator BAPTA-AM and the antioxidant MC1-186 significantly reversed the lipotoxic effect by decreasing the generation of ROS and significantly increasing cell viability. We conclude that lipotoxicity in Schwann cells results in cellular dysfunction and cell death that involves a robust ER stress response, mitochondrial dysfunction and an augmented stated of cellular oxidative stress (ASCOS).

Introduction

Normal cellular functions depend on the availability of adequate levels of free fatty acids (FFA) that are an important for critical normal metabolic activities [2]. However, a significant metabolic disarray and cellular dysfunction can occur when tissues and cells are chronically exposed to elevated levels of FFAs as observed in connection with type 2 diabetes and obesity [11-12, 134-136]. While adipocytes have the proper cellular machinery to store and safely utilize high amount of these FFAs, non-adipose cells are vulnerable to lipid overload which can induce lipotoxicity and apoptotic cell death [5, 22] Lipotoxicity in non-nerve cells is well documented in the literature [39-40, 137-138]. For instance, pancreatic beta cells exposed to high levels of palmitic acid (PA) exhibit a dramatic dysfunction and apoptotic cell death [15-18]. It has been shown that lipotoxicity-induced apoptosis and beta cell death in the pancreas precedes hyperglycemia suggesting that can be an important factor in the onset of type 2 diabetes [19-22].

Nerve cells are exposed to multiple pathologies that can lead to lipid overload and lipotoxicity that can result in lipid peroxidation and lipotoxicity [64, 139-141]. During ischemia, there is a significant increase in lipid peroxidation in membranes of the endoplasmic reticulum (ER), mitochondria and lysosomes which may contribute to the marked dysfunction and cell death observed in this condition [142]. Ceramide, a lipid species that forms from fatty acyl CoA and sphingosine, is elevated in the white matter of post-mortem brains of patients with Alzheimer's disease [143-144]. Traumatic injuries in the brain and spinal cord result in a significant increase of FFAs that is believed to play a

role in the extensive amount of cell death and tissue damage occurring during the secondary phase of the condition [145-147].

Our laboratory is interested in examining the role and impact of lipotoxicity in the nervous system. Previous reports have shown that stearic acid and PA trigger a strong apoptotic cell death response in nerve growth factor differentiated pheochromocytoma cells (PC12 cells) and cortical cells cultures [35, 64]. This lipotoxic effect was shown to be specific for saturated fatty acids such as palmitic (PA) and stearic acid, because treatment with oleic nor arachidonic acids at the same concentrations did not trigger a lipotoxic response [35]. The lipotoxic cell death process is caspase-independent and involves a significant differential expression in gene expression of fas ligand/receptor and members of the Bcl2 family [35]. Caspase-independent cell death can occur through significant dysfunction of vital organelles such as the endoplasmic reticulum, lysosomes and mitochondria suggesting that calcium and oxidative cellular disruptions may play a key cellular role in this process [35, 41, 64] The aim of this study was to assess whether ER stress and ROS production are principal contributors to the PA-LTx process in Schwann cells grown under hyperglycemic and euglycemic conditions. This study shows for the first time that Schwann cells (SC) chronically exposed to PA overload exhibit dramatic lipotoxicity and apoptotic cell death by a mechanism that require ER stress followed by an augmented state of cellular oxidative stress (ASCOS) and mitochondrial dysfunction. Further, PA-LTx occurred in a dose and time dependent manner, and is significantly stimulated by high glucose. These data is important considering that pathological conditions that lead to lipid overload can result in SC dysfunction,

demyelination and axon atrophy, three prominent abnormalities associated with peripheral neuropathies and axon degeneration [85-86, 89, 124]

Experimental Procedure

Cell Culture

The Schwann cells used in this study were a generous gift from Dr. Laurel Bolin [148]. This "spontaneous immortalized SC clone" (iSC) was shown to express markers specific to primary SC such as S100β, p75 ^{NGFR} and Vimentin [148-151]. Cells were maintained in culture medium prepared from Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) 50/50 mix without glutamine (Mediatech, Herndon, VA). This medium contains 17 mM glucose (i.e. the euglycemic condition for this study). Ten percent horse serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Mediatech), 100 units/ml penicillin and 100 μg/ml streptomycin (Mediatech) were supplemented to the media. iSC cells were incubated at 37°C with 5% CO_2. Culture media was changed every three days. Cells were used at a maximum of 7 passages. To achieve desired hyperglycemic and hypoglycemic state of the culture media, we also prepared DMEM beginning with powder DMEM (Mediatech) without glucose, L-glutamine, phenol red, pyruvate or sodium bicarbonate. These were added at the appropriate concentrations after DMEM was dissolved in sterile deionized water. To make hyperglycemic media, 45 mM dextrose (equals to 90 mM glucose) was supplemented and no additional glucose was added to make hypoglycemic media. Next, DMEM was filtered and mixed with equal volume of Ham's F12 (contains 10 mM glucose) to make final DMEM/F12 (50/50) media with 50 mM glucose (hyperglycemic)

or 5 mM glucose (hypoglycemic) respectively. Further, 10% horse serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin were also added.

Fatty Acid Treatment

Treatments were done in serum free media. Serum free media consisted of DMEM/F12 (50/50), 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin, and 1X N-2 supplement (Gibco, Invitrogen Corp.). The iSC cells were plated in full serum media, placed in the incubator and allowed to attach for 5 hr. The media was removed, washed with Dulbecco's Phosphate-Buffered Saline (DPBS) and transferred to serum free media. After 12 hr in serum free media, cells were treated with the selected concentrations of PA complexed with BSA as described previously [35, 64]. The PA (Sigma-Aldrich, St. Louis, MO) stock was prepared in 100% ethanol at a concentration of 300 mM. Fatty acid-free BSA (EMD Biosciences, San Diego, CA) was used as a buffer to ensure that concentrations of unbound free PA in the media were in the 10 nM range during the course of incubation [35]. In brief, serum free medium containing 150 µM fatty acid-free BSA was prepared and warmed up to 37°C to aid in the complete disassociation of PA. Once media was warm, PA stock was added while vortexing to avoid clumping of PA. Two treatment concentrations were used: 150 µM PA (PA:BSA, 1:1) and 300 µM PA (PA:BSA, 2:1). Treatment media were place in the water bath at 37°C for 30 min before they were added to the cells.

Cell Viability Assays

Trypan Blue Exclusion Assay

iSC cells were plated in T25 flask at a density of 2.5 x 10^5 cells/flask. After PA/BSA treatment, cells were trypsinized and centrifuged at 700 x g for 5 min and resuspended in 500 µl of DPBS with 10% glycerol. Trypan Blue 0.4% solution (500 µl, Sigma-Aldrich) was added immediately before counting cells using an inverted microscope. At least 1000 cells were counted for each flask. Percent of blue cells (damaged cells) and clear cells (viable cells) in a given field were recorded.

Crystal Violet Assay

Briefly, iSC cells were plated in 96 well- tissue culture plates at the density of 1.0 x 10^4 cells/well. Subsequent to 24 or 48 hr treatment of PA, 100 µl/well of 4% formaldehyde was added. Following a 5 min incubation period at room temperature, the fixative was removed and cells were washed twice with distilled water. Afterward, cells were post-fixed with 4% formaldehyde for another 30 min. iSC cells were then washed twice with distilled water and allowed to dry completely. Once dried, 100µl/well of crystal violet dye (Accustain®, Sigma-Aldrich) was added and incubated for 30 min at room temperature. Cells were washed with distilled water to remove any unbound stain and dried. The bound crystal violet was then dissolved with 100µl/well of 10% acetic acid solution and place in rocker for 10 min at room temperature. The plate was analyzed by µQuant (Bio-Tek Instruments, Winooski, VT) using an optical density (O.D.) of 570 nm. Data was examined using KCjunior™ software (Bio-Tek Instruments).

WST-1 Assay

Similar to crystal violet assay, iSC cells were plated in 96 well plates. After treatment, WST-1 (10 µl, Roche Applied Science, Indianapolis, IN) in 100 µl of serum free media was added to each well. The plate was incubated at 37°C with 5% CO_2 for 2 hr and O.D. of 450 nm was determined using the µQuant plate reader. Data was examined using KCjunior™ software.

Nuclear Morphology

Hoechst 33258 dye was used to assess chromatin condensation in iSC cells. The dye (10 µg/ml) was added to the cells and incubated for 10 min at 37°C with 5% CO_2. Nuclear morphology of the cells was then photographed using an Olympus fluorescent microscope (excitation/emission wavelength of 365/420 nm). Cells categorized as apoptotic demonstrated apoptotic bodies and increased chromatin condensation.

Assessment of Apoptosis by Flow Cytometry

Annexin V (BD Biosciences, San Diego, CA) and 7AAD (eBioscience, San Diego, CA) were used to detect apoptotic changes occurring in PA-LTx in iSC cells. iSC cells (1.25×10^4) were plated in a 6 well plate. After treatment, cells were trypsinized and collected. They were then resuspended in 40 µl of binding buffer with 2 µl Annexin V-FITC. Cells were incubated for 15 min in the dark at room temperature. After incubation, 160 µl of binding buffer and 2 µl of 7AAD were added. The cells were incubated for 5 min and additional 200 µl of binding buffer was added. Before analyzing, cells were filtered through a cell strainer cap that was fitted to a polystyrene round bottom flow cytometric tube. Cells were analyzed using the Becton-Dikinson

FACSCalibur® flow cytometer (Becton-Dikinson, San Francisco, CA). A total of 100,000 events were measured per sample. Annexin V was detected in the FL-1 channel (530/30nm) while 7AAD was detected in FL-3 channel (650nm). Data was collected in log scale and analyzed using Cell Quest Pro® software and Flow-Jo® software.

Analysis of Mitochondrial Membrane Permeabilization

Disruption of the mitochondrial membrane potential was assessed using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylben-zimidazol-carbocyanine iodide (JC-1, MitoScreen kit, BD Biosciences) as described previously [64]. Briefly, unfixed cells were washed with PBS and resuspended in 1X JC-1 assay buffer supplemented with 10 μg/ml of JC-1. Cells were then incubated for 15 min at 37°C, washed, and resuspended in 1X JC-1 assay buffer for immediate FACSCalibur® flow cytometry analysis. JC-1 in healthy cells was aggregated in mitochondria and detected in the FL-2 channel while JC-1 in apoptotic cells with depolarized mitochondria membrane potential was indicated by a reduced fluorescence in the FL-2 channel. The percentage of cells with disrupted mitochondrial membrane potential was calculated using the Flow-Jo® software.

Measurements of $[Ca^{++}]_{ER}$

To measure the $[Ca^{++}]$ in ER after addition of PA in iSC cells, the Fluo-4 NW Calcium Assay kit (Invitrogen) was used. Briefly, 1.0 $x10^4$ cells were plated in 96 well poly-D- lysine coated plates. Cells were treated accordingly. Afterward, 100 μl of the dye, which included Probenecid (2.5 mM) was added to each well. The plates were

incubated at 37°C for 30 min, then at room temperature for another 30 min. In the last 2 min of room temperature incubation, Thapsigargin (1μM) or DMSO (vehicle) was added to the cells in order to deplete Ca^{++} from ER. Cells were analyzed using the Envison HTS Microplate reader (Perkin-Elmer ® Life and Analytical sciences, Shelton, CT). Excitation/emission parameters were 480 nm and 510 nm respectively.

Ca^{++} Chelating by BAPTA-AM

BAPTA-AM (1,2-bis-(o-Aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester, Invitrogen) was used as a Ca^{++} chelator to reduce intracellular Ca^{++} levels in iSC cells while undergoing PA treatment. BAPTA-AM was dissolved in DMSO and co-treatment of 5 μM BAPTA-AM and 300 μM PA was used. Cell viability was assessed by crystal violet after 24 and 48 hr treatment.

Inhibition of NMDAR by D-AP5

D-AP5, D-(−)-2-amino-5-phosphonopentanoic acid, (Tocris, Bristol, UK) was used to inhibit NMDA receptors in iSC cells. D-AP5 was dissolved in ddH_20 and a co-treatment of 50μM D-AP5 and 300μM PA was utilize. Cell viability was analyzed using crystal violet assays after 24 and 48hr.

RT-PCR

Total RNA was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH). RNA quantification was done by measuring O.D. at 260 nm and stored at -80°C. Two-step RT-PCR was performed. First, RNA (0.5~ 1μg) was used to make

cDNA using the iScrip cDNA synthesis kit (Bio-Rad, Hercules, CA). Next, real-time PCR with Syber Green was conducted using CFX96 Real-Time System (Bio-Rad). For that, CHOP reverse primer 5'-TCC-TCA-TAC-CAG-GCT-TCC-AG-3' and forward primer 5'-CAG-CGA-CAG-AGC-CAA-AAT-AA-3', GRP78 reverse primer 5'-ATA-GGG-CTC-TGC-TGG-AGT-CA-3' and forward primer 5'- CTA-CCC-ACC-TTT-TGC-CAC-TC-3', Xbp1 reverse primer 5'-TTT-CTA-TCT-CGC-GCA-GTC-TGT-3' and forward primer 5'-CCC-CCA-AAG-TGC-TAC-TCC-TA-3' were used. ß-actin was selected as a housekeeping gene (reverse primer 5'-GCG GCA GTG GCC ATC TC-3' and forward primer 5'- GGG AAA TCG TGC GTG ACA TT-3'). The relative amount of mRNA was calculated using the 2 $-\Delta\Delta C_T$ formula.

Reactive Oxygen Species (ROS) Detection

Detection of oxidative stress was done by staining the iSC cells with 20 μM of 2', 7' dichlorodihydrofluorecein deacetate (H_2DCFDA, Invitrogen) for 20 min at 37° C. Cells were then detached by trypsinization and washed twice with DPBS. After filtered through cell strainer cap, cells were analyzed using a FACSCalibur® flow cytometer. A total of 10,000 events were measured per sample. Excitation/Emission wavelengths were 488 nm and 530/30 nm respectively. Data was collected in log scale and analyzed using Cell Quest Pro® software and Flow-Jo® software. Antioxidant MCI-186 (Biomol Research Laboratories, Plymouth Meeting, PA) was used to reduce ROS during PA-LTx. MCI-186 was first dissolved in DMSO and warmed up to 37° C to ensure the complete disassociation. MCI-186 stock solution (or DMSO as a control) was then diluted into the PA/BSA treatment media to attain final concentration of 100 μM or 1 mM.

Western Blots

Western procedures have been described elsewhere [35, 152]. Protein extract from iSC were separate on a SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane. After transfer, the membranes were blocking with 5% milk in Tris-buffered saline (TTBS) with 0.05% Tween 20, PH 7.4 at room temperature for 1 hr. The membranes were then incubated with specific antibodies against different protein, GRP78 and CHOP/Ddit3 (Abcam) in TTBPS containing 0.05% Tween 20 at 4^{o}C overnight. Subsequently the membranes were washed three times with TTBS and incubated with HRP-anti mouse or HRP-anti rabbit (GE Healthcare Bioscience) for 1 hr at room temperature, followed by three washes with TTBS. The signal was then detected by ECL-plus (GE Healthcare Bioscience). Quantitative analysis of the protein was performed by densitometric scanning of the autoradiographs by employing the using ChemiImager™ 4000 (Alpha Innotech Corporation, San Leandro, CA, USA).

Statistical Analysis

All the experiments were repeated independently at least three times. Values represent means ± SE. Statistical comparisons were made using Student t test. Significance was accepted at $p < 0.05$.

Results

Cell Viability and Lipotoxicity in Immortalized Schwann Cells (iSC) Cultures in Different Glucose Concentrations

Chronic elevated plasma levels of saturated FFA have been reported to cause dysfunction and injury in affected tissues and cells [12, 153-156]. Previous reports from

our laboratory have shown that PA (PA:BSA 2:1, see Experimental Procedure) induces lipotoxicity and cell death in nerve growth factor differentiated PC12 cells and rat cortical cells [35, 41, 64]. This experimental paradigm exposed cells to a 10 nM range of unbound FFA concentration which parallel to levels found in conditions such as diabetes and ischemic injury [64, 157-159].

Our initial experiments determined the changes in cell viability under three different conditions: 1) hypoglycemic (5mM glucose), 2) euglycemic (17mM glucose) and 3) hyperglycemic (50 mM glucose) [160]. In all of our studies, cells were cultured in the indicated glucose concentrations for 4 days before the exposure to PA. Our results show that glucose level alone did not affect viability of iSC cells at any of the concentrations tested (Figure 1A, B).

Fig.1

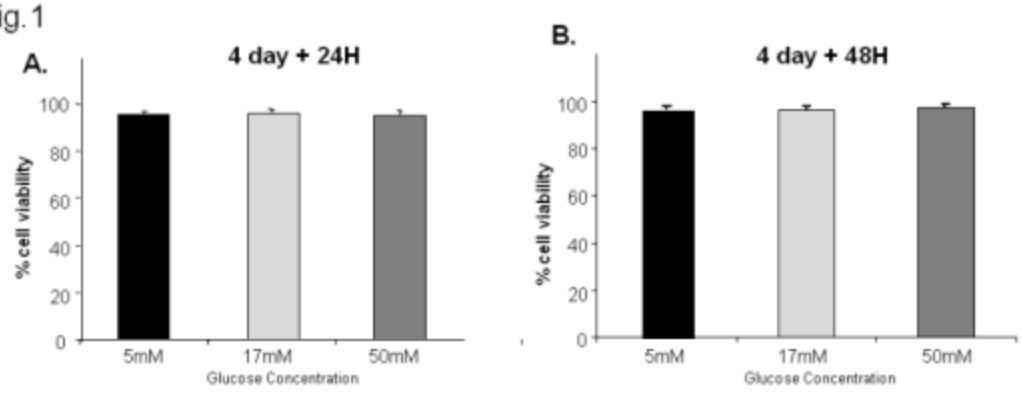
A.
4 day + 24H
% cell viability
100
80
60
40
20
0
5mM
17mM
50mM
Glucose Concentration
B.
4 day + 48H
% cell viability
100
80
60
40
20
0
5mM
17mM
50mM
Glucose Concentration

Figure 1: Cell viability under varied glucose and PA concentrations. iSC cells were cultured in medium containing 5, 17 or 50 mM glucose for 4 days + 24H (A) and 4 days + 48H (B) before the addition of PA. Cell viability was assessed by trypan blue assay after 24 and 48 hr. Statistical analysis was performed using student's t-test.. *$p < 0.05$, **$p<0.005$, N=4

To explore any potential synergistic detrimental effects of FFA and glucose, we performed a series of experiments using trypan blue assays to assess viability of iSC after exposure to PA at a dose of 1:1 or 2:1 together with these three non-damaging glucose concentrations. Figure 1C and 1D shows that under each of these glucose concentrations, PA caused a dose- and time-dependent decrease in cell viability. Cell loss observed in cell cultures exposed to PA under hypoglycemic (5 mM glucose) and euglycemic (17 mM glucose) were similar, however the dose and time-dependent increase in cell death was significantly greater in hyperglycemic cultures (Figure 1C, D).

Fig. 1

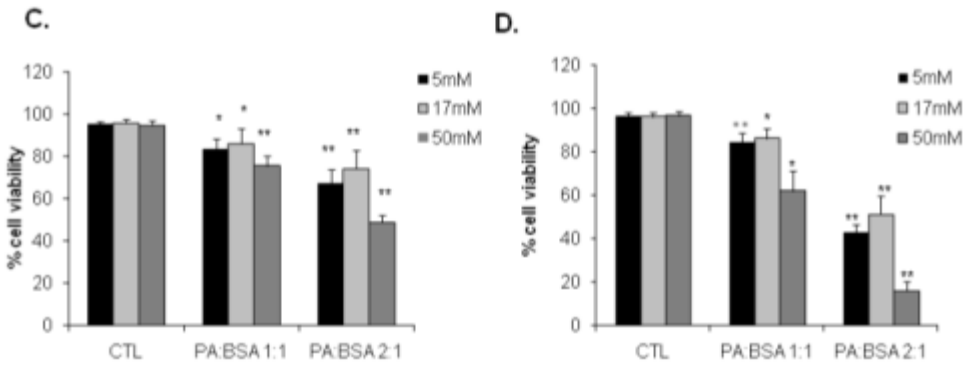
C.
D.
% cell viability
120
100
80
60
40
20
0
5mM
17mM
50mM
CTL
PA:BSA 1:1
PA:BSA 2:1

Figure 1: Cell viability under varied glucose and PA concentrations. iSC cells were cultured in medium containing 5, 17 or 50 mM glucose for 4 days before the addition of PA. After 4 days of pre-conditioning under varied glucose concentrations, PA:BSA (1:1 or 2:1) was added to the cells at 24H (C) and 48H (D). Cell viability was assessed by trypan blue assay after 24 and 48 hr. Statistical analysis was performed using student's t-test.. *$p < 0.05$, **$p < 0.005$, N=4

Cell cultures exposed to PA for 24 hr under hyperglycemic condition showed a 24.3 ± 4.9 % and 60.6 ±19.0 % induction of cell death at PA:BSA 1:1 and 2:1 treatments respectively. At 48 hr, hyperglycemic conditions showed dramatically increased cell death in PA:BSA 1:1 and 2:1 treatments, up to 32.7 ± 12.2 % and 83.9± 4.0 % respectively (Figure 1D).

iSC Cells Undergo Apoptosis During PA-LTx

As shown by Hoescht staining in Figure 2, numerous cells in the PA: BSA 1:1 and PA: BSA 2:1 panels exhibit nucleus condensation and apoptotic bodies while those in untreated cultures show normally dispersed chromatin and intact nuclear membranes. The next series of experiments further quantified this apoptotic process by taking advantage of established flow cytometric methods using Annexin V FITC and 7 AAD to measure early (Annexin V positive cell populations) and late apoptosis (7AAD positive cell populations)..

Fig.2

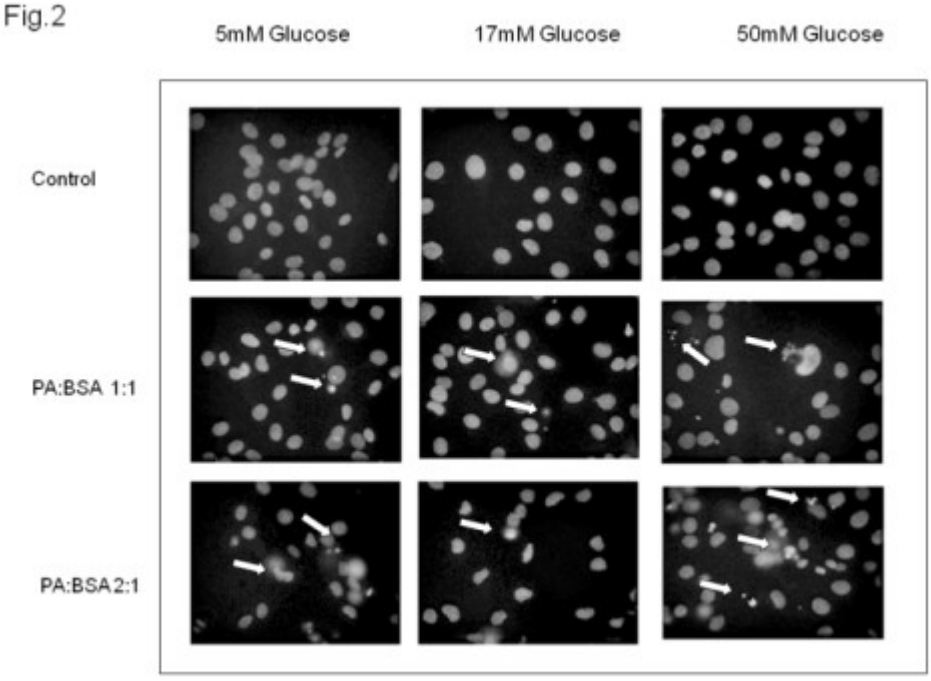
5mM Glucose
17mM Glucose
50mM Glucose
Control
PA:BSA 1:1
PA:BSA 2:1

Figure 2: PA-LTx induced apoptotic features in iSC cells. To determine nuclear morphology, Hoescht staining was performed after 48 hr PA treatment. Nuclear condensation is indicated with white arrows. Representative micrographs of five independent experiments are shown.

Figure 3A shows that there is a significant increase in the number of apoptotic iSC cells at 6 hr following exposure to PA in euglycemic cultures (8.69% early apoptotic, 5.38% late apoptotic cells) and the proportion of apoptotic cells continued to rise up until 48 hr of PA treatment (26.60% early apoptotic, 21.95 % late apoptotic cells). In contrast to the euglycemic conditions, cultures grown in hyperglycemic conditions exhibited an earlier and more pronounced increase in cell death. Cell death in the hyperglycemic conditions occurred as early as 3 hr (3.40% early apoptotic, 4.40% late apoptotic cells) and continued to increase reaching a peak at 48 hr (41.98% early apoptotic, 24.80% late apoptotic cells) Figure 3B.

Fig.3

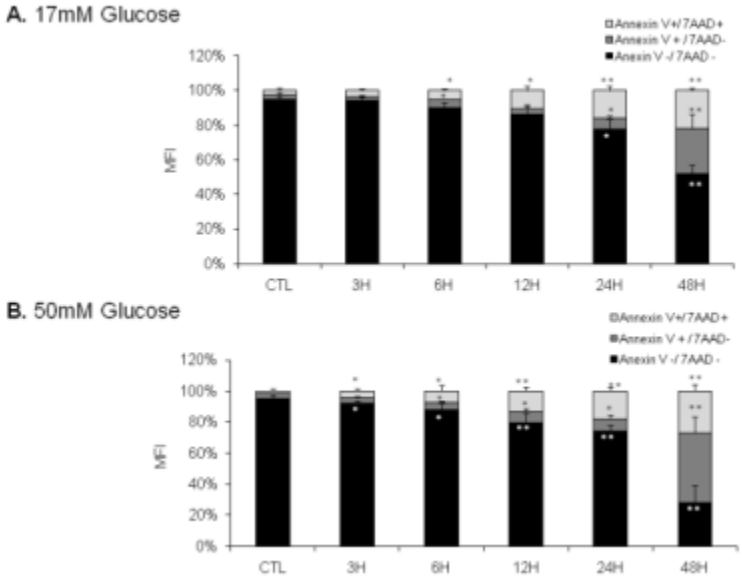
A. 17mM Glucose
Annexin V+/7AAD+
Annexin V + /7AAD-
Anexin V -/7AAD -
120%
100%
80%
60%
40%
20%
0%
MFI
CTL
3H
6H
12H
24H
48H
B. 50mM Glucose
Annexin V+/7AAD+
Annexin V + /7AAD-
Anexin V -/7AAD -
120%
100%
80%
60%
40%
20%
0%
MFI
CTL
3H
6H
12H
24H
48H

Figure 3: Time-dependent induction of apoptosis by PA-LTx. Time course experiments of Annexin V and 7AAD flow cytometric assay of apoptosis were performed on PA treated iSC cells cultured in (A) euglycemic and (B) hyperglycemic media. Early apoptotic translocation of phosphotidyl cistein to the outer cell membrane renders the cell Annexin V+, while late apoptosis is detected by entry and retention of 7AAD in the nucleus. The percentages of cells that are Annexin V-/7AAD- (non apoptotic). Annexin V +/7AAD- (early stage apoptosis) and Annexin V+/7AAD+ (late stage apoptosis) are graphed. Statistical analysis was performed using student's t-test. *$p< 0.05$, **$p<0.005$, N=***4***

Early Mediators of PA-LTx in iSC Cells: Potential Role of Intracellular Calcium

In the nervous system, neurons and associated supportive cells like Schwann cells are vulnerable to conditions that generate calcium overload and ROS as is evident in ischemic and traumatic injuries [121-123]. For example, FFA overload has been associated with significant ER and mitochondrial dysfunction which may result in Ca^{++} release and potential toxicity and cell death [24, 40, 64, 161]. The first series of experiments examined the potential role of calcium overload by examining releasable $[Ca^{++}]$ in the ER, the cellular organelle that is most responsible for controlling free Ca^{++} concentrations in the cytosol. Cell cultures were exposed to 2:1 PA: BSA and Ca^{++} levels in the ER ($[Ca^{++}]_{ER}$) were analyzed at 5 min, 15 min, 30 min and 3, 6, 12 hr. Figure 4A shows that PA-LTx reduced $[Ca^{++}]_{ER}$ in a time dependent manner in both 17 and 50 mM glucose conditions, suggesting that PA was able to impair the capacity of the ER to sequester Ca^{++}. In euglycemic cultures, a significant decrease of $[Ca^{++}]_{ER}$ was present at 30 min after PA treatment, while in hyperglycemic treatment, a significant reduction in $[Ca^{++}]_{ER}$ was observed as early as 15 min after PA treatment and the $[Ca^{++}]_{ER}$ was reduced to approximately 25% by 12 hr (Figure 4A). The limit in the capacity of the ER to store Ca^{++} imposed by PA-LTx results in an increase of free Ca^{++} in the cytoplasm which could lead to cellular toxicity. To test this hypothesis we used a well established Ca^{++} chelator, BAPTA-AM, to assess whether we could reduce the lipotoxic injury. Figure 4B and 4C shows that BAPTA-AM, as expected, reversed the loss of cell viability triggered by PA-LTx in both glucose concentrations and was able to reverse the LTx effect at both 24 and 48 hr (Figure 4B, C). These data suggest that PA-LTx in iSC cells induces an early ER dysfunction that compromises the ability of this organelle to

sequester Ca^{++}; resulting in levels of cytosolic Ca^{++} that can cause cell injury. It is important to note that elevated levels of glucose alone did not significantly affect $[Ca^{++}]_{ER}$ in iSC cells (data not shown).

Fig.4

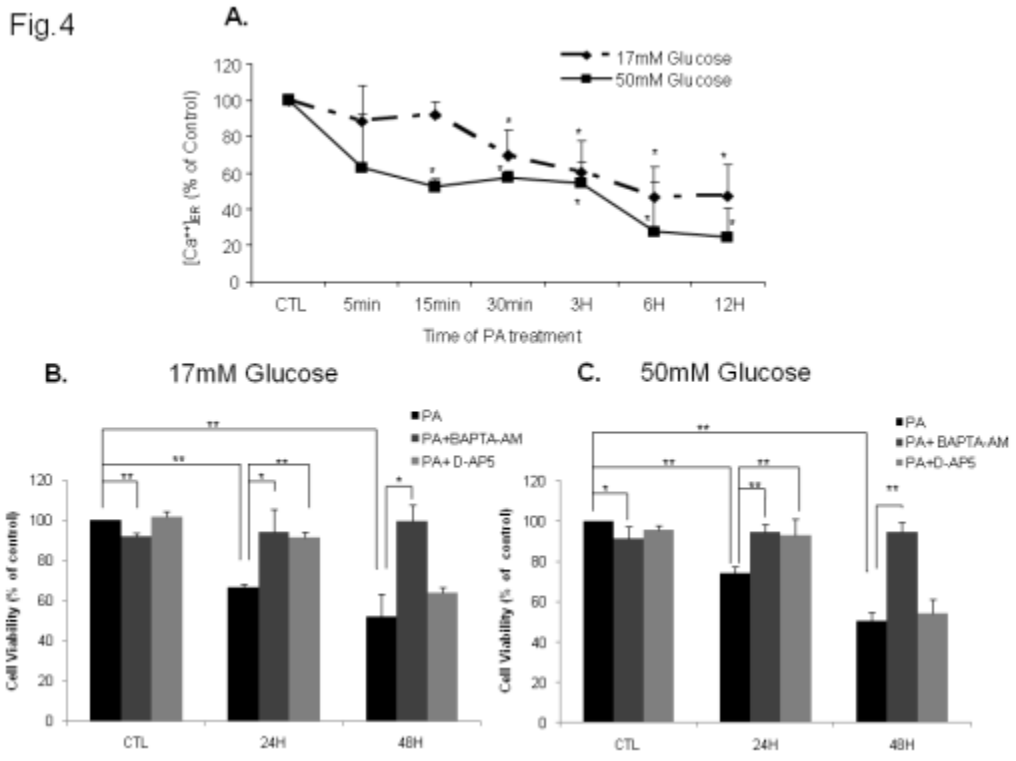
A.
17mM Glucose
50mM Glucose
[Ca++]ER (% of Control)
120
100
80
60
40
20
0
CTL
5min
15min
30min
3H
6H
12H
Time of PA treatment
B.
17mM Glucose
C.
50mM Glucose
PA
PA+BAPTA-AM
PA+ D-AP5
Cell Viability (% of control)
CTL
24H
48H

Figure 4: Ca^{++} concentration in the ER is reduced with PA treatment. (A) The iSC cells were cultured in 17 mM or 50 mM glucose and treated with PA:BSA 2:1. $[Ca^{++}]_{ER}$, reference to control level, was determined at 5, 15, 30 min and 3, 6, 12 hr after treatment. (B)(C) Cells were treated with PA:BSA 2:1 in the presence or absence of BAPTA-AM (5μM) or D-AP5 (50μM) for 24 and 48 hr. Assessment of cell viability was performed by crystal violet assays. Statistical analysis was performed using student's t-test..*p< 0.05, **p< 0.005, N=3

To assess the potential sources of the elevated levels of intracellular Ca^{++} observed under PA-LTx conditions it was important to also examine the possible contribution of influx of Ca^{++} from the extracellular space. A co-treatment of an NMDAR inhibitor, D-AP5 (50μM) with PA (2:1) was performed in order to test for the contribution of NMDA receptors. Our data demonstrated that D-AP5 was able to significantly increase cell viability in euglycemic and hyperglycemic iSC at 24hr only. However, no increase in cell viability was observed at 48hr (Figure 4B, C).

As shown above, the effect of PA-LTx on ER dysfunction and Ca^{++} released into the cytosol is the earliest event of PA-LTx that was observed in iSC cells. To further examine the effects of PA-LTx on the ER, the mRNA expression of well-established ER stress response proteins such as CHOP, GRP78 and Xbp1 were examined [162-164]. CHOP, also known as Ddit3, is a transcription factor that mediates the ER stress-induced apoptotic pathways. The mechanism by which CHOP induces apoptosis remains unclear although it has been implicated in the inhibition of the transcription of anti-apoptotic Bcl-2 proteins [163-164]. ER stress genes were examined using quantitative real time PCR at 0, 3, 6, 12, 24, and 48 hr following exposure to PA:BSA 2:1. We found that PA-LTx triggers a robust up-regulation of CHOP mRNA at 6, 12, and 24 hr in euglycemic and hyperglycemic conditions (Figure 5A and 5B). Spliced Xbp1 is a transcriptional activator that induces genes that encode for ER-associated degradation, chaperones, and lipid synthesis [45, 163]. PA-LTx induced a modest increase in mRNA levels of Xbp1 at 6 hr and reached a peak at 24 hr in euglycemic cells. However, Xbp1 was only significantly up-regulated at 24 and 48hr in hyperglycemic cells (Figure 5A and 5B). Grp78 is a 78 kDa glucose regulated protein that resides in the ER and tends to be up-

regulated when the cell is exposed to environmental stressors [165-167]. Interestingly, mRNA levels of GRP78 were significantly down-regulated at 24 and 48 hr in euglycemic and had a maximum 5-fold up-regulation in hyperglycemic conditions at 48 hr. (Figure 5A and 5B).

Fig.5

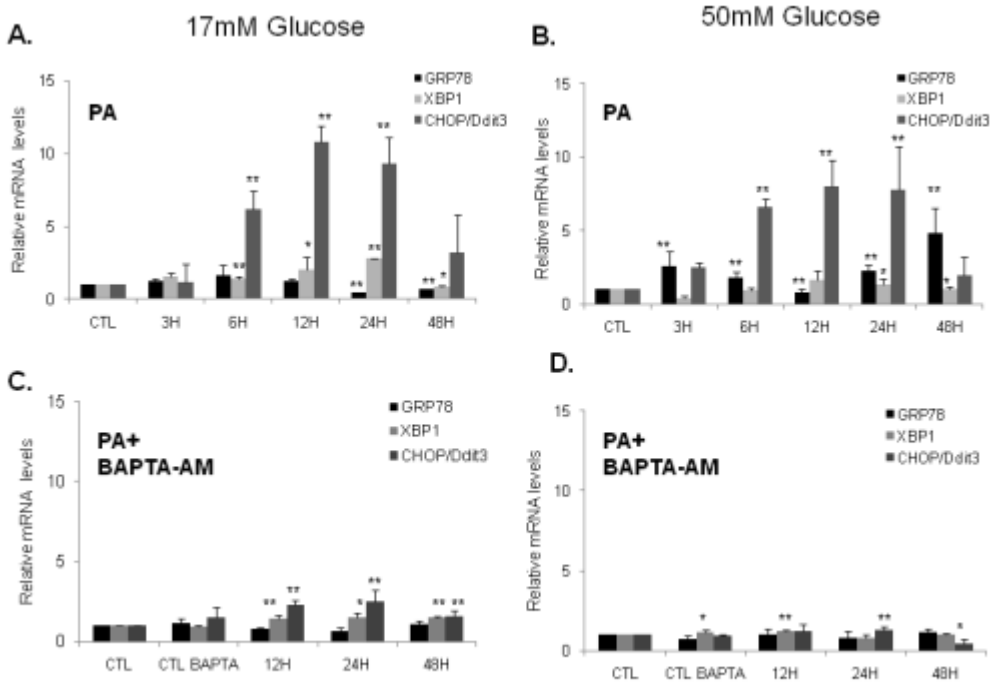
A.
17mM Glucose
PA
GRP78
XBP1
CHOP/Ddit3
Relative mRNA levels
CTL
3H
6H
12H
24H
48H
B.
50mM Glucose
PA
C.
PA+
BAPTA-AM
CTL BAPTA
D.
PA+
BAPTA-AM

Figure 5: ER stress genes are regulated with PA treatment. RT-PCR was performed to measure mRNA levels of ER stress genes CHOP, Xbp1, and Grp78 in iSC cells at 0 (CTL), 3, 6, 12, 24 and 48 hr after adding PA:BSA 2:1 in euglycemic (A) and hyperglycemic conditions (B). In addition, ER stress genes where also evaluated after a co-treatment of PA and BAPTA-AM in both euglycemic (C) and hyperglycemic (D) conditions. Statistical analysis was performed using student's t-test. *$p<0.05$, **$p<0.005$, N=3

Furthermore, we also examined the effect of PA on the protein levels of these ER stress genes at 0, 6, 12, 24, and 48 hr by using Western blots. We found that PA-LTx triggers a robust up-regulation of CHOP protein levels at 12 hrs which returned to normal levels at 24 and 48 hr in euglycemic and hyperglycemic culture conditions (Figure 6A and 6B). In addition, the proteins levels of GRP78 were significantly up-regulated at 12 hr and returned to control expression at 24 hrs and 48 hr in the euglycemic condition (Figure 6C). However, in hyperglycemic condition we observed a significant increased in the protein levels of GRP78 at 24 hrs that remained elevated at 48 hrs (latest time point measured, Figure 6D). We were not able to performed Western experiments for the XBP1 protein because unable to obtain a good antibody.

Fig. 6

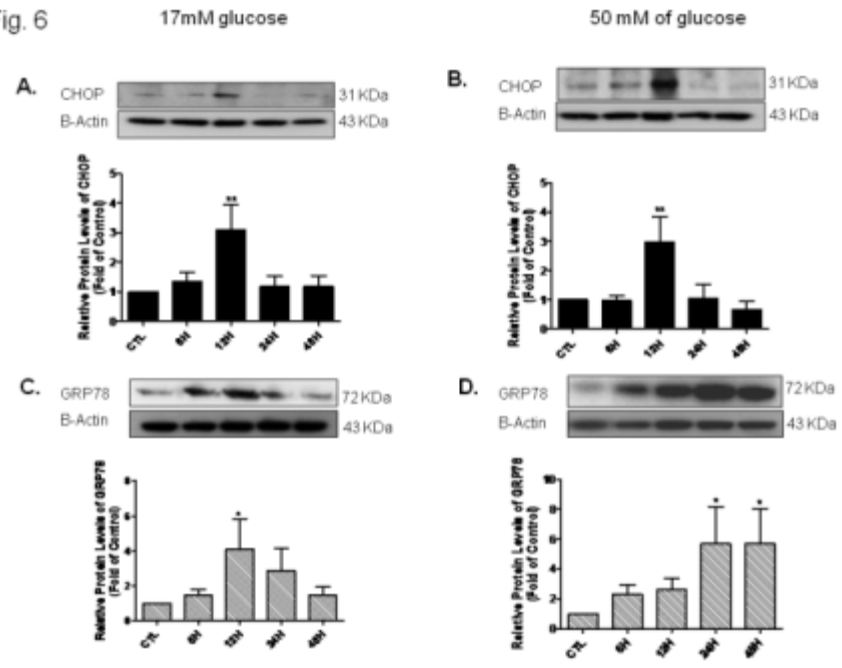
17mM glucose
50 mM of glucose
A.
CHOP
B-Actin
31 KDa
43 KDa
B.
CHOP
B-Actin
31 KDa
43 KDa
Relative Protein Levels of CHOP (Fold of Control)
CTL
6H
12H
24H
48H
C.
GRP78
B-Actin
72 KDa
43 KDa
D.
GRP78
B-Actin
72 KDa
43 KDa
Relative Protein Levels of GRP78 (Fold of Control)

Figure 6: ER stress proteins are altered after PA treatment. Cells lysates of iSC from euglycemic and hyperglycemic condition at 0 (CTL),6, 12, 24 and 48 hr after adding PA:BSA 2:1. were prepared and subjected to Western blot analysis using specific antibodies against CHOP (A,B top) and GRP78(C,D top). The proteins were quantified by densitometric scanning (A–D, bottom). Statistical analysis was performed using student's t-test. *$p<0.05$, **$p<0.005$, N=4

ER stress genes were also examined in the presence of BAPTA-AM. In both euglycemic and hyperglycemic conditions, BAPTA-AM stabilized GRP78 mRNA and maintained its level similar to control through 48 hr after PA treatment (Figure 7A and 7B). For XBP1 mRNA levels, a 50% up-regulation of was observed in euglycemic cells at 12, 24, 48 hr and a 20% increase showed at 12 hr in hyperglycemic cells during co-treatment situation (Figure 7A and 7B). PA in the presence of BAPTA-AM still produced a significant up-regulation of CHOP mRNA in iSC cells cultured in euglycemic environment, however it was not comparable to the levels found with PA treated alone (Figure 7A and 7B). In hyperglycemic conditions, a significant up-regulation of CHOP was observed at 24 hr followed by a significant reduction at 48hr (Figure 7B).

Fig. 7

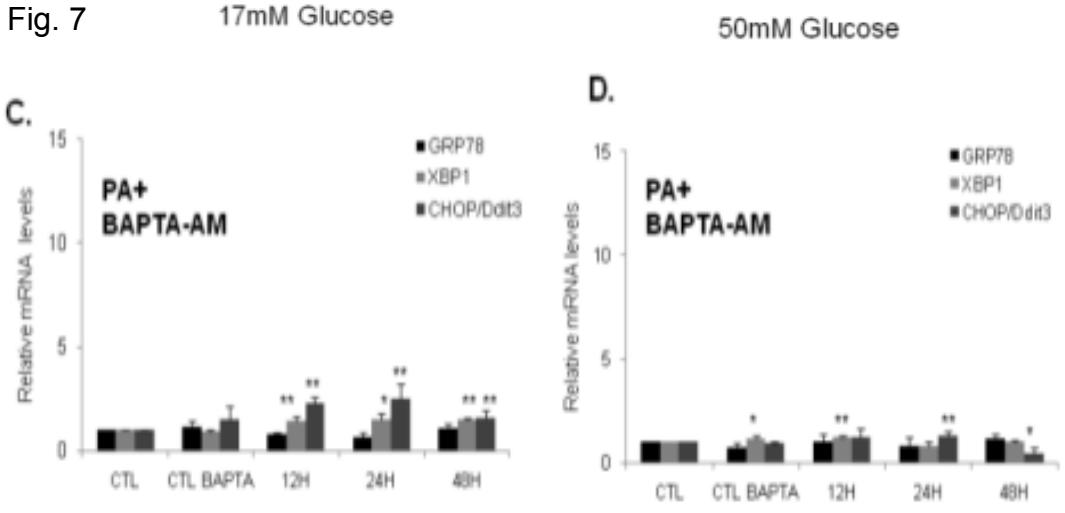
17mM Glucose
50mM Glucose
C.
D.
PA+
BAPTA-AM
GRP78
XBP1
CHOP/Ddit3
Relative mRNA levels
15
10
5
0
CTL
CTL BAPTA
12H
24H
48H

Figure 7: ER stress genes are regulated with PA treatment. RT-PCR was performed to measure mRNA levels of ER stress genes CHOP, Xbp1, and Grp78 in iSC cells at 0 (CTL), 3, 6, 12, 24 and 48 hr after adding after a co-treatment of PA/BSA 2:1 and BAPTA-AM in both euglycemic (C) and hyperglycemic (D). Statistical analysis was performed using student's t-test. *$p<0.05$, **$p<0.005$, N=3

Early Mediators of PA-LTx in iSC Cultures: Potential Role of Reactive Oxygen Species

We assessed the generation of ROS using 2', 7' dichlorodihydrofluorecein diacetate (H_2DCFDA) and flow cytometry. Flow cytometry analysis was performed at 0, 3, 6, 12, 24, and 48 hr following exposure to PA. In agreement with our earlier experiments (see figure 1), hyperglycemic conditions in our cell model did not elicit an increase in ROS levels (Figure7A). However, the addition of PA:BSA 2:1 induced a significant increase of ROS as early as 3hr, followed by further increases at 6, 12, 24, and 48 hr under 17 mM glucose condition (Figure 8B). With a lower dose of PA (PA:BSA 1:1), the ROS levels were shown to significantly increase at 12, 24 and 48 hr only (Figure 8B). In hyperglycemic system, this increase in ROS was observed as early as 3 hr after PA exposure and continued to elevate up to 48 hr (Figure 8C). The magnitude of the ROS increase was also concentration-and time-dependent (Figure 8C).

Fig.8

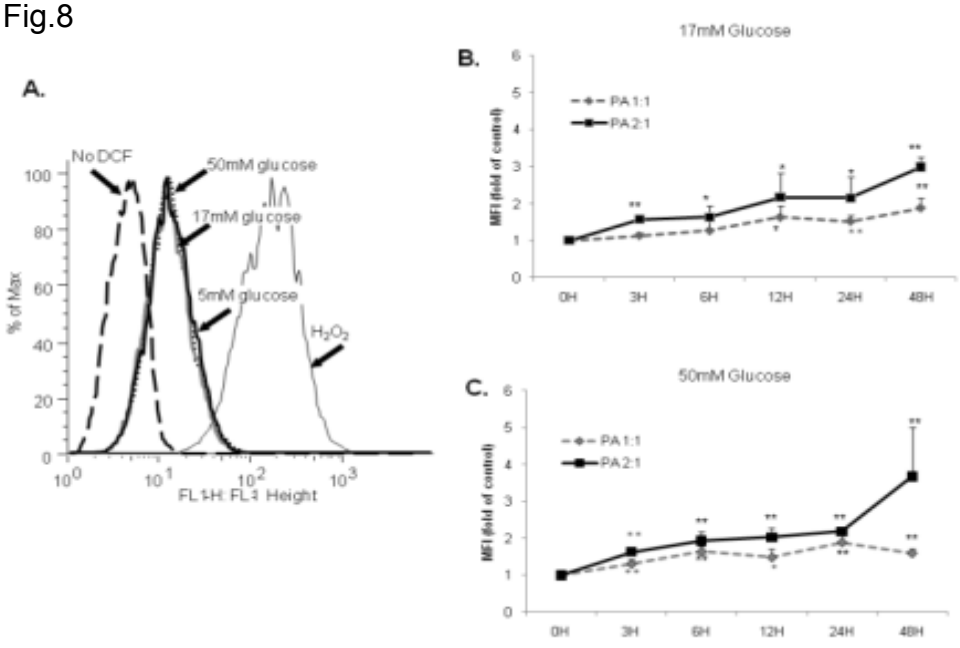
A.
No DCF
50mM glucose
17mM glucose
5mM glucose
H_2O_2
% of Max
FL1-H: FL1 Height
B.
17mM Glucose
PA 1:1
PA 2:1
MFI (fold of control)
0H
3H
6H
12H
24H
48H
C.
50mM Glucose
PA 1:1
PA 2:1
MFI (fold of control)
0H
3H
6H
12H
24H
48H

Figure 8: ROS analysis using H_2DCFDA flow cytometry. iSC cells were cultured in hypoglycemic (5 mM glucose), euglycemic (17 mM glucose), and hyperglycemic (50 mM glucose) media for at least 4 days before analyzing ROS levels by H_2DCFDA assay. The increase in fluorescence is indicative of an increase in ROS within the cell. Cells treated with 300 μM of H_2O_2 for 15 min were used as a positive control. (A) Representative flow cytometry graphs of iSC cells cultured in different concentrations of glucose. No increase in ROS was observed. ROS analysis was performed on iSC cells treated with PA:BSA 1:1 and 2:1 in (B) 17 mM glucose and (C) 50 mM glucose conditions. Flow cytometry data was quantified using Cell Quest Pro® and Flow-Jo® software. Statistical analysis was performed using student's t-test..*$p< 0.05$, **$p< 0.005$, N=3

Next, we evaluated the physiological relevance of this ROS elevation by treating the culture with MCI-186, a well characterized free radical scavenger [168]. Our data show that control euglycemic cell cultures treated with 0.1 mM of MCI-186 exhibited a 38% reduction in the ROS levels with approximately 93% of the cells viable at 24 hr (Figure 9A and 9C). Subsequently, co-treatment of PA and MCI-186 in euglycemic cell cultures resulted in a 46% reduction in ROS as compared to PA treatment alone (Figure 9A). Similarly, the addition of 1 mM MCI-186 to cultures grown under hyperglycemic conditions reduced ROS levels by 16% in control and by 47% in PA-treated cells at 24 hr (Figure 9B). MCI-186 was found to be toxic in both euglycemic and hyperglycemic cultures at 48 hr (data not shown). To examine the effects of this reduction in ROS on viability of PA-treated iSC cells, crystal violet viability assays were performed. Figure 8C shows that iSC cell viability was increased by 20% in PA-LTx cells co-treated with 0.1mM of MCI-186 under euglycemic conditions (Figure 9C) and completely restored to normal levels when 1 mM of MC-186 was added to cells grown in hyperglycemic conditions (Figure 9D).

Given that BAPTA-AM increased cell viability at 24 and 48hr after PA treatment, it was of interest to also evaluate levels of ROS. Our data demonstrates that co-treatment of 5μM BAPTA-AM with 300μM PA was able to significantly decrease ROS levels at 24 and 48hr (Figure 9E and 9F).

Fig. 9

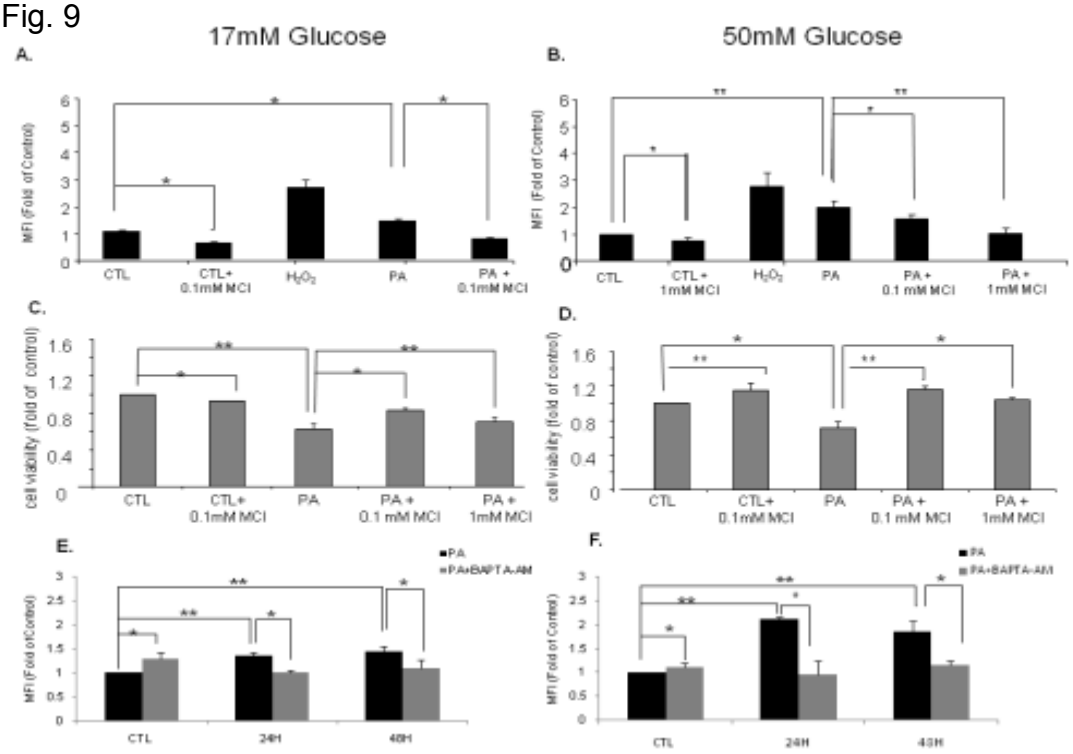
17mM Glucose
50mM Glucose
A.
B.
C.
D.
E.
F.
MFI (Fold of Control)
cell viability (fold of control)
CTL
CTL+ 0.1mM MCl
H2O2
PA
PA + 0.1mM MCl
CTL+ 1mM MCl
PA + 0.1 mM MCl
PA + 1mM MCl
PA
PA+BAPTA-AM
24H
48H

Figure 9: MCI-186 and BAPTA-AM reduce ROS levels and increases cell viability in PA-LTx iSC cells. The iSC cells were cultured in 17 mM glucose (A, C, E) or 50 mM glucose (B,D, F) concentrations and treated with PA:BSA 2:1 (PA) in the presence or absence of 100 μM or 1 mM MCI-186 or 5μM BAPTA-AM. ROS analysis using H_2DCFDA flow cytometry (A, B) and Crystal violet assay for cell viability (C, D) were performed at 24 hr. Cells cultured in euglycemic (E) and hyperglycemic (F) conditions were co-treated with BAPTA-AM for 24 and 48H and analyzed for ROS levels. Statistical analysis was performed using student's t-test.*$p< 0.05$, **$p< 0.005$, N=3

The significant generation of ROS observed after exposure to PA suggests a potential increase in mitochondrial permeability and dysfunction. We performed a series of flow cytometry experiments using JC-1 to evaluate mitochondrial function. Cell cultures were treated with PA:BSA 2:1 and analyzed by JC-1 flow cytometry at 30 min, 1, 3, 6 and 12 hr after the initial exposure to PA. Figure 10A and 10C show that cells grown in euglycemic conditions exhibit significant mitochondria membrane depolarization at 6 hr and reached a peak at 12 hr after the initial exposure to PA (Figures 10A and 10C). Interestingly, cells grown under hyperglycemic conditions show this dysfunction as early as one hour after exposure to PA:BSA 2:1 treatment (Figure 10B and 10D). Hyperglycemia alone did not result in significant mitochondrial depolarization in iSC cells (data not shown).

Fig. 10

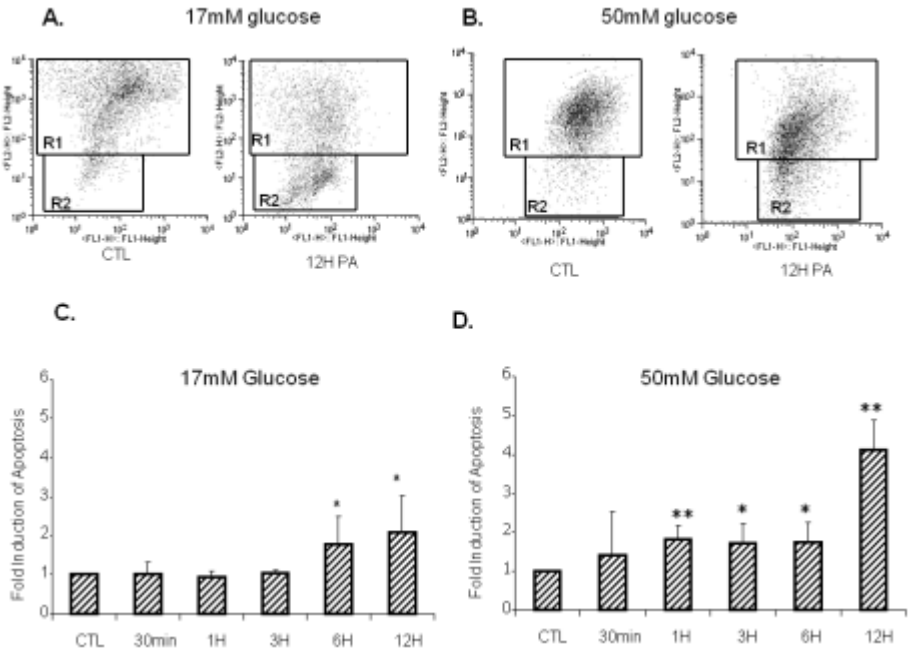
A.
17mM glucose
R1
R2
CTL
12H PA
B.
50mM glucose
R1
R2
CTL
12H PA
C.
17mM Glucose
Fold Induction of Apoptosis
CTL
30min
1H
3H
6H
12H
D.
50mM Glucose
Fold Induction of Apoptosis
CTL
30min
1H
3H
6H
12H

Figure 10: PA-LTx induces mitochondrial depolarization in iSC cells. Mitochondrial membrane depolarization was examined by flow cytometric analysis using JC-1 fluorescent dye. Representative flow cytometric plots of control (CTL) and 12 hr PA:BSA 2:1 treatment (12H PA) under (A) 17 mM glucose condition and (B) 50 mM glucose condition are shown. R1 quadrant indicates cells with intact mitochondria while R2 quadrant represents cells with depolarized mitochondria, i.e. apoptotic cells. Quantitative analysis of JC-1 data using Flow JO® software is shown in (C) 17 mM glucose condition and (D) 50 mM glucose condition. Statistical analysis was performed using student's t-test..*$p<0.05$, **$p<0.005$ N=4

Discussion

The present study shows for the first time that exposure to high levels of PA results in a strong lipotoxic process that result in apoptotic cell death and is mediated though ER stress, ROS generation and mitochondria depolarization. This lipotoxic insult increases when cells are cultured under high glucose conditions. Interestingly, the calcium chelator BAPTA-AM and free radical scavenger MC1-186 are powerful inhibitors of PA-LTx and cell death. Our findings also show that ER stress precedes mitochondrial dysfunction and ROS generation after exposure to PA.

Previous studies have examined the role of high glucose in inducing Schwann cell (SC) dysfunction and elucidated its potential role in the development of peripheral neuropathy (PN), a common type 2 diabetes morbidity [120, 160] [132]. However, because of controversies as to whether hyperglycemia is the primary upstream factor responsible of SC dysfunction in PN, another contributor, dyslipidemia, is attracting further attention [84, 87, 125-126]. Type 2 diabetes is characterized by a serious dysregulation in lipid metabolism resulting in chronic elevated levels of FFA in the plasma [12, 136, 153-155]. Thus, it is important to assess the pathological implications of hyperglycemia in combination with high levels of FFA in SC viability. This report shows that chronic hyperglycemia alone does not induce iSC cell death but its presence strongly magnified the effects of lipotoxic injury. This is consistent with observations that cultures treated with high glucose alone retard neurite outgrowth, slows down proliferation and delays migration without inducing cell death in isolated neonatal SC cultures [108]. PA-LTx has also been shown to be enhanced by high glucose in pancreatic β-cell cell death [169]. The failure of hyperglycemia to induce death of iSC

may be due to the activation of cellular pro-survival pathways known to be present when cells are exposed to low stress insults. This induced protective status includes the generation of anti-apoptotic pro-survival proteins that counteract pro-apoptotic pathways stimulated by the low threshold stimuli. The exposure to an additional insult such as lipid overload may result in an override of the cell defensive response and result in cell death and apoptosis [170-172].

Long chain saturated free fatty acids (LCSFFA) like stearic acid and PA are strong inducers of lipotoxicity and cell death in neurons and other cells [35, 41, 64]. Cell membranes containing phospholipids enriched with LCSFFA exhibit lower fluidity [24]. These LCSFFA may accumulate in membrane lipid rafts, alter the lipid environment and affect important functions of key receptors and other membrane proteins [173-176]. These LCSFFA also undergo esterification with less efficiency as a result of possible low efficiency of the enzymes involved [5, 25, 30, 156]. Furthermore, these LCFFA accumulate in the cell and form diaglycerides and ceramide, which may contribute to the cellular dysfunction observed [26, 28-29]. Ceramide is a byproduct produced by saturated fatty acids and is a key mediator of cytotoxicity and apoptosis [177-179]. This may be one of the apoptotic pathways that are involved in the PA-induced cell death demonstrated in numerous studies using non-nerve cells and in the present study [169, 180-181].

Our data show that PA-LTx in the presence of high glucose results in an early and more robust release of Ca^{++} into the cytosol from the ER. The data points to the ER as the earliest organelles to be affected by the lipid overload. Studies have shown that PA-LTx may affect cellular organelles like the ER and the mitochondria [39, 64, 182]. In

the mitochondria, Ca^{++} is important for the generation of ATP and Ca^{++} sensing chaperones and binding proteins are equally important in regulating free cytosolic Ca^{++} concentrations [183-185]. The data is consistent with a model in which PA initially injures the ER membranes, resulting in the release of Ca^{++} into the cytosol and overloading the mitochondrial matrix. Although not measured in detail in the present study, these series of events would lead to the translocation of AIF and EndoG to the nucleus, thus inducing chromatin condensation and DNA fragmentation [186]. Moreover, injury to the mitochondria induces the release of cytrochrome c into the cytosol, which may contribute to the formation of the apoptosome (cyto-c +Apaf-1 + caspase 9) [61, 187-188].

Previous work from our laboratory demonstrated that inhibition of caspase activation did not block the lipotoxic process but the present study found that reducing excessive cytosolic Ca^{++} levels using BAPTA-AM inhibited the cell death process. Furthermore, our data demonstrates that BAPTA-AM is able to reduce ROS level in both euglycemic and hyperglycemic iSC at 24 and 48hr. Previous studies in the literature have shown that buffering calcium with agents such as BAPTA-AM prevented the induction of apoptosis in different cellular models by preventing mitochondrial membrane depolarization [188-191]. Deniaud et al (2008) et al examined the inner mitochondrial membrane permeabilization of HeLa cells and found that after 24 hr treatment with ER-stress inducers, such as thapsigargin and tunicamycin, inner mitochondrial membrane permeabilization was reduced by BAPTA-AM. [191]. This effect of BAPTA-AM was highly significant even though this drug has a modest effect on ROS levels in control cells in control group [192-193].

Besides BAPTA-AM, another factor that can increase cell viability is the inhibition of NMDA receptors [194-196]. A study by Yu et al., (2002) found that activation of NMDA receptors are needed in order to induce the translocation of AIF from the mitochondria to the nucleus, thus leading to apoptosis. In our hands, inhibiting NMDA receptors with D-AP5 increased cell viability at 24hr in both euglycemic and hyperglycemic conditions. We speculate that by inhibiting the NMDA receptors, translocation of AIF to the nucleus may have been prevented and future experiments will address this question. The inability of D-AP5 to increase cell viability at 48hr may be explained by the overall severity of cellular injury that drives the cell towards cellular death regardless of the inhibition of these receptors.

We found that the depletion of $[Ca^{++}]_{ER}$ by PA not only affects normal mitochondrial function but also alters the expression of ER stress genes and proteins such as CHOP, Xbp1, and GRP78. In euglycemic conditions, PA-LTx triggers the up-regulation of Xbp1 and CHOP mRNA levels, simultaneously, before significant cell death was observed. It was also observed that protein levels of CHOP were increased at 12hr. The pattern of gene regulation observed in cells undergoing PA-LTx suggests that SC increases the expression of genes that encode for the ER stress response, such as chaperones, and transcription factors. These in turn function to decrease the load on the ER, therefore promoting cell survival [162-163, 197-198]. In conjunction, these cells also promote cell death through up-regulation of CHOP, which down-regulates Bcl-2 expression, depletes cellular levels of glutathione, translocates BAX from the cytosol to the mitochondria, and induces TRB3, an AKT inhibitor that plays a role in ER stress-induced cell death [163-164, 199]. In addition, the unfolding protein response can

activate IRE-1, a transmembrane ER stress sensor, that will engage TNF receptor-associated factor 2 (TRAF2) which can mobilize apoptosis signal-regulating kinase (ASK1) resulting in the activation of c-Jun amino terminal kinase (JNK), a powerful pro-apoptotic kinase that interacts with Bcl-2 family proteins [52, 200-202]. Hence, there is a period of time where the cell promotes cell survival and cell death simultaneously [203-204]. Ultimately, as time progresses, one pathway will dominate and govern the fate of the cell. In this manner, PA treated cells will begin to express significantly higher mRNA levels of CHOP, in which Xbp-1 can no longer exert its influence, resulting in cellular death.

An interesting finding was the up-regulation of GRP78 mRNA and protein expression in the hyperglycemic state at 24 and 48 hr after PA treatment since up-regulation of GRP78 is frequently correlated with cell survival [205-206]. Elevation of GRP78 expression has also been observed at times when cells are undergoing apoptosis [39, 201, 207]. In the spontaneous hypertensive rat model, cardiomyocytes that suffered from prolonged hypertension (32 weeks) demonstrated an increase in apoptotic cells and expression of significant levels of GRP78, caspase 3 and caspase 12 [208].

Treatment of iSC cells with BAPTA-AM resulted in the stabilization of mRNA levels of pro-survival genes such as GRP78, upregulation of XBP1 in a euglycemic and hyperglycemic conditions. The decrease in pro-apoptotic ER stress genes such as CHOP was observed in both euglycemic and hyperglycemic conditions. Thus, reducing the stress on the ER by BAPTA-AM, may assist the cell in its attempt to survive by promoting anti-apoptotic pathways.

The mechanisms by which hyperglycemia potentiates PA injury has not been studied in SC. Cell culture studies using beta cells have found that PA increases nitric oxide (NO) while high glucose increases levels of superoxide (O_2^-). However, when the cells are exposed to both insults the production of both radicals can lead to the production of peroxynitrite ($OOON^-$), a powerful free radical known to cause DNA damage and apoptosis [169]. Hyperglycemia can also activate PARP which can deplete NAD levels and lead to dysfunction of glycolitic and mitochondrial respiration pathways in diabetic neuropathy [103-106].

Our findings indicate that the ER response is the earliest event that follows PA-LTx. The ER response results in Ca^{++} to be released into the cytosol, affecting the mitochondria and leading to mitochondrial membrane depolarization. Consequently, mitochondrial dysfunction will result in an increase in levels of ROS and cellular metabolic derangements. Moreover, pro-apoptotic and anti-apoptotic pathways will be activated simultaneously. As the injury continues, a full ASCOS condition will be in place affecting proteins, lipids, and DNA. Further, when high glucose levels are incorporated, other factors such as a decrease in anti-oxidant molecules, disruption of metabolic activities, including glycolysis and respiratory pathways, and possibly the activation of inflammatory mediators can dramatically augment the injury caused by PA. Increasing number of studies are linking ER stress to a decrease in myelin production in the nervous system [44, 209]. The data is consistent with these studies and agrees with these published results and further show that the combination of hyperglycemia and palmitic acid overload can cause SC dysfunction and SC death. We propose that similar

mechanism maybe happening *in vivo* that may result in SC dysfunction, cell death, focal demyelination resulting in abnormal nerve conduction and synaptic transmission.

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References

1. Bezuglov, V.V., M. Bobrov, and A.V. Archakov, *Bioactive amides of fatty acids.* Biochemistry (Mosc), 1998. **63**(1): p. 22-30.

2. Ghosh, S. and B. Rodrigues, *Cardiac cell death in early diabetes and its modulation by dietary fatty acids.* Biochim Biophys Acta, 2006. **1761**(10): p. 1148-62.

3. Wilding, J.P., *The importance of free fatty acids in the development of Type 2 diabetes.* Diabet Med, 2007. **24**(9): p. 934-45.

4. Ioannidis, I., *The road from obesity to type 2 diabetes.* Angiology, 2008. **59**(2 Suppl): p. 39S-43S.

5. Unger, R.H., *Reinventing type 2 diabetes: pathogenesis, treatment, and prevention.* JAMA, 2008. **299**(10): p. 1185-7.

6. Kusminski, C.M., et al., *Diabetes and apoptosis: lipotoxicity.* Apoptosis, 2009. **14**(12): p. 1484-95.

7. Listenberger, L.L., et al., *Triglyceride accumulation protects against fatty acid-induced lipotoxicity.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3077-82.

8. van Herpen, N.A. and V.B. Schrauwen-Hinderling, *Lipid accumulation in non-adipose tissue and lipotoxicity.* Physiol Behav, 2008. **94**(2): p. 231-41.

9. El-Assaad, W., et al., *Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death.* Endocrinology, 2003. **144**(9): p. 4154-63.

10. Wei, Y., et al., *Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells.* Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E275-81.

11. Rho, M.C., et al., *Sensitization of vascular smooth muscle cell to TNF-alpha-mediated death in the presence of palmitate.* Toxicol Appl Pharmacol, 2007. **220**(3): p. 311-9.

12. Li, Z., et al., *Using dynamic gene module map analysis to identify targets that modulate free fatty acid induced cytotoxicity.* Biotechnol Prog, 2008. **24**(1): p. 29-37.

13. Shimabukuro, M., et al., *Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2498-502.

14. Lupi, R., et al., *Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is*

caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated. Diabetes, 2002. **51**(5): p. 1437-42.

15. Kato, A., et al., *Protective effects of dietary chamomile tea on diabetic complications.* J Agric Food Chem, 2008. **56**(17): p. 8206-11.

16. Koshkin, V., et al., *Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic beta-cells.* J Biol Chem, 2008. **283**(12): p. 7936-48.

17. Unger, R.H., *How obesity causes diabetes in Zucker diabetic fatty rats.* Trends Endocrinol Metab, 1997. **8**(7): p. 276-82.

18. Unger, R.H. and Y.T. Zhou, *Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover.* Diabetes, 2001. **50 Suppl 1**: p. S118-21.

19. Schaffer, J.E., *Lipotoxicity: when tissues overeat.* Curr Opin Lipidol, 2003. **14**(3): p. 281-7.

20. Simonian, N.A. and J.T. Coyle, *Oxidative stress in neurodegenerative diseases.* Annu Rev Pharmacol Toxicol, 1996. **36**: p. 83-106.

21. Ilieva, E.V., et al., *Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis.* Brain, 2007. **130**(Pt 12): p. 3111-23.

22. Almaguel, F.G., et al., *Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid.* J Neurosci Res, 2009. **87**(5): p. 1207-18.

23. Taghibiglou, C., et al., *Mechanisms involved in cholesterol-induced neuronal insulin resistance.* Neuropharmacology, 2009. **57**(3): p. 268-76.

24. Hayashi, T., et al., *Damage to the endoplasmic reticulum and activation of apoptotic machinery by oxidative stress in ischemic neurons.* J Cereb Blood Flow Metab, 2005. **25**(1): p. 41-53.

25. Grimm, M.O., et al., *Regulation of cholesterol and sphingomyelin metabolism by amyloid-beta and presenilin.* Nat Cell Biol, 2005. **7**(11): p. 1118-23.

26. He, X., et al., *Deregulation of sphingolipid metabolism in Alzheimer's disease.* Neurobiol Aging, 2010. **31**(3): p. 398-408.

27. Conti, A.C., et al., *Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period.* J Neurosci, 1998. **18**(15): p. 5663-72.

28. Newcomb, J.K., et al., *Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat.* Exp Neurol, 1999. **158**(1): p. 76-88.

29. Malecki, A., et al., *4-Hydroxynonenal induces oxidative stress and death of cultured spinal cord neurons.* J Neurochem, 2000. **74**(6): p. 2278-87.

30. Ulloth, J.E., C.A. Casiano, and M. De Leon, *Palmitic and stearic fatty acids induce caspase-dependent and -independent cell death in nerve growth factor differentiated PC12 cells.* J Neurochem, 2003. **84**(4): p. 655-68.

31. Almaguel, F.G., et al., *Lipotoxicity-mediated cell dysfunction and death involve lysosomal membrane permeabilization and cathepsin L activity.* Brain Res, 2010. **1318**: p. 133-43.

32. Welcher, A.A., et al., *Molecular approaches to nerve regeneration.* Philos Trans R Soc Lond B Biol Sci, 1991. **331**(1261): p. 295-301.

33. Dyck, P.J. and C. Giannini, *Pathologic alterations in the diabetic neuropathies of humans: a review.* J Neuropathol Exp Neurol, 1996. **55**(12): p. 1181-93.

34. Song, Z., et al., *Transgenic mice overexpressing aldose reductase in Schwann cells show more severe nerve conduction velocity deficit and oxidative stress under hyperglycemic stress.* Mol Cell Neurosci, 2003. **23**(4): p. 638-47.

35. Sango, K., et al., *High glucose-induced activation of the polyol pathway and changes of gene expression profiles in immortalized adult mouse Schwann cells IMS32.* J Neurochem, 2006. **98**(2): p. 446-58.

36. Reaven, G.M., et al., *Lowering of plasma glucose in diabetic rats by antilipolytic agents.* Am J Physiol, 1988. **254**(1 Pt 1): p. E23-30.

37. Paolisso, G., et al., *A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM.* Diabetologia, 1995. **38**(10): p. 1213-7.

38. McGarry, J.D., *Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes.* Diabetes, 2002. **51**(1): p. 7-18.

39. Cnop, M., *Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes.* Biochem Soc Trans, 2008. **36**(Pt 3): p. 348-52.

40. Bazan, N.G., Jr., *Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain.* Biochim Biophys Acta, 1970. **218**(1): p. 1-10.

41. Cistola, D.P., J.C. Sacchettini, and J.I. Gordon, *13C NMR studies of fatty acid-protein interactions: comparison of homologous fatty acid-binding proteins*

produced in the intestinal epithelium. Mol Cell Biochem, 1990. **98**(1-2): p. 101-10.

42. Thomas, M.E., et al., *Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinuria.* Am J Physiol Renal Physiol, 2002. **283**(4): p. F640-7.

43. Vincent, A.M., et al., *Short-term hyperglycemia produces oxidative damage and apoptosis in neurons.* Faseb J, 2005. **19**(6): p. 638-40.

44. Dugan, L.L., et al., *IL-6 mediated degeneration of forebrain GABAergic interneurons and cognitive impairment in aged mice through activation of neuronal NADPH oxidase.* PLoS One, 2009. **4**(5): p. e5518.

45. Lu, Q., et al., *Guattegaumerine protects primary cultured cortical neurons against oxidative stress injury induced by hydrogen peroxide concomitant with serum deprivation.* Cell Mol Neurobiol, 2009. **29**(3): p. 355-64.

46. Barber, S.C. and P.J. Shaw, *Oxidative stress in ALS: Key role in motor neuron injury and therapeutic target.* Free Radic Biol Med, 2010. **48**(5): p. 629-641.

47. Borradaile, N.M., et al., *Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death.* J Lipid Res, 2006. **47**(12): p. 2726-37.

48. Karaskov, E., et al., *Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis.* Endocrinology, 2006. **147**(7): p. 3398-407.

49. Xu, C., B. Bailly-Maitre, and J.C. Reed, *Endoplasmic reticulum stress: cell life and death decisions.* J Clin Invest, 2005. **115**(10): p. 2656-64.

50. Malhotra, J.D. and R.J. Kaufman, *The endoplasmic reticulum and the unfolded protein response.* Semin Cell Dev Biol, 2007. **18**(6): p. 716-31.

51. Eizirik, D.L., A.K. Cardozo, and M. Cnop, *The role for endoplasmic reticulum stress in diabetes mellitus.* Endocr Rev, 2008. **29**(1): p. 42-61.

52. Yasuda, H., et al., *Diabetic neuropathy and nerve regeneration.* Prog Neurobiol, 2003. **69**(4): p. 229-85.

53. Rao, R.V. and D.E. Bredesen, *Misfolded proteins, endoplasmic reticulum stress and neurodegeneration.* Curr Opin Cell Biol, 2004. **16**(6): p. 653-62.

54. Dey, A., I.G. Kessova, and A.I. Cederbaum, *Decreased protein and mRNA expression of ER stress proteins GRP78 and GRP94 in HepG2 cells over-expressing CYP2E1.* Arch Biochem Biophys, 2006. **447**(2): p. 155-66.

55. Hiramatsu, N., et al., *Real-time detection and continuous monitoring of ER stress in vitro and in vivo by ES-TRAP: evidence for systemic, transient ER stress during endotoxemia.* Nucleic Acids Res, 2006. **34**(13): p. e93.

56. Takahashi, G., et al., *MCI-186 reduces oxidative cellular damage and increases DNA repair function in the rabbit spinal cord after transient ischemia.* Ann Thorac Surg, 2004. **78**(2): p. 602-7.

57. Eckersley, L., *Role of the Schwann cell in diabetic neuropathy.* Int Rev Neurobiol, 2002. **50**: p. 293-321.

58. Yu, C., S. Rouen, and R.T. Dobrowsky, *Hyperglycemia and downregulation of caveolin-1 enhance neuregulin-induced demyelination.* Glia, 2008. **56**(8): p. 877-87.

59. Vincent, A.M., et al., *Dyslipidemia-induced neuropathy in mice: the role of oxLDL/LOX-1.* Diabetes, 2009. **58**(10): p. 2376-85.

60. Wiggin, T.D., et al., *Elevated triglycerides correlate with progression of diabetic neuropathy.* Diabetes, 2009. **58**(7): p. 1634-40.

61. Cameron, N.E. and M.A. Cotter, *Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway.* Curr Drug Targets, 2008. **9**(1): p. 60-7.

62. Obrosova, I.G., et al., *High-fat diet induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition.* Diabetes, 2007. **56**(10): p. 2598-608.

63. Gumy, L.F., E.T. Bampton, and A.M. Tolkovsky, *Hyperglycaemia inhibits Schwann cell proliferation and migration and restricts regeneration of axons and Schwann cells from adult murine DRG.* Mol Cell Neurosci, 2008. **37**(2): p. 298-311.

64. Okuyama, R., T. Fujiwara, and J. Ohsumi, *High glucose potentiates palmitate-induced NO-mediated cytotoxicity through generation of superoxide in clonal beta-cell HIT-T15.* FEBS Lett, 2003. **545**(2-3): p. 219-23.

65. Sango, K., et al., *Diabetes is not a potent inducer of neuronal cell death in mouse sensory ganglia, but it enhances neurite regeneration in vitro.* Life Sci, 2002. **71**(20): p. 2351-68.

66. Cheng, C. and D.W. Zochodne, *Sensory neurons with activated caspase-3 survive long-term experimental diabetes.* Diabetes, 2003. **52**(9): p. 2363-71.

67. Kamiya, H., W. Zhangm, and A.A. Sima, *Apoptotic stress is counterbalanced by survival elements preventing programmed cell death of dorsal root ganglions in subacute type 1 diabetic BB/Wor rats.* Diabetes, 2005. **54**(11): p. 3288-95.

68. Innis, S.M. and M.T. Clandinin, *Dynamic modulation of mitochondrial membrane physical properties and ATPase activity by diet lipid.* Biochem J, 1981. **198**(1): p. 167-75.

69. Stubbs, C.D. and A.D. Smith, *Essential fatty acids in membrane: physical properties and function.* Biochem Soc Trans, 1990. **18**(5): p. 779-81.

70. Horrobin, D.F., *The membrane phospholipid hypothesis as a biochemical basis for the neurodevelopmental concept of schizophrenia.* Schizophr Res, 1998. **30**(3): p. 193-208.

71. Hon, G.M., et al., *Red blood cell membrane fluidity in the etiology of multiple sclerosis.* J Membr Biol, 2009. **232**(1-3): p. 25-34.

72. Gelb, A.M., M.I. Davidson, and J.I. Kessler, *Effect of Fasting on Esterification of Fatty Acids by the Small Intestine in Vitro.* Am J Physiol, 1964. **207**: p. 1207-10.

73. Maedler, K., et al., *Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function.* Diabetes, 2003. **52**(3): p. 726-33.

74. Hardy, S., et al., *Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin.* J Biol Chem, 2003. **278**(34): p. 31861-70.

75. Prentki, M. and S.R. Madiraju, *Glycerolipid metabolism and signaling in health and disease.* Endocr Rev, 2008. **29**(6): p. 647-76.

76. Ricchi, M., et al., *Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes.* J Gastroenterol Hepatol, 2009. **24**(5): p. 830-40.

77. Brugg, B., et al., *Ceramide induces apoptosis in cultured mesencephalic neurons.* J Neurochem, 1996. **66**(2): p. 733-9.

78. Wiesner, D.A. and G. Dawson, *Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway.* J Neurochem, 1996. **66**(4): p. 1418-25.

79. German, O.L., et al., *Ceramide is a mediator of apoptosis in retina photoreceptors.* Invest Ophthalmol Vis Sci, 2006. **47**(4): p. 1658-68.

80. Belosludtsev, K., et al., *On the mechanism of palmitic acid-induced apoptosis: the role of a pore induced by palmitic acid and Ca2+ in mitochondria.* J Bioenerg Biomembr, 2006. **38**(2): p. 113-20.

81. Chakrabandhu, K., et al., *Palmitoylation is required for efficient Fas cell death signaling.* EMBO J, 2007. **26**(1): p. 209-20.

82. Borradaile, N.M. and J.E. Schaffer, *Lipotoxicity in the heart.* Curr Hypertens Rep, 2005. **7**(6): p. 412-7.

83. Paschen, W., *Shutdown of translation: lethal or protective? Unfolded protein response versus apoptosis.* J Cereb Blood Flow Metab, 2003. **23**(7): p. 773-9.

84. Csordas, G., et al., *Structural and functional features and significance of the physical linkage between ER and mitochondria.* J Cell Biol, 2006. **174**(7): p. 915-21.

85. Pinton, P., et al., *Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis.* Oncogene, 2008. **27**(50): p. 6407-18.

86. van Gurp, M., et al., *Mitochondrial intermembrane proteins in cell death.* Biochem Biophys Res Commun, 2003. **304**(3): p. 487-97.

87. Kroemer, G., N. Zamzami, and S.A. Susin, *Mitochondrial control of apoptosis.* Immunol Today, 1997. **18**(1): p. 44-51.

88. McConkey, D.J. and S. Orrenius, *The role of calcium in the regulation of apoptosis.* Biochem Biophys Res Commun, 1997. **239**(2): p. 357-66.

89. Kruman, II and M.P. Mattson, *Pivotal role of mitochondrial calcium uptake in neural cell apoptosis and necrosis.* J Neurochem, 1999. **72**(2): p. 529-40.

90. Kruman, I., Q. Guo, and M.P. Mattson, *Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells.* J Neurosci Res, 1998. **51**(3): p. 293-308.

91. Paschen, W., S. Hotop, and C. Aufenberg, *Loading neurons with BAPTA-AM activates xbp1 processing indicative of induction of endoplasmic reticulum stress.* Cell Calcium, 2003. **33**(2): p. 83-9.

92. Deniaud, A., et al., *Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis.* Oncogene, 2008. **27**(3): p. 285-99.

93. Takadera, T., M. Ohtsuka, and H. Aoki, *Chelation of extracellular calcium-induced cell death was prevented by glycogen synthase kinase-3 inhibitors in PC12 cells.* Cell Mol Neurobiol, 2010. **30**(2): p. 193-8.

94. Ahluwalia, J.P., et al., *A role for calcium in stabilizing transport vesicle coats.* J Biol Chem, 2001. **276**(36): p. 34148-55.

95. Yu, S.W., et al., *Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor.* Science, 2002. **297**(5579): p. 259-63.

96. Novelli, A., et al., *Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced.* Brain Res, 1988. **451**(1-2): p. 205-12.

97. Peng, L.L., et al., *Inhibition of NMDA receptors underlies the neuroprotective effect of ginsenoside Rb3.* Am J Chin Med, 2009. **37**(4): p. 759-70.

98. Paschen, W., et al., *Peroxidative stress selectively down-regulates the neuronal stress response activated under conditions of endoplasmic reticulum dysfunction.* J Neurochem, 2001. **76**(6): p. 1916-24.

99. Sundar Rajan, S., et al., *Endoplasmic reticulum (ER) stress & diabetes.* Indian J Med Res, 2007. **125**(3): p. 411-24.

100. Schapansky, J., et al., *NF-kappaB activated by ER calcium release inhibits Abeta-mediated expression of CHOP protein: enhancement by AD-linked mutant presenilin 1.* Exp Neurol, 2007. **208**(2): p. 169-76.

101. Urano, F., et al., *Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1.* Science, 2000. **287**(5453): p. 664-6.

102. Lee, A.H., N.N. Iwakoshi, and L.H. Glimcher, *XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response.* Mol Cell Biol, 2003. **23**(21): p. 7448-59.

103. Yoneda, Y., et al., *A cell-penetrating peptidic GRP78 ligand for tumor cell-specific prodrug therapy.* Bioorg Med Chem Lett, 2008. **18**(5): p. 1632-6.

104. Szegezdi, E., et al., *Bcl-2 family on guard at the ER.* Am J Physiol Cell Physiol, 2009. **296**(5): p. C941-53.

105. Chauhan, D. and K.C. Anderson, *Mechanisms of cell death and survival in multiple myeloma (MM): Therapeutic implications.* Apoptosis, 2003. **8**(4): p. 337-43.

106. Lee, S.Y., R.P. Cherla, and V.L. Tesh, *Simultaneous induction of apoptotic and survival signaling pathways in macrophage-like THP-1 cells by Shiga toxin 1.* Infect Immun, 2007. **75**(3): p. 1291-302.

107. Jamora, C., G. Dennert, and A.S. Lee, *Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME.* Proc Natl Acad Sci U S A, 1996. **93**(15): p. 7690-4.

108. Zhang, J., et al., *Association of elevated GRP78 expression with increased lymph node metastasis and poor prognosis in patients with gastric cancer.* Clin Exp Metastasis, 2006. **23**(7-8): p. 401-10.

109. Ohse, T., et al., *Albumin induces endoplasmic reticulum stress and apoptosis in renal proximal tubular cells.* Kidney Int, 2006. **70**(8): p. 1447-55.

110. Sun, Y., et al., *Upregulation of GRP78 and caspase-12 in diastolic failing heart.* Acta Biochim Pol, 2008. **55**(3): p. 511-6.

111. Obrosova, I.G., et al., *Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy.* Diabetes, 2004. **53**(3): p. 711-20.

112. Virag, L., et al., *Nitric oxide-peroxynitrite-poly(ADP-ribose) polymerase pathway in the skin.* Exp Dermatol, 2002. **11**(3): p. 189-202.

113. Pacher, P., et al., *Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies.* Curr Med Chem, 2005. **12**(3): p. 267-75.

114. Sharma, S.S., A. Kumar, and R.K. Kaundal, *Protective effects of 4-amino1,8-napthalimide, a poly (ADP-ribose) polymerase inhibitor in experimental diabetic neuropathy.* Life Sci, 2008. **82**(11-12): p. 570-6.

115. Lin, W., et al., *Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress.* Brain, 2006. **129**(Pt 5): p. 1306-18.

116. Shin, Y.K., et al., *Pathological adaptive responses of Schwann cells to endoplasmic reticulum stress in bortezomib-induced peripheral neuropathy.* Glia, 2010. **58**(16): p. 1961-76.

117. Bolin, L.M., T.P. Iismaa, and E.M. Shooter, *Isolation of activated adult Schwann cells and a spontaneously immortal Schwann cell clone.* J Neurosci Res, 1992. **33**(2): p. 231-8.

118. Cochard, P. and D. Paulin, *Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo.* J Neurosci, 1984. **4**(8): p. 2080-94.

119. Heumann, R., et al., *Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection.* J Cell Biol, 1987. **104**(6): p. 1623-31.

120. De Leon, M., L.J. Van Eldik, and E.M. Shooter, *Differential regulation of S100 beta and mRNAs coding for S100-like proteins (42A and 42C) during development and after lesion of rat sciatic nerve.* J Neurosci Res, 1991. **29**(2): p. 155-62.

CHAPTER THREE

HYPERGLYCEMIC MODULATION OF LIPOTOXICITY IN SCWANN CELLS

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Abstract

Schwann cells (SC) cultured in euglycemic and hyperglycemic conditions undergo lipotoxicity (LTx) when treated with elevated levels of PA. The purpose of this study is to elucidate the mechanisms of palmitic acid lipotoxicity (PA-LTx) in primary SCs and further uncover the influence of hyperglycemia during the lipotoxic response. Our study demonstrated that primary Schwann cells (pSC) exposed to elevated levels of PA exhibit a loss in cell viability in a dose and time-dependent manner. Crystal violet assays demonstrated that elevated levels of glucose alone did not result in a significant decrease in cell viability in pSCs, however a significant decrease in cell viability was exhibited after PA exposure. Hoescht and Annexin V/7AAD staining demonstrated an apoptotic cell death in euglycemic and hyperglycemic pSC treated with PA. An early decrease in Ca^{++} levels in the endoplasmic reticulum (ER, $[Ca++]_{ER}$) was also observed in euglycemic and hyperglycemic pSCs undergoing PA-LTx. In euglycemic pSC treated with PA, flow cytometric experiments using acridine orange (AO) demonstrated lysosomal membrane destabilization (LMD) that preceded mitochondrial membrane depolarization (MMD). In addition, colometric assays demonstrated that PA-LTx was able to induce the activation of caspase 3/7 after a 3 hour exposure. Interestingly, pSCs cultured in a hyperglycemic condition demonstrated MMD, and caspase 3/7 activation that was followed by LMD. As a result, it is suggested that hyperglycemia may be able to delay the involvement of lysosomes in PA-LTx, and increase the intensity to the damage of surrounding cellular organelles, thus leading to increased levels of cellular death.

Introduction

An interruption in homeostatic levels of free fatty acids (FFAs) has been implicated in many disorders. Adequate levels of FFAs are needed to ensure proper cellular functions but elevated lipid levels can cause havoc to the surrounding tissues and cells, leading to debilitating complications, such as those demonstrated by individuals with type 2 diabetes [95]. Lipotoxicity (LTx) is the pathological response of cells to elevated and toxic levels of FFAs. It has been shown that LTx can lead to cellular dysfunction in many cell types, such as cardiomyocytes, pancreatic beta cells and, neurons [41, 53, 210].

Our lab and others have shown that saturated fatty acids, such as palmitic (PA) and stearic acid, can induce LTx that can lead to cellular death [35, 53, 211]. However, the mechanisms involving LTx continue to be unclear. Recent studies in LTx have revealed the involvement of organelles such as the endoplasmic reticulum (ER), mitochondria and lysosomes [41, 53, 212-213]. A study conducted by Li, Z.Z. et al., (2008) revealed that treatment with 200-500μM of PA induced lysosomal membrane destabilization (LMD), mitochondrial dysfunction and cell death in two hepatocyte cell lines (HepG2 and McNtcp.24) [39]. Similarly, mice fed a high fat diet demonstrated a derangement in lipid levels leading to an increase in lipid oxidation and DRG injury, thus contributing to the development of diabetic peripheral neuropathy [84].

Previous work from our laboratory demonstrated that elevated levels of PA resulted in mitochondrial membrane depolarization (MMD), oxidative stress (ROS), and LMD in PC12 cells and cortical cells [35, 41]. In addition, in an immortalized Schwann cell clone, PA-LTx caused an initial injury to the ER resulting in a significant efflux of

Ca^{++} into the cytosol, followed by MMD and ROS production. This study also demonstrated that the preconditioning of iSC in a hyperglycemic environment dramatically augmented PA cellular injury [213].

The aim of this study is to further elucidate the mechanisms of LTx in primary SCs (pSC) and to further examine the impact of hyperglycemia in the lipotoxic response. Our data show that lipotoxicity induces an early reduction of Ca^{++} stores from the ER ($[Ca^{++}]_{ER}$), followed by lysosomal membrane destabilization (LMD), mitochondrial membrane depolarization (MMD) and an increase in oxidative stress (ROS) in euglycemic (5mM) pSC exposed to PA:BSA 2:1 (300μM: 150μM). Interestingly, pSC cultured in hyperglycemic (17mM) conditions demonstrated a robust and earlier depletion of $[Ca^{++}]_{ER}$ stores, followed by MMD, an increase in ROS levels, and the delay in the induction of LMD. As a result, it is suggested that an alternate pathway in PA-LTx is activated when pSC are cultured in hyperglycemic conditions.

It is of importance to understand the mechanism and the ramifications of LTx, considering that SC injury is frequently observed in individuals suffering from peripheral neuropathies and axonal degenerations [85-86, 89, 124]

Experimental Procedures

Cell Culture

Primary Schwann cells (pSC) and Schwann cell medium (SCM) used in this study were purchased from Science Cell Research Laboratories™ (Carlsbad, CA). Cell medium consisted of basal medium with a final concentration of 5.5 mM glucose (i.e. the euglycemic condition for this study). SCM was supplemented with 1% Schwann cell

growth supplement (SCGS), 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin which were also provided by the company.

pSC cells were incubated at 37°C with 5% CO_2. Culture media was changed every three days. To achieve desired hyperglycemic states (17mM), 11.5mM dextrose (equals to 17 mM final concentration) was added to the SCM and filtered before the addition of FBS, SCGs, and penicillin/streptomycin.

Fatty Acid Treatment

Treatments were done in serum free media conditions. Serum free media consisted of SCM, 1% SCGS, and 1% penicillin/streptomycin (ScienceCell Research Laboratories™.). The pSC cells were plated in full serum media, placed in the incubator and allowed to attach for 5 hr. The media was removed, washed with Dulbecco's Phosphate-Buffered Saline (DPBS) and transferred to serum free media. After 12 hr in serum free media, cells were treated with the selected concentration of PA complexed with BSA as described previously [35, 64]. PA (Sigma-Aldrich, St. Louis, MO) stock was prepared in 100% ethanol at a concentration of 300 mM. Fatty acid-free BSA (EMD Biosciences, San Diego, CA) was used as a buffer to ensure that concentrations of unbound free PA in the media were in the 10 nM range during the course of incubation [35]. In brief, serum free medium containing 150 μM fatty acid-free BSA was prepared and warmed up to 37°C to aid in the complete disassociation of PA. Once media was warm, PA stock was added while vortexing to avoid crystalization of PA. A final treatment concentration of 300 μM PA (PA:BSA, 2:1) was used. Treatment media was place in the water bath at 37°C for 30 min before it was added to the cells.

Cell Viability Assays

Crystal Violet Assay

Briefly, pSC cells were plated in 96 well- tissue culture plates coated with Poly-L lysine (0.1%) (Sigma-Aldrich, Inc., St. Louis, MO), at a density of 1.0 x 10^4 cells/well. Subsequent to 0, 24, 48, 72, 96 hour treatments with PA, 100 µl/well of 4% formaldehyde was added. Following a 5 min incubation period at room temperature, the fixative was removed. Cells were post-fixed with 4% formaldehyde for another 30 min. PSC cells were then washed twice with distilled water and allowed to dry completely. Once dried, 100µl/well of crystal violet dye (Accustain®, Sigma-Aldrich) was added and incubated for 30 min at room temperature. Cells were washed with distilled water to remove any unbound stain and dried. The bound crystal violet was then dissolved with 100µl/well of 10% acetic acid solution and place in rocker for 10 min at room temperature. The plate was analyzed by µQuant (Bio-Tek Instruments, Winooski, VT) using an optical density (O.D.) of 570 nm. Data was examined using KCjunior™ software (Bio-Tek Instruments).

Nuclear Morphology

Hoechst 33258 dye was used to assess chromatin condensation in iSC cells. The dye (10 µg/ml) was added to the cells and incubated for 10 min at 37°C with 5% CO_2. The nuclear morphology of cells was observed and photographed using an Olympus fluorescent microscope (excitation/emission wavelength of 365/420 nm). Cells categorized as apoptotic demonstrated apoptotic bodies and increased chromatin condensation.

Assessment of Apoptosis by Flow Cytometry

Annexin V (BD Biosciences, San Diego, CA) and 7AAD (eBioscience, San Diego, CA) were used to detect apoptotic changes occurring in PA-LTx in pSC cells. PSC cells (1.25×10^4) were plated in a 6 well Poly-L lysine plate. After treatment, cells were trypsinized and collected. They were then resuspended in 40 µl of binding buffer with 2 µl Annexin V FITC. Cells were incubated for 15 min in the dark at room temperature. After incubation, 160 µl of binding buffer and 2 µl of 7AAD were added. The cells were incubated for 5 min and additional 200 µl of binding buffer was added. Before analyzing, cells were filtered through a cell strainer cap that was fitted to a polystyrene round bottom flow cytometric tube. Cells were analyzed using the Becton-Dikinson FACSCalibur® flow cytometer (Becton-Dikinson, San Francisco, CA). A total of 100,000 events were measured per sample. Annexin V was detected in the FL-1 channel (530/30nm) while 7AAD was detected in FL-3 channel (650nm). Data was collected in log scale and analyzed using Cell Quest Pro® software and Flow-Jo® software.

Analysis of Mitochondrial Membrane Permeabilization

Disruption of the mitochondrial membrane potential was assessed using the lipophilic cationic probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylben-zimidazol-carbocyanine iodide (JC-1, MitoScreen kit, BD Biosciences) as described previously [64]. Briefly, unfixed cells were washed with PBS and resuspended in 1X JC-1 assay buffer supplemented with 10 µg/ml of JC-1. Cells were then incubated for 15 min at 37°C, washed, and resuspended in 1X JC-1 assay buffer for immediate analysis using the

FACSCalibur® software program. JC-1 in healthy cells aggregated in the mitochondria and was detected in the FL-2 channel while JC-1 with depolarized mitochondria membrane potential demonstrated a reduced fluorescence in the FL-2 channel. The percentage of cells with disrupted mitochondrial membrane potential was calculated using the Flow-Jo® software.

Measurements of $[Ca^{++}]_{ER}$

To measure the $[Ca^{++}]$ in ER during PA treatment, the Fluo-4 NW Calcium Assay kit (Invitrogen) was used. Briefly, 1.0 $x10^4$ cells were plated in 96 well poly-D- lysine coated plates. Cells were treated accordingly. Afterward, 90 µl of the dye, which included Probenecid (2.5 mM) was added to each well. The plates were incubated at 37°C for 30 min. In the last 2 min of incubation thapsigargin (1µM) or DMSO (vehicle) (10ul/well) was added to the cells in order to deplete Ca^{++} from the ER. Cells were analyzed using the Envison HTS Microplate reader (Perkin-Elmer ® Life and Analytical sciences, Shelton, CT). Excitation/emission parameters were 480 nm and 510 nm respectively.

Reactive Oxygen Species (ROS) Detection

Detection of oxidative stress was done by staining the pSC cells with 20 µM of 2', 7' dichlorodihydrofluorecein deacetate (H_2DCFDA, Invitrogen) for 20 min at 37° C. Cells were then detached by trypsinization and washed twice with DPBS. After filtered through cell strainer cap, cells were analyzed using a FACSCalibur® flow cytometer. A total of 10,000 events were measured per sample. Excitation/Emission wavelengths were

488 nm and 530/30 nm respectively. Data was collected in log scale and analyzed using Cell Quest Pro® software and Flow-Jo® software.

Analysis of Lysosomal Membrane Destabilization

Acridine Orange (AO, Sigma Aldrich, St. Louis, MO) was used to examine lysosomal membrane destabilization (LMD). AO, which is permeable to the cell and accumulates in the lysosome, due to proton trapping, emits a red/orange fluorescence at high concentrations (lysosomes) and green fluorescence at low concentrations (nucleus and cytoplasm). Cells with intact lysosomes demonstrate red speckles while cells with compromised lysosomes show a yellow diffused color.

Briefly, AO (5 μg/ml) was added to pSC culture media and counterstained with Hoechst 33342 (1 μg/ml) for 20 min at 37°C. Cells were then examined under an Olympus BX50 epifluorescence microscope using a water immersion objective. Images were acquired using a digital Spot camera system. Flow cytometry using AO was also utilized. PSC were stained with AO (5 μg/ml) in culture media for15 min at 37^0C. After the incubation period, pSC were washed twice with PBS and trypzinised. SCM (2ml) was used to stop the trypzinization process and centrifuged at 6,000 rpms for 5min. Cells were resuspended and washed with PBS and centrifuged for another 5min. The cellular pellet was resuspsended with 500μl of PBS and filtered through cell strainer cap. Cells were analyzed using a FACSCalibur® flow cytometer. Ten thousand events were collected and recorded on a logarithmic scale using the Beacon Dickinson FACScan instrument (excitation 488nm (argon laser). Detectors for the green (FL1) and red (FL3)

fluorescence were utilized to identify cells with intact lysosomes (FL-3 positive) or disrupted lysosomes (Fl-3 negative).

Detection of Cathepsin L and B Activity

The detection of Cathepsin L and B activity was examined by using the fluorogenic substrate-based assay Magic Red L MR-(FR)2 and (MR-(RR)2 for cathepsin L and cathepsin B, respectively (Immunochemistry Technologies, Bloomington, MN). Magic Red was reconstituted with DMSO and further diluted (1:5 ratio) with distilled water. Treated pSC were exposed to Magic Red substrate (for 20min) and counterstained with Hoechst 33342 (for 5 min) and washed once with medium. Cells were examined under an Olympus BX50 epifluorescence microscope using a water immersion objective. Images were acquired using a digital Spot camera system.

Caspase Activity Assays

Assays to examine caspase 3/7 were performed on pSC treated with PA. Cells (10,000 /well with the addition of 100ul of SCM/well) were plated in 96 well Poly-L lysine coated plate and treated with PA. Caspase-Glo® 3/7 assay kit (Promega, Madison, WI) was utilized as well as Caspase 3 inhibitor (Promega, Madison, WI). Briefly, Caspase –Glo® 3/7 substrate was activated with Caspase-Glo® 3/7 buffer, in addition to the preparation of 1mg/ml of caspase inhibitor. After the completion of treatment, 50ul/well of media was removed for pSC and replaced with 50ul/well of substrate or substrate plus caspase 3/7 inhibitor. The plate was then placed on a shaker and protected

from light at room temperature for 1H. Cells were analyzed using the MicroLumatPlus illuminometer and quantified using the Win Glow Software.

Statistical Analysis

All the experiments were repeated independently at least three times. Values represent means ± SD. Statistical comparisons were made using One Way ANOVA with Bonferroni's Multiple Comparison test. Significance was accepted at $p < 0.05$.

Results

PA Decreases Cell Viability and Induces Apoptosis in pSC Cells Cultured in Different Glucose Concentrations

Normal metabolic functions require a balance of cellular FFAs and glucose concentrations [12, 214-215]. Studies have demonstrated that chronic and elevated levels of FFAs result in tissue toxicity and cellular death, also known as LTx [12, 14, 21-23, 84, 216].

Our laboratory has shown that the ratio of PA:BSA 2:1 was able to generate a 10nM range of unbound FFAs, a concentration observed in conditions such as diabetes and ischemia, that was able to induce LTx in nerve growth factor differentiated PC12 cells, cortical cells and iSCs [35, 41, 64, 157-159, 213].

PSC were cultured in different concentrations of glucose, 5.5mM (euglycemic) or 17mM (hyperglycemic), for 5 days prior to exposure to PA. To identify the effects of glucose alone on pSCs, cell viability assays using crystal violet and Hoescht staining where performed at 96H following the 5 day incubation period with their prospective glucose concentrations. Our results demonstrated that glucose alone did not cause a

significant amount of cell death to pSC (Figure 1A). In addition, nucleic staining revealed normally dispersed chromatin with an intact cellular membrane (Figure 1B). However, the addition of PA (300μM) triggered apoptotic changes, such as chromatin condensation, DNA fragmentation, and apoptotic bodies that were detected by Hoescht staining (Figure. 1B).

Fig. 1

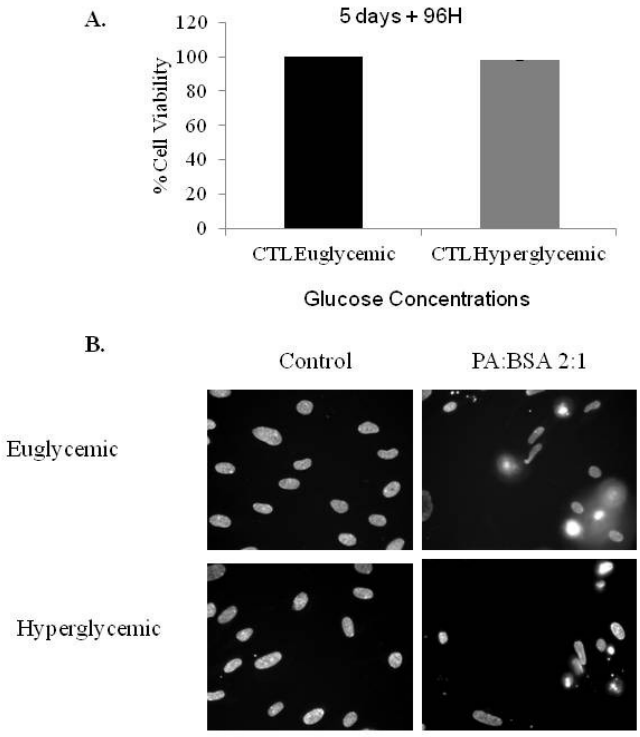

A.
5 days + 96H
% Cell Viability
120
100
80
60
40
20
0
CTL Euglycemic
CTL Hyperglycemic
Glucose Concentrations
B.
Control
PA:BSA 2:1
Euglycemic
Hyperglycemic

Figure 1: Analysis of pSC viability and the affects of PA on cellular morphology. pSC cells were cultured in medium containing 5mM (euglycemic) or 17 mM glucose (hyperglycemic) for 5 days before the addition of PA. After 5 days of pre-conditioning (A) crystal violet cell viability assays detected no significant cell death in euglycemic and hyperglycemic controls (BSA+0.1% ETOH) (B) To determine nuclear morphology, Hoescht staining was performed after 72H of PA:BSA 2:1 treatment. Nuclear condensation is indicated with white arrows. Representative micrographs were taken of four independent experiments. Statistical analysis was performed using one way ANOVA *$p< 0.05$, **$p<0.005$, N=4

To further understand the effects of PA on pSC, a time course using crystal violet assays and AnnexinV/ 7AAD flow cytometric analysis were performed (Figure 2A). demonstrates a significant decrease in cell viability at 24H and 48H in pSC cultured in euglycemic and hyperglycemic environments, respectively. To confirm PA's induction of apoptosis, the established flow cytometric methods using Annexin V FITC and 7 AAD to measure early (Annexin V positive cell populations) and late apoptosis (7AAD positive cell populations) were performed. Upon closer inspection, our study revealed a significant amount of apoptosis (5.38% early apoptosis ± 1.58 and 3.92% ± 0.957late apoptosis) at 24H in euglycemic cells increasing by 72H (22.00% ± 11.68 early apoptosis, and 6.07 % ± 1.57 late apoptosis). In hyperglycemic conditions a significant amount of cell death (3.17% early apoptosis ± 0.78, 5.25% ± 2.35 late apoptosis) was observed at 12H and continued to increase at 72H (35.67% ± 7.87 early apoptosis, 12.17 ± 1.45 late apoptosis) (Figure. 2B, 2D).

Fig. 2

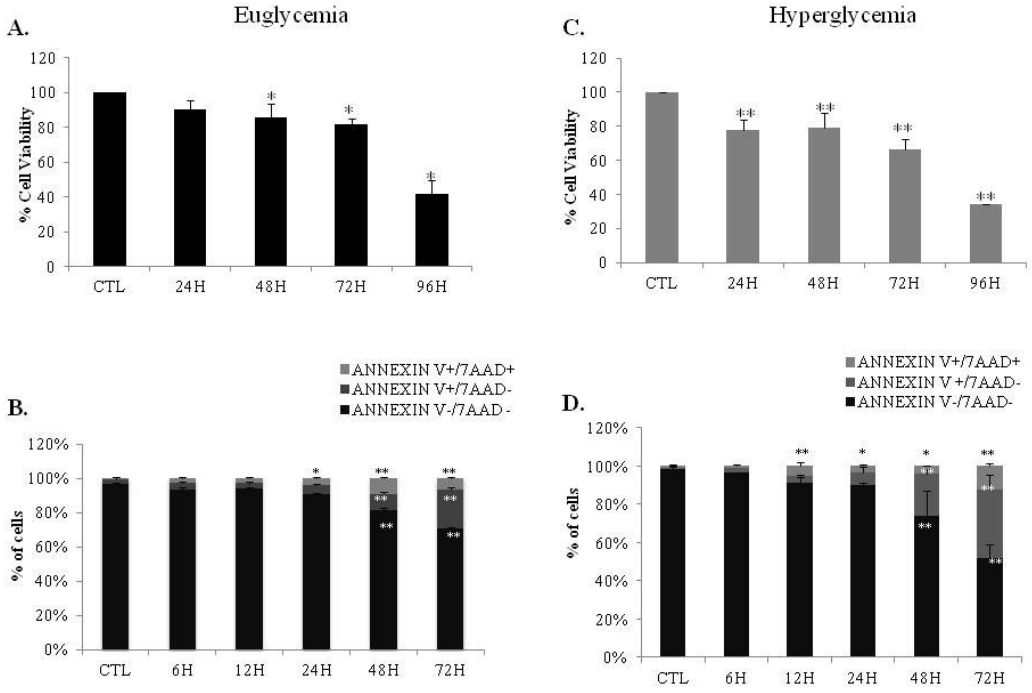

A.
Euglycemia
% Cell Viability
120
100
80
60
40
20
0
CTL
24H
48H
72H
96H
C.
Hyperglycemia
% Cell Viability
CTL
24H
48H
72H
96H
B.
ANNEXIN V+/7AAD+
ANNEXIN V+/7AAD-
ANNEXIN V-/7AAD -
% of cells
120%
100%
80%
60%
40%
20%
0%
CTL
6H
12H
24H
48H
72H
D.
ANNEXIN V+/7AAD+
ANNEXIN V +/7AAD-
ANNEXIN V-/7AAD-
% of cells
CTL
6H
12H
24H
48H
72H

Figure 2: Time course of PA-LTx induction of apoptosis in pSC. Crystal violet and Annexin V/7AAD flow cytometric assays were performed at different time points on PA treated pSC cells cultured in (A and B) euglycemic and (C and D) hyperglycemic conditions. Early apoptotic translocation of phosphotidyl cistein to the outer cell membrane renders the cell Annexin V+, while late apoptosis is detected by entry and retention of 7AAD in the nucleus. The percentages of cells that are Annexin V-/7AAD- (non apoptotic). Annexin V +/7AAD- (early stage apoptosis) and Annexin V+/7AAD+ (late stage apoptosis) are graphed. Statistical analysis was performed using one way ANOVA, with Bonferroni's exclusion test. *$p< 0.05$, **$p<0.001$, N=4

PA-LTx Affects the ER and Results in an Increase in Intracellular Calcium

Borradaile, et al., (2006) demonstrated that PA (500μM) was able to incorporate itself into the membrane lipid rafts in the ER of Chinese hamster ovarian cells, causing injury to the ER membrane and resulting in the efflux of Ca^{++} from the ER ($[Ca^{++}]_{ER}$) into the cytosol [24]. This study found a significant decrease in $[Ca^{++}]_{ER}$ within the first 5H of PA treatment. In accordance this study, our data shows that PA also caused an early dysfunction of the ER with a significant decrease in $[Ca^{++}]_{ER}$ stores as early as 3H in euglycemic and 15min in hyperglycemic conditions (23.62 ± 5.85% and 23.59 ± 2.39% reduction in $[Ca^{++}]_{ER}$ concentrations respectively) (Figure 3A).

Fig. 3

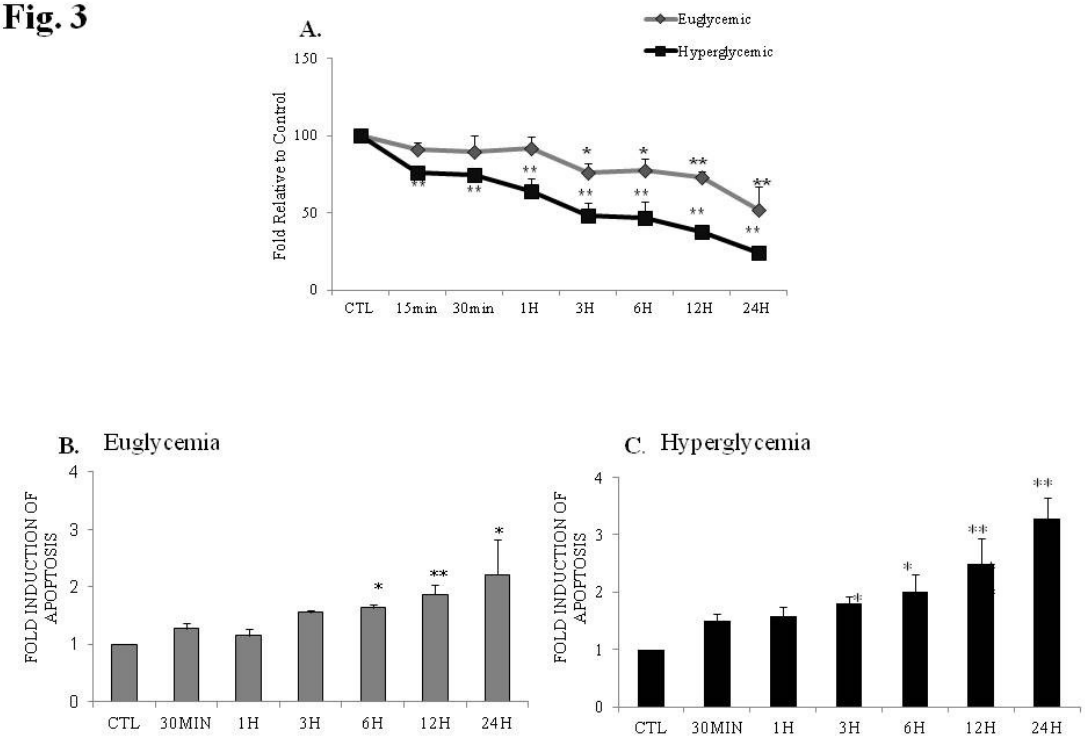

A.
Euglycemic
Hyperglycemic
Fold Relative to Control
150
100
50
0
CTL
15min
30min
1H
3H
6H
12H
24H
B. Euglycemia
FOLD INDUCTION OF APOPTOSIS
4
3
2
1
0
CTL
30MIN
1H
3H
6H
12H
24H
C. Hyperglycemia
FOLD INDUCTION OF APOPTOSIS
4
3
2
1
0
CTL
30MIN
1H
3H
6H
12H
24H

Figure 3: PALTX reduced Ca^{++} concentration in the ER and increases mitochondrial depolarization in pSC. (A) pSC cells were cultured in 5 mM or 17 mM glucose and treated with PA:BSA 2:1. $[Ca^{++}]_{ER}$, reference to control level, was determined at 5, 15, 30 min and 3, 6, 12, 24 H after treatment. Mitochondrial membrane depolarization was examined by flow cytometric analysis using JC-1 fluorescent dye in (B) euglycemic and (C) hyperglycemic conditions in combination with PA:BSA 2:1 treatments. Quantitative analysis of JC-1 data using Flow JO® software is shown. Statistical analysis was performed using one way ANOVA with Bonferroni's exclusion test *$p<0.05$, **$p<0.001$ N=3

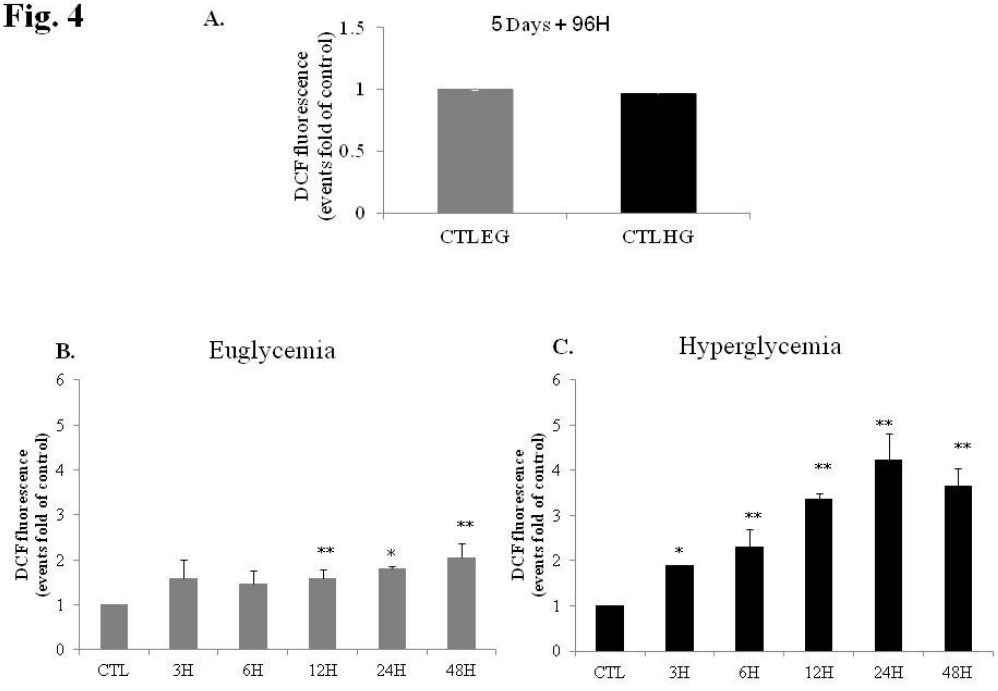
Mitochondrial Depolarization Follows ER Stress Damage in PA-LTx

To assess mitochondrial membrane potential and the overall health of the mitochondria, JC-1, a lipophilic, cationic dye was used [217-218]. JC-1 assays using flow cytometric analysis demonstrated a significant amount of MMD in pSC treated with PA at 6H in euglycemic cells and 1H in hyperglycemic cells (1.72 ± 0.22 and 1.97 ± 0.68 fold of control respectively) (Figure 3A,3B). Interestingly, a significant amount of MMD was not observed in pSC cultured in different concentrations of glucose alone (data not shown).

PA-LTx Induced an Augmented State of Oxidative Stress in pSC

To assess the generation of ROS on PA treated pSCs, 2'7' dichlorodihydrofluorecein dictate (H_2DCFDA) was used and analyzed through flow cytometry. A time course of 0, 3, 6, 12, 24, 48H was performed following PA exposure. Our data demonstrates that different glucose concentrations alone did not increase reactive oxidative stress (ROS) levels (Figure. 4A). However, once PA was administered an increase in ROS levels was detected at 12H in euglycemic and at 3H in hyperglycemic concentrations (1.59± 0.21, 1.90 ± 0.008 fold of control) (Figure. 4B, 4C).

Fig. 4


A.
5 Days + 96H
DCF fluorescence (events fold of control)
1.5
1
0.5
0
CTLEG
CTLHG
B.
Euglycemia
DCF fluorescence (events fold of control)
6
5
4
3
2
1
0
CTL
3H
6H
12H
24H
48H
**
*
**
C.
Hyperglycemia
DCF fluorescence (events fold of control)
6
5
4
3
2
1
0
CTL
3H
6H
12H
24H
48H
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**
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Figure 4: PA-LTx increases ROS in pSC cultured in euglycemic and hyperglycemic conditions. ROS was observed using H2DCFDA and analyzed through flow cytometry. PSC cells were cultured in euglycemic (5 mM glucose), and hyperglycemic (17mM glucose) conditions for 5 days prior to PA treatment (PA:BSA 2:1). The increase in fluorescence is indicative of an increase in ROS levels within the cell. Cells treated with 300 μM of H_2O_2 for 15 min were used as a positive control. No increase in ROS was observed in pSC cultured in different glucose concentrations alone (A). However, following PA treatment pSC cultured in (B) euglycemic and (C) hyperglycemic conditions demonstrated a significant increase in ROS levels as early as 12H in euglycemic and 3H in hyperglycemic conditions. Flow cytometry data was quantified using Cell Quest Pro® and Flow-Jo® software. Statistical analysis was performed using one way ANOVA with Bonferroni's exclusion test t.*$p< 0.05$, **$p< 0.001$, N=3

Lysosomal Involvement During PA-LTx

Other studies and previous work in our lab have demonstrated that PA-LTx can induce lysosomal membrane destabilization (LMD) that can lead to protease release into the cytosol, resulting in cellular damage [39, 63, 70]. Acridine orange (AO) is able to detect LMD through its red/ green fluorescence when stimulated by green light [219-220]. PSC cells were treated with PA at 0, 15min, 30min, 1, 3, 6, 12, 24, 48, and 72H and analyzed by flow cytometry. Interestingly, our data suggests an early induction of LMD in euglycemic pSC that begins at 3H and reaches a peak at 72H after treatment (1.83± 0.46, 4.69± 1.36 fold of control, respectively) Figure 5A. In contrast, LMD in hyperglycemic conditions was not detected until 24H after PA treatment and reached a peak at 72H (Figure 6A) (2.41 ± 0.58 fold of control, 7.09 ± 2.56 fold of control, respectively).

Fluorescent microscopy analysis of LMD and the possible release of cathepsins into the cytosol were also performed with AO or Magic Red for cathepsin B or L. Magic red uses the cresyl violet fluorophore that becomes fluorescent upon cathepsin B or L cleavage. Fluorescent microscopy with AO revealed LMD at 6, 12 and 24H after PA exposure in pSC cultured in euglycemic environment. (Figure 5B**)** However, in hyperglycemic conditions, observable LMD occurred after 12H of PA treatment (Figure. 6B). Furthermore, protease activity, like cathepsin B and L were significantly observed at 24and 48H in euglycemic and hyperglycemic conditions (Figures 5a,5b,6a,6b).

Fig. 5

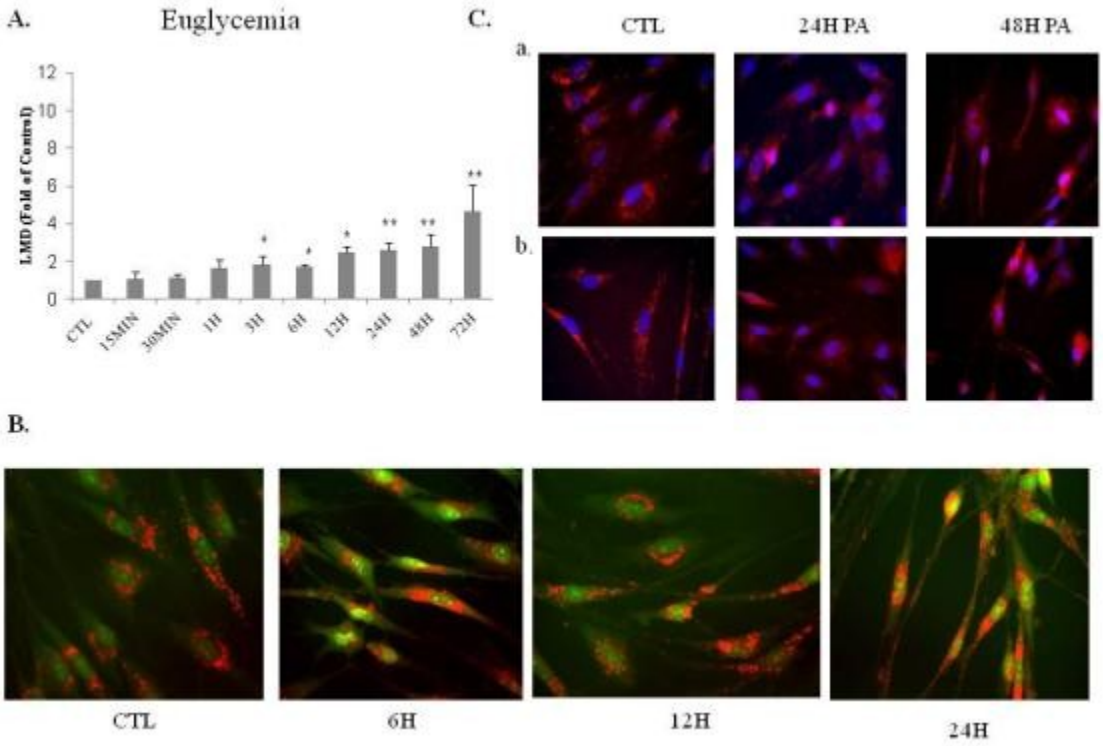

A.
Euglycemia
LMD (Fold of Control)
12
10
8
6
4
2
0
CTL
15MIN
30MIN
1H
3H
6H
12H
24H
48H
72H
C.
CTL
24H PA
48H PA
a.
b.
B.
CTL
6H
12H
24H

Figure 5: PA-LTx induces lysosomal membrane destabilization (LMD) in pSC cultured in euglycemic conditions. (A)Time course using acridine orange (AO) and flow cytometric analysis was used to quantify LMD in euglycemic pSC. Flow cytometric analysis demonstrates LMD as early as 3H after PA treatment. (B) AO staining was performed in pSC 0, 6, 12, 24H, in addition to Magic red staining for cathepsin B [221] and L (C.b) activity at 24 and 48 hours. Cells were counterstained with Hoechst staining and examined by fluorescent microscopy. Merged images are shown. Representative micrographs were taken of five independent experiments. Flow cytometry data was quantified using Cell Quest Pro® and Flow-Jo® software. Statistical analysis was performed using one way ANOVA with Bonferroni's exclusion test t.*$p < 0.05$, **$p < 0.001$, N=4

Fig. 6

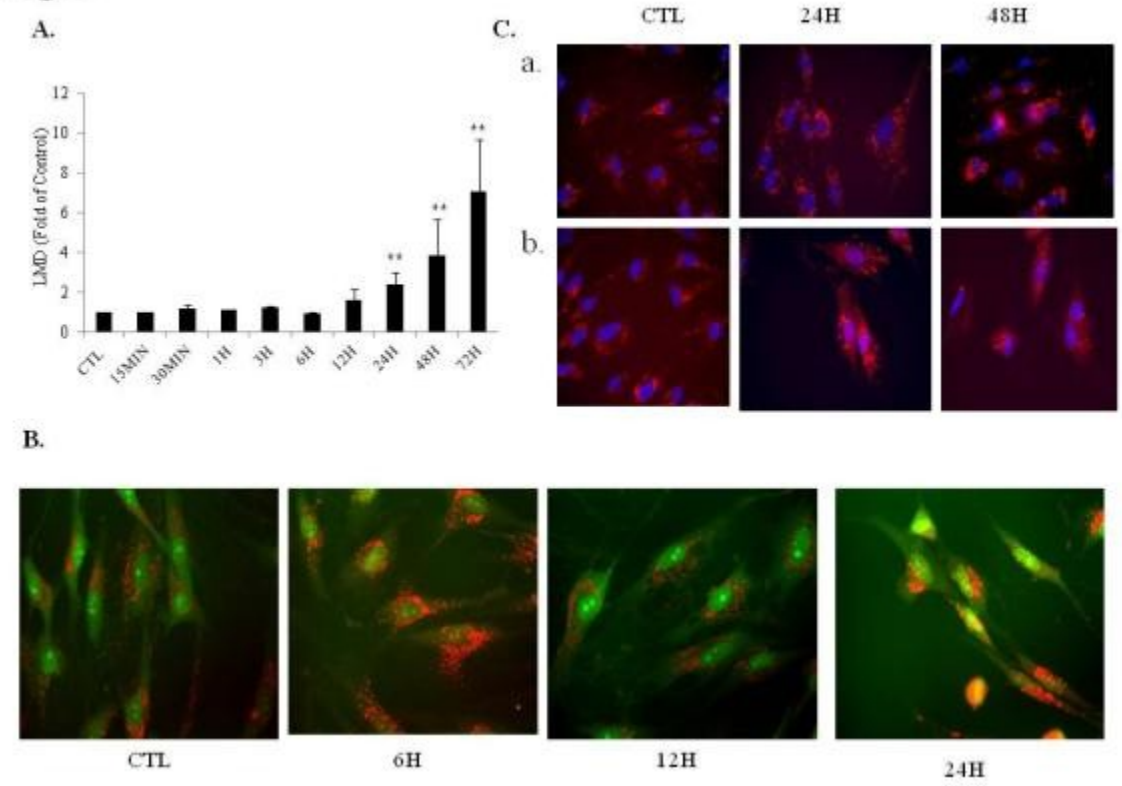

A.
LMD (Fold of Control)
12
10
8
6
4
2
0
CTL
15MIN
30MIN
1H
3H
6H
12H
24H
48H
72H
**
**
**
C.
CTL
24H
48H
a.
b.
B.
CTL
6H
12H
24H

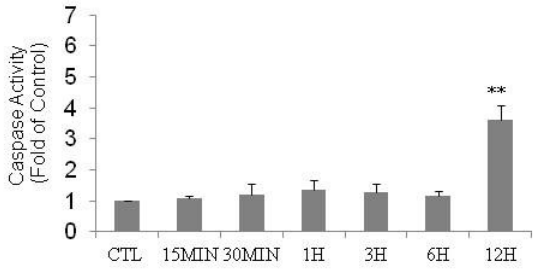
Figure 6: PA-LTx induction of lysosomal membrane destabilization (LMD) in pSC cultured in hyperglycemic conditions. (A) Flow cytometric analysis using acridine orange (AO) to examine LMD in pSC.. Flow cytometric analysis demonstrated a significant increase in LMD at 24H after treatment. (B) AO staining was performed in pSC 0, 6, 12, 24H, in addition to (C) Magic red staining for cathepsin B [221] and L (C.b) activity at 24 and 48 hours. Representative micrographs were taken of five independent experiments. Cells were counterstained with Hoechst staining and examined by fluorescent microscopy. Merged images are shown. Flow cytometry data was quantified using Cell Quest Pro® and Flow-Jo® software. Statistical analysis was performed using one way ANOVA with Bonferroni's exclusion test t.*$p < 0.05$, **$p < 0.001$, N=3

PA-LTx Induced Caspase 3/7 in pSC

The activation of caspases has been shown to be a characteristic of PA-LTx [35, 64, 222]. Therefore, caspase 3/7 activity was examined to further confirm and understand the mechanism of apoptosis by PA-LTx. In euglycemic conditions, caspase 3/7 activity significantly increased at 12H (3.62± 0.47 fold relative to control) (Figure. 6A). In hyperglycemic conditions, an early activation of caspase 3/7 activity (3H) was demonstrated (1.59 ± 0.18 fold induction) with levels reaching 4.72 ± 0.26 fold of control by 12H (Figure B).

Fig. 7

A. Euglycemic



B. Hyperglycemic

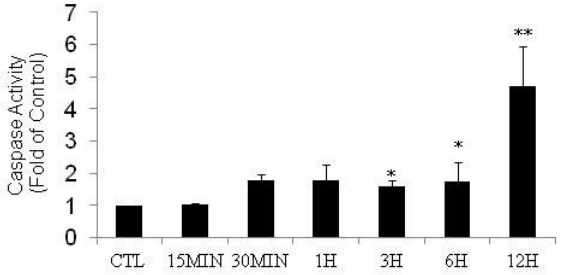


Figure 7: PA-LTx induces caspase 3/7 activation in pSC cultured in euglycemic and hyperglycemic conditions. A colometric assay analyzing caspase 3/7 activity was performed on pSC cultured in (A) euglycemic and (B) hyperglycemic conditions and treated with PA:BSA 2:1 for 0, 15 min 30min, 1, 3, 6, 12H. The data demonstrates a significant detection of caspase 3/7 activity at 12H and 3H in euglycemic and hyperglycemic conditions respectively. Statistical analysis was performed using one way ANOVA with Bonferroni's exclusion test t.*$p < 0.05$, **$p < 0.001$, N=3

Discussion

Peripheral neuropathy (PN) is a debilitating complication experienced by many individuals, including those with type 2 diabetes [223-225]. Many studies on the development of diabetic peripheral neuropathy have focused on hyperglycemia and its detrimental effects on SCs [120, 160]. However, research is attempting to uncover the potential role of lipids in the induction of such disorders [12, 84, 213].

Elevated and chronic levels of plasma FFAs can result in a derangement of cellular function that can lead to the injury and death of the affected cell [153-156]. Damage to SCs can lead to segmental demyelination and axon atrophy, which are disorders that are associated with axonal degeneration and neuropathies [226]. This study sought to comprehend the contributions of PA-LTx in SC dysfunction and the possible association it may have to segmental demyelination and the development of peripheral neuropathies.

Our data demonstrated that elevated levels of glucose alone did not result in observable pSC injury. However, when exposed to PA, pSC underwent PA-LTx that resulted in apoptotic cellular changes and significant amounts of cell death beginning at 24H in euglycemic and 12H in hyperglycemic conditions. Furthermore, it was found that PA was able to incite a marked decrease in $[Ca^{++}]_{ER}$, induce MMD, LMD injury and generate an increase in cellular ROS levels in both euglycemic and hyperglycemic environments. **Of significance,** hyperglycemic pSC seemed to demonstrate an altered sequence of events as an earlier injury to the ER, the mitochondria, and significant increase in caspase activity and ROS levels followed LMD.

In agreement with previous studies, the findings of our investigation also pointed to the detrimental effects of PA on cellular function [35, 64, 227-228]. A significant finding of this study is that it reports for the first time that elevated levels of glucose in the context of PA-LTx enhance the injury process by altering the sequence of events associated with this process in pSC.

The main role of hyperglycemia in the injury to SCs and axons that leads to the development of peripheral neuropathy has been debated for a long time. It has been suggested that hyperglycemia alone is able to increase oxidative stress and cause mitochondrial dysfunction in SCs resulting in peripheral neuropathy [102, 229]. However, others have observed that glucose alone was unable to significantly increase ROS levels or induce apoptosis in SC or DRG models [108, 170]. Gumy, et al., (2008) found that hyperglycemia affected SC proliferation, migration, and hindered axonal and SC regeneration, but did not induce apoptosis [108]. In accordance to these studies, our lab did not detect a significant decrease in cell viability or an increase in ROS levels in pSC cultured in elevated levels of glucose alone. It is believed that pSC cultured in a hyperglycemic condition may possess the ability to adapt to the mild and chronic stimulus presented by glucose. A study by Vincent, et al., (2009) found that the adaption of DRGs to elevated levels of glucose is achieved by reducing aconitase and increasing antioxidant levels, thus decreasing cellular stress [90]. Studies have suggested that upon encountering a mild and chronic insult, cells can initiate pro-survival as well as pro-apoptotic pathways simultaneously. Thus, the fate of the cell is decided by the stronger of the two pathways [90, 170-172]. Nevertheless, the discrepancy between the studies may be explained by considering the concentrations of glucose being utilized, the period

of time the cells are exposed to elevated glucose levels and the type of SCs (embryonic vs. adult) that are used in the study.

Since glucose alone did not affect cell viability or increase ROS levels, our focus was on discerning the mechanism of PA-LTx in pSC . A time course using crystal violet and Annexin V/7AAD, assays were performed after PA treatment. The results show that a significant amount of apoptosis occurred in pSC cultured in euglycemic and hyperglycemic conditions after PA treatment. The toxicity and the reduction of cell viability brought upon by PA treatment can be explained in three forms. 1) PA is a long chain saturated fatty acids (LCSFFA) that lacks double bonds and can tightly compact itself into lipid membrane rafts. The compaction of PA into lipid membrane rafts can result in a less flexible and rigid membrane that losses its ability to function properly 2) esterification of LCSFFA is less efficient, leading to the accumulation of nonesterefied FAs in the cytosol and resulting in organelle injury and cellular dysfunction 3) LCSFFA generate toxic by-products such as diaglycerides and ceramides which can also result in cellular failure [24, 28, 176, 230].

The next series of experiments focused on elucidating the mechanisms of PA-LTx on pSC. Studies have demonstrated that PA is able to injure the ER early on, generating an increase in intracellular Ca^{++} ($[Ca^{++}]_i$) and activating cell death pathways [39, 79, 182]. A study performed in human beta cells and MIN6 cells found that a 10min exposure of PA (100uM) caused a rapid increase of $[Ca^{++}]_i$ [231]. The affects of PA on the ER were also observed in Chinese hamster ovarian cells which demonstrated that 500uM PA exposure is able to significantly induce a decrease in $[Ca^{++}]_{ER}$ stores as early as 5H after treatment and is followed by release of chaperones, such as GRP78, into the

cytosol [24]. In accordance to these studies, our data demonstrates that in euglycemic pSCs a significant decrease in $[Ca^{++}]_{ER}$ was observed after 3H of PA (300uM) treatment (Figure. 3A). By contrast, hyperglycemic pSCs exhibited an earlier depletion of $[Ca^{++}]_{ER}$ stores detected as early as 15min after PA exposure (Figure 3A).

Current research has elucidated different pathways in the attempt to explain the rapid affect of PA on cellular Ca^{++} homeostasis and ER damage. Borradaile, et al (2006) demonstrated that elevated levels of PA accumulated in the lipid membrane rafts of the ER leading to membrane rigidity, loss of membrane fluidity and ER dysfunction [24]. Yet, a study using hypothalamic neurons found that a 4H PA exposure lead to an increase JNK phosphorylation leading to FOXO1 activation and the possible upregulation in the transcription of pro-apoptotic genes that can induce ER stress [51, 53]. Furthermore, other studies have suggested that PA can also stimulate voltage-sensitive channels that result in Ca^{++} influx from the extracellular environment causing an amplification in the concentration of intracellular Ca^{++} and leading to the activation of cell death pathways [231-233].

Interestingly, following the injury to the ER by PA, the cell death pathways of pSCs cultured in euglycemic environments diverge from pSC cultured in hyperglycemic conditions. Our results revealed that pSCs cultured in euglycemic environments and exposed to PA resulted in an increase in $[Ca^{++}]_i$, followed by LMD, MMD, ROS production and caspase 3/7 activation. However, in hyperglycemic conditions, pSCs exhibited an earlier involvement of the ER, followed by MMD, ROS, production, caspase 3/7 activation and a late emergence of LMD. Our data suggests a modification in the cascade of events following PA-LTx by hyperglycemic conditions.

Previous studies in our lab found that LMD is an early event of PA-LTx and the inhibition of cathepsin L significantly inhibited MMD and apoptosis in nerve growth factor differentiated PC12 cells (NGFDPC12) [41]. In addition, Li, et al.,(2008) demonstrated that PA was able to cause lysosomal damage which resulted in the release of cathepsin B into the cytosol, thus triggering mitochondrial dysfunction and an increase in ROS production [39]. In euglycemic pSCs exposed to PA, our data appears to support such pathway since LMD precedes MMD, ROS production and caspase 3/7 activation. Feldstein, et al (2006) found that a 3H PA exposure to mouse hepatocytes was able to induce the translocation of Bax to the lysosome causing LMD the release of cathepsin B, leading to MMD and caspase activation. The mechanism of PA activation of Bax remains unclear, however, this study found that the induction of Bax is not specific to PA, as another LCSFFA, stearic acid, had similar effects. In addition, the treatment with 4-bromopalmitate did not prevent Bax translocation to the lysosome [70]. To take this a step further, elevated levels Ca^{++} have also been associate with the activation and translocation of Bax to membranes resulting in permeabilization [234-235]. In summary, the upstream LMD may be through 1) the direct activation of Bax by PA or 2) a consequence of ER injury and the increase in $[Ca^{++}]_i$ after PA exposure, thus activating Bax . It is also possible that PA may be incorporating itself in the ER and at the same time stimulating Bax. The increase in $[Ca^{++}]_i$,which along with Bax, is also able to destabilize the lysosome causing a release in cathepsins. It is possible that the increase in $[Ca++]_i$ and the release of cathepsins by the lysosome can injure the mitochondria, resulting in ROS production, caspase activation and cell death.

However, our data found that in pSC cultured in hyperglycemic conditions, a delayed in LMD was observed after PA exposure. Similarly, Russell, et. al., (1999) observed that in diabetic rats, apoptotic changes were detected in DRGs and SC, however lysosomes remained intact [236]. The alternation of events in hyperglycemic environments can be explained in three ways 1) the ER's proximity to the mitochondria, thus injuring the mitochondria first 2) hyperglycemic stabilization of the lysosomal membrane and 3) affects of hyperglycemia on lysosomal enzymes.

The functional and physical interactions between the mitochondria and the ER have been described by many. Studies have suggested that the transfer of Ca^{++} from the ER to the mitochondria occurs in "hot spots" or areas where the ER and the mitochondria are in close approximation [237]. Czardas, et al., (2006) demonstrated that the increase in the approximation of the mitochondria and the ER resulted in the increased sensitivity of the mitochondria to Ca^{++} overload and activation of cell death pathways [184, 238]. Therefore, it possible that the robust and earlier release of Ca^{++} from the ER is able to significantly overload the mitochondria matrix causing an early MMD, activation of caspases and production of ROS prior to the destabilization of the lysosome.

A second explanation may be the stabilization of the lysosomal membrane by elevated glucose levels. Studies have found that levels of Hsp70, a stress induced protein that promotes cell survival by inhibiting LMD , were significantly higher in diabetic patients [42, 239]. It is suggested that Hsp70 binds to the endolysosomal anionic phospholipid bis (monoacylglycerol) phosphate (BMP) which can then inhibit the activity of acid shingomyelinase [240-242]. Thus, it is possible that the delay in LMD found in hyperglycemia pSC may be through the possible upregulation of Hsp70.

In addition, hyperglycemia has also been shown to affect lysosomal enzymes and reduce the low molecular pool of iron radicals resulting in lysosomal stabilization [243]. Studies have found a decrease in lysosomal enzyme activity like, cathepsin B and L, in tissues of diabetic streptozotocin rats [244-245]. In addition, through the production of advance glycation endproducts the reduction of lysosomal enzyme activity in retinal pigment epithelium has been observed [246]. However, the exact mechanism on how glucose decreases lysosomal enzyme activity continues to be unclear.

Glucose can reduce low molecular iron levels in lysosomes, thus rendering them more stable and less sensitive to insults. It has been shown an increase in blood glucose levels was able to prevent or significantly reduce the toxic effects of alloxan, a glucose analogue used to induce diabetes in animals. It is believed that alloxan increases free radicals in β cells [247-248]. A study conducted by, Olenjnicka, et al., (1999) observed that in alloxan treated animals, β cells cultured in low concentrations of glucose demonstrated higher redox active iron levels and signs of autophagy. In addition, β cells were more sensitive to oxidative stress and exhibited unstable lysosomes. In contrast, cells cultured in a hyperglycemic condition had a decrease in redox active iron and did not exhibit sighs of autophagy. Moreover, hyperglycemic β cells were less sensitive to oxidative stress and did not demonstrate LMD after alloxan treatments. The above study suggests that the stability of the lysosome may be directly correlated to the amount of intralysosomal redox active iron which is inversely proportional to glucose concentrations [249].

The delay in LMD in hyperglycemic pSCs may explain the early and elevated levels of caspase 3/7 after PA treatment. Han, et. al., (2010) found that inhibition of

autophagy in β cells cultured in chronic hyperglycemic conditions increased the activation of capase-3. Therefore, the lack of involvement of the lysosome in the apoptotic process may influence the cell to utilize an alternative pathway, resulting in the early and robust activation of caspases to initiate cell death [250].

Our data suggests that hyperglycemic conditions place a chronic and mild insult on pSC. However, a second insult, such as the addition of PA, will drive the pSC beyond its capacity to survive, resulting in ER injury and an early and robust release of Ca^{++} into the cytosol. Due to the close approximation of the mitochondria to the ER, the mitochondrial matrix will undergo Ca^{++} overload resulting in MMD. The increase in mitochondrial damage leads to ROS production and an increase in caspase activation initiating cellular death. A prolonged PA exposure (more than 24H) will override the hyperglycemic stability of the lysosome and LMD will occur releasing cathepsin B and L and further damaging the cell.

Our studies have shown for the first time the ability of hyperglycemia to alter the cell death pathway initiated by PA-LTx. The mechanism of hyperglycemic stabilization of lysosomes continues to be unclear. Further studies need to examine the influence of glucose, such as Hsp70 and iron, on lysosomal stability. In addition, early events such as JNK and Bax involvement also need to be addressed to further elucidate the effects of PA-LTx on pSC. Understanding the mechanisms of PA-LTx in SCs is of great importance as the comprehension of these pathways can aid in the discovery of innovative therapies for the effective treatment and prevention of neuropathies, such diabetic peripheral neuropathy.

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References

1. Bezuglov, V.V., M. Bobrov, and A.V. Archakov, *Bioactive amides of fatty acids.* Biochemistry (Mosc), 1998. **63**(1): p. 22-30.

2. Boulton, A.J. and L. Vileikyte, *The diabetic foot: the scope of the problem.* J Fam Pract, 2000. **49**(11 Suppl): p. S3-8.

3. Mayer, C.M. and D.D. Belsham, *Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons: rescue of resistance and apoptosis through adenosine 5′ monophosphate-activated protein kinase activation.* Endocrinology, 2010. **151**(2): p. 576-85.

4. Almaguel, F.G., et al., *Lipotoxicity-mediated cell dysfunction and death involve lysosomal membrane permeabilization and cathepsin L activity.* Brain Res, 2010. **1318**: p. 133-43.

5. Giacca, A., et al., *Lipid-induced pancreatic beta-cell dysfunction: focus on in vivo studies.* Am J Physiol Endocrinol Metab, 2011. **300**(2): p. E255-62.

6. Mantzaris, M.D., E.V. Tsianos, and D. Galaris, *Interruption of triacylglycerol synthesis in the endoplasmic reticulum is the initiating event for saturated fatty acid-induced lipotoxicity in liver cells.* FEBS J, 2011. **278**(3): p. 519-30.

7. Ulloth, J.E., C.A. Casiano, and M. De Leon, *Palmitic and stearic fatty acids induce caspase-dependent and -independent cell death in nerve growth factor differentiated PC12 cells.* J Neurochem, 2003. **84**(4): p. 655-68.

8. Henique, C., et al., *Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis.* J Biol Chem, 2010. **285**(47): p. 36818-27.

9. Padilla, A., et al., *Hyperglycemia magnifies Schwann cell dysfunction and cell death triggered by PA-induced lipotoxicity.* Brain Res, 2011. **1370**: p. 64-79.

10. Li, Z., et al., *Using dynamic gene module map analysis to identify targets that modulate free fatty acid induced cytotoxicity.* Biotechnol Prog, 2008. **24**(1): p. 29-37.

11. Vincent, A.M., et al., *Dyslipidemia-induced neuropathy in mice: the role of oxLDL/LOX-1.* Diabetes, 2009. **58**(10): p. 2376-85.

12. Welcher, A.A., et al., *Molecular approaches to nerve regeneration.* Philos Trans R Soc Lond B Biol Sci, 1991. **331**(1261): p. 295-301.

13. Dyck, P.J. and C. Giannini, *Pathologic alterations in the diabetic neuropathies of humans: a review.* J Neuropathol Exp Neurol, 1996. **55**(12): p. 1181-93.

14. Song, Z., et al., *Transgenic mice overexpressing aldose reductase in Schwann cells show more severe nerve conduction velocity deficit and oxidative stress under hyperglycemic stress.* Mol Cell Neurosci, 2003. **23**(4): p. 638-47.

15. Sango, K., et al., *High glucose-induced activation of the polyol pathway and changes of gene expression profiles in immortalized adult mouse Schwann cells IMS32.* J Neurochem, 2006. **98**(2): p. 446-58.

16. Almaguel, F.G., et al., *Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid.* J Neurosci Res, 2009. **87**(5): p. 1207-18.

17. Randle, P.J., et al., *The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus.* Lancet, 1963. **1**(7285): p. 785-9.

18. McGarry, J.D., *What if Minkowski had been ageusic? An alternative angle on diabetes.* Science, 1992. **258**(5083): p. 766-70.

19. Unger, R.H., *Reinventing type 2 diabetes: pathogenesis, treatment, and prevention.* JAMA, 2008. **299**(10): p. 1185-7.

20. Song, Z., et al., *Silymarin prevents palmitate-induced lipotoxicity in HepG2 cells: involvement of maintenance of Akt kinase activation.* Basic Clin Pharmacol Toxicol, 2007. **101**(4): p. 262-8.

21. Unger, R.H., *Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications.* Diabetes, 1995. **44**(8): p. 863-70.

22. Gaemers, I.C., et al., *Lipotoxicity and steatohepatitis in an overfed mouse model for non-alcoholic fatty liver disease.* Biochim Biophys Acta, 2011. **1812**(4): p. 447-58.

23. Schaffer, J.E., *Lipotoxicity: when tissues overeat.* Curr Opin Lipidol, 2003. **14**(3): p. 281-7.

24. van Herpen, N.A. and V.B. Schrauwen-Hinderling, *Lipid accumulation in non-adipose tissue and lipotoxicity.* Physiol Behav, 2008. **94**(2): p. 231-41.

25. Bazan, N.G., Jr., *Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain.* Biochim Biophys Acta, 1970. **218**(1): p. 1-10.

26. Cistola, D.P., J.C. Sacchettini, and J.I. Gordon, *13C NMR studies of fatty acid-protein interactions: comparison of homologous fatty acid-binding proteins produced in the intestinal epithelium.* Mol Cell Biochem, 1990. **98**(1-2): p. 101-10.

27. Thomas, M.E., et al., *Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinuria.* Am J Physiol Renal Physiol, 2002. **283**(4): p. F640-7.

28. Borradaile, N.M., et al., *Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death.* J Lipid Res, 2006. **47**(12): p. 2726-37.

29. Salvioli, S., et al., *JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis.* FEBS Lett, 1997. **411**(1): p. 77-82.

30. Korper, S., et al., *The K+ channel openers diazoxide and NS1619 induce depolarization of mitochondria and have differential effects on cell Ca2+ in CD34+ cell line KG-1a.* Exp Hematol, 2003. **31**(9): p. 815-23.

31. Almaguel, F., *Mechanisms of DHA neuroprotection from PA indiuced lipotoxicity*. 2010. p. 223.

32. Feldstein, A.E., et al., *Bax inhibition protects against free fatty acid-induced lysosomal permeabilization.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(6): p. G1339-46.

33. Robbins, E. and P.I. Marcus, *Dynamics of Acridine Orange-Cell Interaction. I. Interrelationships of Acridine Orange Particles and Cytoplasmic Reddening.* J Cell Biol, 1963. **18**: p. 237-50.

34. Li, W., et al., *Induction of cell death by the lysosomotropic detergent MSDH.* FEBS Lett, 2000. **470**(1): p. 35-9.

35. De Leon, M., Welcher, A A., Nahin R.H., Liu, Y., Ruda, M.A., Shooter, E.M. Molina, C.A. , *Fatty acid binding protein is induced in neurons of the dorsal root ganglia after peripheral nerve injury.* Journal of Neuroscience Research, 1996. **44**(3): p. 283-92.

36. Boslem, E., et al., *A lipidomic screen of palmitate-treated MIN6 beta-cells links sphingolipid metabolites with endoplasmic reticulum (ER) stress and impaired protein trafficking.* Biochem J, 2011. **435**(1): p. 267-76.

37. Lupachyk, S., et al., *PARP inhibition alleviates diabetes-induced systemic oxidative stress and neural tissue 4-hydroxynonenal adduct accumulation: Correlation with peripheral nerve function.* Free Radic Biol Med, 2011.

38. Yousry, A.H. and A.M. Abdalhady, *Management of diabetic neuropathic ankle arthropathy by arthrodesis using an Ilizarov frame.* Acta Orthop Belg, 2010. **76**(6): p. 821-6.

39. Pham, M., et al., *Proximal neuropathic lesions in distal symmetric diabetic polyneuropathy: findings of high-resolution magnetic resonance neurography.* Diabetes Care, 2011. **34**(3): p. 721-3.

40. Eckersley, L., *Role of the Schwann cell in diabetic neuropathy.* Int Rev Neurobiol, 2002. **50**: p. 293-321.

41. Vincent, A.M., et al., *Short-term hyperglycemia produces oxidative damage and apoptosis in neurons.* Faseb J, 2005. **19**(6): p. 638-40.

42. Reaven, G.M., et al., *Lowering of plasma glucose in diabetic rats by antilipolytic agents.* Am J Physiol, 1988. **254**(1 Pt 1): p. E23-30.

43. Paolisso, G., et al., *A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM.* Diabetologia, 1995. **38**(10): p. 1213-7.

44. McGarry, J.D., *Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes.* Diabetes, 2002. **51**(1): p. 7-18.

45. Cnop, M., *Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes.* Biochem Soc Trans, 2008. **36**(Pt 3): p. 348-52.

46. Ikota, H., et al., *Neuromyelitis optica with intraspinal expansion of Schwann cell remyelination.* Neuropathology, 2010. **30**(4): p. 427-33.

47. Cacicedo, J.M., et al., *Activation of AMP-Activated Protein Kinase (AMPK) Prevents Lipotoxicity in Retinal Pericytes.* Invest Ophthalmol Vis Sci, 2011.

48. Ishiyama, J., et al., *Unsaturated FAs prevent palmitate-induced LOX-1 induction via inhibition of ER stress in macrophages.* J Lipid Res, 2011. **52**(2): p. 299-307.

49. Obrosova, I.G., *Increased sorbitol pathway activity generates oxidative stress in tissue sites for diabetic complications.* Antioxid Redox Signal, 2005. **7**(11-12): p. 1543-52.

50. Kamboj, S.S., R.K. Vasishta, and R. Sandhir, *N-acetylcysteine inhibits hyperglycemia-induced oxidative stress and apoptosis markers in diabetic neuropathy.* J Neurochem, 2010. **112**(1): p. 77-91.

51. Sango, K., et al., *Diabetes is not a potent inducer of neuronal cell death in mouse sensory ganglia, but it enhances neurite regeneration in vitro.* Life Sci, 2002. **71**(20): p. 2351-68.

52. Gumy, L.F., E.T. Bampton, and A.M. Tolkovsky, *Hyperglycaemia inhibits Schwann cell proliferation and migration and restricts regeneration of axons and*

Schwann cells from adult murine DRG. Mol Cell Neurosci, 2008. **37**(2): p. 298-311.

53. Vincent, A.M., et al., *Sensory neurons and schwann cells respond to oxidative stress by increasing antioxidant defense mechanisms.* Antioxid Redox Signal, 2009. **11**(3): p. 425-38.

54. Cheng, C. and D.W. Zochodne, *Sensory neurons with activated caspase-3 survive long-term experimental diabetes.* Diabetes, 2003. **52**(9): p. 2363-71.

55. Kamiya, H., W. Zhangm, and A.A. Sima, *Apoptotic stress is counterbalanced by survival elements preventing programmed cell death of dorsal root ganglions in subacute type 1 diabetic BB/Wor rats.* Diabetes, 2005. **54**(11): p. 3288-95.

56. Kessler, J.I., et al., *Lipoprotein lipase inhibition in rabbits with experimental pancreatitis.* Proc Soc Exp Biol Med, 1962. **110**: p. 24-6.

57. Hon, G.M., et al., *Red blood cell membrane fluidity in the etiology of multiple sclerosis.* J Membr Biol, 2009. **232**(1-3): p. 25-34.

58. Prentki, M. and S.R. Madiraju, *Glycerolipid metabolism and signaling in health and disease.* Endocr Rev, 2008. **29**(6): p. 647-76.

59. Borradaile, N.M. and J.E. Schaffer, *Lipotoxicity in the heart.* Curr Hypertens Rep, 2005. **7**(6): p. 412-7.

60. Green, A.Q., et al., *Altered C-fiber function as an indicator of early peripheral neuropathy in individuals with impaired glucose tolerance.* Diabetes Care, 2010. **33**(1): p. 174-6.

61. Gwiazda, K.S., et al., *Effects of palmitate on ER and cytosolic Ca2+ homeostasis in beta-cells.* Am J Physiol Endocrinol Metab, 2009. **296**(4): p. E690-701.

62. Martinez, S.C., et al., *Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis.* Diabetes, 2008. **57**(4): p. 846-59.

63. Remizov, O., et al., *Palmitate-induced Ca2+-signaling in pancreatic beta-cells.* Mol Cell Endocrinol, 2003. **212**(1-2): p. 1-9.

64. Olofsson, C.S., et al., *Palmitate increases L-type Ca2+ currents and the size of the readily releasable granule pool in mouse pancreatic beta-cells.* J Physiol, 2004. **557**(Pt 3): p. 935-48.

65. Epand, R.F., et al., *Membrane perturbations induced by the apoptotic Bax protein.* Biochem J, 2002. **367**(Pt 3): p. 849-55.

66. Epand, R.F., et al., *Direct evidence for membrane pore formation by the apoptotic protein Bax.* Biochem Biophys Res Commun, 2002. **298**(5): p. 744-9.

67. Russell, J.W., et al., *Neurons undergo apoptosis in animal and cell culture models of diabetes.* Neurobiol Dis, 1999. **6**(5): p. 347-63.

68. Rizzuto, R., et al., *Mitochondria as biosensors of calcium microdomains.* Cell Calcium, 1999. **26**(5): p. 193-9.

69. Csordas, G., et al., *Structural and functional features and significance of the physical linkage between ER and mitochondria.* J Cell Biol, 2006. **174**(7): p. 915-21.

70. Kawamata, H. and G. Manfredi, *Mitochondrial dysfunction and intracellular calcium dysregulation in ALS.* Mech Ageing Dev, 2010. **131**(7-8): p. 517-26.

71. Nakhjavani, M., et al., *Serum heat shock protein 70 and oxidized LDL in patients with type 2 diabetes: does sex matter?* Cell Stress Chaperones, 2011. **16**(2): p. 195-201.

72. Yabunaka, N., et al., *Elevated levels of heat-shock protein 70 (HSP70) in the mononuclear cells of patients with non-insulin-dependent diabetes mellitus.* Diabetes Res Clin Pract, 1995. **30**(2): p. 143-7.

73. Kolter, T. and K. Sandhoff, *Principles of lysosomal membrane digestion: stimulation of sphingolipid degradation by sphingolipid activator proteins and anionic lysosomal lipids.* Annu Rev Cell Dev Biol, 2005. **21**: p. 81-103.

74. Petersen, N.H., et al., *Connecting Hsp70, sphingolipid metabolism and lysosomal stability.* Cell Cycle, 2010. **9**(12).

75. Kirkegaard, T., et al., *Hsp70 stabilizes lysosomes and reverts Niemann-Pick disease-associated lysosomal pathology.* Nature, 2010. **463**(7280): p. 549-53.

76. Olejnicka, B.T., K. Ollinger, and U.T. Brunk, *A short exposure to a high-glucose milieu stabilizes the acidic vacuolar apparatus of insulinoma cells in culture to ensuing oxidative stress.* APMIS, 1997. **105**(9): p. 689-98.

77. Comper, W.D., et al., *Disease-dependent mechanisms of albuminuria.* Am J Physiol Renal Physiol, 2008. **295**(6): p. F1589-600.

78. Olbricht, C.J. and B. Geissinger, *Renal hypertrophy in streptozotocin diabetic rats: role of proteolytic lysosomal enzymes.* Kidney Int, 1992. **41**(4): p. 966-72.

79. Glenn, J.V., et al., *Advanced glycation end product (AGE) accumulation on Bruch's membrane: links to age-related RPE dysfunction.* Invest Ophthalmol Vis Sci, 2009. **50**(1): p. 441-51.

80. Malaisse-Lagae, F., A. Sener, and W.J. Malaisse, *Contrasting modes of action of D-glucose and 3-O-methyl-D-glucose as protectors of the rat pancreatic B-cell against alloxan.* Biochim Biophys Acta, 1983. **762**(1): p. 36-43.

81. Pipeleers, D. and M. Van de Winkel, *Pancreatic B cells possess defense mechanisms against cell-specific toxicity.* Proc Natl Acad Sci U S A, 1986. **83**(14): p. 5267-71.

82. Olejnicka, B.T., et al., *Beta-cells, oxidative stress, lysosomal stability, and apoptotic/necrotic cell death.* Antioxid Redox Signal, 1999. **1**(3): p. 305-15.

83. Han, D., et al., *Activation of autophagy through modulation of 5'-AMP-activated protein kinase protects pancreatic beta-cells from high glucose.* Biochem J, 2010. **425**(3): p. 541-51.

CHAPTER FOUR

LIPOTOXICITY AND THE DISRUPTION OF PMP22: POSSIBLE ASSOCIATION IN THE DEVELOPMENT OF PERIPHERAL NEUROPATHY

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Abstract

Elevated levels of free fatty acids (FFA) have been demonstrated in individuals suffering from obesity and type 2 Diabetes. Chronic and high FFA concentrations can damage the nervous system leading to lipotoxicity (LTx) and neuronal dysfunction. LTx is the pathological response of the cell to chronic and elevated levels of fatty acids. Our lab is interested in determining whether LTx plays a role in the development of peripheral nerve injury resulting in peripheral neuropathy, a common complication of individuals who are obese and/or suffer from type 2 Diabetes. The focus of the present study is to determine whether pathological concentrations of palmitic acid (PA), a long chain saturated FFA, affects Schwann cell's (SC) ability to produce and maintain adequate myelin protein levels for proper myelin formation. A disturbance in myelin formation leads to segmental demyelination resulting in peripheral neuropathy. Our experimental model is an immortalized Schwann (iSC) cell line that has similar morphology, immunohystochemistry, and gene expression features to primary rat SC. Due to its importance in myelin function, levels of peripheral myelin protein (PMP22) were analyzed. ISC cells were cultured at glucose concentrations of 17mM and 50mM., to mimic a euglycemic and hyperglycemic state respectively. ISC were treated with PA dissolved in ETOH and Bovine Serum Albumin (BSA) (PA:BSA 2:1 (300uM/150uM)) in serum free media. Real Time PCR (RTPCR) experiments demonstrated a downregulation of PMP22 mRNA levels within the first 12H of PA exposure which was more pronounced in iSC cultured in a hyperglycemic environment. Western blot analysis also demonstrated a significant decrease in PMP22 protein levels at 12H, which was more evident in hyperglycemic conditions. A co treatment of PA with MCI-186, an

antioxidant used to reduce ROS concentrations prevented the downregulation of PMP22 mRNA levels. Our data suggests that the expression of PMP22 mRNA levels in iSC undergoing PA-LTx may be associated with cellular oxidative stress. It is of importance to understand the effects of PA-LTx on myelin proteins in iSC in order to offer novel therapies to halt the development and the progression of peripheral neuropathies.

Introduction

Over 20 million people suffer from peripheral neuropathy, which is considered to be one of the most common and chronic diseases in the United States [75]. Peripheral neuropathy describes the neuronal dysfunction and damage caused by disease or trauma that leads to nerve conduction disturbances. Adequate myelin compaction and formation are essential for proper nerve function. In the peripheral nervous system Schwann cells (SCs) produce myelin, which enhances the nerve conduction velocity (NCV) of the axon. Myelin, the modified plasma membrane of the SC, wraps around the axon forming a cover called the myelin sheath. The myelin sheath is composed of cholesterol, phospholipids, and proteins, such as protein zero (P0), myelin basic protein (MBP) and peripheral myelin protein 22 (PMP22). The structures of SC membranes consist of integral proteins that are secured within the membrane and extrinsic proteins that are attached to the surfaces of the membrane. As the cell myelinates, opposition from the two extracellular and cytosolic areas in the membrane come together, the glycolipids and myelin proteins in the membranes come closer and fuse providing the stability and compaction needed for proper myelin function. While proteins such as P0 and MBP are

necessary for myelin compaction, PMP22 is critical for myelin function, assembly and maintenance [119, 251-255].

Myelin increases axonal conductance by decreasing the energy requirements for axonal membrane depolarization, leading to an increase in conduction velocity. Therefore, the enhancement of the NCV allows for more advanced and accelerated processing to take place in the human nervous system.

However, genetic and metabolic abnormalities can damage SCs resulting in the inadequate production, transportation and increased degradation of myelin proteins, which can lead to the development of peripheral neuropathies. Studies have demonstrated that a decrease in myelin protein concentrations can lead to a decrease in myelin thickness, loose uncompacted myelin, myelin "loops" and infoldings/outfoldings leading to the accumulation of non functional myelin at the nodes of Ranvier [256-257].

A critical component of myelin is PMP22. PMP22 makes 2-5% of the total protein, it is considered to be one of the most important myelin proteins in the peripheral nervous system. PMP22 mRNA levels are significantly downregulated in crush nerve injuries and upregulated during SC myelination [119, 258]. However, during the quiescent periods of the SC, approximately 80% of PMP22 is misfolded and degraded by the unfolded protein response in the endoplasmic reticulum (ER) [259].

Studies have shown that the overexpression or underexpression of PMP22 affects myelin stability and function, resulting in peripheral neuropathies [252-253, 260-263]. Charcot-Marie Tooth disease (CMT-1A) and autosomal dominant hereditary peripheral neuropathy with liability to pressure palsies (HNPP) are examples of neuropathies that are associated with PMP22 overexpression and underexpression respectively. CMT-1A

is caused by a duplication of PMP22 gene that is found in human chromosome 17 [254, 262]. Studies have demonstrated that PMP22 overexpression will form perinuclear aggregosomes in SCs resulting in the upregulation of the lysosomal pathway. In addition, the accumulation and degradation of PMP22 in the cytosol can reduce its incorporation into myelin and contribute to the demyelination observed in CMT-1A [253, 257]. Furthermore, the downregulation of PMP22 observed in HNPP is characterized by demyelination and hypermyelination of axonal areas called tomacula [263-264].

Metabolic abnormalities can also lead to demyelination. Peripheral nerves of obese individuals and people with diabetes type 2 have also demonstrated segmental demyelination. Studies have observed "onion-bulb" formations, disruptions and ballooning of myelin sheaths in obese and diabetic animal models. In addition, SCs were observed to have "reactive, degenerative and proliferative changes" in peripheral nerves of diabetic animal models [115, 229].

Previous studies from our lab found that palmitic acid (PA), a long chain saturated fatty acid, induced lipotoxicity (LTx) that activated an apoptotic pathway in immortalized and primary Schwann cells [78, 213]. The pathological response of the cell to the toxic levels of fatty acids (FA) has been termed LTx [5, 22]. Our lab has also demonstrated that hyperglycemia is able to magnify the lipotoxic response [213]. Therefore, this study sought to elucidate the possible effects of PA-LTx on myelin proteins, such as PMP22 , in iSCs cultured in euglycemic and hyperglycemic conditions.

Our data shows that iSCs exposed to PA demonstrated mRNA levels of PMP22 that were downregulated in a time and concentration dependent manner. In addition, iSCs cultured in hyperglycemic conditions demonstrated an earlier downregulation of PMP22

mRNA levels when compared to euglycemic environments. Western blot analysis demonstrated a decrease in protein levels that correlated with mRNA levels of PMP22. The co- treatment of PA and MCI- 186, an antioxidant, was able to significantly reduce oxidative stress and stabilize mRNA levels of PMP22. Therefore, our study suggests that the expression of PMP22 mRNA levels in iSC undergoing PA-LTx may be mediated through cellular oxidative stress.

Experimental Procedures

Cell Culture

Schwann cells used in this study were a generous gift from Dr. Laurel Bolin [148]. The immortalized SC clone (iSC) was shown to express markers specific to primary SC such as S100β, p75 ^{NGFR} and Vimentin [148-151] Cells were maintained in culture medium prepared from Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12) 50/50 mix without glutamine (Mediatech, Herndon, VA). This medium contains 17 mM glucose (i.e. the euglycemic condition for this study). Ten percent horse serum (Invitrogen, Carlsbad, CA), 2 mM L-glutamine (Mediatech), 100 units/ml penicillin and 100 µg/ml streptomycin (Mediatech) were supplemented to the media. iSC cells were incubated at 37° C with 5% CO_2. Culture media was changed every three days. Cells were used at a maximum of 7 passages. To achieve a desired hyperglycemic state of the culture media, we also prepared DMEM beginning with powder DMEM (Mediatech) without glucose, L-glutamine, phenol red, pyruvate or sodium bicarbonate. These were added at the appropriate concentrations after DMEM was dissolved in sterile deionized water. To make hyperglycemic media, 45 mM dextrose was supplemented.

Next, DMEM was filtered and mixed with equal volume of Ham's F12 (contains 10 mM glucose) to make final DMEM/F12 (50/50) media with 50 mM glucose (hyperglycemic). Further, 10% horse serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin were also added.

Fatty Acid Treatment

Treatments were done in serum free media. Serum free media consisted of DMEM/F12 (50/50), 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin, and 1X N-2 supplement (Gibco, Invitrogen Corp.). The iSC cells were plated in full serum media, placed in the incubator and allowed to attach for 5 hr. The media was removed, washed with Dulbecco's Phosphate-Buffered Saline (DPBS) and transferred to serum free media. After 12 hr in serum free media, cells were treated with the selected concentrations of PA complexed with bovine serum albumin (BSA) as described previously [35, 64]. PA (Sigma-Aldrich, St. Louis, MO) stock was prepared in 100% ethanol at a concentration of 300 mM. fatty acid-free BSA (EMD Biosciences, San Diego, CA) was used as a buffer to ensure that concentrations of unbound free PA in the media were in the 10 nM range during the course of incubation [35]. In brief, serum free medium containing 150 μM fatty acid-free BSA was prepared and warmed up to 37°C to aid in the complete disassociation of PA. Once media was warm, PA stock was added while vortexing to avoid clumping of PA. Two treatment concentrations were used: 150 μM PA (PA:BSA, 1:1) and 300 μM PA (PA:BSA, 2:1). Treatment media were place in the water bath at 37°C for 30 min before they were added to the cells.

Antioxidant Treatment

Antioxidant MCI-186 (Biomol Research Laboratories, Plymouth Meeting, PA) was used to reduce ROS during PA-LTx. MCI-186. MCI-186 was first dissolved in DMSO and warmed up to 37° C to ensure the complete disassociation. MCI-186 stock solution (or DMSO as a control) was then diluted into the PA/BSA treatment media to attain final concentration of 100 μM or 1 mM.

RTPCR Experiments

Total RNA was extracted using TRI-Reagent (Molecular Research Center, Cincinnati, OH). RNA quantification was done by measuring O.D. at 260 nm and stored at -80° C. Two-step RT-PCR was performed. First, RNA (0.5~ 1μg) was used to make cDNA using the iScrip cDNA synthesis kit (Bio-Rad, Hercules, CA). Next, real-time PCR with Syber Green was conducted using CFX96 Real-Time System (Bio-Rad). PMP22 reverse primer was 5'CACTGTGTCTCACTGTGTAGATGGC3' and forward primer 5'TGTCCCTGTTCCTGTTCTTCTGC'3. ß-actin was selected as a housekeeping gene (reverse primer 5'-GCG GCA GTG GCC ATC TC-3' and forward primer 5'- GGG AAA TCG TGC GTG ACA TT-3'). The relative amount of mRNA was calculated using the 2 $-\Delta\Delta C_T$ formula.

Western Blots

Protein extract from iSC were separate on a SDS-PAGE gel and electrophoretically transferred to a nitrocellulose membrane. After transfer, 5% milk in Tris-buffered saline (TTBS) with 0.05% Tween 20, PH 7.4 at room temperature for 1 hr

was performed to block the membrane. Subsequently, the membrane was washed and then incubated with primary antibody against PMP22 (Abcam, Cambridge, MA) in TTBS 1X containing 0.05% and Tween 20 at 4^0C overnight. The membrane wash washed three times with TTBS 1X and incubated with HRP-anti mouse (GE Healthcare Bioscience) for 1 hr at room temperature, followed by three washes with TTBS. The signal was then detected by ECL-plus (GE Healthcare Bioscience).

Statistical Analysis

All the experiments were repeated independently at least three times. Values represent means ± SD. Statistical comparisons were made using One Way ANOVA with Bonferroni's Multiple Comparison test. Significance was accepted at $p < 0.05$.

Results

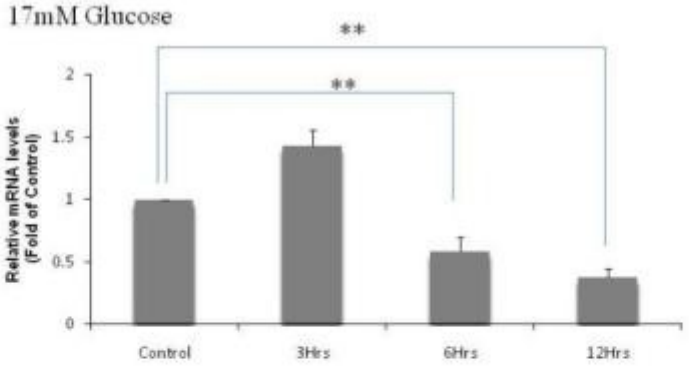
PA-LTx is Able to Downregulate mRNA of PMP22

Previous work demonstrated that PA-LTx was able to induce apoptosis in iSCs with significant cell death at 24 and 48Hrs [213]. To test the possible effects of PA-LTx on myelin genes, RTPCR experiments were performed to analyze changes in PMP22 mRNA levels. Our data demonstrates a significant decrease in mRNA concentration in iSCs treated with PA:BSA 2:1 ratio in both euglycemic (17mM glucose) and hyperglycemic (50mM glucose) conditions (Figure. 1A and 1B). After 6hrs of treatment, there was a 40% decrease in PMP22 mRNA levels that reached a 67% reduction at 12hrs in iSCs cultured in a euglycemic environment. In hyperglycemic iSCs a similar trend

was observed with a significant decrease in mRNA levels beginning earlier at 3hrs (45%) and continued to 12hrs (63%) (Figure. 1B).

FIG. 1

A. 17mM Glucose



B. 50mM Glucose

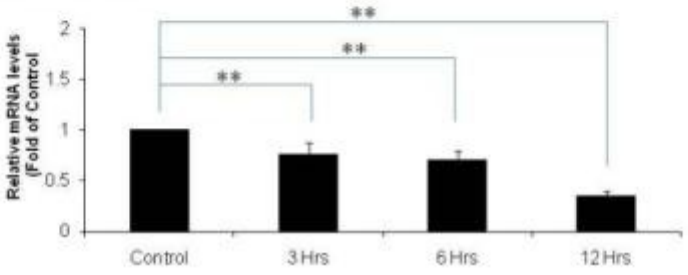


Figure 1. Levels of PMP22 mRNA in iSC cells treated with PA
Levels of mRNA were analyzed by RT- PCR. (A) iSC cells cultured in euglycemic state (17mM glucose concentration) demonstrated a decrease in PMP22 mRNA levels following treatment with PA. (B) A similar trend was demonstrated in iSC cells cultured in a hyperglycemic state (50mM glucose concentration). *$p < 0.05$, $p < 0.001$

PA-LTx Induces a Decrease in PMP22 Protein Levels

Western blot analysis demonstrated a significant decrease in protein levels at 12hrs of PA treatment in both euglycemic and hyperglycemic conditions (Figure. 2A and 2B**).**

FIG. 2

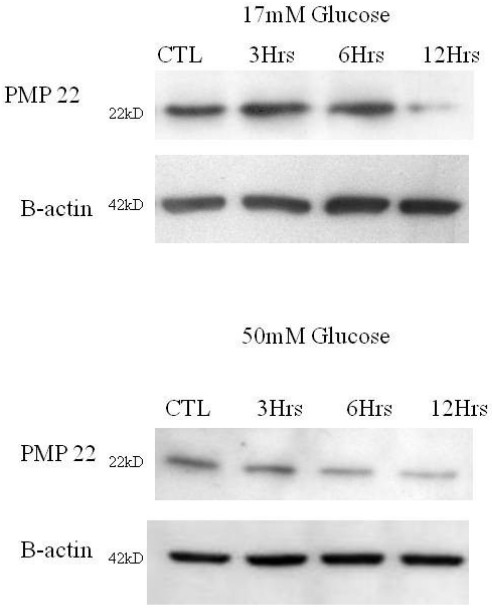

17mM Glucose
CTL
3Hrs
6Hrs
12Hrs
PMP 22
22kD
B-actin
42kD
50mM Glucose
CTL
3Hrs
6Hrs
12Hrs
PMP 22
22kD
B-actin
42kD

Figure 2: PA-LTx induced a decrease in protein levels of PMP22
ISC were treated with PA:BSA 2:1 concentrations and analyzed for PMP22 protein concentrations. Western blot analysis reveals a significant decrease in PMP 22 protein levels at12hrs in both euglycemic and hyperglycemic conditions

PMP22 mRNA Levels are Restored with the Addition of MCI-186 in PA-LTx iSC Cells

PA-LTx significantly increase ROS levels as early as 3Hrs after treatment and peaks at 48H [213]. Previous work from our lab demonstrated that the addition of MCI-186, an antioxidant, significantly reduced ROS levels and prevented cellular death during the first 24Hrs of PA exposure [213]. To better understand the role of ROS in the disturbance of myelin proteins, PMP22 mRNA levels were analyzed in the presence of MCI-186. Our data demonstrates that PMP 22 mRNA levels in euglycemic cells co treated with 100uM MCI-186 and PA:BSA 2:1 were not altered through the 12H time course. A significant drop was evident at 12hr 2:1 PA/BSA treatment with no MCI-186; however, this decrease was significant inhibited with the addition of MCI-186 (Figure 3A). In hyperglycemic cells co-treated with PA:BSA 2:1 and 1mM MCI-186 mRNA levels of PMP22 were also stabilized (Figure 2B).

FIG. 3

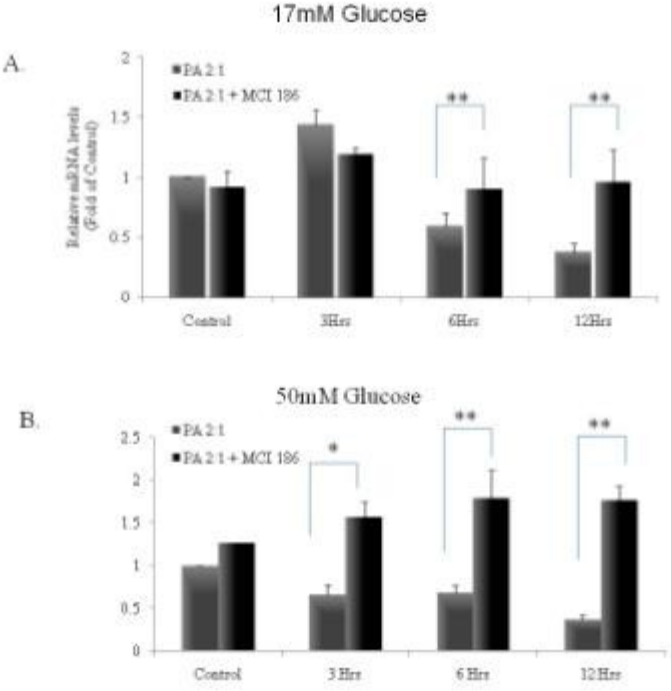

A.
17mM Glucose
PA 2:1
PA 2:1 + MCI 186
Relative miRNA levels (Fold of Control)
2
1.5
1
0.5
0
**
**
Control
3Hrs
6Hrs
12Hrs
B.
50mM Glucose
PA 2:1
PA 2:1 + MCI 186
2.5
2
1.5
1
0.5
0
*
**
**
Control
3 Hrs
6 Hrs
12 Hrs

Figure 3. PMP22 mRNA levels in iSC cultures treated with MCI-186. iSC cells in A) euglycemic and B) hyperglycemic media were treated with PA with or without MCI-186. Cells in euglycemic media were treated with 100uM MCI-186, while cells in hyperglycemic media were treated with 1mM MCI-186. Results demonstrate that MCI-186 prevented the decline of PMP22 mRNA levels in both euglycemic and hyperglycemic iSC cells. *$p< 0.05$, **$p<0.001$

Discussion

Proper levels of myelin proteins are required for precise myelin compaction and function. Studies have shown that SC dysfunction can lead to a disruption of myelin proteins, such as PMP22, resulting in demyelination and the development of peripheral neuropathies, such as CMT-1A and HNPP. Since, demyelination has also been observed in individuals demonstrating diabetic peripheral neuropathy, the purpose of this study was to investigate a possible disruption of PMP22 levels in SCs undergoing PA-LTx.

The current study found that PA-LTx induced a significant downregulation of PMP22 mRNA levels in iSCs cultured in euglycemic and hyperglycemic conditions. In addition, earlier disruptions of mRNA levels were observed in a hyperglycemic environment. Studies have shown that the manufacturing of proteins that are unnecessary for cell survival will become restrained when the cell encounters an insult, such as PA. It is possible that PMP22 is not essential for cellular survival, thus its downregulation is initiated in cells undergoing PA-LTx.

However, ROS levels are also known to have the ability to influence gene expression [265]. A study found that ROS was able to regulate gene expression by activating different transcription factors, such as activator protein 1 (AP-1), which can affect cellular proliferation, differentiation and morphogenesis [265]. Our studies demonstrated that PA-LTx is able to significantly increase ROS concentrations in SCs cultured in euglycemic and hyperglycemic conditions [213]. Furthermore, other studies have shown that intracellular Ca++ disturbances can also play a major role gene expression [266-268]. Since, work from our lab found that PA-LTx resulted in elevated

intracellular Ca++ levels, the downregulation of mRNA concentrations of PMP22 may be due to factors such as AP-1 [213, 265].

The downregulation of mRNA levels correlated with the decrease in protein levels of PMP22 after PA exposure. A decrease in mRNA levels can directly affect protein expression in the cells, however, the disruption in protein expression can also involve the ER. Studies have shown that upon ER stress, the unfolded protein response [44] or the ER- assisted degradation response [45] can be activated. The purpose of the UPR and ERAD response is to relive the load on the ER by decreasing the translation of proteins and increasing the degradation of improperly folded proteins, respectively [3, 45, 253]. Work from our lab found that PA-LTx is able to induce ER stress and activate ER stress genes [44]. Therefore, the decrease in PMP22 protein levels can be the result of the induction of the UPR or ERAD pathways.

The co- treatment of MCI-186, an antioxidant, and PA was able to significantly reduce oxidative stress and prevent the downregulation of PMP22mRNA levels in iSC cultured in a euglycemic and hyperglycemic environments. The decrease in ROS levels by MCI-186 may prevent oxidant damage and severe intracellular Ca^{++} disruptions, thus normal transcription of genes, such as PMP22, are able to be produced. In conclusion, the ability of MCI-186 to stabilize PMP22 mRNA levels in PA-LTx iSC cells suggests that the regulation of PMP22 mRNA expression may be mediated through cellular oxidative stress.

The current study demonstrates that 1) iSCs exposed to PA demonstrated a downregulation of mRNA levels of PMP22 in a time and concentration dependent manner 2) PA-LTx in hyperglycemic conditions exhibited an earlier downregulation of

PMP22 mRNA levels when compared to euglycemic environments 3) the co- treatment of PA and MCI- 186, an antioxidant, was able to significantly reduce oxidative stress and stabilize mRNA levels of PMP22 in euglycemic and hyperglycemic conditions. Therefore, our study proposes that the expression of PMP22 mRNA levels in iSC cultured in euglycemic and hyperglycemic conditions undergoing PA-LTx may be mediated through cellular oxidative stress.

It is also suggested that a decrease in production or the increase in degradation of PMP22 can lead to the inability of this critical myelin protein to reach the plasma membrane of SCs. Thus, the inadequate levels of PMP22 in the membrane will yield an improper compaction of myelin, resulting in axonal demyelination and the possible development of peripheral neuropathy. Furthermore, hyperglycemic conditions may magnify PA-LTx by driving the cell to a more severe and earlier cellular dysfunction. In conclusion, the data supports the notion that elevated levels of FFAs may play a role in segmental demyelination through the disruption of myelin proteins, like PMP22, leading to the development of peripheral neuropathy.

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References

1. Available from: http://www.neuropathy.org/site/PageServer?pagename=About_Facts.

2. Hui-Chou, H.G., et al., *Clinical implications of peripheral myelin protein 22 for nerve compression and neural regeneration: a review.* J Reconstr Microsurg, 2011. **27**(1): p. 67-74.

3. Amici, S.A., et al., *Peripheral myelin protein 22 is in complex with alpha6beta4 integrin, and its absence alters the Schwann cell basal lamina.* J Neurosci, 2006. **26**(4): p. 1179-89.

4. Fortun, J., et al., *Alterations in degradative pathways and protein aggregation in a neuropathy model based on PMP22 overexpression.* Neurobiol Dis, 2006. **22**(1): p. 153-64.

5. Taioli, F., et al., *Inherited demyelinating neuropathies with micromutations of peripheral myelin protein 22 gene.* Brain, 2011. **134**(Pt 2): p. 608-17.

6. De Leon, M., et al., *SR13/PMP-22 expression in rat nervous system, in PC12 cells, and C6 glial cell lines.* J Neurosci Res, 1994. **38**(2): p. 167-81.

7. De Leon, M., et al., *Identification of transcriptionally regulated genes after sciatic nerve injury.* J Neurosci Res, 1991. **29**(4): p. 437-48.

8. Robinson, F.L., et al., *Loss of the inactive myotubularin-related phosphatase Mtmr13 leads to a Charcot-Marie-Tooth 4B2-like peripheral neuropathy in mice.* Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4916-21.

9. Notterpek, L., E.M. Shooter, and G.J. Snipes, *Upregulation of the endosomal-lysosomal pathway in the trembler-J neuropathy.* J Neurosci, 1997. **17**(11): p. 4190-200.

10. Haney, C., et al., *Ultrastructural distribution of PMP22 in Charcot-Marie-Tooth disease type 1A.* J Neuropathol Exp Neurol, 1996. **55**(3): p. 290-9.

11. Pareek, S., et al., *Neurons promote the translocation of peripheral myelin protein 22 into myelin.* J Neurosci, 1997. **17**(20): p. 7754-62.

12. Hasse, B., et al., *Peripheral myelin protein 22 kDa and protein zero: domain specific trans-interactions.* Mol Cell Neurosci, 2004. **27**(4): p. 370-8.

13. Nave, K.A., *Myelin genetics: new insight into old diseases.* Brain Pathol, 1995. **5**(3): p. 231-2.

14. Lupski, J.R., et al., *DNA duplication associated with Charcot-Marie-Tooth disease type 1A.* Cell, 1991. **66**(2): p. 219-32.

15. Roa, B.B., C.A. Garcia, and J.R. Lupski, *Charcot-Marie-Tooth disease type 1A: molecular mechanisms of gene dosage and point mutation underlying a common inherited peripheral neuropathy.* Int J Neurol, 1991. **25-26**: p. 97-107.

16. Thomas, F.P., et al., *Tomaculous neuropathy in chromosome 1 Charcot-Marie-Tooth syndrome.* Acta Neuropathol, 1994. **87**(1): p. 91-7.

17. Mizisin, A.P., et al., *Myelin splitting, Schwann cell injury and demyelination in feline diabetic neuropathy.* Acta Neuropathol, 1998. **95**(2): p. 171-4.

18. Kamboj, S.S., R.K. Vasishta, and R. Sandhir, *N-acetylcysteine inhibits hyperglycemia-induced oxidative stress and apoptosis markers in diabetic neuropathy.* J Neurochem, 2010. **112**(1): p. 77-91.

19. Miscio, G., et al., *Obesity and peripheral neuropathy risk: a dangerous liaison.* J Peripher Nerv Syst, 2005. **10**(4): p. 354-8.

20. Padilla, A., et al., *Hyperglycemia magnifies Schwann cell dysfunction and cell death triggered by PA-induced lipotoxicity.* Brain Res, 2011. **1370**: p. 64-79.

21. Listenberger, L.L., et al., *Triglyceride accumulation protects against fatty acid-induced lipotoxicity.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3077-82.

22. van Herpen, N.A. and V.B. Schrauwen-Hinderling, *Lipid accumulation in non-adipose tissue and lipotoxicity.* Physiol Behav, 2008. **94**(2): p. 231-41.

23. Bolin, L.M., T.P. Iismaa, and E.M. Shooter, *Isolation of activated adult Schwann cells and a spontaneously immortal Schwann cell clone.* J Neurosci Res, 1992. **33**(2): p. 231-8.

24. Cochard, P. and D. Paulin, *Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo.* J Neurosci, 1984. **4**(8): p. 2080-94.

25. Heumann, R., et al., *Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection.* J Cell Biol, 1987. **104**(6): p. 1623-31.

26. De Leon, M., L.J. Van Eldik, and E.M. Shooter, *Differential regulation of S100 beta and mRNAs coding for S100-like proteins (42A and 42C) during development and after lesion of rat sciatic nerve.* J Neurosci Res, 1991. **29**(2): p. 155-62.

27. Ulloth, J.E., C.A. Casiano, and M. De Leon, *Palmitic and stearic fatty acids induce caspase-dependent and -independent cell death in nerve growth factor differentiated PC12 cells.* J Neurochem, 2003. **84**(4): p. 655-68.

28. Almaguel, F.G., et al., *Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid.* J Neurosci Res, 2009. **87**(5): p. 1207-18.

29. Xanthoudakis, S. and T. Curran, *Redox regulation of AP-1: a link between transcription factor signaling and DNA repair.* Adv Exp Med Biol, 1996. **387**: p. 69-75.

30. Suzuki, Y., et al., *Different utilization of Ca2+ in the contractile action of endothelin-1 on cerebral, coronary and mesenteric arteries of the dog.* Eur J Pharmacol, 1992. **219**(3): p. 401-8.

31. Lin, W.N., et al., *Regulation of COX-2 and cPLA(2) gene expression by LPS through the RNA-binding protein HuR: involvement of NADPH oxidase, ROS and MAPKs.* Br J Pharmacol, 2011.

32. Kayano, M., et al., *ROS-DET: robust detector of switching mechanisms in gene expression.* Nucleic Acids Res, 2011.

33. Yasuda, H., et al., *Diabetic neuropathy and nerve regeneration.* Prog Neurobiol, 2003. **69**(4): p. 229-85.

34. Fortun, J., et al., *Impaired proteasome activity and accumulation of ubiquitinated substrates in a hereditary neuropathy model.* J Neurochem, 2005. **92**(6): p. 1531-41.

35. Lin, W., et al., *Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress.* Brain, 2006. **129**(Pt 5): p. 1306-18.

CHAPTER FIVE

DISCUSSION

Summary of Findings

The present study explored the role of PA-LTx in SCs cultured in different concentrations of glucose. The aim of this study was to elucidate the potential contributions of FFAs in the development of peripheral nerve damage. It is essential to understand the mechanisms of PA-LTx in SCs since this may lead to the development of innovative and effective therapies that will prevent or halt the progression of peripheral neuropathy, a common complication of diabetes type 2.

Previous work from our lab demonstrated that PA and stearic acid, at concentrations similar to those found in traumatic brain injuries (FFA:BSA 2:1), were able to induce cellular death [64, 213]. By contrast, oleic and arachodonic acids showed no significant decrease in cell viability. LCSFAs, such as PA and stearic acid activated caspase 3 and 8, and cleavage of poly (ADP-ribose) polymerase (PARP). Toxicity by PA and stearic acid was found to be caspase independent and involved the disruption in gene expression of fas ligand/receptor [35]. Further work in NGFDPC12 and rat cortical cells showed that PA-LTx induced apoptosis, the ASCOS response, MMD, and an increase in BNIP3, a proapoptotic member of the Bcl-2 family. Lysosomal membrane destabilization and cathepsin L leakage into the cytosol was detected and deemed imperative in the apoptotic process due to the hindrance in cell death with the addition of cathepsin L inhibitors [41, 64].

The findings of this study demonstrated that exposure to high levels of PA, a LCSFA, resulted in a powerful lipotoxic response in immortalized and primary SCs. Both immortalized (iSC) and primary SCs (pSC) were cultured in euglycemic and hyperglycemic environments for 4-5 days before PA exposure to mimic a chronic glucose environment. Furthermore, full serum media was changed to serum free before exposing cells to PA. This was done to remove any confounding factors that are added by full serum and to decrease the proliferation rate of the cells.

In a euglycemic conditions the addition of PA decreased cell viability of iSC and pSC in a dose and time dependent manner. The decrease of endoplasmic reticulum (ER) Ca^{++} stores was the first indication of PA cellular injury (approximately 1H after PA exposure). Lysosomal injury (3H) followed ER damage and included the release in cathepsin L and B . A prolonged PA exposure to iSC and pSC resulted in mitochondrial membrane depolarization (6H) leading to a significant increase in reactive oxidative stress (ROS) (12H) and the activation of caspase 3 (12H).

Upon a closer examination to the ER, PA-LTx induced the up-regulation of ER stress genes Xbp1 and CHOP mRNA levels, while significantly downregulating GRP78 with increased cell death. In addition, mRNA and protein levels of myelin protein 22 (PMP22) were also downregulated after PA exposure.

Interestingly, it was observed that chronic hyperglycemia alone does not induce iSC or pSC cell death but its presence strongly magnifies that apoptotic process. In addition, 2'7' DCF and JC-1 assays found no significant increase in ROS production or MMD with elevated glucose concentrations alone in both iSC and pSC. However, upon the addition of PA, a robust and earlier detection of ER Ca^{++} efflux into the cytosol was

observed after 15min. ER stress genes were also disrupted in iSC cells, with an up-regulation of GRP78 mRNA and protein expression in the hyperglycemic state when iSC were undergoing cellular death.

In contrast to the euglycemic conditions, PA-LTx caused MMD (1H) and significantly increased ROS production (3H), and caspase activation (3H). After 24H of PA exposure, flow cytometric analysis was able to detect a significant LMD with cathepsin B and L release into the cytosol. Similarly, PMP 22 mRNA and protein levels were decreased with PA treatments, however the downregulation of mRNA was significant at an earlier time when compared to euglycemic conditions. Overall, the apoptotic events observed in hyperglycemic conditions were encountered at earlier time points when compared to euglycemic conditions, except for lysosomal injury. The delay in lysosomal damage in hyperglycemic conditions continues to be unclear and needs to be further examined.

In the attempt to understand the role of Ca^{++} and ROS in cellular damage, a calcium chelator, BAPTA-AM, and an antioxidant, MCI-186, were used as a co-treatment with PA. Our study found that reducing excessive cytosolic Ca^{++} levels with a Ca^{++} chelator (BAPTA-AM) inhibited cell death in euglycemic and hyperglycemic Schwann cells. Furthermore, our data demonstrates that BAPTA-AM is able to reduce ROS level in both euglycemic and hyperglycemic iSC, thus possible suggesting a major role of Ca^{++} in the lipotoxic response. Furthermore, BAPTA-AM resulted in the stabilization of mRNA levels of pro-survival genes such as GRP78, upregulation of XBP1 in a euglycemic and hyperglycemic conditions. The decrease in pro-apoptotic ER stress genes such as CHOP was observed in both euglycemic and hyperglycemic

conditions. The data indicates that reducing the stress on the ER may assist the cell in its attempt to survive. To further examine the role of Ca^{++} in PA-LTx, the inhibition of N-methyl D-aspartate (NMDA) receptors with D-AP5 was utilized as a co-treatment with PA. D-AP5 was able to increased iSC cell viability at 24H only in both euglycemic and hyperglycemic conditions.

The antioxidant MCI-186 was able to significantly decrease oxidative stress in both euglycemic and hyperglycemic conditions and prevented the downregulation of mRNA levels of PMP22 in iSC during PA exposure. MCI 186 also increased euglycemic and hyperglycemic iSC viability at 24 hours, however, MCI-186 could not prevent cellular death after a prolonged PA exposure greater than 24 hours.

Proposed PA-LTx Mechanism

The data generated by this study leads to the proposition of two distinct pathways of PA-LTx that is modulated by elevated levels of glucose. The first pathway involves the induction of PA-LTx in euglycemic conditions beginning with the injury to the ER causing the efflux of Ca^{++} into the cytosol. Weather PA directly or indirectly injures the ER membrane continues to remain unclear. However, possibly through PA's activation of proapoptotic proteins, such as Bax or thought the toxic release of Ca^{++} into the cytosol, LMD and the release cathepsins are the next events that occur after PA exposure. The release of cathepsins, in addition to the increase in cytosolic Ca^{++} may directly affect the mitochondrial membrane leading to MMD. The increase in ROS levels and caspase follows MMD, in addition to a decrease in PMP22 mRNA and protein levels possibly due to increase in ROS or the activation of the ER stress response. Consequently,

cellular injury by PA-LTx will result in the initiation of apoptotic pathways leading to cell death.

The alternate pathway involves the influence of hyperglycemia in the cascade of apoptotic events. Hyperglycemia alone can disrupt the cell by 1) modifying proteins and protein-DNA interactions 2) influencing FA metabolism by the accumulation of malonyl-CoA 3) and disrupting the lysosomal degradation pathway [269]-[270]. However, if the insult is not great, the cells are able to readjust with the stress without cellular death [90].

Nonetheless, the addition of a second insult can result in severe cellular damage. As observed in our study, the addition of PA to SCs cultured in hyperglycemia resulted in an early and strong ER response followed by MMD. The earlier elevation of intracellular Ca^{++} may explain the early MMD observed in our study.

The damage to the mitochondria can result in the generation of ROS and caspase activation. Since, SCs have already increased their production of factors such as antioxidants to cope with elevated glucose levels, the insult by PA may have pushed the SC to an earlier and robust generation of ROS. The increase in caspase activation may also be explained by the elevated levels of ROS and mitochondrial damage after PA exposure. In addition, the disruption of PMP22 mRNA and protein levels at an earlier time can also be explained by the negative cellular influence of hyperglycemia.

LMD occurred at a later time point in the lipotoxic process possibly due to the involvement of an increase in proteins secondary to hyperglycemia, such as the anti apoptotic chaperone heat shock protein 70 (Hsp 70). The delay in lysosomal involvement may have contributed to a disruption in the autophagic process resulting in the strong increase in caspase activation that is observed in our studies.

In conclusion, the earlier and stronger elevation of levels of cytosolic Ca^{++} concentrations, ROS levels, possible impairments to autophagic pathways due to lysosomal delay, and the release in cathepsins L and B, greatly increase the severity of SC damage. Figure 1.

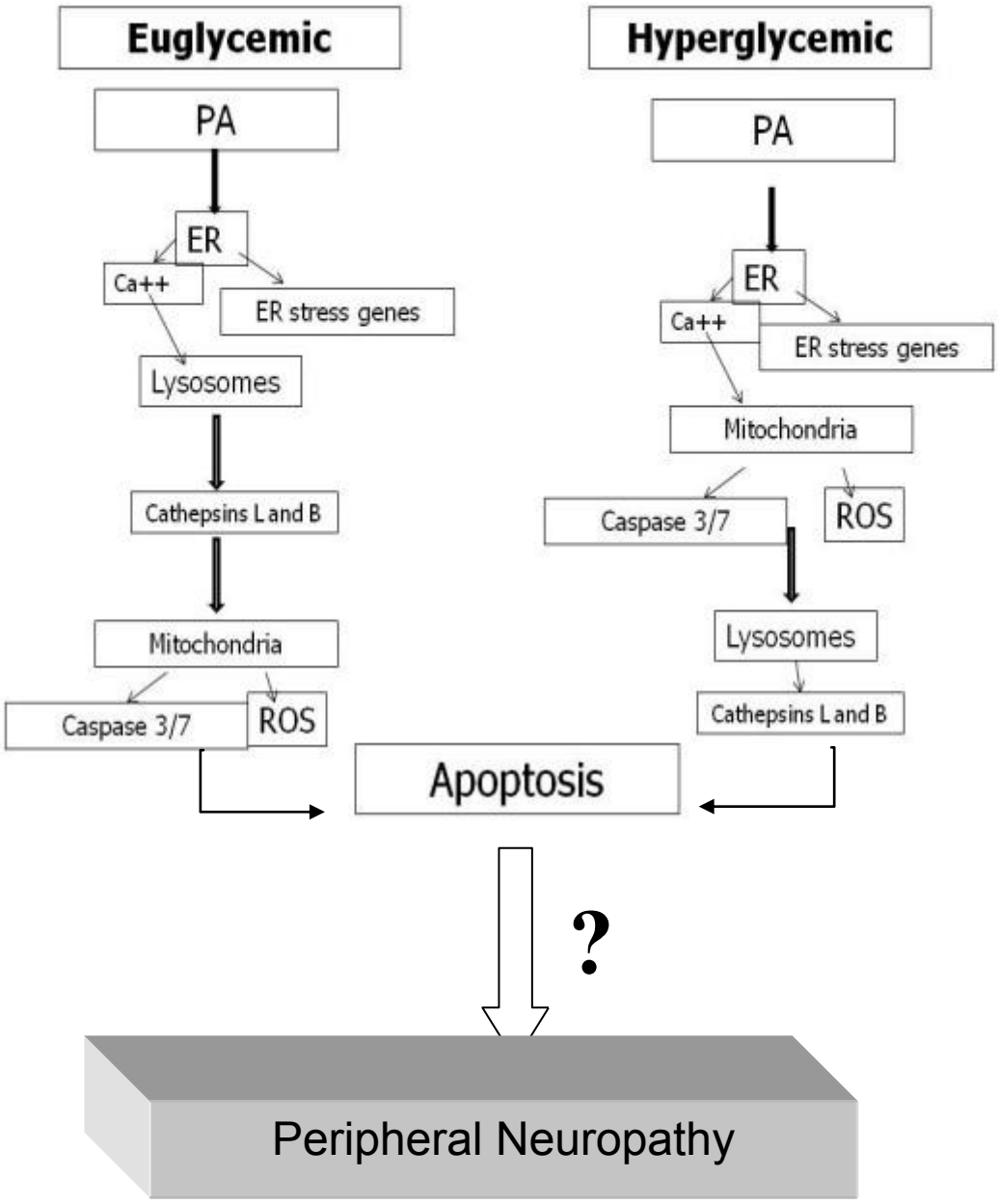
Euglycemic
PA
ER
Ca++
ER stress genes
Lysosomes
Cathepsins L and B
Mitochondria
Caspase 3/7
ROS
Hyperglycemic
PA
ER
Ca++
ER stress genes
Mitochondria
Caspase 3/7
ROS
Lysosomes
Cathepsins L and B
Apoptosis
?
Peripheral Neuropathy

Figure 1. Proposed Mechanisms of PA-LTx in Schwann Cells.
Based on our studies, PA-LTx occurs in both euglycemic and hyperglycemic conditions in SC. PA will injure the ER causing the activation of ER stress genes and an efflux of Ca^{++} from the ER into the cytosol. In euglycemic conditions, lysosomal membrane destabilization will follow with the release of cathepsins into the cytosol. Mitochondrial damage, increase in ROS and activation of caspase 3/7 ensues. In hyperglycemic conditions, following ER damage, mitochondrial injury, ROS, caspase activation,. lysosomal damage and the release of cathepsins is observed The delay in the lysosomal pathway suggests an altered cell death pathway in hyperglycemic conditions. However, both pathways lead to apoptosis, thus potentially contributing to the development of peripheral neuropathy. PA, palmitic acid; ER, endoplasmic reticulum; Ca^{++}, cytosolic calcium; ROS, reactive oxidative species.

Future Studies

A closer examination of PA-LTx and its direct or indirect injury to critical cellular organelles in the initial stages of apoptosis would be of vast benefit. The understanding of early events in PA-LTx may lead to the capability to prevent the full activation of the apoptotic pathway and thus inhibiting cell death. In addition, the role of hyperglycemia in PA-LTx and its ability to delay LMD and drive the cell into an earlier and severe cellular dysfunction would aid in our attempt to understand the multifactorial aspects of diabetic peripheral neuropathy. In addition, a closer examination in the disruption of myelin proteins and the possible additional roles that PMP22 may have in the apoptotic process can further aid in the prevention of myelin damage and axonal injury.

Ultimately, the goal is to explore these finding and extrapolate them into an *in vivo* model. The female C57BL/6J mouse model has been shown to be a good model since mice will develop diabetes base on diet alone. Mice fed a high fat diet (HFD)(58% fat, 25.6% carbohydrate, and 16.4% protein, total of 12.6 kJ/g) will develop peripheral neuropathies which can be examined through nerve conduction velocities and behavioral tests: (tactile responses and thermal analgesia). Sciatic nerves and lumbar dorsal root ganglions (DRGs) from control and HFD animals would be examined using RPTC to analyze mRNA levels of ER stress genes and myelin proteins. Immunohistochemical studies would also be performed to analyze the myelin disturbances or nerve disruptions that may lead to segmental demyelination and neuropathy. In addition, in vivo studies could also explore the consequences of the reduction in fat in the mice diet and the administration of antioxidants (MCI-186) or Ca^{++} chelators (BAPTA-AM) within the diet.

Conclusion

In conclusion this study demonstrated that SC can undergo LTx in euglycemic and hyperglycemic environments with earlier and more severe changes occurring in hyperglycemic conditions. The implications of these findings point to the ability of circulating elevated levels of LCSFAs to cause Schwann cells injury resulting in SC death. The significant dysfunction and decrease in SC viability by LTx may result in the disruption of myelin proteins leading to segmental demyelination of axon. The loss of myelin, SC-axonal survival interactions and the environment essential for axonal health and regeneration, can become a major factor in the development of peripheral neuropathy. In addition, the ability of hyperglycemia to drive the SC into a more severe and earlier apoptotic resolution emphasizes the importance in finding a treatment that will not only reduce the pain, but will more importantly inhibit neuronal damage and the development or the progression diabetic peripheral neuropathy.

References

1. Bell, G.H., *The metabolism of long-chain fatty acids and alcohols by Candida tropicalis and Saccharomyces cerevisiae.* Antonie Van Leeuwenhoek, 1973. **39**(1): p. 137-49.

2. Bezuglov, V.V., M. Bobrov, and A.V. Archakov, *Bioactive amides of fatty acids.* Biochemistry (Mosc), 1998. **63**(1): p. 22-30.

3. Fortun, J., et al., *Impaired proteasome activity and accumulation of ubiquitinated substrates in a hereditary neuropathy model.* J Neurochem, 2005. **92**(6): p. 1531-41.

4. Stephens, F.B., D. Constantin-Teodosiu, and P.L. Greenhaff, *New insights concerning the role of carnitine in the regulation of fuel metabolism in skeletal muscle.* J Physiol, 2007. **581**(Pt 2): p. 431-44.

5. Listenberger, L.L., et al., *Triglyceride accumulation protects against fatty acid-induced lipotoxicity.* Proc Natl Acad Sci U S A, 2003. **100**(6): p. 3077-82.

6. Shah, A. *Obesity*. 2010; Available from: http://www.globalissues.org/article/558/obesity.

7. Carroll, C.O.a.M. (June 2010) *Prevalence of Obesity Among Children and Adolescents: United States, Trends 1963–1965 Through 2007–200*.

8. Ahrens, W., et al., *The IDEFICS cohort: design, characteristics and participation in the baseline survey.* Int J Obes (Lond), 2011. **35 Suppl 1**: p. S3-15.

9. Lee, A., M.M. Ho, and V.M. Keung, *Global epidemics of childhood obesity is hitting a 'less industrialized' corner in Asia: a case study in Macao.* Int J Pediatr Obes, 2011. **6**(2-2): p. e252-6.

10. Yanovski, S.Z. and J.A. Yanovski, *Obesity prevalence in the United States--up, down, or sideways?* N Engl J Med, 2011. **364**(11): p. 987-9.

11. Kusminski, C.M., et al., *Diabetes and apoptosis: lipotoxicity.* Apoptosis, 2009. **14**(12): p. 1484-95.

12. Unger, R.H., *Reinventing type 2 diabetes: pathogenesis, treatment, and prevention.* JAMA, 2008. **299**(10): p. 1185-7.

13. Ho, A.J., et al., *Obesity is linked with lower brain volume in 700 AD and MCI patients.* Neurobiol Aging, 2010. **31**(8): p. 1326-39.

14. Unger, R.H., *Lipotoxicity in the pathogenesis of obesity-dependent NIDDM. Genetic and clinical implications.* Diabetes, 1995. **44**(8): p. 863-70.

15. Shimabukuro, M., et al., *Fatty acid-induced beta cell apoptosis: a link between obesity and diabetes.* Proc Natl Acad Sci U S A, 1998. **95**(5): p. 2498-502.

16. Lupi, R., et al., *Prolonged exposure to free fatty acids has cytostatic and pro-apoptotic effects on human pancreatic islets: evidence that beta-cell death is caspase mediated, partially dependent on ceramide pathway, and Bcl-2 regulated.* Diabetes, 2002. **51**(5): p. 1437-42.

17. Kato, A., et al., *Protective effects of dietary chamomile tea on diabetic complications.* J Agric Food Chem, 2008. **56**(17): p. 8206-11.

18. Koshkin, V., et al., *Limited mitochondrial permeabilization is an early manifestation of palmitate-induced lipotoxicity in pancreatic beta-cells.* J Biol Chem, 2008. **283**(12): p. 7936-48.

19. Unger, R.H., *How obesity causes diabetes in Zucker diabetic fatty rats.* Trends Endocrinol Metab, 1997. **8**(7): p. 276-82.

20. Unger, R.H. and Y.T. Zhou, *Lipotoxicity of beta-cells in obesity and in other causes of fatty acid spillover.* Diabetes, 2001. **50 Suppl 1**: p. S118-21.

21. Schaffer, J.E., *Lipotoxicity: when tissues overeat.* Curr Opin Lipidol, 2003. **14**(3): p. 281-7.

22. van Herpen, N.A. and V.B. Schrauwen-Hinderling, *Lipid accumulation in non-adipose tissue and lipotoxicity.* Physiol Behav, 2008. **94**(2): p. 231-41.

23. Song, Z., et al., *Silymarin prevents palmitate-induced lipotoxicity in HepG2 cells: involvement of maintenance of Akt kinase activation.* Basic Clin Pharmacol Toxicol, 2007. **101**(4): p. 262-8.

24. Borradaile, N.M., et al., *Disruption of endoplasmic reticulum structure and integrity in lipotoxic cell death.* J Lipid Res, 2006. **47**(12): p. 2726-37.

25. Gelb, A.M., M.I. Davidson, and J.I. Kessler, *Effect of Fasting on Esterification of Fatty Acids by the Small Intestine in Vitro.* Am J Physiol, 1964. **207**: p. 1207-10.

26. Hardy, S., et al., *Saturated fatty acid-induced apoptosis in MDA-MB-231 breast cancer cells. A role for cardiolipin.* J Biol Chem, 2003. **278**(34): p. 31861-70.

27. Moffitt, J.H., et al., *Adverse physicochemical properties of tripalmitin in beta cells lead to morphological changes and lipotoxicity in vitro.* Diabetologia, 2005. **48**(9): p. 1819-29.

28. Prentki, M. and S.R. Madiraju, *Glycerolipid metabolism and signaling in health and disease.* Endocr Rev, 2008. **29**(6): p. 647-76.

29. Ricchi, M., et al., *Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes.* J Gastroenterol Hepatol, 2009. **24**(5): p. 830-40.

30. Maedler, K., et al., *Monounsaturated fatty acids prevent the deleterious effects of palmitate and high glucose on human pancreatic beta-cell turnover and function.* Diabetes, 2003. **52**(3): p. 726-33.

31. Goswami, R., et al., *Differential regulation of ceramide in lipid-rich microdomains (rafts): antagonistic role of palmitoyl:protein thioesterase and neutral sphingomyelinase 2.* J Neurosci Res, 2005. **81**(2): p. 208-17.

32. Argia, T., R.K. Yu, and T. Miyatake, *Characterization of gangliosides by direct inlet chemical ionization mass spectrometry.* J Lipid Res, 1984. **25**(10): p. 1096-101.

33. Blazquez, C., et al., *The AMP-activated protein kinase prevents ceramide synthesis de novo and apoptosis in astrocytes.* FEBS Lett, 2001. **489**(2-3): p. 149-53.

34. Suzuki, J., et al., *Palmitate induces apoptosis in Schwann cells via both ceramide-dependent and independent pathways.* Neuroscience, 2011. **176**: p. 188-98.

35. Ulloth, J.E., C.A. Casiano, and M. De Leon, *Palmitic and stearic fatty acids induce caspase-dependent and -independent cell death in nerve growth factor differentiated PC12 cells.* J Neurochem, 2003. **84**(4): p. 655-68.

36. Chilton, F.H., et al., *Depression and altered serum lipids in cynomolgus monkeys consuming a Western diet.* Physiol Behav, 2011.

37. Kanoski, S.E. and T.L. Davidson, *Western diet consumption and cognitive impairment: Links to hippocampal dysfunction and obesity.* Physiol Behav, 2011. **103**(1): p. 59-68.

38. Schmid, A., *The role of meat fat in the human diet.* Crit Rev Food Sci Nutr, 2011. **51**(1): p. 50-66.

39. Li, Z., et al., *Using dynamic gene module map analysis to identify targets that modulate free fatty acid induced cytotoxicity.* Biotechnol Prog, 2008. **24**(1): p. 29-37.

40. Wei, Y., et al., *Saturated fatty acids induce endoplasmic reticulum stress and apoptosis independently of ceramide in liver cells.* Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E275-81.

41. Almaguel, F.G., et al., *Lipotoxicity-mediated cell dysfunction and death involve lysosomal membrane permeabilization and cathepsin L activity.* Brain Res, 2010. **1318**: p. 133-43.

42. Nakhjavani, M., et al., *Serum heat shock protein 70 and oxidized LDL in patients with type 2 diabetes: does sex matter?* Cell Stress Chaperones, 2011. **16**(2): p. 195-201.

43. Alkhourl, N.D., L.J.; Feldstein, A.E., *Lipotoxicity in Nonalcoholic Fatty Liver Disease: Not All Lipids Are Created Equal.* Expert Rev Gastroenterol Hepatol., 2009. **3**(4): p. 445-451.

44. Lin, W., et al., *Interferon-gamma inhibits central nervous system remyelination through a process modulated by endoplasmic reticulum stress.* Brain, 2006. **129**(Pt 5): p. 1306-18.

45. Yasuda, H., et al., *Diabetic neuropathy and nerve regeneration.* Prog Neurobiol, 2003. **69**(4): p. 229-85.

46. Greber, U.F. and L. Gerace, *Depletion of calcium from the lumen of endoplasmic reticulum reversibly inhibits passive diffusion and signal-mediated transport into the nucleus.* J Cell Biol, 1995. **128**(1-2): p. 5-14.

47. Lam, M., G. Dubyak, and C.W. Distelhorst, *Effect of glucocorticosteroid treatment on intracellular calcium homeostasis in mouse lymphoma cells.* Mol Endocrinol, 1993. **7**(5): p. 686-93.

48. Nutt, L.K., et al., *Bax and Bak promote apoptosis by modulating endoplasmic reticular and mitochondrial Ca2+ stores.* J Biol Chem, 2002. **277**(11): p. 9219-25.

49. Brodsky, J.L. and A.A. McCracken, *ER-associated and proteasomemediated protein degradation: how two topologically restricted events came together.* Trends Cell Biol, 1997. **7**(4): p. 151-6.

50. Kaufman, R.J., *Orchestrating the unfolded protein response in health and disease.* J Clin Invest, 2002. **110**(10): p. 1389-98.

51. Martinez, S.C., et al., *Inhibition of Foxo1 protects pancreatic islet beta-cells against fatty acid and endoplasmic reticulum stress-induced apoptosis.* Diabetes, 2008. **57**(4): p. 846-59.

52. Urano, F., et al., *Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1.* Science, 2000. **287**(5453): p. 664-6.

53. Mayer, C.M. and D.D. Belsham, *Palmitate attenuates insulin signaling and induces endoplasmic reticulum stress and apoptosis in hypothalamic neurons:*

rescue of resistance and apoptosis through adenosine 5′ monophosphate-activated protein kinase activation. Endocrinology, 2010. **151**(2): p. 576-85.

54. Mayer, C.M. and D.D. Belsham, *Central insulin signaling is attenuated by long-term insulin exposure via insulin receptor substrate-1 serine phosphorylation, proteasomal degradation, and lysosomal insulin receptor degradation.* Endocrinology, 2010. **151**(1): p. 75-84.

55. Sekido, H., et al., *Reduced cell replication and induction of apoptosis by advanced glycation end products in rat Schwann cells.* Biochem Biophys Res Commun, 2004. **320**(1): p. 241-8.

56. Turrens, J.F., *Mitochondrial formation of reactive oxygen species.* J Physiol, 2003. **552**(Pt 2): p. 335-44.

57. Schrauwen, P. and M.K. Hesselink, *Oxidative capacity, lipotoxicity, and mitochondrial damage in type 2 diabetes.* Diabetes, 2004. **53**(6): p. 1412-7.

58. Schrauwen, P., et al., *Etomoxir-induced increase in UCP3 supports a role of uncoupling protein 3 as a mitochondrial fatty acid anion exporter.* FASEB J, 2002. **16**(12): p. 1688-90.

59. Green, K., M.D. Brand, and M.P. Murphy, *Prevention of mitochondrial oxidative damage as a therapeutic strategy in diabetes.* Diabetes, 2004. **53 Suppl 1**: p. S110-8.

60. Kirkland, R.A. and J.L. Franklin, *Bax, reactive oxygen, and cytochrome c release in neuronal apoptosis.* Antioxid Redox Signal, 2003. **5**(5): p. 589-96.

61. Kroemer, G., N. Zamzami, and S.A. Susin, *Mitochondrial control of apoptosis.* Immunol Today, 1997. **18**(1): p. 44-51.

62. Peterson, J.M., et al., *Bax signaling regulates palmitate-mediated apoptosis in C(2)C(12) myotubes.* Am J Physiol Endocrinol Metab, 2008. **295**(6): p. E1307-14.

63. Almaguel, F., *Mechanisms of DHA neuroprotection from PA indiuced lipotoxicity*. 2010. p. 223.

64. Almaguel, F.G., et al., *Activation and reversal of lipotoxicity in PC12 and rat cortical cells following exposure to palmitic acid.* J Neurosci Res, 2009. **87**(5): p. 1207-18.

65. Roberts, D.L., et al., *Molecular basis of lysosomal enzyme recognition: three-dimensional structure of the cation-dependent mannose 6-phosphate receptor.* Cell, 1998. **93**(4): p. 639-48.

66. Turk, B., D. Turk, and V. Turk, *Lysosomal cysteine proteases: more than scavengers.* Biochim Biophys Acta, 2000. **1477**(1-2): p. 98-111.

67. Bidere, N., et al., *Cathepsin D triggers Bax activation, resulting in selective apoptosis-inducing factor (AIF) relocation in T lymphocytes entering the early commitment phase to apoptosis.* J Biol Chem, 2003. **278**(33): p. 31401-11.

68. Boya, P., et al., *Lysosomal membrane permeabilization induces cell death in a mitochondrion-dependent fashion.* J Exp Med, 2003. **197**(10): p. 1323-34.

69. Schotte, P., et al., *Cathepsin B-mediated activation of the proinflammatory caspase-11.* Biochem Biophys Res Commun, 1998. **251**(1): p. 379-87.

70. Feldstein, A.E., et al., *Bax inhibition protects against free fatty acid-induced lysosomal permeabilization.* Am J Physiol Gastrointest Liver Physiol, 2006. **290**(6): p. G1339-46.

71. Sugimoto, K., Y. Murakawa, and A.A. Sima, *Diabetic neuropathy--a continuing enigma.* Diabetes Metab Res Rev, 2000. **16**(6): p. 408-33.

72. McLeod, J.G., *Investigation of peripheral neuropathy.* J Neurol Neurosurg Psychiatry, 1995. **58**(3): p. 274-83.

73. Dyck, P.J., et al., *Fiber loss is primary and multifocal in sural nerves in diabetic polyneuropathy.* Ann Neurol, 1986. **19**(5): p. 425-39.

74. Said, G., D. Baudoin, and K. Toyooka, *Sensory loss, pains, motor deficit and axonal regeneration in length-dependent diabetic polyneuropathy.* J Neurol, 2008. **255**(11): p. 1693-702.

75. Available from: http://www.neuropathy.org/site/PageServer?pagename=About_Facts). .

76. Dumitru, D. and J.C. King, *Median/ulnar premotor potential identification and localization.* Muscle Nerve, 1995. **18**(5): p. 518-25.

77. Greene, D.A., et al., *Complications: neuropathy, pathogenetic considerations.* Diabetes Care, 1992. **15**(12): p. 1902-25.

78. Miscio, G., et al., *Obesity and peripheral neuropathy risk: a dangerous liaison.* J Peripher Nerv Syst, 2005. **10**(4): p. 354-8.

79. Green, A.Q., et al., *Altered C-fiber function as an indicator of early peripheral neuropathy in individuals with impaired glucose tolerance.* Diabetes Care, 2010. **33**(1): p. 174-6.

80. Smith, A.G., et al., *Lifestyle intervention for pre-diabetic neuropathy.* Diabetes Care, 2006. **29**(6): p. 1294-9.

81. Bland, J.D., *The relationship of obesity, age, and carpal tunnel syndrome: more complex than was thought?* Muscle Nerve, 2005. **32**(4): p. 527-32.

82. Buschbacher, R.M., *Body mass index effect on common nerve conduction study measurements.* Muscle Nerve, 1998. **21**(11): p. 1398-404.

83. Chen, X.J., et al., *Proprioceptive sensory neuropathy in mice with a mutation in the cytoplasmic Dynein heavy chain 1 gene.* J Neurosci, 2007. **27**(52): p. 14515-24.

84. Vincent, A.M., et al., *Dyslipidemia-induced neuropathy in mice: the role of oxLDL/LOX-1.* Diabetes, 2009. **58**(10): p. 2376-85.

85. Dyck, P.J. and C. Giannini, *Pathologic alterations in the diabetic neuropathies of humans: a review.* J Neuropathol Exp Neurol, 1996. **55**(12): p. 1181-93.

86. Song, Z., et al., *Transgenic mice overexpressing aldose reductase in Schwann cells show more severe nerve conduction velocity deficit and oxidative stress under hyperglycemic stress.* Mol Cell Neurosci, 2003. **23**(4): p. 638-47.

87. Wiggin, T.D., et al., *Elevated triglycerides correlate with progression of diabetic neuropathy.* Diabetes, 2009. **58**(7): p. 1634-40.

88. Ray, L., et al., *Mechanisms of association between obesity and chronic pain in the elderly.* Pain, 2011. **152**(1): p. 53-9.

89. Sango, K., et al., *High glucose-induced activation of the polyol pathway and changes of gene expression profiles in immortalized adult mouse Schwann cells IMS32.* J Neurochem, 2006. **98**(2): p. 446-58.

90. Vincent, A.M., et al., *Sensory neurons and schwann cells respond to oxidative stress by increasing antioxidant defense mechanisms.* Antioxid Redox Signal, 2009. **11**(3): p. 425-38.

91. Turpin, S.M., et al., *Apoptosis in skeletal muscle myotubes is induced by ceramides and is positively related to insulin resistance.* Am J Physiol Endocrinol Metab, 2006. **291**(6): p. E1341-50.

92. Rachek, L.I., et al., *Palmitate induced mitochondrial deoxyribonucleic acid damage and apoptosis in l6 rat skeletal muscle cells.* Endocrinology, 2007. **148**(1): p. 293-9.

93. Poncelet, A.N., *An algorithm for the evaluation of peripheral neuropathy.* Am Fam Physician, 1998. **57**(4): p. 755-64.

94. Wild, S., et al., *Global prevalence of diabetes: estimates for the year 2000 and projections for 2030.* Diabetes Care, 2004. **27**(5): p. 1047-53.

95. Boulton, A.J. and L. Vileikyte, *The diabetic foot: the scope of the problem.* J Fam Pract, 2000. **49**(11 Suppl): p. S3-8.

96. Amos, A.F., D.J. McCarty, and P. Zimmet, *The rising global burden of diabetes and its complications: estimates and projections to the year 2010.* Diabet Med, 1997. **14 Suppl 5**: p. S1-85.

97. Gupta, S.K., et al., *Diabetes prevalence and its risk factors in rural area of Tamil Nadu.* Indian J Community Med, 2010. **35**(3): p. 396-9.

98. Llewelyn, J.G., *Diabetic neuropathy.* Curr Opin Neurol, 1995. **8**(5): p. 364-6.

99. Said, G., G. Slama, and J. Selva, *Progressive centripetal degeneration of axons in small fibre diabetic polyneuropathy.* Brain, 1983. **106 (Pt 4)**: p. 791-807.

100. Dyck, P.J. and P.C. O'Brien, *Quantitative sensation testing in epidemiological and therapeutic studies of peripheral neuropathy.* Muscle Nerve, 1999. **22**(6): p. 659-62.

101. Obrosova, I.G., *How does glucose generate oxidative stress in peripheral nerve?* Int Rev Neurobiol, 2002. **50**: p. 3-35.

102. Obrosova, I.G., *Increased sorbitol pathway activity generates oxidative stress in tissue sites for diabetic complications.* Antioxid Redox Signal, 2005. **7**(11-12): p. 1543-52.

103. Obrosova, I.G., et al., *Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy.* Diabetes, 2004. **53**(3): p. 711-20.

104. Virag, L., et al., *Nitric oxide-peroxynitrite-poly(ADP-ribose) polymerase pathway in the skin.* Exp Dermatol, 2002. **11**(3): p. 189-202.

105. Pacher, P., et al., *Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies.* Curr Med Chem, 2005. **12**(3): p. 267-75.

106. Sharma, S.S., A. Kumar, and R.K. Kaundal, *Protective effects of 4-amino1,8-napthalimide, a poly (ADP-ribose) polymerase inhibitor in experimental diabetic neuropathy.* Life Sci, 2008. **82**(11-12): p. 570-6.

107. Urban, M.J., et al., *Inhibiting heat-shock protein 90 reverses sensory hypoalgesia in diabetic mice.* ASN Neuro, 2010. **2**(4).

108. Gumy, L.F., E.T. Bampton, and A.M. Tolkovsky, *Hyperglycaemia inhibits Schwann cell proliferation and migration and restricts regeneration of axons and Schwann cells from adult murine DRG.* Mol Cell Neurosci, 2008. **37**(2): p. 298-311.

109. Encyclopedia, B., *Action Potential Conduction.*

110. Nguyen, T., et al., *Axonal protective effects of the myelin-associated glycoprotein.* J Neurosci, 2009. **29**(3): p. 630-7.

111. Brant-Zawadzki, M. and D.R. Enzmann, *Using computed tomography of the brain to correlate low white-matter attenuation with early gestational age in neonates.* Radiology, 1981. **139**(1): p. 105-8.

112. Fricker, F.R., et al., *Axonally derived Neuregulin-1 is required for remyelination and regeneration after nerve injury in adulthood.* J Neurosci, 2011. **31**(9): p. 3225-33.

113. Sugimoto, K., M. Yasujima, and S. Yagihashi, *Role of advanced glycation end products in diabetic neuropathy.* Curr Pharm Des, 2008. **14**(10): p. 953-61.

114. Verheijen, M.H., et al., *Local regulation of fat metabolism in peripheral nerves.* Genes Dev, 2003. **17**(19): p. 2450-64.

115. Mizisin, A.P., et al., *Myelin splitting, Schwann cell injury and demyelination in feline diabetic neuropathy.* Acta Neuropathol, 1998. **95**(2): p. 171-4.

116. Yu, C.L., Z.G. Zhu, and L.S. Lei, *[Study of dynamic changes of blood sugar and body signs in streptozotocin-induced diabetic animal models].* Nan Fang Yi Ke Da Xue Xue Bao, 2008. **28**(1): p. 132-3.

117. Veves, A., et al., *The relationship between sural nerve morphometric findings and measures of peripheral nerve function in mild diabetic neuropathy.* Diabet Med, 1991. **8**(10): p. 917-21.

118. Laura, M., et al., *Rapid progression of late onset axonal Charcot-Marie-Tooth disease associated with a novel MPZ mutation in the extracellular domain.* J Neurol Neurosurg Psychiatry, 2007. **78**(11): p. 1263-6.

119. De Leon, M., et al., *SR13/PMP-22 expression in rat nervous system, in PC12 cells, and C6 glial cell lines.* J Neurosci Res, 1994. **38**(2): p. 167-81.

120. Eckersley, L., *Role of the Schwann cell in diabetic neuropathy.* Int Rev Neurobiol, 2002. **50**: p. 293-321.

121. Dugan, L.L., et al., *IL-6 mediated degeneration of forebrain GABAergic interneurons and cognitive impairment in aged mice through activation of neuronal NADPH oxidase.* PLoS One, 2009. **4**(5): p. e5518.

122. Lu, Q., et al., *Guattegaumerine protects primary cultured cortical neurons against oxidative stress injury induced by hydrogen peroxide concomitant with serum deprivation.* Cell Mol Neurobiol, 2009. **29**(3): p. 355-64.

123. Barber, S.C. and P.J. Shaw, *Oxidative stress in ALS: Key role in motor neuron injury and therapeutic target.* Free Radic Biol Med, 2010. **48**(5): p. 629-641.

124. Welcher, A.A., et al., *Molecular approaches to nerve regeneration.* Philos Trans R Soc Lond B Biol Sci, 1991. **331**(1261): p. 295-301.

125. Cameron, N.E. and M.A. Cotter, *Pro-inflammatory mechanisms in diabetic neuropathy: focus on the nuclear factor kappa B pathway.* Curr Drug Targets, 2008. **9**(1): p. 60-7.

126. Obrosova, I.G., et al., *High-fat diet induced neuropathy of pre-diabetes and obesity: effects of "healthy" diet and aldose reductase inhibition.* Diabetes, 2007. **56**(10): p. 2598-608.

127. Sultan, A., et al., *Duloxetine for painful diabetic neuropathy and fibromyalgia pain: systematic review of randomised trials.* BMC Neurol, 2008. **8**: p. 29.

128. Lindsay, T.J., et al., *Treating diabetic peripheral neuropathic pain.* Am Fam Physician, 2010. **82**(2): p. 151-8.

129. Davis, T.M., et al., *Prevalence, characteristics, and prognostic significance of HFE gene mutations in type 2 diabetes: the Fremantle Diabetes Study.* Diabetes Care, 2008. **31**(9): p. 1795-801.

130. Davis, T.M., et al., *Lipid-lowering therapy and peripheral sensory neuropathy in type 2 diabetes: the Fremantle Diabetes Study.* Diabetologia, 2008. **51**(4): p. 562-6.

131. Smith, A.G. and J.R. Singleton, *Impaired glucose tolerance and neuropathy.* Neurologist, 2008. **14**(1): p. 23-9.

132. Yu, C., S. Rouen, and R.T. Dobrowsky, *Hyperglycemia and downregulation of caveolin-1 enhance neuregulin-induced demyelination.* Glia, 2008. **56**(8): p. 877-87.

133. Adibhatla, R.M. and J.F. Hatcher, *Altered Lipid Metabolism in Brain Injury and Disorders.* Subcell Biochem, 2008. **48**: p. nihpa41041.

134. Ghosh, S. and B. Rodrigues, *Cardiac cell death in early diabetes and its modulation by dietary fatty acids.* Biochim Biophys Acta, 2006. **1761**(10): p. 1148-62.

135. Wilding, J.P., *The importance of free fatty acids in the development of Type 2 diabetes.* Diabet Med, 2007. **24**(9): p. 934-45.

136. Ioannidis, I., *The road from obesity to type 2 diabetes.* Angiology, 2008. **59**(2 Suppl): p. 39S-43S.

137. El-Assaad, W., et al., *Saturated fatty acids synergize with elevated glucose to cause pancreatic beta-cell death.* Endocrinology, 2003. **144**(9): p. 4154-63.

138. Rho, M.C., et al., *Sensitization of vascular smooth muscle cell to TNF-alpha-mediated death in the presence of palmitate.* Toxicol Appl Pharmacol, 2007. **220**(3): p. 311-9.

139. Simonian, N.A. and J.T. Coyle, *Oxidative stress in neurodegenerative diseases.* Annu Rev Pharmacol Toxicol, 1996. **36**: p. 83-106.

140. Ilieva, E.V., et al., *Oxidative and endoplasmic reticulum stress interplay in sporadic amyotrophic lateral sclerosis.* Brain, 2007. **130**(Pt 12): p. 3111-23.

141. Taghibiglou, C., et al., *Mechanisms involved in cholesterol-induced neuronal insulin resistance.* Neuropharmacology, 2009. **57**(3): p. 268-76.

142. Hayashi, T., et al., *Damage to the endoplasmic reticulum and activation of apoptotic machinery by oxidative stress in ischemic neurons.* J Cereb Blood Flow Metab, 2005. **25**(1): p. 41-53.

143. Grimm, M.O., et al., *Regulation of cholesterol and sphingomyelin metabolism by amyloid-beta and presenilin.* Nat Cell Biol, 2005. **7**(11): p. 1118-23.

144. He, X., et al., *Deregulation of sphingolipid metabolism in Alzheimer's disease.* Neurobiol Aging, 2010. **31**(3): p. 398-408.

145. Conti, A.C., et al., *Experimental brain injury induces regionally distinct apoptosis during the acute and delayed post-traumatic period.* J Neurosci, 1998. **18**(15): p. 5663-72.

146. Newcomb, J.K., et al., *Temporal profile of apoptotic-like changes in neurons and astrocytes following controlled cortical impact injury in the rat.* Exp Neurol, 1999. **158**(1): p. 76-88.

147. Malecki, A., et al., *4-Hydroxynonenal induces oxidative stress and death of cultured spinal cord neurons.* J Neurochem, 2000. **74**(6): p. 2278-87.

148. Bolin, L.M., T.P. Iismaa, and E.M. Shooter, *Isolation of activated adult Schwann cells and a spontaneously immortal Schwann cell clone.* J Neurosci Res, 1992. **33**(2): p. 231-8.

149. Cochard, P. and D. Paulin, *Initial expression of neurofilaments and vimentin in the central and peripheral nervous system of the mouse embryo in vivo.* J Neurosci, 1984. **4**(8): p. 2080-94.

150. Heumann, R., et al., *Changes of nerve growth factor synthesis in nonneuronal cells in response to sciatic nerve transection.* J Cell Biol, 1987. **104**(6): p. 1623-31.

151. De Leon, M., L.J. Van Eldik, and E.M. Shooter, *Differential regulation of S100 beta and mRNAs coding for S100-like proteins (42A and 42C) during development and after lesion of rat sciatic nerve.* J Neurosci Res, 1991. **29**(2): p. 155-62.

152. Liu, J.W., et al., *Expression of E-FABP in PC12 cells increases neurite extension during differentiation: involvement of n-3 and n-6 fatty acids.* J Neurochem, 2008. **106**(5): p. 2015-29.

153. Reaven, G.M., et al., *Lowering of plasma glucose in diabetic rats by antilipolytic agents.* Am J Physiol, 1988. **254**(1 Pt 1): p. E23-30.

154. Paolisso, G., et al., *A high concentration of fasting plasma non-esterified fatty acids is a risk factor for the development of NIDDM.* Diabetologia, 1995. **38**(10): p. 1213-7.

155. McGarry, J.D., *Banting lecture 2001: dysregulation of fatty acid metabolism in the etiology of type 2 diabetes.* Diabetes, 2002. **51**(1): p. 7-18.

156. Cnop, M., *Fatty acids and glucolipotoxicity in the pathogenesis of Type 2 diabetes.* Biochem Soc Trans, 2008. **36**(Pt 3): p. 348-52.

157. Bazan, N.G., Jr., *Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain.* Biochim Biophys Acta, 1970. **218**(1): p. 1-10.

158. Cistola, D.P., J.C. Sacchettini, and J.I. Gordon, *13C NMR studies of fatty acid-protein interactions: comparison of homologous fatty acid-binding proteins produced in the intestinal epithelium.* Mol Cell Biochem, 1990. **98**(1-2): p. 101-10.

159. Thomas, M.E., et al., *Fatty acids exacerbate tubulointerstitial injury in protein-overload proteinuria.* Am J Physiol Renal Physiol, 2002. **283**(4): p. F640-7.

160. Vincent, A.M., et al., *Short-term hyperglycemia produces oxidative damage and apoptosis in neurons.* Faseb J, 2005. **19**(6): p. 638-40.

161. Karaskov, E., et al., *Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis.* Endocrinology, 2006. **147**(7): p. 3398-407.

162. Xu, C., B. Bailly-Maitre, and J.C. Reed, *Endoplasmic reticulum stress: cell life and death decisions.* J Clin Invest, 2005. **115**(10): p. 2656-64.

163. Malhotra, J.D. and R.J. Kaufman, *The endoplasmic reticulum and the unfolded protein response.* Semin Cell Dev Biol, 2007. **18**(6): p. 716-31.

164. Eizirik, D.L., A.K. Cardozo, and M. Cnop, *The role for endoplasmic reticulum stress in diabetes mellitus.* Endocr Rev, 2008. **29**(1): p. 42-61.

165. Rao, R.V. and D.E. Bredesen, *Misfolded proteins, endoplasmic reticulum stress and neurodegeneration.* Curr Opin Cell Biol, 2004. **16**(6): p. 653-62.

166. Dey, A., I.G. Kessova, and A.I. Cederbaum, *Decreased protein and mRNA expression of ER stress proteins GRP78 and GRP94 in HepG2 cells over-expressing CYP2E1.* Arch Biochem Biophys, 2006. **447**(2): p. 155-66.

167. Hiramatsu, N., et al., *Real-time detection and continuous monitoring of ER stress in vitro and in vivo by ES-TRAP: evidence for systemic, transient ER stress during endotoxemia.* Nucleic Acids Res, 2006. **34**(13): p. e93.

168. Takahashi, G., et al., *MCI-186 reduces oxidative cellular damage and increases DNA repair function in the rabbit spinal cord after transient ischemia.* Ann Thorac Surg, 2004. **78**(2): p. 602-7.

169. Okuyama, R., T. Fujiwara, and J. Ohsumi, *High glucose potentiates palmitate-induced NO-mediated cytotoxicity through generation of superoxide in clonal beta-cell HIT-T15.* FEBS Lett, 2003. **545**(2-3): p. 219-23.

170. Sango, K., et al., *Diabetes is not a potent inducer of neuronal cell death in mouse sensory ganglia, but it enhances neurite regeneration in vitro.* Life Sci, 2002. **71**(20): p. 2351-68.

171. Cheng, C. and D.W. Zochodne, *Sensory neurons with activated caspase-3 survive long-term experimental diabetes.* Diabetes, 2003. **52**(9): p. 2363-71.

172. Kamiya, H., W. Zhangm, and A.A. Sima, *Apoptotic stress is counterbalanced by survival elements preventing programmed cell death of dorsal root ganglions in subacute type 1 diabetic BB/Wor rats.* Diabetes, 2005. **54**(11): p. 3288-95.

173. Innis, S.M. and M.T. Clandinin, *Dynamic modulation of mitochondrial membrane physical properties and ATPase activity by diet lipid.* Biochem J, 1981. **198**(1): p. 167-75.

174. Stubbs, C.D. and A.D. Smith, *Essential fatty acids in membrane: physical properties and function.* Biochem Soc Trans, 1990. **18**(5): p. 779-81.

175. Horrobin, D.F., *The membrane phospholipid hypothesis as a biochemical basis for the neurodevelopmental concept of schizophrenia.* Schizophr Res, 1998. **30**(3): p. 193-208.

176. Hon, G.M., et al., *Red blood cell membrane fluidity in the etiology of multiple sclerosis.* J Membr Biol, 2009. **232**(1-3): p. 25-34.

177. Brugg, B., et al., *Ceramide induces apoptosis in cultured mesencephalic neurons.* J Neurochem, 1996. **66**(2): p. 733-9.

178. Wiesner, D.A. and G. Dawson, *Staurosporine induces programmed cell death in embryonic neurons and activation of the ceramide pathway.* J Neurochem, 1996. **66**(4): p. 1418-25.

179. German, O.L., et al., *Ceramide is a mediator of apoptosis in retina photoreceptors.* Invest Ophthalmol Vis Sci, 2006. **47**(4): p. 1658-68.

180. Belosludtsev, K., et al., *On the mechanism of palmitic acid-induced apoptosis: the role of a pore induced by palmitic acid and Ca2+ in mitochondria.* J Bioenerg Biomembr, 2006. **38**(2): p. 113-20.

181. Chakrabandhu, K., et al., *Palmitoylation is required for efficient Fas cell death signaling.* EMBO J, 2007. **26**(1): p. 209-20.

182. Borradaile, N.M. and J.E. Schaffer, *Lipotoxicity in the heart.* Curr Hypertens Rep, 2005. **7**(6): p. 412-7.

183. Paschen, W., *Shutdown of translation: lethal or protective? Unfolded protein response versus apoptosis.* J Cereb Blood Flow Metab, 2003. **23**(7): p. 773-9.

184. Csordas, G., et al., *Structural and functional features and significance of the physical linkage between ER and mitochondria.* J Cell Biol, 2006. **174**(7): p. 915-21.

185. Pinton, P., et al., *Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis.* Oncogene, 2008. **27**(50): p. 6407-18.

186. van Gurp, M., et al., *Mitochondrial intermembrane proteins in cell death.* Biochem Biophys Res Commun, 2003. **304**(3): p. 487-97.

187. McConkey, D.J. and S. Orrenius, *The role of calcium in the regulation of apoptosis.* Biochem Biophys Res Commun, 1997. **239**(2): p. 357-66.

188. Kruman, II and M.P. Mattson, *Pivotal role of mitochondrial calcium uptake in neural cell apoptosis and necrosis.* J Neurochem, 1999. **72**(2): p. 529-40.

189. Kruman, I., Q. Guo, and M.P. Mattson, *Calcium and reactive oxygen species mediate staurosporine-induced mitochondrial dysfunction and apoptosis in PC12 cells.* J Neurosci Res, 1998. **51**(3): p. 293-308.

190. Paschen, W., S. Hotop, and C. Aufenberg, *Loading neurons with BAPTA-AM activates xbp1 processing indicative of induction of endoplasmic reticulum stress.* Cell Calcium, 2003. **33**(2): p. 83-9.

191. Deniaud, A., et al., *Endoplasmic reticulum stress induces calcium-dependent permeability transition, mitochondrial outer membrane permeabilization and apoptosis.* Oncogene, 2008. **27**(3): p. 285-99.

192. Takadera, T., M. Ohtsuka, and H. Aoki, *Chelation of extracellular calcium-induced cell death was prevented by glycogen synthase kinase-3 inhibitors in PC12 cells.* Cell Mol Neurobiol, 2010. **30**(2): p. 193-8.

193. Ahluwalia, J.P., et al., *A role for calcium in stabilizing transport vesicle coats.* J Biol Chem, 2001. **276**(36): p. 34148-55.

194. Yu, S.W., et al., *Mediation of poly(ADP-ribose) polymerase-1-dependent cell death by apoptosis-inducing factor.* Science, 2002. **297**(5579): p. 259-63.

195. Novelli, A., et al., *Glutamate becomes neurotoxic via the N-methyl-D-aspartate receptor when intracellular energy levels are reduced.* Brain Res, 1988. **451**(1-2): p. 205-12.

196. Peng, L.L., et al., *Inhibition of NMDA receptors underlies the neuroprotective effect of ginsenoside Rb3.* Am J Chin Med, 2009. **37**(4): p. 759-70.

197. Paschen, W., et al., *Peroxidative stress selectively down-regulates the neuronal stress response activated under conditions of endoplasmic reticulum dysfunction.* J Neurochem, 2001. **76**(6): p. 1916-24.

198. Sundar Rajan, S., et al., *Endoplasmic reticulum (ER) stress & diabetes.* Indian J Med Res, 2007. **125**(3): p. 411-24.

199. Schapansky, J., et al., *NF-kappaB activated by ER calcium release inhibits Abeta-mediated expression of CHOP protein: enhancement by AD-linked mutant presenilin 1.* Exp Neurol, 2007. **208**(2): p. 169-76.

200. Lee, A.H., N.N. Iwakoshi, and L.H. Glimcher, *XBP-1 regulates a subset of endoplasmic reticulum resident chaperone genes in the unfolded protein response.* Mol Cell Biol, 2003. **23**(21): p. 7448-59.

201. Yoneda, Y., et al., *A cell-penetrating peptidic GRP78 ligand for tumor cell-specific prodrug therapy.* Bioorg Med Chem Lett, 2008. **18**(5): p. 1632-6.

202. Szegezdi, E., et al., *Bcl-2 family on guard at the ER.* Am J Physiol Cell Physiol, 2009. **296**(5): p. C941-53.

203. Chauhan, D. and K.C. Anderson, *Mechanisms of cell death and survival in multiple myeloma (MM): Therapeutic implications.* Apoptosis, 2003. **8**(4): p. 337-43.

204. Lee, S.Y., R.P. Cherla, and V.L. Tesh, *Simultaneous induction of apoptotic and survival signaling pathways in macrophage-like THP-1 cells by Shiga toxin 1.* Infect Immun, 2007. **75**(3): p. 1291-302.

205. Jamora, C., G. Dennert, and A.S. Lee, *Inhibition of tumor progression by suppression of stress protein GRP78/BiP induction in fibrosarcoma B/C10ME.* Proc Natl Acad Sci U S A, 1996. **93**(15): p. 7690-4.

206. Zhang, J., et al., *Association of elevated GRP78 expression with increased lymph node metastasis and poor prognosis in patients with gastric cancer.* Clin Exp Metastasis, 2006. **23**(7-8): p. 401-10.

207. Ohse, T., et al., *Albumin induces endoplasmic reticulum stress and apoptosis in renal proximal tubular cells.* Kidney Int, 2006. **70**(8): p. 1447-55.

208. Sun, Y., et al., *Upregulation of GRP78 and caspase-12 in diastolic failing heart.* Acta Biochim Pol, 2008. **55**(3): p. 511-6.

209. Shin, Y.K., et al., *Pathological adaptive responses of Schwann cells to endoplasmic reticulum stress in bortezomib-induced peripheral neuropathy.* Glia, 2010. **58**(16): p. 1961-76.

210. Giacca, A., et al., *Lipid-induced pancreatic beta-cell dysfunction: focus on in vivo studies.* Am J Physiol Endocrinol Metab, 2011. **300**(2): p. E255-62.

211. Mantzaris, M.D., E.V. Tsianos, and D. Galaris, *Interruption of triacylglycerol synthesis in the endoplasmic reticulum is the initiating event for saturated fatty acid-induced lipotoxicity in liver cells.* FEBS J, 2011. **278**(3): p. 519-30.

212. Henique, C., et al., *Increased mitochondrial fatty acid oxidation is sufficient to protect skeletal muscle cells from palmitate-induced apoptosis.* J Biol Chem, 2010. **285**(47): p. 36818-27.

213. Padilla, A., et al., *Hyperglycemia magnifies Schwann cell dysfunction and cell death triggered by PA-induced lipotoxicity.* Brain Res, 2011. **1370**: p. 64-79.

214. Randle, P.J., et al., *The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus.* Lancet, 1963. **1**(7285): p. 785-9.

215. McGarry, J.D., *What if Minkowski had been ageusic? An alternative angle on diabetes.* Science, 1992. **258**(5083): p. 766-70.

216. Gaemers, I.C., et al., *Lipotoxicity and steatohepatitis in an overfed mouse model for non-alcoholic fatty liver disease.* Biochim Biophys Acta, 2011. **1812**(4): p. 447-58.

217. Salvioli, S., et al., *JC-1, but not DiOC6(3) or rhodamine 123, is a reliable fluorescent probe to assess delta psi changes in intact cells: implications for studies on mitochondrial functionality during apoptosis.* FEBS Lett, 1997. **411**(1): p. 77-82.

218. Korper, S., et al., *The K+ channel openers diazoxide and NS1619 induce depolarization of mitochondria and have differential effects on cell Ca2+ in CD34+ cell line KG-1a.* Exp Hematol, 2003. **31**(9): p. 815-23.

219. Robbins, E. and P.I. Marcus, *Dynamics of Acridine Orange-Cell Interaction. I. Interrelationships of Acridine Orange Particles and Cytoplasmic Reddening.* J Cell Biol, 1963. **18**: p. 237-50.

220. Li, W., et al., *Induction of cell death by the lysosomotropic detergent MSDH.* FEBS Lett, 2000. **470**(1): p. 35-9.

221. De Leon, M., Welcher, A A., Nahin R.H., Liu, Y., Ruda, M.A., Shooter, E.M. Molina, C.A. , *Fatty acid binding protein is induced in neurons of the dorsal root ganglia after peripheral nerve injury.* Journal of Neuroscience Research, 1996. **44**(3): p. 283-92.

222. Boslem, E., et al., *A lipidomic screen of palmitate-treated MIN6 beta-cells links sphingolipid metabolites with endoplasmic reticulum (ER) stress and impaired protein trafficking.* Biochem J, 2011. **435**(1): p. 267-76.

223. Lupachyk, S., et al., *PARP inhibition alleviates diabetes-induced systemic oxidative stress and neural tissue 4-hydroxynonenal adduct accumulation: Correlation with peripheral nerve function.* Free Radic Biol Med, 2011.

224. Yousry, A.H. and A.M. Abdalhady, *Management of diabetic neuropathic ankle arthropathy by arthrodesis using an Ilizarov frame.* Acta Orthop Belg, 2010. **76**(6): p. 821-6.

225. Pham, M., et al., *Proximal neuropathic lesions in distal symmetric diabetic polyneuropathy: findings of high-resolution magnetic resonance neurography.* Diabetes Care, 2011. **34**(3): p. 721-3.

226. Ikota, H., et al., *Neuromyelitis optica with intraspinal expansion of Schwann cell remyelination.* Neuropathology, 2010. **30**(4): p. 427-33.

227. Cacicedo, J.M., et al., *Activation of AMP-Activated Protein Kinase (AMPK) Prevents Lipotoxicity in Retinal Pericytes.* Invest Ophthalmol Vis Sci, 2011.

228. Ishiyama, J., et al., *Unsaturated FAs prevent palmitate-induced LOX-1 induction via inhibition of ER stress in macrophages.* J Lipid Res, 2011. **52**(2): p. 299-307.

229. Kamboj, S.S., R.K. Vasishta, and R. Sandhir, *N-acetylcysteine inhibits hyperglycemia-induced oxidative stress and apoptosis markers in diabetic neuropathy.* J Neurochem, 2010. **112**(1): p. 77-91.

230. Kessler, J.I., et al., *Lipoprotein lipase inhibition in rabbits with experimental pancreatitis.* Proc Soc Exp Biol Med, 1962. **110**: p. 24-6.

231. Gwiazda, K.S., et al., *Effects of palmitate on ER and cytosolic Ca2+ homeostasis in beta-cells.* Am J Physiol Endocrinol Metab, 2009. **296**(4): p. E690-701.

232. Remizov, O., et al., *Palmitate-induced Ca2+-signaling in pancreatic beta-cells.* Mol Cell Endocrinol, 2003. **212**(1-2): p. 1-9.

233. Olofsson, C.S., et al., *Palmitate increases L-type Ca2+ currents and the size of the readily releasable granule pool in mouse pancreatic beta-cells.* J Physiol, 2004. **557**(Pt 3): p. 935-48.

234. Epand, R.F., et al., *Membrane perturbations induced by the apoptotic Bax protein.* Biochem J, 2002. **367**(Pt 3): p. 849-55.

235. Epand, R.F., et al., *Direct evidence for membrane pore formation by the apoptotic protein Bax.* Biochem Biophys Res Commun, 2002. **298**(5): p. 744-9.

236. Russell, J.W., et al., *Neurons undergo apoptosis in animal and cell culture models of diabetes.* Neurobiol Dis, 1999. **6**(5): p. 347-63.

237. Rizzuto, R., et al., *Mitochondria as biosensors of calcium microdomains.* Cell Calcium, 1999. **26**(5): p. 193-9.

238. Kawamata, H. and G. Manfredi, *Mitochondrial dysfunction and intracellular calcium dysregulation in ALS.* Mech Ageing Dev, 2010. **131**(7-8): p. 517-26.

239. Yabunaka, N., et al., *Elevated levels of heat-shock protein 70 (HSP70) in the mononuclear cells of patients with non-insulin-dependent diabetes mellitus.* Diabetes Res Clin Pract, 1995. **30**(2): p. 143-7.

240. Kolter, T. and K. Sandhoff, *Principles of lysosomal membrane digestion: stimulation of sphingolipid degradation by sphingolipid activator proteins and anionic lysosomal lipids.* Annu Rev Cell Dev Biol, 2005. **21**: p. 81-103.

241. Petersen, N.H., et al., *Connecting Hsp70, sphingolipid metabolism and lysosomal stability.* Cell Cycle, 2010. **9**(12).

242. Kirkegaard, T., et al., *Hsp70 stabilizes lysosomes and reverts Niemann-Pick disease-associated lysosomal pathology.* Nature, 2010. **463**(7280): p. 549-53.

243. Olejnicka, B.T., K. Ollinger, and U.T. Brunk, *A short exposure to a high-glucose milieu stabilizes the acidic vacuolar apparatus of insulinoma cells in culture to ensuing oxidative stress.* APMIS, 1997. **105**(9): p. 689-98.

244. Comper, W.D., et al., *Disease-dependent mechanisms of albuminuria.* Am J Physiol Renal Physiol, 2008. **295**(6): p. F1589-600.

245. Olbricht, C.J. and B. Geissinger, *Renal hypertrophy in streptozotocin diabetic rats: role of proteolytic lysosomal enzymes.* Kidney Int, 1992. **41**(4): p. 966-72.

246. Glenn, J.V., et al., *Advanced glycation end product (AGE) accumulation on Bruch's membrane: links to age-related RPE dysfunction.* Invest Ophthalmol Vis Sci, 2009. **50**(1): p. 441-51.

247. Malaisse-Lagae, F., A. Sener, and W.J. Malaisse, *Contrasting modes of action of D-glucose and 3-O-methyl-D-glucose as protectors of the rat pancreatic B-cell against alloxan.* Biochim Biophys Acta, 1983. **762**(1): p. 36-43.

248. Pipeleers, D. and M. Van de Winkel, *Pancreatic B cells possess defense mechanisms against cell-specific toxicity.* Proc Natl Acad Sci U S A, 1986. **83**(14): p. 5267-71.

249. Olejnicka, B.T., et al., *Beta-cells, oxidative stress, lysosomal stability, and apoptotic/necrotic cell death.* Antioxid Redox Signal, 1999. **1**(3): p. 305-15.

250. Han, D., et al., *Activation of autophagy through modulation of 5'-AMP-activated protein kinase protects pancreatic beta-cells from high glucose.* Biochem J, 2010. **425**(3): p. 541-51.

251. Hui-Chou, H.G., et al., *Clinical implications of peripheral myelin protein 22 for nerve compression and neural regeneration: a review.* J Reconstr Microsurg, 2011. **27**(1): p. 67-74.

252. Amici, S.A., et al., *Peripheral myelin protein 22 is in complex with alpha6beta4 integrin, and its absence alters the Schwann cell basal lamina.* J Neurosci, 2006. **26**(4): p. 1179-89.

253. Fortun, J., et al., *Alterations in degradative pathways and protein aggregation in a neuropathy model based on PMP22 overexpression.* Neurobiol Dis, 2006. **22**(1): p. 153-64.

254. Taioli, F., et al., *Inherited demyelinating neuropathies with micromutations of peripheral myelin protein 22 gene.* Brain, 2011. **134**(Pt 2): p. 608-17.

255. De Leon, M., et al., *Identification of transcriptionally regulated genes after sciatic nerve injury.* J Neurosci Res, 1991. **29**(4): p. 437-48.

256. Robinson, F.L., et al., *Loss of the inactive myotubularin-related phosphatase Mtmr13 leads to a Charcot-Marie-Tooth 4B2-like peripheral neuropathy in mice.* Proc Natl Acad Sci U S A, 2008. **105**(12): p. 4916-21.

257. Notterpek, L., E.M. Shooter, and G.J. Snipes, *Upregulation of the endosomal-lysosomal pathway in the trembler-J neuropathy.* J Neurosci, 1997. **17**(11): p. 4190-200.

258. Haney, C., et al., *Ultrastructural distribution of PMP22 in Charcot-Marie-Tooth disease type 1A.* J Neuropathol Exp Neurol, 1996. **55**(3): p. 290-9.

259. Pareek, S., et al., *Neurons promote the translocation of peripheral myelin protein 22 into myelin.* J Neurosci, 1997. **17**(20): p. 7754-62.

260. Hasse, B., et al., *Peripheral myelin protein 22 kDa and protein zero: domain specific trans-interactions.* Mol Cell Neurosci, 2004. **27**(4): p. 370-8.

261. Nave, K.A., *Myelin genetics: new insight into old diseases.* Brain Pathol, 1995. **5**(3): p. 231-2.

262. Lupski, J.R., et al., *DNA duplication associated with Charcot-Marie-Tooth disease type 1A.* Cell, 1991. **66**(2): p. 219-32.

263. Roa, B.B., C.A. Garcia, and J.R. Lupski, *Charcot-Marie-Tooth disease type 1A: molecular mechanisms of gene dosage and point mutation underlying a common inherited peripheral neuropathy.* Int J Neurol, 1991. **25-26**: p. 97-107.

264. Thomas, F.P., et al., *Tomaculous neuropathy in chromosome 1 Charcot-Marie-Tooth syndrome.* Acta Neuropathol, 1994. **87**(1): p. 91-7.

265. Xanthoudakis, S. and T. Curran, *Redox regulation of AP-1: a link between transcription factor signaling and DNA repair.* Adv Exp Med Biol, 1996. **387**: p. 69-75.

266. Suzuki, Y., et al., *Different utilization of Ca2+ in the contractile action of endothelin-1 on cerebral, coronary and mesenteric arteries of the dog.* Eur J Pharmacol, 1992. **219**(3): p. 401-8.

267. Lin, W.N., et al., *Regulation of COX-2 and cPLA(2) gene expression by LPS through the RNA-binding protein HuR: involvement of NADPH oxidase, ROS and MAPKs.* Br J Pharmacol, 2011.

268. Kayano, M., et al., *ROS-DET: robust detector of switching mechanisms in gene expression.* Nucleic Acids Res, 2011.

269. Meyer, M., H.L. Pahl, and P.A. Baeuerle, *Regulation of the transcription factors NF-kappa B and AP-1 by redox changes.* Chem Biol Interact, 1994. **91**(2-3): p. 91-100.

270. Pahl, H.L. and P.A. Baeuerle, *Oxygen and the control of gene expression.* Bioessays, 1994. **16**(7): p. 497-502.