Targeting Heat Shock Protein 70 to Improve Oxidative Stress and Mitochondrial Bioenergetics in Diabetic Sensory Neurons

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Abstract

Neuronal mitochondrial dysfunction is a key pathophysiologic mechanism of diabetic peripheral neuropathy (DPN). KU-596 is a small molecule modulator of heat shock protein 90 (Hsp90) that can reverse clinically relevant measures of DPN in diabetic animal models. Mechanistically, drug efficacy requires Hsp70 and correlates with improving mitochondrial bioenergetics (mtBE) in diabetic sensory neurons. The goal of this study was to determine if KU-596 improves mtBE by decreasing glucose-induced oxidative stress in an Hsp70-dependent manner. Sensory neurons were isolated from non-diabetic or diabetic mice wild type (WT) or Hsp70 knockout (Hsp70 KO) mice and treated *ex vivo* with KU-596 in the presence of low or high glucose concentrations. In diabetic WT and Hsp70 KO neurons, hyperglycemia significantly increased superoxide levels, but KU-596 only decreased superoxide in WT neurons. Similarly, KU-596 significantly improved mtBE in hyperglycemically stressed diabetic WT neurons but did not improve mtBE in diabetic Hsp70 KO under the same conditions. Since manganese superoxide dismutase (MnSOD) is the main mechanism to detoxify mitochondrial superoxide radicals, we determined the cause and effect relationship between improved respiration and decreased oxidative stress by knocking down MnSOD. Downregulating MnSOD in diabetic WT neurons increased hyperglycemia-induced superoxide levels as measured by electron paramagnetic resonance spectroscopy and blocked the ability of KU-596 to enhance mtBE. In diabetic neurons, knockdown of MnSOD increased maximal respiratory capacity (MRC) and this was significantly decreased by mito-TEMPO-H after scavenging overproduced superoxide radicals. Following MnSOD knockdown, KU-596 decreased mitochondrial superoxide but was unable to improve MRC. Overall, this study shows that the Hsp90 inhibitor KU-596

improves mtBE and decreases mitochondrial oxidative stress of diabetic sensory neurons in an Hsp70-dependent manner. However, the drug-induced improvement of mtBE is not necessarily dependent on decreasing mitochondrial oxidative stress and other mechanisms can be modulated by novologue therapy to improve mtBE. This work furthers our mechanistic understanding by which novologue therapy can improve metabolic aspects of neuronal function that contribute to the efficacy of KU-596 for treating diabetic peripheral neuropathy.

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List of Abbreviations

17-AAG	17-allylamino-17-demethoxygeldanamycin
ACE	angiotensin-converting enzyme
AD	Alzheimer's disease
AGE	advanced glycation endproducts
Akt	protein kinase B
ALA	alpha lipoic acid
AMPK	AMP-activated protein kinase
ANG II	angiotensin II
APC	antigen-presenting cells
Apaf-1	apoptosis-activating factor 1
APP	amyloid precursor protein
AR	aldose reductase
ATP	adenosine triphosphate
AVONA	analysis of variance
BAX	Bcl-2-like protein 4
Bcl-2	B-cell lymphoma 2
BG	basal glucose
Bim	Bcl-2-like protein 11
CML	carboxymethyl-lysine
CNS	central nervous system
COX-2	cyclooxygenase-2
CoQ	coenzyme Q10

CREB	cAMP responsive element binding protein
CRP	c-reactive peptides
CVD	cardiovascular diseases
DAG	diacylglycerol
DHE	dihydroethidine
DNA	deoxyribonucleic acid
DPN	diabetic peripheral neuropathy
DPP-4	dipeptidyl peptidase 4
DRG	dorsal root ganglion
Drp1	dynamin-related protein 1
ECAR	extracellular acidification rate
ECM	extracellular matrix
EMG	electromyogram
EPR	electron paramagnetic resonance
ESRD	end-stage renal disease
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
ESRD	end-stage renal disease
FCCP	carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone
FBG	fasting blood glucose
FDA	Food and Drug Administration
Fis1	mitochondrial fission 1 protein
FPG	fasting plasma glucose

FGT	fasting glucose test
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GDA	geldanamycin
GFAP	glutamine-fructose-6-phosphate amidotransferase
GFAT	fructose-6-phosphate amidotranferase
GlcN-6-P	glucosamine-6-phosphate
GLP-1	glucagon-like peptide-1
GLUT	glucose transporter
GLUT2	Type 2 glucose transporter
GRB	growth factor receptor-bound protein
Grp94	glucose regulated protein 94
GSH	glutathione
GSSH	oxidized glutathione
HbA1c	glycated hemoglobin
HD	Huntington’s disease
HFD	high fat diet
HG	hyperglycemic
HOP	Hsp70-Hsp90 Organizing Protein
HSF	heat shock factor
HSP	heat shock protein
HSR	heat shock response
IDF	International Diabetes Federation
iENF	intraepidermal nerve fiber

IGF	insulin-like growth factor
IGFR	insulin-like growth factor receptor
IKK	inhibitor κB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	intraperitoneal
IR	insulin receptor
IRS	insulin receptor substrates
IV	intravenous
JNK	c-jun N-terminal kinase
KO	knockout
Lep	leptin
Lepr	leptin receptor
MAPK	mitogen-activated protein kinase
MBP	myelin basic protein
MCP-1	monocyte chemoattractant protein-1
MMP	mitochondrial action potential
MNCV	motor nerve conduction velocity
MRC	maximum respiratory capacity
MTP18	Mitochondrial protein 18 kDa
mtBE	mitochondrial bioenergetics
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin

NADP+	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide reduced
NC	nerve conduction
NCV	nerve conduction velocity
NF-κB	nuclear factor kappa B
NGF	nerve growth factor
NK	natural killer
NO	nitric oxide
NOX	NADPH oxidases
Novologue	novobiocin analogue
NPDR	non-proliferative diabetic retinopathy
NPH	neutral protamine hagedorn
NRG1	neuregulin-1
NT-3	neurotrophin 3
OCR	oxygen consumption rate
OGTT	oral glucose tolerance test
P0	myelin protein zero
PARP	poly (ADP-ribose) polymerase
PDK1	pyruvate dehydrogenase kinase 1
PGC-1α	proliferator-activated receptor-γ coactivator α
PI3-K	phosphatidylinositol 3-kinase
PINK-1	PTEN-induced putative kinase 1
PIP3	phosphatidylinositol 3,4,5-triphosphate

PKC	protein kinase C
PMP22	peripheral myelin protein-22
PNS	peripheral nervous system
PPARγ	peroxisome proliferator-activated receptor γ
PTP	permeability transition pore
PTPase	protein tyrosine phosphatases
QST	quantitative sensory testing
qRT-PCR	quantitative real-time PCR
RAGE	receptor for AGEs
RAS	renin-angiotensin system
RNA-Seq	RNA sequencing
ROS	reactive oxygen species
SCs	schwann cells
SDH	sorbitol dehydrogenase
SH2	Src-homology-2
SHC	Src homology 2 domain containing
shRNA	small hairpin RNA
SIRT1	sirtuin (silent mating type information regulation 2 homolog) 1
SNCV	sensory nerve conduction velocity
SNRIs	serotonin-norepinephrine reuptake inhibitors
SOD	superoxide dismutase
SOCS	suppressor of cytokine signaling
SP1	specificity protein 1

SRC	spare respiratory capacity
SSNRIs	selective serotonin-norepinephrine reuptake inhibitors
STAT	signal transducer and activator of transcription
STZ	streptozotocin
T2DM	Type 2 diabetes
TCA	tricarboxylic acid
TGF-β	transforming growth factor-β
TNF-α	tumor necrosis factor-α
TRAP	tumor necrosis factor receptor Type 1-associated protein
Trx	thioredoxin
TXNIP	thioredoxin interacting/inhibiting protein
UDP-GlcNAc	uridine 5-diphosphate-N-acetylglucosamine
VEGF	vascular endothelial growth factor
WHO	World Health Organization
WT	wild type
2-h PG	2-hour plasma glucose

Chapter 1. Background

1.1 Diabetes Mellitus Overview

Diabetes mellitus is a metabolic disease that develops when the body cannot secrete enough insulin or cannot respond to insulin effectively. Insulin is a hormone produced by pancreatic β cells, which is required to transport glucose from blood into the cells for energy. The lack or ineffectiveness of insulin in diabetic patients could lead to hyperglycemia, manifested as elevated levels of blood glucose. Over time, hyperglycemia causes damage to many tissues, leading to diabetic complications such as diabetic nephropathy, retinopathy and neuropathy.

There are three major types of diabetes: Type 1 diabetes, Type 2 diabetes and gestational diabetes [1-3] that can be diagnosed based on fasting plasma glucose (FPG) levels or a 2-h plasma glucose (2-h PG) value after a 75-g oral glucose tolerance test (OGTT) [1]. Diabetes can also be diagnosed based on the attachment of glucose to hemoglobin using the A1c test, also called hemoglobin A1c or HbA1c test. Since the lifespan of hemoglobin is three months, HbA1c indicates the average blood glucose levels over this period. The HbA1c test result is reported as a percentage and the criteria point for a diagnosis of diabetes is set at 6.5% (Table 1.1.1). The A1c test has several advantages over plasma glucose tests, since it doesn't require fasting, has greater analytical stability and less fluctuation. However, according to the American Diabetes Association, FPG is the preferred method for diagnosing diabetes since it is more convenient and less expensive. The standard diagnosis of diabetes is based on two separate blood glucose tests with FPG levels greater than 126 mg/dL. For individuals with a FPG of 110 - 124 mg/dL or an A1c of 6.0%–6.4%, a 2h-PG after 75-g OGTT tests should be given to verify diabetes [4].

Table 1.1.1: Diagnosis criteria for diabetes mellitus

	Normal	Pre-Diabetes	Diabetes
A1c (%)	Below 5.7	5.7 - 6.4	Above 6.5
FPG (mg/dL)	Below 110	111 - 125	Above 126
2h-PG (mg/dL) during an 75-g OGTT	Below 140	140 - 200	Above 200

1.1.1 Type 1 Diabetes

Type 1 diabetes usually occurs in children or young adults and is caused by autoimmune destruction of pancreatic β cells, after which the body cannot produce insulin. Type 1 diabetes is usually diagnosed by an elevated FPG level, together with sudden weight loss, abnormal thirst, dry mouth, frequent urination, lack of energy, and blurred vision. Type 1 diabetes patients need insulin every day to control their blood glucose levels for survival. Regular quick-acting human insulin and intermediate NPH (Neutral protamine Hagedorn) insulin and long acting insulin analogs are top options. With regular blood glucose monitoring and maintaining a healthy diet and lifestyle, Type 1 diabetes patients can lead a very normal and healthy life [5].

The number of Type 1 diabetes patients is increasing. According to the National Diabetes Statistics Report of 2014, there are about 1.25 million Americans living with Type 1 diabetes, including about 200,000 youth (less than 20 years old) and over a million adults (20 years old and older) [6]. More than 40,000 people are diagnosed each year in the U.S and about 14 billion dollars are spent on Type 1 diabetes in the US [7].

Immune factors and immune responses may induce and exacerbate the damage of pancreatic β cells to cause Type 1 diabetes. β-cell antigens can be recognized by antigen-presenting cells (APCs) to stimulate insulin reactive $CD4^+$ and $CD8^+$ T cells differentiation

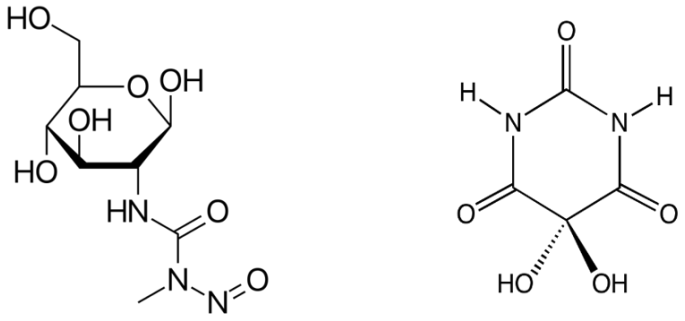
[8]. Activated T cells can directly kill β cells through a cytotoxic process. They can also induce the pro-inflammatory cytokines, granzyme B, perforin and even activate programmed cell death pathways. Other immune cells including B lymphocytes, natural killer (NK) cells and dendritic cells may also involve in the damage of pancreatic β cells, which ultimately lead to Type 1 diabetes [9].

Type 1 diabetes can be induced by high dose streptozotocin (STZ) injections in rodents. STZ (Fig.1.1.1.1) is an antibiotic derived from the bacterium Streptomyces achromogens. It contains a deoxy glucose molecule to direct the chemical to the GLUT2 transporter on the pancreatic β cells, while linking with a highly reactive methylnitrosourea moiety to mediate STZ's cytotoxic effects by damaging DNA [10].

It is generally assumed that the cytotoxic effect of STZ is dependent upon the DNA alkylating activity of its methylnitrosourea moiety [11, 12]. The transfer of the methyl group from STZ to the DNA molecule could cause a chain of damage, resulting in the fragmentation of the DNA. In compensation to repair DNA, poly (ADP-ribose) polymerase (PARP) is overstimulated resulting in a diminishment of cellular NAD^+ and ATP reserves. The depletion of the cellular energy can ultimately lead to β cell necrosis. Furthermore, STZ can also methylate and glycosylate proteins to cause β cell defects [13].

Though GLUT2 is mainly expressed on pancreatic β cells, lower levels also exist in the liver and kidneys. After IV administration, STZ has a half-life of 15 minutes. It is quickly metabolized in the liver and is rapidly eliminated by kidneys [14, 15]. Though high doses of STZ might impair liver and kidney functions, its long-term toxicity to the liver and kidneys is attributed to its induction of hyperglycemia [16]. This is further confirmed by Grossman's report that insulin treatment to STZ diabetic mice can promote the

regeneration of diabetic pancreatic β cells. This suggests that after STZ elimination, further β cell dysfunction is due to hyperglycemia [17].



Streptozotocin (STZ) Alloxan

Figure 1.1.1.1: Chemical structures of drugs inducing Type-1 diabetes.

Alloxan (Fig.1.1.1.1) is another chemical to induce Type 1 diabetes. Alloxan is quickly taken up by β cells and reduced into dialuric acid, which is then re-oxidized to alloxan to produce a redox cycle. This redox cycle generates superoxide radicals that can be further converted into hydroxyl radicals and hydrogen peroxide, resulting in β cell DNA fragmentation. However, compared with STZ, alloxan induces diabetes in a narrow dose range, and slight overdosing can cause systemic toxicity, especially to the kidneys [18].

1.1.2 Type 2 Diabetes

Type 2 diabetes is the most common type of diabetes that accounts for 90-95% of diagnosed cases. In 2012, over 29.1 million people in the United States had Type 2 diabetes, but 8.1 million may be undiagnosed and unaware of their condition. This disease usually affects adults, but the number of children and adolescents with diabetes is increasing rapidly. At the onset of Type 2 diabetes, the body is resistant to insulin, even with sufficient production by β cells. Over time, insulin levels may become inadequate and both the insulin resistance and deficiency can lead to hyperglycemia in Type 2 diabetes [3].

Many Type 2 diabetics are unaware of their condition for many years because hyperglycemia develops gradually and the classic symptoms are generally less marked than in Type 1 diabetes. However, during this process, the body is already being damaged by excess hyperglycemia and develops microvascular and macrovascular complications. Although the exact reasons for developing Type 2 diabetes are still not known, obesity, physical inactivity, poor nutrition, family history, previous diagnosis of gestational diabetes and advancing age could be associated with disease development [19].

Unlike Type 1 diabetes, most Type 2 diabetes patients do not require daily insulin therapy to survive, and can improve their glycemic regulation by adopting a healthy diet, increasing physical activity and controlling obesity. However, in the absence of adequate management of blood glucose by diet and exercise, a number of "blood glucose controlling" drugs can be prescribed. Metformin and sulfonylureas are on the World Health Organization list of essential medicines for diabetes [20, 21]. Other commonly used treatments for Type 2 diabetes include glucagon-like peptide 1 (GLP-1) analogues and dipeptidy peptidase 4 (DPP4) inhibitors. Both treatments can enhance the body's natural response to ingested food and reduce blood glucose levels after eating [22].

Animal models of Type 2 diabetes tend to include insulin resistance models or β cell failure models. Since Type 2 diabetes development is closely associated with obesity in humans, most animal models of Type 2 diabetes are obese models. This can be achieved with genetic manipulation or high fat diet approaches. Although human obesity is rarely caused by a single gene mutation, monogenic mutations in leptin signaling to cause obesity are commonly used in Type 2 diabetes studies [23]. Leptin mediates satiety, and leptin deficiency leads to hyperphagia and subsequent obesity. Two mouse models of leptin

deficiency are $Lep^{ob/ob}$ and $Lepr^{db/db}$ mice. In $Lep^{ob/ob}$ mice, the leptin protein is mutated. Body weight starts to increase at 2 weeks, and the mice begin to develop hyperinsulinemia. By 4 weeks, hyperglycemia becomes apparent, and blood glucose continues to rise, peaking at 3-5 months. Other diabetic symptoms include hyperlipidemia and lower physical activity [24]. A second model is the $Lepr^{db/db}$ mouse that has a recessive mutation in the leptin receptor [25]. Hyperinsulinemia is apparent at around 2 weeks, obesity is evident starting from 3-4 weeks and hyperglycemia develops at 4-8 weeks [26].

High fat diet can also lead to obesity, hyperinsulinemia and hyperglycemia due to impaired glucose tolerance. In a normal diet, about 26% of energy comes from protein, 63% from carbohydrates and 11% from fat. However, in a high fat diet, about 58% of energy comes from fat [27]. Within the first week of a high fat diet, mice are heavier than those on a normal diet, and several weeks of a high fat diet induces substantial weight gain. This gain of weight is associated with lack of β cell compensation and insulin resistance. Since obesity is usually manipulated by environmental factors rather than gene mutations in humans, the high fat diet model is a more relevant model than those based on genetically-induced diabetes [23].

1.1.3 Gestational Diabetes

Women with slightly elevated blood glucose levels are classified as having gestational diabetes and this tends to occur from the 24th week of pregnancy. Even though this normally disappears after birth, patients diagnosed with gestational diabetes are more likely to develop Type 2 diabetes later in pregnancy and later life. Infants born to mothers with gestational diabetes also have a higher risk of developing Type 2 diabetes in adolescents or early adulthood [28]. 4.6 to 9.2 Percent of pregnancies may be affected by gestational

diabetes with up to 10 percent diagnosed with Type 2 diabetes just after the pregnancy. The rest of these women have a 35 to 60 percent chance of developing Type 2 diabetes within 10 to 20 years. This risk decreases if the woman leads an active lifestyle and maintains an ideal weight [29].

1.1.4 Insulin Signaling

Insulin resistance in patients with Type 2 diabetes is mainly due to the loss of cell signaling in response to insulin, which regulates glucose, lipid, and energy homeostasis in liver, adipose and skeletal muscle. Insulin and Insulin-like Growth Factor-1 (IGF-1) bind to tyrosine kinase-linked insulin and IGF-1 receptors (Fig.1.1.4.1). This initiates a cascade of phosphorylation reactions that activate phosphatidylinositol 3 kinase (PI3K) and protein kinase B (also called Akt) to regulate cellular metabolism. Perturbations in these signaling pathways can lead to insulin resistance.

Upon binding with insulin, the receptors undergo a conformational change and auto-phosphorylation which permits subsequent phosphorylation of downstream targets such as the insulin receptor substrate 1 (IRS-1) and the adapter protein, Shc. IRS-1 is responsible for many of the anabolic aspects of insulin signaling by activating the PI3K-Akt pathway. PI3K increases the membrane concentration of PI 3,4 bis-phosphate and PI 3,4,5 trisphosphate which promotes the activity of phosphoinositol dependent kinase (PDK-1), the upstream activator of Akt. Akt mediates most of insulin's metabolic effects, by increasing glucose transport, lipid synthesis, and glycogen synthesis while suppressing gluconeogenesis and glycogenolysis. On the other hand, Shc activates the RAS-MAPK (mitogen-activated protein kinase) pathway, which controls cellular proliferation and gene transcription.

Insulin signaling can be attenuated by several phosphatases that dephosphorylate receptors, IRS-1, MAPK and PI3,4 or 3,4,5 polyphosphates. Moreover, stress kinases, such as JNK (c-Jun N-terminal kinase), IKK (IκB kinase), PKC (Protein kinase C) and ERK (Extracellular signal-regulated protein kinases) can inhibit insulin/IGF-1 signaling pathway by inducing inhibitory serine/threonine phosphorylation of IR/IGFR and IRS proteins [30].

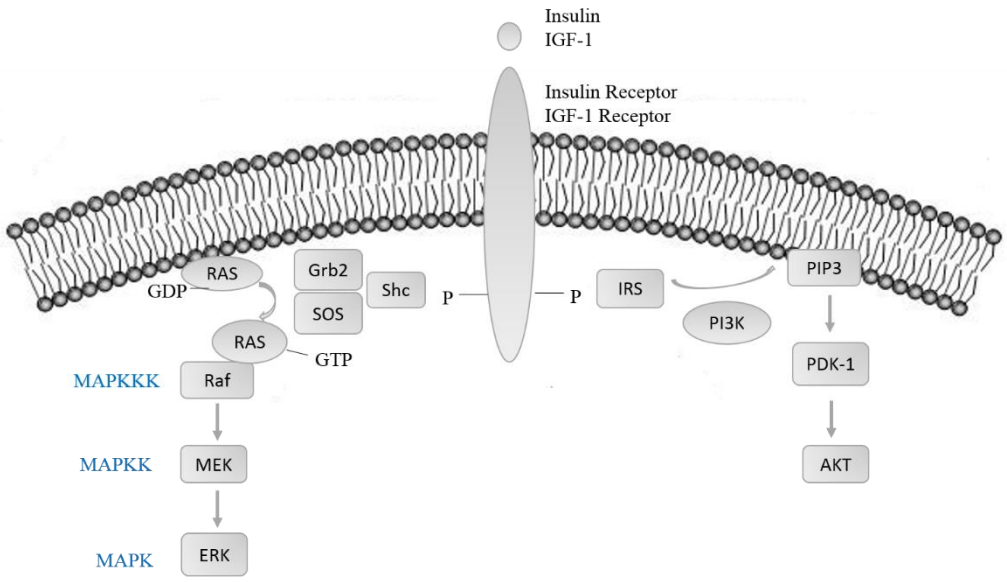


Figure 1.1.4.1: Positive pathway of insulin signaling

1.1.5 Glucose Metabolism

To generate energy, glucose needs to be metabolized inside the cells by glycolysis first. Once transported into the cell, glucose can be phosphorylated by hexokinases to glucose-6-phosphate. Glucose-6-P can undergo two routes, it can enter the pentose phosphate pathway to produce NADPH for use in anabolic reactions (Fig.1.1.5.1), or be converted into fructose-6-P by phosphoglucose isomerase to continue into glycolysis. The product of glycolysis is pyruvate, which enters the mitochondria to form acetyl CoA for use in the Krebs cycle [31].

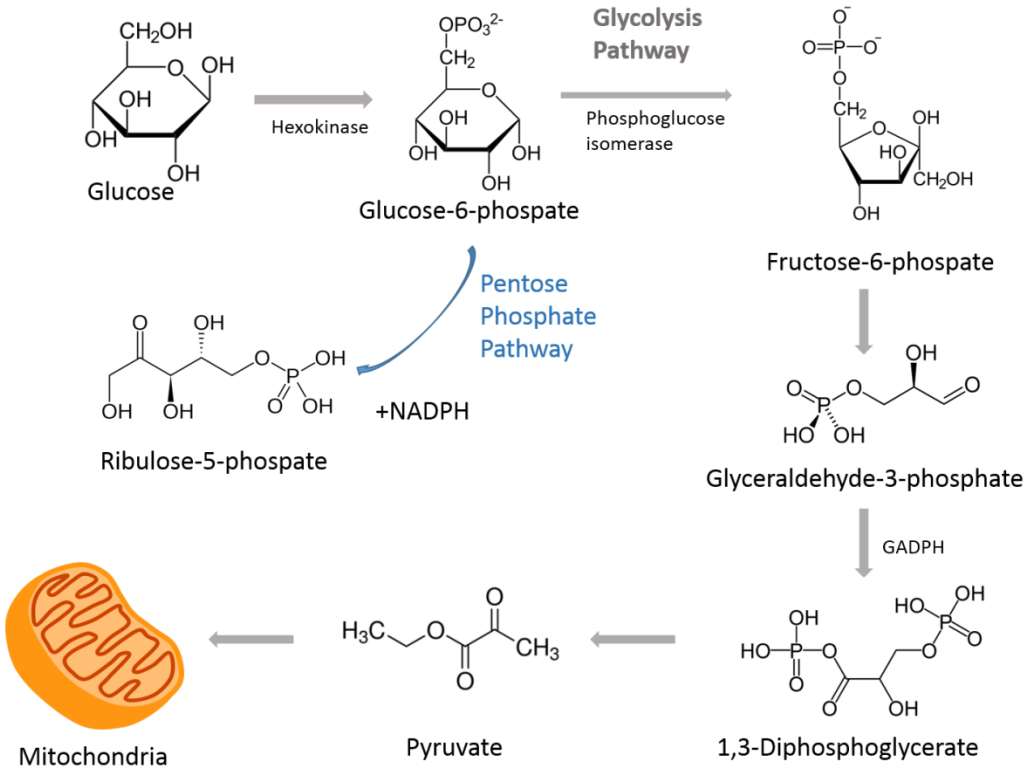


Figure 1.1.5.1: Glycolysis pathway

Glucose overload in diabetes can increase its flux through a minor metabolic pathway called the polyol pathway (Fig.1.1.5.2). In the polyol pathway, excess glucose is reduced into sorbitol by aldose reductase (AR) and oxidized to fructose by sorbitol dehydrogenase. This process consumes NADPH, a co-factor that is also essential for the conversion of oxidized glutathione (GSSH) to glutathione (GSH) via glutathione reductase. The utilization of NADPH in the polyol pathway can cause or exacerbate intracellular oxidative stress [32]. Additionally, an increase in fructose promotes excess formation of fructose 6-phosphate, which is an entry substrate for flux through the hexosamine pathway. In the hexosamine pathway, fructose 6-phosphate is converted by the enzyme glutamine-fructose-6-phosphate amidotransferase (GFAP) to UDP-N-Acetylglucosamine, which can

modify numerous proteins on Ser and Thr residues by forming O-linked N-acetylglucosamine [33].

Glucose accumulation can also over produce glyceraldehyde 3-phosphate, which can activate two major pathways involved in hyperglycemic complications. An increase in this triose phosphate can activate the glycation pathway by driving glyceraldehyde-3-phosphate and dihydroxyacetone-3-phosphate into non-enzymatic synthesis of methylglyoxal. Advanced glycation end products (AGEs) are formed by the non-enzymatic reaction between the ketone or aldehyde groups of these trioses and the amino groups of proteins. Research has shown that accumulation of AGEs in diabetes is accelerated and activates the receptor of AGEs (RAGE). This can lead to the intracellular generation of Reactive oxygen species (ROS) by NADPH oxidase and causes downstream activation of redox-sensitive transcription factors such as the nuclear factor-κB (NF-κB) and vascular endothelial growth factor (VEGF) [34]. In the other pathway, glyceraldehyde 3-phosphate accumulation can increase diacylglycerol production which activates PKC [35].

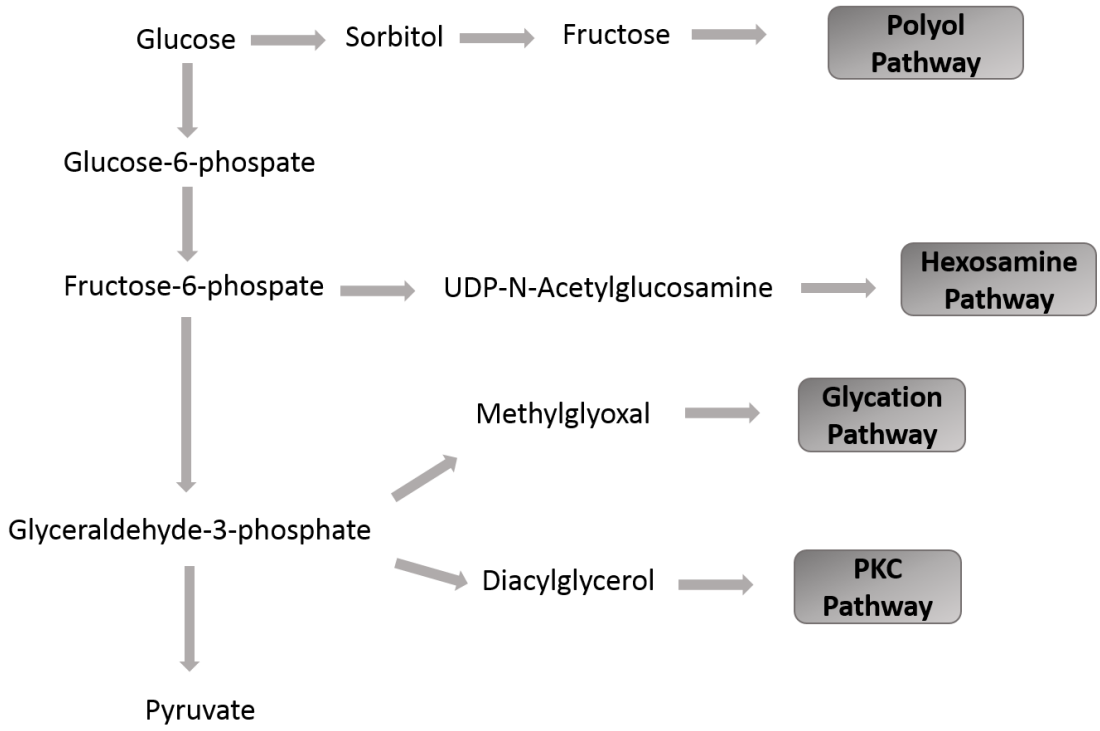


Figure 1.1.5.2: Hyperglycemic damage mechanism

1.1.6 Diabetic Complications

Prolonged hyperglycemia can lead to serious complications affecting the heart, kidneys, nerves and eyes. In diabetes, damage to small blood vessels can lead to microvascular complications, while damage to arteries can lead to macrovascular complications. Microvascular complications include retinopathy, nephropathy, and neural damage. The major macrovascular complications are cardiovascular disease resulting in myocardial infarction and strokes [36].

Retinopathy

Diabetic retinopathy is a leading cause of blindness in adults and is associated with the development of progressively severe retinal lesions that damage vision. Diabetic retinopathy develops for many years, and almost all Type 1 diabetes patients [37], and most

Type 2 diabetes patients [38], can exhibit retinal lesion symptoms after more than 15 years of diabetes. However, retinal disorder is more severe in Type 1 than Type 2 diabetes.

The pathologic signs of retinopathy include changes in vascular permeability, capillary micro-aneurysms, capillary degeneration, and excessive intra-retinal hemorrhage, accompanied with the formation of lipid exudates that causes regional failure of retinal microvascular circulation, blurred vision and blindness. Clinically, diabetic retinopathy can be classified into non-proliferative and proliferative stages. Non-proliferative diabetic retinopathy (NPDR) is the initial stage in which symptoms are mild and likely un-noticeable. In NPDR, the blood vessels in the retina are weakened, and micro-aneurysms can be formed that may leak fluid into the retina. This leakage can lead to swelling of the macula, which is responsible for straight ahead vision. In the advanced proliferative stage, new blood vessels are formed and extend to the vitreous and cause hemorrhage and fluid accumulation within the retina. This leads to macula edema to cause visual impairment [39]. In more severe conditions, there can be bleeding due to fibrosis in retinal vascular membranes which can lead to retinal detachment [40].

There are three common treatments for diabetic retinopathy to improve vision loss, in addition to controlling blood pressure and glucose levels. These include retinal laser photocoagulation therapy, vitrectomy to remove the vitreous, or injection of VEGF antagonists into the eye since VEGF increases retinal angiogenesis [40, 41].

Nephropathy

Diabetic nephropathy is one of the leading causes of end-stage renal disease (ESRD) due to microangiopathy, glomerular hypertrophy, thickening of glomerular basement membrane and enlarged glomerular mesangium [42, 43]. It is characterized by a chronic

inflammation, progressive loss of renal function, glomerular/tubular-interstitial scarring and albuminuria. Progression of the nephropathy can be classified into three stages. The first stage in early diabetes is glomerular hypertrophy characterized as glomerular hyperfiltration [44]. Glomerular hyperfiltration causes the damage to the preglomerular and glomerulus vessels, which are the filtering components of the kidney [45]. The kidneys then filter increased amounts of glucose, fatty acids, proteins, amino acids, growth factors, and cytokines which can trigger many pathologic pathways leading to energy imbalances, redox anomalies, inflammation and fibrosis. This leads to the second stage of inflammation within glomerular and tubulointerstitial regions. The third stage is a reduction of cell number by apoptosis and accumulation of extracellular matrix (ECM). The deposition of ECM in the tubular component of the kidney can cause tubulointerstitial fibrosis, which can be used to determine the progression of diabetic nephropathy [46].

Besides glycemic control, the most widely used therapies for the treatment of diabetic nephropathy are through controlling systemic or intra-glomerular hypertension. The first line treatment of diabetic nephropathy targets the renin-angiotensin system (RAS) including angiotensin II (ANG II) receptor antagonists and angiotensin converting enzyme (ACE) inhibitors [47]. It is noteworthy that early nephropathy is a major risk factor for initiating cardiovascular disease including heart attacks and stroke in diabetic patients [48]. This indicates that more attention should be paid to diabetic nephropathy development from the early stages.

Cardiovascular Disease

Diabetic Cardiovascular Disease (CVD) is ventricular dysfunction accounting for over half of diabetes related deaths [49]. In Type 2 diabetes, nephropathy is a major risk

factor for cardiomyopathy, along with poor glycemic control, continued high blood pressure and dyslipidemia [50]. Diabetic CVD symptoms include atherosclerosis, cardiac and diastolic dysfunction as well as myocardial infarction and stroke [51].

Cardiac endothelium is critical to controlling heart adhesiveness, permeability and integrity. Endothelial dysregulation drives atherosclerosis, leading to the entry of low-density lipoproteins into the subendothelial space causing fatty streak formation. Eventually, this can lead to necrosis, and the formation of atherosclerotic plaques, which may block the blood vessel at the formation site. In the coronary or femoral circulation, embolization of blood vessels that obstruct the distant sites may destabilize and rupture, leading to unstable angina, myocardial infarction or stroke [52, 53]. In addition to myocardial damage, coronary artery disease and cardiomyopathy can also occur in diabetes [54]. Cardiomyopathy is characterized by diastolic dysfunction of the heart, an inability to relax and fill during diastole of the cardiac cycle. Myocardial sclerosis caused by hypertrophy and neuronal abnormalities leads to diastolic dysfunction due to cross-linking and ECM deposition. The main clinical consequence of diastolic dysfunction is exercise-induced dyspnea, which hinders the ability of diabetic patients to exercise, an important aspect of obesity management in diabetes [55].

Diabetic individuals with CVD should be under strict glycemic control, taking anti-hypertension drugs and consider lipid lowering therapy with statins or fibrates as well as antithrombotic agents, such as aspirin [56].

1.2 Diabetic Peripheral Neuropathy (DPN)

Diabetic neuropathy is the most common diabetic complication and is characterized by both autonomic and somatic nerve disorders. According to National Institute of Diabetes,

Digestive and Kidney Diseases, about 60 to 70 percent of diabetic patients have some type of neuropathy. Nerve damage can occur in the feet, legs, hands, arms, digestive tract, heart and basically every organ system. Diabetic patients can experience neurological problems at any time, but risk increases with age, the duration of diabetes and problems controlling blood glucose levels. The highest incidence of neuropathy is among diabetic patients for at least 25 years [57].

The autonomic nervous system consists of nerves innervating the heart, lungs, bone, fat, sweat glands, blood vessels, gastrointestinal and genitourinary systems. In diabetic autonomic neuropathy, the most commonly affected systems include the gastrointestinal tract, genitourinary and cardiovascular systems [58]. Gastrointestinal neuropathy can affect the esophagus, stomach and intestines causing pain, nausea, vomiting, diarrhea and constipation. Delayed gastric emptying can lead to slowed medication effect while sluggish peristalsis of the small intestine can cause bacterial overgrowth, worsened by the presence of hyperglycemia. Gastroparesis is alleviated by eating smaller, more frequent meals. However, it can be enhanced by drugs that slow gastric emptying, like incretin/amylin mimetics. Antibiotics can be used for bacterial suppression.

Peripheral neuropathy is the most common type of diabetic neuropathy. It is distal, symmetric and bilateral damage to nerves of the feet, legs, hands, and arms, with a distal-to-proximal gradient of severity, known as stocking-glove sensorimotor neuropathy [59]. DPN is a length-dependent disorder, and feet and legs are often affected before hands and arms. Symptoms of sensory neuropathy are numbness or insensitivity to pain or temperature change, tingling, burning, or prickling sensation, sharp pain or cramps, and extreme sensitivity to touch. Motor neuropathy is less common and may occur at a later

stage. Symptoms may include muscle weakness and loss of reflexes, especially at the ankle, affecting the way people walk [60]. Foot deformities, such as hammertoes may also occur. Infection and ulceration may happen on numb areas of the foot due to injury goes unnoticed. The cumulative lifetime incidence of diabetic foot ulcers may be as high as 25%. If the infection is not treated promptly, it may spread to the bone, and the foot may have to be amputated. Foot ulcers precede 84% of all diabetes-related lower leg amputations [61]. Many amputations are preventable if we can catch and treat minor problems in timely manner [62]. However, DPN is still the first leading cause of non-traumatic amputations, representing a serious medical, social and economic problem all over the world.

1.2.1 Diagnosis of DPN

Besides controlling blood glucose level, diabetic patients should have an annual foot exam to identify high-risk foot conditions. This exam should assess sensation, foot structure, vascular status, skin integrity and ankle reflexes [63]. Sensory perceptions should be assessed, particularly the sensation of vibration and touch due to damage to thinly myelinated Aδ and unmyelinated C fibers. Proprioception, the perception of position, may also be affected due to damage to large myelinated Aα and Aβ nerve fibers (Table.1.2.1) [64].

There are several quantitative diagnostic tests can be used to determine the presence and type of diabetic peripheral neuropathy. Quantitative sensory testing (QST) can be used to determine a loss of neural sensation or sensitivity. Nerve conduction velocity studies can detect possible nerve damage by assessing the transmission of nerve impulses. Slowed transmission may indicate nerve damage. Electromyogram (EMG) can be used to assess how muscles respond to nerve impulses to determine if there is damage to muscle or neural

innervation [59, 65]. Loss of unmyelinated intra-epidermal nerve fibers (iENFs) in the feet is associated with loss of thermal sensitivity. Thus, assessing iENF density is becoming an important clinical measure of DPN [66].

Table 1.2.1: Sensory neuropathy test

Sensory Modality	**Nerve Fiber**	**Test Approach**
Pressure	Aα, Aβ	1 and 10 g monofilament
Light touch	Aα, Aβ	Wisp of cotton
Vibration	Aβ	128-Hz tuning fork
Cold	Aδ	Cold tuning fork
Pain (pin prick)	C	Neuro-tips

1.2.2 Current Therapies for DPN

Despite advanced understanding of DPN development, prevention is still a key goal of diabetes care due to the lack of effective treatments for peripheral nerve injury. Prevention of diabetic neuropathies includes tight glycemic control and lifestyle changes. Strict glycemic control in Type 1 diabetes patients significantly reduces the incidence of distal symmetric polyneuropathy. While in Type 2 diabetes patients with multiple complications, glycemic control alone is less effective at preventing distal symmetric polyneuropathy. For patients with pre-diabetes, Type-1 diabetes and Type-2 diabetes, lifestyle interventions such as exercise and dietary change are recommended for distal DPN prevention [67].

Although significant progress has been made in understanding the pathogenesis of diabetic neuropathy, pharmacotherapies are still limited. Antidepressant drugs, both of the tricyclic type and serotonin-norepinephrine reuptake inhibitors (SNRIs), are widely used

and are effective treatments for neuropathic pain, although only duloxetine (Cymbalta) is FDA approved for DPN [68]. Duloxetine is a selective norepinephrine and serotonin reuptake inhibitor. In multicenter clinical trials, doses of 60 and 120 mg/day showed strong efficacy in the treatment of DPN pain, despite its high drop-out rate [69]. Duloxetine can also improve diabetes-related quality of life. However, in longer-term studies, duloxetine treatment may cause a mild increase in A1c in diabetic patients compared with placebo treatment [70].

Anticonvulsants drugs have also been effectively used in treating of neuropathic pain. The FDA has approved pregabalin (Lyrica) for treating painful DPN. It is widely used in treating abnormal pain, such as pins or needles sensations and burning. Pregabalin is a voltage-gated calcium channel α2-δ subunit ligand to reduce inappropriate calcium influx into the hypersensitive cells. It is an effective therapy for DPN neuropathic pain. By far, it is the most widely studied drug for DPN, with 30%–50% positive response rate for pain improvement [71].

The opioids, in general, are effective in treating neuropathic pain, though they are not recommended as first-line treatments. Traditional opioids act on the mu-opioid receptor primarily. Tramadol has a dual effect of blocking norepinephrine uptake and acting on the mu-opioid receptor. Although there is no FDA approval for the treatment of DPN, these drugs are effective [72]. The problem with opioids are side effects, and very few people can tolerate particularly high doses. Nausea, constipation and even habituation are commonly observed [73].

In addition to these systemic therapies, topical medications like lidocaine patches or capsaicin cream may also be used to treat neuropathic pain. The lidocaine patch is a 5%

impregnation into a patch that you place on the skin. It is thought to act by blocking sodium channels, and because the sodium channels set up the afferent signal traffic, ectopic nerve impulses are suppressed. Capsaicin, which is the active component in hot pepper oil, acts differently. It depletes substance P in the skin, which mediates pain. It also blocks the vanilloid receptor to reduce pain [74, 75].

1.2.3 Peripheral Nervous System (PNS) Composition

The two major components of the nervous system are the central nervous system (CNS) and peripheral nervous system (PNS). The PNS consists of 12 cranial nerves and 31 pairs of spinal nerves. Efferent axons from motor neurons transmit information from the CNS to muscles and glands, whereas afferent axons from sensory neurons carry information from peripheral sensory receptors to the CNS [76].

The sciatic nerve is the longest nerve in PNS, extending from the rear of the pelvis to the back of the thigh. It is the main nerve of the leg controlling the muscles of the hip and lower extremities. Other major peripheral nerves include the tibial nerve and sural nerve. The tibial nerve is a branch of the sciatic nerve that passes sensation to the soles. It controls the calf muscles, allowing the toes and feet to flex. The sural nerve consisting of medial and lateral branches is the sensory nerve in the leg. It passes sensory signals from feet and legs to the spinal cord and brain [77].

DPN is a length-dependent, sensory more than motor, axonal neuropathy. At least two explanations have been made to illustrate why longer nerves and their axons usually get wounded before shorter ones. Sensory axons can be up to 3 feet or longer. The long length of these axons make them more vulnerable to blood-born or systemic damage. Another reason may be due to the supply of molecules and/or nutrients is harder to be transported

to more distal nerve terminals. These nerve ends therefore shrink and then disappear [78]. Diabetic sensory neuropathy usually precedes the involvement of motor neurons, which affects either or both the small and large nerve fibers in limbs [79].

As summarized in Table.1.2.2 [80], peripheral sensory nerve fibers are classified into large myelinated Aβ-fibers, small thinly myelinated Aδ-fibers and unmyelinated C-fibers. Aβ-nerve fibers transmit sensation of proprioception, vibration, and pressure, while C-fibers can sense warm and cold temperature change and harmful input with high threshold [80]. There are more unmyelinated than myelinated axons in the PNS, and these C fibers are majorly involved in PNS impairment. C fibers carry information for the autonomic nervous system as well as afferent impulses in response to temperature and noxious stimuli that could cause tissue damage. The earliest pathological nerve changes in DPN occur at unmyelinated C fibers, with initial degeneration and regeneration of C fibers resulting in pain, allodynia, and hyperesthesias [81]. As DPN progresses, degeneration exceeds regeneration, leading to C fibers loss. This C-fiber loss can also be observed in pre-diabetic patients. Comparing with myelinated fibers, unmyelinated C fibers are more vulnerable to metabolic damage mainly due to the lack of protection and nutritional supplementation by Schwann cells (SCs). After C-fiber atrophy, myelinated axons may undergo segmental demyelination and remyelination [81, 82]. As demyelination overrides remyelination, there is axonal degeneration of myelinated fibers. This temporal course of nerve pathology in DPN argues for a loss of protective and nutrient flow from the SCs to the myelinated axons, leading to eventual axonal loss. Furthermore, DPN develops in a distal-to-proximal manner along the nerve [83].

Table 1.2.2: Motor and Sensory Nerve Fibers in PNS

	Motor	Sensory		
Classification	Myelinated Aα	Thickly Myelianted Aβ	Thinly Myelinated Aδ	Unmyelinated C fibers
Function	Motor	Proprioception	Cold	Burning pain
		Vibration		Warmth
			Sharp pain	
		Pressure		Autonomic function
Clinical sign	Decreased ankle and knee reflexes	Impaired perception	Hyperalgesia	Hyperalgesia
		Hyperalgesia	Hypoalgesia	Hypoalgesia
		Hypoesthesia	Hypoesthesia	Hypoesthesia

1.2.4 Pathogenesis of Diabetic Neuropathy Development

Schwann Cell Pathology

SCs are glial cells that myelinate axons to ensure rapid and efficient nerve conduction. The myelin sheath electrically insulates axonal membranes and allows fast propagation of nerve impulses by saltatory conduction. SCs maintain neuronal structure and function, nourish axons, and promote survival and growth upon injury and this is disturbed in diabetes. SCs express a wide range of receptors, and they upregulate the synthesis and secretion of neuroprotecitve factors that promote neuron regrowth and remyelination [84,

85]. Schwannopathy accompanied by axonal degeneration is a key pathology in DPN development [86].

Proliferating SCs are more glycolytic, delivering pyruvate and lactate to axons, but SC mitochondrial dysfunction can cause SC degeneration leading to demyelination [87-89]. During diabetes, SCs can lose their ability to provide energy to myelinated and unmyelinated axons and transfer toxic lipid species to the axons they contact. Disruption of their metabolism results in the accumulation of neurotoxic intermediates and compromises production of neuronal support factors, contributing to axonal degeneration, endothelial dysfuction and diabetic neuropathy [87].

Polyol Pathway

Excessive glucose in diabetes can activate the polyol pathway to produce sorbitol by the action of aldose reductase. The accumulation of sorbitol results in an osmotic imbalance in the cell to cause cellular and organ injury. Increased sorbitol decreases myo-inositol in peripheral nerves, which is an essential component of sodium/potassium (Na/K) ATPase. Aldose reductase activity also consumes NADPH, which can decrease GSH levels [90]. Since GSH is an important ROS scavenger, reduced GSH can cause or exacerbate intracellular oxidative stress. Excessive ROS can impair SC as well as endothelial functions that can reduce and contribute to poor blood flow in DPN [91].

Several drugs have been designed to inhibit aldose reductase and target the polyol pathway to treat DPN. Examples of such compounds include spirohydantoins (sorbinil) and carboxylic acid derivatives (tolrestat, fidarestat, and epalrestat). Clinical trials over the past decade have assessed the efficacy of these agents in treating diabetic retinopathy,

neuropathy, and nephropathy. Although some positive results have been reported, the benefits have generally been minimal and of no substantive clinical importance [92].

Sorbinil is efficacious in preventing neurological dysfunction in the STZ mouse model. It reverses the accumulation of sorbitol, promotes the recovery of myo-inositol and slightly improves nerve conduction velocity (NCV). Although nerve conduction velocities were increased by sorbinil in the peroneal nerve — but not in the median motor or sensory nerves — no amelioration of early clinical signs or symptoms of diabetic neuropathy was observed [93]. Moreover, it has been withdrawn from further use because of its severe and frequent toxicity [94].

Tolrestat was also withdrawn from the worldwide market in 1998 after the appearance of severe liver toxicity (including one case of hepatic necrosis reported from Italy) and the failure of clinical trials to demonstrate clear benefit [95, 96]. Fidarestat is a specific and potent aldose reductase inhibitor [97]. Significant improvement in electrophysiological measures of nerve conduction in motor and sensory nerves was observed with fidarestat treatment along with an improvement in numbness, spontaneous pain, sensation of rigidity, paresthesia in the soles upon walking, foot heaviness, and hyperesthesia. However, no other clinical trials of fidarestat have been reported since 2005. Epalrestat was well tolerated and significantly prevented deterioration of median nerve MNCV while preserving vibration perception threshold seen in the control group [98]. Overall, the lack of strong efficacy of aldose reductase inhibitors in clinical trials has limited the use of these drugs in treating DPN.

Hexosamine Pathway

Excessive flux of glucose through glycolysis can cause the accumulation of fructose-6-phosphate. This glycolytic intermediate can be metabolized into glucosamine-6-phosphate (GlcN-6-P) by fructose-6-phosphate amidotranferase (GFAT). GlcN-6-P can be further converted into uridine 5-diphosphate-N-acetylglucosamine (UDP-GlcNAc) to enter the hexosamine biosynthesis pathway. UDP-GlcNac is one of the sugar moieties that can cause protein and lipid glycosylation. UDP-GlcNac binds to O-linkage of serine/threonine residues of various proteins, including transcription factors like Sp1, c-myc and CREB [99-102]. Overstimulation of the hexosamine pathway is related with insulin resistance, lipid dyshomeostasis, inflammation, and vascular complications in peripheral nerves [103]. No therapeutics that directly target this pathway to treat DPN have been developed.

Protein Kinase C (PKC) Pathway

Increased glycolysis can also cause the accumulation of glyceraldehyde-3-phosphate which can be metabolized to diacylglycerol (DAG). An increase in DAG can activate neuronal PKC and phosphorylate transcription factors to regulate protein expressions [104]. PKC is a family of serine/threonine protein kinases, including the isoforms of PKC-α, PKC-β1, PKC-β2, PKC-γ and PKC-ε. Activated PKC can cause changes in blood flow, endothelial dysfunction, increased vascular permeability, angiogenesis, cell growth and apoptosis, vessel dilation, basement membrane thickening and extracellular matrix expansion, abnormal angiogenesis, altered MAPK activity and disrupted Na/K ATPase activity [105]. Reduced Na/K ATPase activity leads to reduced nerve conduction and neuroregeneration. All PKC isoforms are expressed in nerve [106] and the PKC-β inhibitor ruboxiastaurin (LY333531, RBX) is a type of bisindoylmaleimide that was found to reverse

diabetic retinopathy and nephropathy. Ruboxiastaurin has a slight beneficial effect in the treatment of neuropathy sensory symptoms [107] but it is not approved to treat DPN.

Advanced Glycation Pathway

Another significant alteration induced by hyperglycemia is the non-enzymatic glycation of proteins via AGE formation [108]. Glucose and some glycolytic intermediates can be metabolized into α, β-dicarbonyls through Schiff base formation, Amadori and Maillard reactions. Dicarbonyls can react with amino groups of proteins, nucleotides and lipids to form AGEs. Proteins with more abundant lysine, arginine and cysteine residues are more easily glycated, which can cause changes in protein structure and function. Nucleotides with deoxyguanosines and lipids with basic phospholipids may also undergo glycation which can cause DNA mutation and affect membrane integrity [109, 110]. AGEs can also interact with their membrane receptors - RAGE to activate pro-inflammatory NFκB pathway and its downstream factors like MAPK, p21 and JNK [111, 112], causing multiple pathological changes.

In diabetic patients, there is an increase in both intra- and extracellular AGEs [113]. A few drugs have been found to alleviate symptoms of diabetes by reducing AGEs. For example, metformin which is a common drug for Type 2 diabetes, can reduce serum reactive dicarbonyls and AGEs levels in Type 2 diabetic patients [114]. Pyridoxamine also relieves diabetic nephropathy by reducing carboxymethyllysine (CML) formation [115]. Pioglitazone and aminoguanidine also have anti-AGE effects [116]. AGEs have been found to play a critical role in the pathology of DPN. In diabetic dorsal root ganglia, AGEs can decrease mitochondrial respiratory chain activity and mitochondrial membrane potential [117-119]. AGEs can not only modify mitochondrial components and affect cellular

bioenergetics, but also modify matrix protein like laminin and fibronectin to blunt axonal regeneration and collateral stretching [117].

1.3 Oxidative Stress

Poloyl pathway flux, overactivity of the hexosamine pathway, activation of PKC isoforms and increased AGEs formation can increase oxidative stress in both the cytoplasm and mitochondria. Another major endogenous source of ROS within most mammalian cells is through mitochondrial respiratory chain activity, which transports electrons to react with oxygen and generate ATP. However, about 0.1-2% of electrons may leak from complex I and complex III and incompletely reduce about 1-5% of total oxygen, producing superoxide radical O_2^- to cause oxidative damage [120, 121].

1.3.1 Overview of Cellular Oxidative Stress

In cellular oxidative metabolism, ROS mainly refer to superoxide anion (O_2^-), hydroxyl radical (OH), hydrogen peroxide (H_2O_2), singlet oxygen (O), and organic hydroperoxides (ROOH) [122]. Cellular antioxidant defense systems act to detoxify these species at low concentrations. Low levels of ROS can be beneficial because they are required for certain biological processes including enzyme activation, gene expression and signal transduction. But over-accumulation of ROS can overwhelm antioxidant defenses leading to excessive oxidative stress and subsequent damage to many intracellular molecules including DNA, lipids, and proteins [123, 124]. More specifically, ROS can cause DNA mutations by oxidizing purines and pyrimidines [125]. As cell membranes are rich in polyunsaturated lipids, their integrity can be disrupted when oxidized by ROS [126]. Proteins, especially those with a high abundance of arginine, proline, threonine and

cysteine residues, can also be oxidized by ROS to alter their structure and function [127, 128].

Oxidative stress is the result of the imbalance between ROS production and detoxification. As electrons traverse the mitochondrial respiratory chain, electrons may leak from complexes I and III, to reduce oxygen into superoxide radical $O_2^{\bullet-}$ [120, 121]. As superoxide contains one electron on the outermost orbital, it cannot penetrate lipid membranes, thus the majority of $O_2^{\bullet-}$ remains in the mitochondria [129]. To protect cells against oxidative damage, defensive mechanisms have evolved to eliminate surplus ROS. Mitochondrial manganese-dependent superoxide dismutase (MnSOD), cytoplasmic Cu/Zn-SOD and extracellular ecSOD can dismutate $O_2^{\bullet-}$ radicals to H_2O_2 or molecular oxygen. Even though H_2O_2 can be processed into oxygen and water by catalase and glutathione peroxidase, excessive H_2O_2 can still diffuse through mitochondria and further initiate many types of cellular injury [130]. For example, increased H_2O_2 is found in melanoma, ovarian and colon carcinomas [131] and it is recognized as a key messenger molecule to regulate cancer signal pathways, like NFκB activation [132]. When reduced transition metals (ions of Fe^{2+}, Cu^{2+} or Co^{2+}) react with H_2O_2 through the Fenton reaction, the •OH is the major product [133]. •OH is able to damage the deoxyribose backbone of DNA causing strand breaks and modify all four DNA bases, resulting in damage to mtDNA and mitochondria dysfunction [134].

Other sources of ROS production include peroxisomes and oxidation enzymes. Peroxisomes contain oxidative enzymes such as D-amino acid oxidase, and uric acid oxidase. They can remove hydrogen atoms from specific organic substrates to react with oxygen and produce hydrogen peroxide. In addition, ROS can also be produced by a family of membrane-bound enzymes NADPH oxidases, which can affect cell proliferation and

apoptosis [135]. NADPH oxidases can be found in the plasma membrane as well as in phagosomal membranes, which are used by neutrophils to engulf microorganisms. During inflammation, leukocytes and mast cells are recruited to the damage site, which leads to a respiratory burst by increasing the uptake of oxygen and an accumulation of ROS [136]. Other enzymatic sources of ROS include cyclooxygenase, xanthine and nitric oxide synthase.

1.3.2 Mitochondrial Oxidative Stress

Mitochondria are crucial cell organelles to regulate cellular functions, metabolism, and cell death in neurodegenerative diseases. They are not only the major source of ROS generation but also sensitive targets of oxygen radicals [137, 138]. In mitochondrial inner membrane, Complex I (NADH coenzyme Q reductase) receives electrons from NADH, and delivers them to coenzyme Q, which also receives electrons from complex II (succinate dehydrogenase). Coenzyme Q transports electrons to cytochrome c. Cytochrome c then transfers electrons to complex IV, which uses hydrogen ions and electrons to reduce oxygen into water. Meanwhile, complex I, complex II and complex IV pump hydrogen ions into the intermembrane space, creating an electrochemical gradient across the inner membrane that is essential for generating ATP. Because of electron leakage, complexes I and III of the electron-transport chain are the major sites for ROS production. Blockade of complex I by rotenone increases the reduction of the NADH dehydrogenase site of complex I, increasing electron leak to ROS [139]. Antimycin A obstructs complex III at the Q_i center and increases superoxide generation from the Q_o center [140-142]. The superoxide produced by complexes I and III can in turn inactivate NADH dehydrogenase, succinate dehydrogenase and ATP synthase activities. Other ROS, like hydrogen peroxide, can also partially inhibit NADH dehydrogenase and cytochrome oxidase [143, 144]. Thus,

oxidative stress can inhibit electron transport and decrease oxygen consumption and ATP generation.

Mitochondrial membrane potential ($\Delta\psi$) is also a crucial parameter for ATP formation and mitochondria function. ROS can oxidize unsaturated phospholipids on the inner mitochondrial membrane leading to increased permeability of the membrane that impairs mitochondria function [145]. In addition, Jendrach's group showed that H_2O_2 can enhance fission factors including Drp1, Fis1 and MTP18 while increasing Mfn1 slightly [146, 147]. Thus, ROS generation can cause mitochondrial fragmentation. As most of the mitochondrial proteins are not synthesized in mitochondria, the intake of preproteins is also a major parameter to assess mitochondrial bioenergetics. Gary's group showed that paraquat, a superoxide-generating molecule, could inhibit the import process of mitochondrial preproteins which are synthesized in the cytosol [148].

The main non-mitochondrial pathway for superoxide production is through NADPH oxidase. This enzyme is a membrane-bound electron transport complex. It receives electrons from NADPH and transport electrons across membrane into intracellular and extracellular compartments, such as mitochondria, nucleus, ER, endosomes, phagosomes and the extracellular space. It is the only major enzyme that produces superoxide and/or hydrogen peroxide and is used primarily to prevent pathogen spreading and kill bacteria. NAD(P)H oxidase activity generates superoxide radicals that promotes mitochondrial dysfunction and apoptosis in neurodegeneration. Overtime, accumulating ROS impairs neurological function and results in diabetic neuropathy development.

SOD is the major ROS defense system. MnSOD localizes in mitochondria, while CuZn-SOD is expressed in cytosol to detoxify superoxide radicals. H_2O_2 is degraded by GSH peroxidase or catalase. GSH peroxidase reduces H_2O_2, in which two gluthathione

(GSH) molecules are oxidized to glutathione disulfide (GSSG). GSH-reductase is used to regenerate GSH, consuming NADPH to $NADP^+$. Catalase is localized in peroxisomes to detoxify H_2O_2 into water and oxygen in the cytosol.

Fluorescent measurements can be used to determine ROS production. Mitochondria-targeted dihydroethidine (DHE), known as MitoSOX can be used to determine mitochondrial superoxide levels by staining with live cells. Triphenylphosphonium cation on MitoSOX allows its accumulation in the negatively charged mitochondrial matrix depending on mitochondrial membrane potential [149]. Another specific method to measure ROS is through Electron Paramagnetic Resonance (EPR) spectroscopy. The ROS level can be determined by wave signals generated by the interaction of free radicals with spin traps, like mitoTEMPO [150].

1.3.3 Antioxidant Therapy

In addition to the antioxidant enzymes including SOD, glutathione peroxidase and catalase, there are several small antioxidant molecules that are of importance in the antioxidant defense system, especially where antioxidant enzymes are absent or present only in small amount. The lipid-soluble antioxidants are localized at cellular membranes and lipoproteins, whereas the water-soluble antioxidants are present in aqueous fluids, such as blood and the fluids within cells and surrounding them.

α-Lipoic acid (ALA)

α-Lipoic acid (ALA), also known as thioctic acid, is a cofactor for pyruvate dehydrogenase to decarboxylate pyruvate into acetyl-CoA [151]. It is taken up from the diet and is permeable to the blood-brain barrier. ALA contains two thiol groups that can be oxidized or reduced into dihydrolipoic acid. Both reduced and oxidized forms are strong antioxidants to scavenge ROS, regenerate other antioxidants such as vitamin C, vitamin E,

chelating metal ions and regulating gene transcription [152]. ALA treatment improves nerve conduction deficits in STZ diabetic rat but has shown limited efficacy in humans [153].

Vitamins E and C

Vitamins E and C scavenge free radicals. Vitamin E is a lipophilic antioxidant which includes tocopherols and tocotrienols. Tocopherol, the biologically and chemically most active form of vitamin E, is by far the most abundant lipid-soluble antioxidant in humans [154]. Vitamin E reacts with hydroxyl radicals to form a stabilized phenolic radical which can be reduced back to the phenol by ascorbate and NAD(P)H dependent reductase enzymes [155]. Vitamin E has been reported to alleviate symptoms of diabetes and diabetic complications in animal models through reducing oxidative stress [156-158]. Treatment of Vitamin E reduced the incidence of sensory neuropathy [159-161]. Vitamin C, also known as ascorbic acid, is the prominent water-soluble antioxidant effectively scavenging ROS. In ob/ob Type 2 diabetic mice, vitamin C supplementation can cause significant reductions in food intake, HbA1c levels and plasma glucose levels, when compared with untreated control ob/ob mice [162].

Ubiquinone (Coenzyme Q10)

Coenzyme Q10 also known as ubiquinone is an important vitamin-like micronutrient acting on the mitochondrial electron transport chain of ATP synthesis. CoQ transfers electrons from NADH at complexes I, succinate at complex II, and glycerol-3-phosphate of complex III to generate ATP. Coenzyme Q10 deficiency is common in Type 2 diabetic patients. In addition, CoQ10 is also a lipid-soluble antioxidant, that can be reduced to ubiquinol and redistributed into lipoproteins, to scavenge free radicals and protect cells from oxidation [163]. Plasma levels of CoQ10 are significantly lower in T2DM patients

than in healthy individuals [164]. Exogenous treatment of CoQ10 can potentially reduce oxidative stress-induced mitochondrial dysfunction, and improve glycemic control in Type 2 diabetic patients [165]. However, its efficacy in human DPN is limited.

Tempol and mitoTEMPO

Tempol is a small molecule, water-soluble and membrane permeable SOD mimetic that is widely used in electron spin resonance spectroscopy [166]. Tempol reacts with both intracellular and extracellular superoxide radicals, to protect cells against oxidative stress [167]. Tempol can be used as an antioxidant in animal models to prevent many diseases that are caused by ROS such as ischemic-reperfusion injury, inflammation and diabetes [168, 169]. *In vivo* treatment with Tempol reduces NADPH oxidase activity, attenuates renal ER stress, inflammation and subsequent kidney damage in the STZ induced Type-1 diabetes rat model [170].

Mito-TEMPO is also a SOD mimetic, which passes through lipid bilayers easily and accumulates selectively in mitochondria. mito-TEMPO is a mitochondria-targeted antioxidant with superoxide and alkyl radical scavenging properties [171]. *In vivo* treatment with mito-TEMPO decreases mitochondrial oxidative stress in cardiomyocytes and improves cardiomyopathy in Type-1 and Type-2 mouse models of diabetes [172].

1.3.4 Oxidative Stress in DPN Development

Increased ROS generation and impaired defense systems result in excessive oxidative stress and are major contributors to the pathogenesis of DPN [173]. In Type 1 and Type 2 diabetic patients, plasma lipid peroxidation products increase while GSH and GSH-metabolizing enzymes decrease with disease development. This is closely related with the onset of diabetic complications [174-177]. Persistent hyperglycemia leads to ROS-induced injury in diabetic neuropathy, and the relationship between ROS and neuropathy

in DM has been assessed using *in vitro* and *in vivo* models. In DPN, increased oxidative stress can disturb neural functions, decrease neurotrophic support and mediate apoptosis of neurons and Schwann cells [178, 179].

Glucose-induced oxidative stress and changes in antioxidant capacity have been studied in different *in vitro* models. 10 - 20 mM additional glucose in rat DRG sensory neurons promotes superoxide and hydrogen peroxide overproduction. This can lead to lipid oxidation and even neuronal death, which can be prevented in part through decreasing ROS production with IGF-I [180]. *In vitro* incubation of sciatic nerve in 20 mM glucose medium increased lipid peroxidation up to four-fold that was partially prevented by the addition of α-lipoic acid to the medium [181]. Lipid peroxidation of nerve membrane could cause peripheral nerve ischemia and hypoxia, features of DPN [182].

Glutathione and SOD levels were decreased in SCs cultured under high glucose and the $NADH/NAD^+$ ratio progressively increased with higher glucose concentrations [183, 184]. In adult sensory neurons isolated from STZ diabetic rats, hyperglycemia induced a robust increase in superoxide levels and this was associated with neurite degeneration and apoptosis [185, 186].

Excessive oxidative stress can activate several inflammatory pathways and peripheral nerves undergo a pro-inflammatory process in DPN [187]. Inflammatory factors such as C-reactive protein (CRP), interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and tumor necrosis factor-α (TNF-α) are present in both Type 1 and Type 2 diabetic patients and correlate with the development of DPN [188]. TNF-α, transforming growth factor-β (TGF-β), and NF-κB can be activated by several glucose-induced pathways [189]. Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are important

enzymes that could be up-regulated by NF-κB in diabetic peripheral nerves and consecutively generate prostaglandin E2 and ROS that trigger further activation of NF-κB. Infiltration of macrophages and granulocytes takes place in diabetic peripheral nerves after ischemia reperfusion [190]. NF-κB activation induces cytokines in neurons, SCs and endothelial cells leading to absorption of macrophages in the diabetic nerves. Macrophages promote DPN via releasing cytokines, ROS, and proteases, which could lead to cellular oxidative damage, myelin breakdown and impair nerves regeneration in DPN [187, 191, 192].

Excessive oxidative stress can disrupt mitochondrial membrane potentials which leads to swelling of neural mitochondria and mitochondrial dysfunction [179]. High glucose in DRG culture medium can lead to ROS accumulation in sensory neurons, which can initiate mitochondria swelling, hyperpolarization, and then depolarization, to release cytochrome c into the cytosol. Cytochrome c efflux activates apoptosis-activating factor 1 (Apaf-1), caspase-3 and -9, with the formation of a cytochrome c/caspase-9/Apaf-1 complex (the apoptosome), resulting in DRG apoptosis [193]. In primary sensory neurons from Type-1 diabetic rats, there was a down-regulation of Bcl-2 to initiate apoptosis [180, 194]. High glucose induced neuronal apoptosis both *in vivo* and *in vitro* [195, 196], but apoptosis is not thought to be an important pathogenetic feature for the development of DPN [179].

1.4 Mitochondria Dysfunction in Diabetes

Mitochondria are important organelles that produce energy through oxidizing products of glucose metabolism. Mitochondrial bioenergetics are important for cellular energy generation via ATP synthesis and has become a keystone of research since the late 1940s

[197]. In diabetes, both metabolic and vascular insults can increase cellular oxidative stress to cause impaired glucose tolerance, insulin resistance and β-cell dysfunction [198]. In mitochondria, oxidative stress can modify mitochondrial DNA, proteins, and membrane lipids, to impair mitochondrial functions in sensory neurons. It should be noted that axons are disposed to hyperglycemic damage owing to their large content of mitochondria and diabetic mitochondria often show a decrease in respiratory function and mitochondrial membrane potential (MMP) [199].

1.4.1 Mitochondrial Morphology

Mitochondria are dynamic organelles that change in number and shape during development. Mitochondria constantly fuse and divide and an imbalance of the two processes can cause an overall change in mitochondrial morphology [200]. Recent studies have shown that abnormal changes in mitochondrial morphology can reduce respiratory capacity and increase ROS formation. Normal mitochondria appear as thin filaments microscopically [201], while under diabetic conditions, the mitochondria swell, and change from a filamentous shape to a spherical and dilated shape. In the STZ-induced Type 1 diabetic model, there was a loss of cristae within mitochondria in skeletal muscle. This was accompanied with an increase of electron-dense granules and lipid droplets formation around the mitochondria [202]. In alloxan-induced diabetic rats, there was a decrease in mitochondrial number and mitochondrial membrane damage in hepatocytes and cardiomyocytes. The impaired mitochondria were round, swelled, had shorter cristae and less dense granules as well as exhibited a foamy and vacuolated matrix. These shape changes may indicate that the mitochondria are losing regular respiratory functions under diabetic conditions [203].

1.4.2 Mitochondrial Membrane Potential

Mitochondrial membrane potential is generated by protons pumped into the intermembrane space at complexes I, III, and IV and the resultant proton flux back into matrix. The flow of protons into the matrix that is not coupled to ATP generation is referred to as proton leak or uncoupled respiration. However, in most cells including neurons, most of the proton flux into the matrix is coupled to ATP generation.

The mitochondria membrane potential ($\Delta\psi$) is an important parameter to assess mitochondria bioenergetics, as it reflects the pumping of hydrogen ions across the inner mitochondrial membrane during the process of electron transport and oxidative phosphorylation, which is the driving force behind ATP production [204]. Mitchell's chemiosmotic theory explains that mitochondrial respiration can create a transmembrane electric potential by generating a electrochemical gradient of protons across the inner membrane, which can in turn regulates the overall electron transport rate in the respiratory chain to generate ATP [205, 206].

In adult sensory neurons isolated from STZ induced diabetic rats, there was depolarization of the mitochondrial $\Delta\psi$ at 3- to 6-week of diabetes. Sensory neurons from STZ diabetic animals had a 50% decrease in the level of the mitochondrial $\Delta\psi$ compared with age-matched control animals and this could be prevented by insulin treatment [194], indicating that depolarization of the mitochondrial membrane in diabetes was due to hyperglycemia. In embryonic sensory neurons, treatment of hyperglycemia also induced depolarization of the mitochondrial $\Delta\psi$ [179]. In neurons, a loss of neurotrophic support induced the translocation of proapoptotic members of the Bcl-2 family of proteins, such as Bim and Bax, to the mitochondrial outer membrane causing mitochondrial membrane

depolarization and cytochrome c release [207-209]. In STZ-diabetic rats, impaired mitochondrial function was associated with reduced Bcl-2 expression [194]. In SCs exposed to high glucose, the resulting degeneration was linked to impaired functioning of Bcl-x [210].

According to the chemiosmotic theory [206], ADP-to-ATP turnover rate is determined by the mitochondrial $\Delta\psi$. Proton leak, opening of the mitochondrial permeability transition pore (MPTP), fatty acids interacting with mitochondrial proteins, or disruption of the inner membrane can cause mitochondrial $\Delta\psi$ loss [211, 212]. Mitochondrial $\Delta\Psi$ potential can be measured by radioisotope, voltammetry or fluorimetry [213], based on the Nernstian equilibrium distribution of lipophilic cations [214]. In mitochondria, these cations accumulate inside negatively charged mitochondrial matrix binding with lipids and proteins to aggregate and even shift spectra [215-218]. Particular fluorophores that enter mitochondria can be used to determine potentials conveniently. Mitochondrial $\Delta\psi$ can be measured in isolated mitochondria or intact cells with various mitochondria-targeted potential-sensitive fluorescent compounds, such as methyltriphenylphosphonium (TPMP), tetraphenylphosphonium (TPP) or tetramethylrhodamine methyl ester (TMRM) [213, 219].

1.4.3 Diabetes Impairs Mitochondrial Bioenergetics and Biogenesis

In the mitochondrial respiratory chain, NADH coenzyme Q reductase (Complex I) receives electrons from the Krebs cycle electron carrier NADH, and passes them to coenzyme Q (ubiquinone), which also receives electrons from succinate dehydrogenase (complex II). Coenzyme Q passes electrons to cytochrome bc1 complex (complex III), which passes them to cytochrome c (Cyt c). Cyt c passes electrons to cytochrome c oxidase

(Complex IV), which uses the electrons to reduce oxygen into water. During this electron transport process, hydrogen ions are transported into the transmembrane area. This mitochondrial membrane potential allows the formation of ATP by ATP synthase with hydrogen ions flowing back into the matrix through the F_0 component of ATP synthase. This reverse flux releases free energy generated during electron carriers (NAD^+ and Q) oxidation driving ATP synthesis, catalyzed by the F_1 component of ATP synthase [220].

Mitochondria respiration, known as aerobic respiration, is an oxygen-dependent process for ATP production. Thus, oxygen consumption of mitochondria is often used to evaluate mitochondrial bioenergetics. Oxygen consumption rate (OCR) can be assessed in whole cells or isolated mitochondria by measuring the decrease of O_2 in the medium. OCR can be widely used to study mitochondrial function by adding various of mitochondrial respiratory inhibitors, ATP synthase activators or both [221].

The basal OCR reflects coupled mitochondrial respiration as well as uncoupled consumption of oxygen forming ROS at mitochondrial and non-mitochondrial enzymatic sites. Coupled and uncoupled respiration can be distinguished by examining the effects of oligomycin, which is an ATP synthase inhibitor. Maximal oxygen consumption rate, provoked by addition of a mitochondrial uncoupling agent such as carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP), provides an index of maximal energetic capacity. FCCP is protonophore and promotes the transport of hydrogen ions into transmembrane areas before they can be used for ATP generation. Since the rate of electron transport is limited by the MMP, dissipation of the MMP by FCCP removes the electrochemical gradient allowing electron transport to occur at its maximal rate and increasing OCR. The increase in OCR induced by FCCP is referred to as the Maximal

respiratory capacity (MRC) and this assesses the functional integrity of respiratory chain once electron transfer is no longer constrained by the proton gradient. Spare respiratory capacity (SRC) is the difference between MRC and basal respiration and reflects the energetic reserve that is available to cells to respond to stress [222, 223]. Diabetes often decreases both MRC and SRC indicating a decline in overall mitochondrial bioenergetics.

MRC, SRC and mitochondrial oxidative phosphorylation protein expression were reduced in cultured sensory neurons from diabetic compared to non-diabetic animals [224-226]. In addition, in cultured diabetic sensory neurons, there was an abnormal mitochondrial fission-fusion equilibrium, resulting in small mitochondria fragmentation [185, 227]. *In vitro* hyperglycemic stress to sensory neurons can also reduce mitochondrial protein translation and decrease mitochondrial OCR [228]. In peripheral nerves, DRG sensory neurons are not the only target of hyperglycemic stress. In SCs cultured under hyperglycemic conditions, there was reduced coupled mitochondrial respiration efficiency and altered mitochondrial protein expression [229]. Hyperglycemia-induced mitochondrial dysfunction in SCs may additionally contribute to sensory neuron dysfunction and mediate DPN development in diabetic animals and humans.

Disruption of mitochondrial biogenesis has also been suggested to cause reduced mitochondrial number, depolarized mitochondrial $\Delta\psi$ and reduced oxidative phosphorylation capacity in diabetes.

Mitochondrial biogenesis and function can be partially modulated, through the AMP-activated protein kinase (AMPK), sirtuin (SIRT), and peroxisome proliferator-activated receptor-γ coactivator α (PGC-1α) signaling axis that senses cellular requirements for energy [186, 230, 231]. These factors regulate the expression of mitochondrial replication

and oxidative phosphorylation genes [232]. PGC-1α activity is enhanced by AMPK phosphorylation and SIRT1 deacetylation. Energy expenditure, such as caloric intake and exercise reduction, leads to an increase in the ratio of AMP to ATP, which activates AMPK [231, 233]. In turn, this increases PGC-1α transcription and phosphorylation. Caloric restriction or exercise also increased NAD^+ relative to NADH and activates the NAD^+-dependent histone deacetylase, SIRT1. SIRT1 enhances PGC-1α by deacetylation at specific lysine residues [234]. AMPK signaling and PGC-1α expression were depressed in DRG sensory neurons in animal models of Type 1 or Type 2 diabetes [235]. This means under diabetic conditions within the perikarya, the high intracellular glucose concentration triggers the down-regulation of the AMPK/SIRT/PGC-1α pathway [236].

PGC-1α is mainly expressed in tissues with high oxidative demands such as heart, liver, brain, skeletal muscle and brown adipose [237]. PGC-1α activation mediates the regulation of gluconeogenesis, glycolysis, lipogenesis, peroxisomal and mitochondrial fatty acid oxidation, and mitochondrial respiration efficiency [238-240]. Over-expression of PGC-1α induces an increase in the number and function of mitochondrial [241]. In contrast, the absence of PGC-1α results in abnormal glucose homeostasis and decreased mitochondrial function [242].

In DRG sensory neuron from STZ-induced diabetic mice, the protein expression of p-AMPK and PGC-1α were significantly decreased by 8 weeks of diabetes [230]. The decreased activation of AMPK and PGC1-α is associated with the down-regulation of a series of mitochondrial biogenesis proteins, including oxidative phosphorylation enzymes (NDUFS3, COX IV), the Kreb's cycle enzyme (citrate synthase) and the mitochondrial ROS scavenger MnSOD [186, 230, 243]. This down-regulated AMPK/SIRT/PGC-1α

pathway correlated with mitochondrial dysfunction in sensory neurons during DPN development [230].

Unfortunately, all drug candidates for DPN so far, showed modest efficacy or adverse side effects in clinical trials. Identification of novel therapeutic targets for DPN is urgent. Instead of focusing on only one target, our group proposes that through the induction of cytoprotective chaperones, DPN development can be delayed or reversed.

1.5 Heat Shock Proteins

Heat shock proteins (Hsps) are molecular chaperones that help cells through stressful conditions. Hsps are important for the folding of newly synthesized proteins and refolding denatured ones [244]. They function constitutively to stabilize and traffic nascent peptides during cell growth, prevent protein aggregation, or target improperly folded proteins to specific pathways for degradation. Hsps can function as housekeeping proteins or can also be induced by environmental, physical and chemical stresses, such as UV irradiation, heat or cytotoxic drugs [245]. Under stressful conditions such as heat shock or hypoxia, increased expression of Hsps protects the cell by stabilizing unfolded or misfolded peptides, allowing the cell to repair or re-synthesize damaged proteins. They also aid the transport of proteins and proteolytic degradation of damaged proteins [246].

Functional proteins within cells are usually in a fully folded form. However, during protein biogenesis or translocation, proteins can become temporarily unfolded. During this conformational change, regions buried within the mature form may be exposed and interact with other proteins causing unwanted aggregation. To prevent this unwanted aggregation, Hsps can bind with interactive surfaces and facilitate the folding of nascent or unfolded polypeptides. As heat shock and other stress conditions could cause cellular proteins to

unfold partially, the ability of Hsps to protect cells from stress is a key feature of molecular chaperones [247, 248].

Hsps play an important role in neurodegenerative diseases, cancer and metabolic disease. Heat shock can induce Hsp expression through heat shock factor 1 (HSF1) to protect cells against stressful conditions [249]. However, the heat shock response is hampered in diabetes, which is characterized by reduced induction of endogenous protective responses [250].

1.5.1 Heat Shock Protein Classification

Hsps can be classified into five major families based on molecular weight, including Hsp100, Hsp90, Hsp70, Hsp60 and small heat shock proteins; Hsp90, Hsp70 and Hsp60 are the most abundant Hsps [251].

Hsp90

The Hsp90 family contains four isoforms, including inducible cytosolic Hsp90α, constitutive cytosolic Hsp90β, endoplasmic reticulum localized glucose regulated protein 94 (Grp94), and mitochondrial tumor necrosis factor receptor type 1-associated protein (TRAP1). Hsp90 is a dimeric protein consisting of three domains: an N-terminal ATP-binding domain, a middle region, and a C-terminal domain involved in homodimerization [252, 253]. Although the ATPase activity of Hsp90 is weak, ATP binding and hydrolysis is crucial to chaperone function [254].

Hsp90 normally binds to and suppresses HSF1. Denatured proteins can initiate the heat shock response by binding with Hsp90, which results in the dissociation of Hsp90 from HSF1. The dissociation of Hsp90 from HSF1 can lead to the phosphorylation of HSF1, which leads to HSF1 activation. The activated HSF1 forms a homo-trimer that binds to the

promoter of Hsp70 and other chaperone genes to stimulate their transcription [255, 256]. This leads to an increase in Hsp70 mRNA and protein levels in the stressed cells. The induction of Hsp70 upon proteotoxic stress aids the refolding and clearance of mis-folded or damaged proteins.

Hsp70

Hsp70 assists in the folding of newly synthesized proteins, refolding of mis-folded or aggregated proteins, transporting proteins across membranes and controlling regulatory proteins [257-259]. Hsp70 is composed of three major domains: the ATPase N-terminal domain, the substrate binding domain and the C-terminal domain that provides a lid for substrate binding domain [260]. Co-chaperones including Hsp40, Hsp70-interacting protein (HIP), Hsp70-Hsp90 organizing protein (HOP) and Bag-1 can regulate Hsp70 activity [261]. Hsp40 delivers the client protein to Hsp70 and stimulates ATP hydrolysis [262]. HIP assists Hsp70 in retaining the client protein [262] while Bag-1 and HspBP1 increase the rate of ADP dissociation and the release of the client protein [263, 264].

The Hsp70 family is composed of 13 proteins [265]. The most widely studied forms include the constitutively expressed Hsc70, inducible Hsp72 (Hsp70 in mouse), the endoplasmic reticulum localized Grp78 (BiP), and the mitochondrial Grp75 (mtHsp70). Cytoplasmic isoforms include the constitutively expressed Hsc70 and the stress-inducible Hsp72 [266]. Hsc70 is consistently expressed in most tissues and is considered to be the major isoform involved in the housekeeping functions associated with the Hsp70 family. In comparison, Hsp72 is present only in low amounts in unstressed cells but its expression is rapidly increased in response to stress through the transcriptional activation of the gene encoding Hsp72 by HSF1 [259]. Hsp70 can bind to denatured proteins to restore the tertiary

structure and enzymatic activity of proteins in a cycle that is driven by ATP hydrolysis. Mitochondrial targeted proteins can also bind to Hsp70 before being transported into mitochondria. Cytoplasmic Hsp70 helps the proteins to remain a linear state of the protein chain rather than folding, so that the protein can penetrate through the mitochondrial membrane. Once across the mitochondrial membrane, mtHsp70 draws the protein chain into the mitochondrial matrix by consuming ATP [267].

Hsp70 participates in two pathways of disposing wrongly folded proteins: namely proteasomal and lysosomal degradation. Ubiquitiantion labels proteins for degradation by the 26S proteasome. Protein ubiquitination requires ubiquitin-activating enzyme E1 binding to ubiquitin and transfer to the enzyme-carrier E2. E2 can associate with E3 ubiquitin ligase which delivers ubiquitin to the protein designated for degradation. After these three steps, the proteasome recognizes and degrade the ubiquitin labelled proteins. The ubiquitin-proteasome system needs Hsp70 and Hsp40 for degradation of short-lived, mutant or aged proteins [268].

Several studies have reported that expression of Hsp70 can reduce cellular toxicity in several neurodegenerative aggregation disease models. In Alzheimer's disease (AD) models, increased Hsp70 can increase amyloid precursor protein (APP) clearance, inhibit Aβ aggregation and reduce Aβ toxicity on neurons [269-271]. Hsp70 also promotes tau solubility and facilitates tau binding to microtubules [272]. In Huntington’s disease (HD), Hsp70 and its cochaperone Hsp40 can suppress the assembly of the huntingtin protein into spherical and annular polyglutamine oligomers [273].

Hsp60 & Small Hsps

Unlike Hsp70, Hsp60 is the only member in Hsp60 family. It can ensure the correct folding of newly synthesized or imported mitochondrial proteins, and may also promote the refolding of mis-folded proteins generated under stress [274]. It is a mitochondrial chaperonin that is responsible for the transport of linear proteins from the cytoplasm into the mitochondrial matrix for refolding [275]. The capacity of Hsp60 to stabilize mitochondrial proteins, promote mitochondrial protein biosynthesis and prevent induction of mitochondrial apoptosis seems to be crucial for its cytoprotective functions [275, 276]. In the cytoplasm, Hsp60 plays a key role in preventing apoptosis. Hsp60 may inhibit caspase-3 or facilitate the maturation of pro-caspase-3 to its active form [277].

Small Hsps (sHsps) also exhibit chaperone-like activity in preventing unwanted aggregation of target proteins, ensuring the correct folding and refolding of denatured ones alone or in cooperation with other ATP-dependent chaperones [278].

1.5.2 Hsp90 Inhibitors Development

N-terminal Inhibitors

Numerous Hsp90 N-terminal inhibitors have been designed to target the ATP-binding pocket. These compounds block the Hsp90 chaperone activity and cause the release of the premature, unfolded client proteins [279].

Geldanamycin (GM) (Fig.1.5.2.1) was discovered as the first Hsp90 N-terminal inhibitor. It binds in the ATP/ADP pocket in the N-terminal domain of Hsp90 and inhibits the Hsp90 chaperone cycle [280]. GM induced a dose-dependent increase of Hsp70 in an AD cell model, as well as in rat primary cortical neurons [272]. In cells exposed to GM, there was accumulation of premature Hsp90 client protein complexes leading to

recruitment of E3 ubiquitin ligases that target Hsp90 clients for degradation in the proteasome [281, 282]. However, GM has been proved to be too toxic for human use [283].

17-AAG (Tanespimycin) is a GM derivative with Hsp90 inhibitory activity but with less toxicity. However, minimal efficacy against melanoma, renal, and prostate cancers was observed in the Phase 1 trials and no patients in these trials achieved either a partial or complete response by Response Evaluation Criteria in Solid Tumors (RECIST) criteria [284]. The drug development was halted after Phase 2 clinical trials.

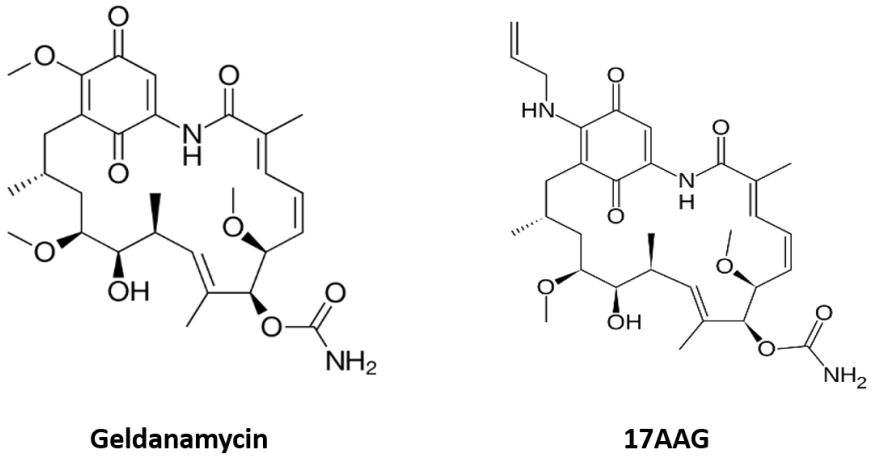


Figure 1.5.2.1: Chemical structures of Hsp90 N-terminal inhibitors

C-terminal Inhibitors

In contrast to N-terminal inhibitors, C-terminal inhibitors prevent formation of the Hsp90 homodimer and prohibit binding with client proteins [285]. Novobiocin was the first Hsp90 C-teriminal inhibitor that was described. Novobiocin (Fig.1.5.2.2) is composed of three distinct parts, including the benzamide side chain, the coumarin core and the noviose sugar [286]. The benzamide side chain binds to the co-chaperone Aha1 and prevents its interaction with Hsp90, while the noviose sugar is critical for interacting with C-terminal domain of Hsp90 [287]. However, novobiocin was reported to bind weakly to the Hsp90 C-terminal nucleotide-binding site and induce degradation of Hsp90 client proteins.

Subsequently, a series of novobiocin analogues were synthesized to improve its Hsp90 inhibitory activity and reduce its cytotoxicity [288].

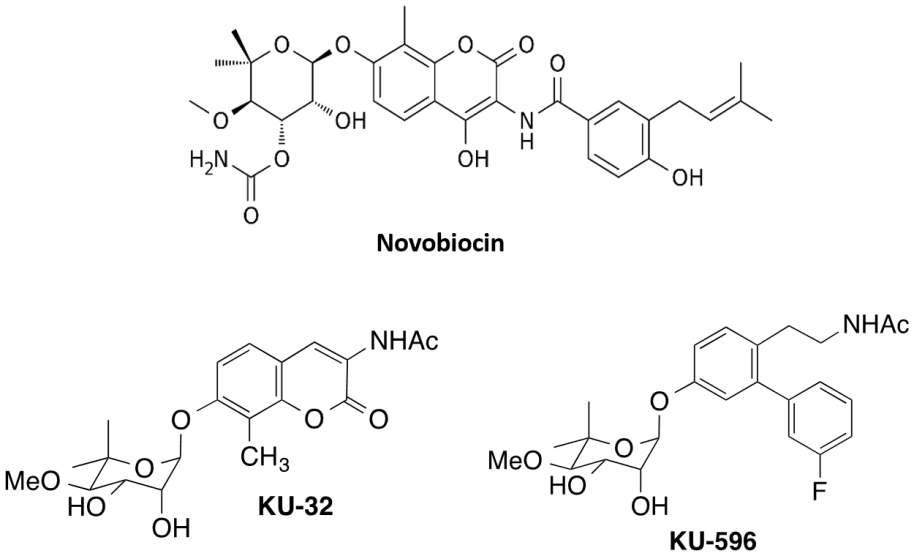


Figure 1.5.2.2: Chemical structures of Hsp90 C-terminal inhibitors

KU-32 and KU-596 were developed as novobiocin-based Hsp90 C-terminal inhibitors. The noviose sugar in KU-32 and KU-596 still allows their binding to Hsp90α [289]. These C-terminal inhibitors can segregate induction of the cytoprotective heat shock response from cytotoxic client protein degradation due to the presence of the acetamide side chain off the coumarin instead of a benzamide or biaryl ring system [290-294]. KU-32 was found to protect against neuronal glucotoxicity and to reverse DPN development in mice [295, 296]. KU-596 was developed as the next generation of novologues, by replacing the coumarin core of KU-32 with a biphenyl ring system and flexible side chain. It has stronger Hsp70 induction ability and enhanced neuroprotective activity against glucotoxicity in sensory neurons [289, 297]. KU-32 and KU-596 can improve mitochondrial bioenergetics in diabetic sensory neurons, and reverse DPN development [298, 299].

1.5.3 Heat Shock Proteins in DPN

Both Type 1 and Type 2 diabetic patients are at high risk of developing macrovascular and microvascular complications. In diabetes, various types of stresses, including dyslipidemia, modification of proteins and lipids, ischemia, hypoxia, apoptosis and oxidative stress, can overwhelm defense system to cause cellular injury [300, 301]. These hyperglycemic stresses can impair protein folding, decrease refolding and induce unwanted aggregation [302]. Hsps may play a crucial role in diabetes by counteracting protein denaturation and facilitating endogenous cellular repair mechanisms. However, hyperglycemic stress may interfere with Hsps synthesis and/or action, to exacerbate diabetic complications [303-305].

In Type 2 diabetes patients with insulin resistance, there were lower levels of Hsp72 mRNA in skeletal muscle [305, 306]. Decreased Hsp72 mRNA levels in skeletal muscle tightly correlated with a poor glucose disposal rate, decreased mitochondrial activity and oxidative capacity [306]. In Type 1 diabetes patients with polyneuropathy, Hsp72 levels in peripheral blood leukocytes were considerably lower than in healthy people [307]. This poor Hsp70 mRNA expression correlated with increased JNK activity [308]. A genetic assessment in diabetic patients identified Hsp70 gene polymorphisms are associated with an increased severity of diabetic foot ulceration, necessity for amputation and longer duration of hospitalization [309].

Overexpression of Hsp72 by transgenic and pharmacologic methods can prevent diet or obesity induced hyperglycemia, hyperinsulinemia, impaired glucose tolerance and insulin resistance. These indicate that Hsp72 plays an essential role in preventing insulin resistance and inflammation in obesity [308]. In addition, long-term hyperglycemia in

diabetes also reduced the HSF-1 mediated heat shock response in heart and muscle [310, 311]. Pharmacological induction of Hsp72 by a hydroxylamine derivative, BGP-15, activated HSF1 and improved insulin-sensitivity in early clinical trials [312]. Bimoclomol can elevate various Hsps levels, typically Hsp72. In STZ-induced Type-1 diabetic rats, bimoclomol can improve motor and sensory nerve conduction velocity (NCV) deficits as well as the ischemic conduction failure in sciatic nerve by anti-ischemic and chaperone-inducing properties [313]. Arimoclomol is another hydroxylamine derivative that is currently under clinical development, with a 4 time longer half life time than bimoclomol. Arimoclomol can dose-dependently improve both the motor and sensory functions in DPN, with more pronounced effects than bimoclomol [314, 315]. Physical exercise can also increase Hsp72 expression and help protect against cell injury and repair damaged nerves in STZ-induced diabetic rats [316].

The novobiocin-derived Hsp90 C-terminal inhibitor KU-32 reversed diabetic mechanical and thermal sensory hypoalgesia along with improving motor and sensory NCV in STZ-induced diabetic mice. KU-32 also prevented the loss of unmyelinated intra-epidermal nerve fibers innervation in diabetic mice to reverse DPN development [226, 295]. However, KU-32 did not change serum glucose or insulin levels, indicating the drug effect is more directly on peripheral nerves [295].

In vivo KU-32 treatment improved mtBE in both the Type 1 and Type 2 models, and this correlated with a reversal of DPN symptoms [299]. Hsp70 is necessary for KU-32 to reverse DPN development since the drug had no effect on reversing DPN sensory hypoalgesia in diabetic Hsp70 knockout (KO) - (Hsp70.1 and 70.3) mice. This is also

associated with the loss of effect on improving mtBE deficits in diabetic Hsp70 KO sensory neurons [295, 299].

Hyperglycemia reduced the translation of a variety of mitochondrial proteins, including MnSOD, which is associated with an increase in mitochondrial superoxide levels and a decrease in mtBE. KU-32 increased the translation of several cytosolic and mitochondrial chaperones, components of the mitochondrial respiration chain and MnSOD in hyperglycemically stressed neurons. These changes correlated with decreased mitochondrial superoxide levels and increased mitochondrial respiratory capacity. These data indicate that KU-32 may improve the sensory defects of DPN by improving mitochondrial function and enhancing chaperone levels [228]. However, the mechanism by which KU-32 can improve mtBE in diabetic sensory neurons has not been determined.

KU-596 is the next generation of noviosylated analogs that has stronger Hsp70 induction and neuroprotective ability. *In vivo* treatment of 2–20 mg/kg KU-596 improved hypoalgesia and mtBE deficits of sensory neurons in STZ-induced Type 1 diabetic mice in a dose-dependent manner. Similar to KU-32, the drug effect is dependent on Hsp70 since KU-596 could not improve these neuropathic deficits in diabetic Hsp70 KO mice [298].

The efficacy of KU-32 and KU-596 in reversing several indices of DPN strongly supports that modulating molecular chaperones might be a promising method to prevent nerve injury in DPN. Although DPN is primarily a metabolic disease, hyperglycemic stress can increase oxidative damage to proteins, impair protein folding, decrease refolding or induce protein aggregation [186, 302]. In this regard, increasing protective chaperones in peripheral nerves may provide a level of quality control by enhancing protein folding or refolding. Thus, modulating chaperone is beneficial to interfere with the progression of

diabetes and its complications. As reported in numerous studies from our own group and other groups, prolonged diabetes can decrease mitochondrial respiratory chain activity in adult sensory neurons [194, 226, 317]. The decrease in SRC means diabetic neurons are more susceptible to stress because they cannot increase energy supply sufficiently to match the demand [221, 318]. Previous studies from our lab have demonstrated that pharmacologically modulating Hsp70 by KU-32 or KU-596, has a beneficial effect on mtBE and improves SRC [226, 298]. Thus, increasing the expression of mitochondrial chaperones might protect against mitochondrial dysfunction in diabetic sensory neurons.

Though our previous publication has suggested *in vivo* treatment of next generation novologue KU-596 can improve mitochondrial bioenergetics in diabetic sensory neurons and reverse DPN in Hsp70 dependent manner, we have not identified the mechanism by which KU-596 improves mitochondrial function in diabetic sensory neurons. Understanding the mechanistic contributions of Hsp70 on improving mtBE will advance novologue development in reversing DPN.

In vivo treatment of KU-596 can improve mtBE and modulate inflammatory response in diabetic sensory neurons. Since there are many factors *in vivo* that can mediate the drug effect on DPN, it is hard to identify if the drug directly or indirectly improving sensory neuron mtBE after *in vivo* treatment. For example, axons of sensory neurons are wrapped with SCs, and SCs may also mediate the drug effect since the drug needs to enter the myelin first and get into the axon. To more directly assess the ability of KU-596 to improve neuronal bioenergetics, the focus of these studies is to use an *ex vivo* approach. In this model, sensory neurons are isolated from non-diabetic and diabetic mice and subsequently

treated with KU-596, to identify direct drug effect on sensory neuron bioenergetics and oxidative stress.

In this current study, we sought to determine if Hsp70 enhances mtBE and decreases oxidative stress in diabetic sensory neurons. In order to determine the cause and effect relationship between mtBE and oxidative stress, we knocked down MnSOD by shRNA to increase oxidative stress in mitochondria and determine if KU-596 can decrease the oxidative stress and improve mitochondrial function. Our goal is to identify whether decreasing superoxide levels is necessary to improve mtBE by the modulation of Hsp70.

Chapter 2. KU-596 Improves Mitochondrial Bioenergetics and Decreases Oxidative Stress in Diabetic Sensory Neurons via Hsp70

2.1 Introduction

Diabetes affected about 415 million adults worldwide in 2015 and about 50-60 percent of these patients are likely to develop diabetic peripheral neuropathy (DPN) [3, 319]. DPN is one of the most prevalent complications of diabetes and manifests as a distal, symmetric and sensorimotor neuropathy that causes positive (sharp pain) and negative (numbness, injury insensitivity) symptoms. Insensate DPN is a main cause of foot ulceration, infection and non-traumatic amputation as the loss of feeling often leads to unnoticed tissue injuries. Unfortunately, other than glycemic control, there is still no approved drugs to help improve nerve function and resolve insensate DPN [67, 320].

The mechanistic pathogenesis of DPN is complex but the developing consensus is that sustained hyperglycemia drives numerous secondary biochemical changes that contribute to a progressive decline in sensory neuron function and the loss of peripheral innervation that characterizes moderate to severe cases of DPN. Though accumulation of advanced glycated end products, abnormal protein kinase C activity, increased oxidative stress, mitochondrial dysfunction and increased flux of glucose through polyol and hexosamine pathways are known to contribute to the development of DPN [321], compounds targeting these pathways have shown little efficacy in clinical trials [322]. However, targeting a single pathway or protein considered to contribute to disease progression may not be an effective approach to manage complex, chronic neurodegenerative diseases. Alternatively, it may prove sufficiently beneficial to pharmacologically enhance the activity of endogenous neuroprotective pathways to help neurons more effectively tolerate recurring

hyperglycemic stress, which may ameliorate the development of DPN or reverse pre-existing insensate symptoms [323]. In this regard, we have provided extensive proof-of-concept that pharmacologic modulation of neuroprotective heat shock proteins may provide a novel, safe and effective approach toward the therapeutic management of DPN [290, 295, 298, 299, 324, 325].

Heat shock proteins (HSPs), such as Hsp70 and Hsp90 function as molecular chaperones that are important for folding newly synthesized proteins and for refolding or clearing damaged/denatured proteins [326, 327]. Hsp90 contains a C-terminal domain that is essential for forming a functional Hsp90 homodimer and an intrinsic N-terminal ATPase activity which, in conjunction with a cohort of co-chaperones, directs the folding of "client" proteins into their biologically active conformations [328-330]. However, Hsp90 also serves another important biologic role since it is a direct regulator of the cellular heat shock response **(HSR).** Hsp90 binds to heat shock factor 1 and suppresses its transactivating capacity [331, 332]. Upon exposure to proteotoxic stress or binding of small molecules, conformational changes in Hsp90 disrupt its interaction with HSF1 and lead to the transcriptional induction of cytoprotective proteins, such as Hsp70 [331].

Though numerous small molecules that function as N-terminal Hsp90 inhibitors have been developed to treat cancer [333], small molecules that interact with the C-terminal of Hsp90 show strong promise as therapeutic candidates to treat diabetic neurodegeneration [290]. Novobiocin was identified as the first ligand to interact with the C-terminal of Hsp90 [334] and the development of diverse libraries of novobiocin derivatives have identified KU-596 as a noviosylated fluoro-biphenyl ethylacetamide "novologue" [335] that demonstrates considerable efficacy in improving insensate DPN [298]. Treating diabetic

mice with KU-596 improved sensory hypoalgesia and this required Hsp70 since the drug could not improve sensory nerve function in diabetic Hsp70 knockout (KO) mice. Moreover, improved neuronal function following KU-596 therapy correlated with enhanced mitochondrial bioenergetics (mtBE) and a decrease in markers of oxidative stress and inflammation in diabetic dorsal root ganglia (DRG) [298]. However, the mechanism by which KU-596 improves mtBE in diabetic sensory neurons remains to be determined.

Mitochondrial dysfunction is a major pathogenic contributor to DPN and reduced respiratory chain activity is observed in sensory neurons [236, 336, 337]. Since an earlier generation of novobiocin derivatives decreased superoxide levels and increased the expression of Mn superoxide dismutase (MnSOD) in hyperglycemically stressed embryonic sensory neurons [338], these data raised the possibility that novologues may require MnSOD to improve mtBE.

Though *in vivo* treatment of KU-596 improved sensory neuron mtBE and decreased genetic indicators of oxidative stress and inflammation in diabetic DRG [298], it was unclear if this may be a direct or indirect effect of the drug. To determine if KU-596 can directly improve mtBE and whether this is related to changes in oxidative stress, sensory neurons were isolated from diabetic mice who had developed insensate DPN and were treated *ex vivo* with KU-596. By manipulating glucose conditions and downregulating the expression of MnSOD, the current study provides strong evidence that KU-596 can directly improve mBE and that this is related to a decrease in glucose-induced superoxide production.

2.2 Materials and Methods

2.2.1 Materials

2.2.1.1 Animals

C57Bl/6N Hsd wild type (WT) mice were obtained from Harlan Laboratories and the Hsp70.1/70.3 double knockout mice on a C57Bl/6 background (Hsp70 KO) mice were obtained from the Mutant Mouse Resource and Research Center. Experimental animals were generated by in-house breeding and were maintained on a 12-hour light/dark cycle with ad libitum access to water and 7022 NIH-07 rodent chow (5.2% fat). All animal procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee and in compliance with standards and regulations for the care and use of laboratory rodents set by the National Institutes of Health.

2.2.1.2 Reagents

Table 2.2.1: List of major reagents used in this study

Name	Company	Catalog Number
Streptozotocin (STZ)	Sigma-Aldrich	572201
Oligomycin	Sigma-Aldrich	75351
Carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP)	Sigma-Aldrich	C2920
Rotenone	Sigma-Aldrich	R8875
Antimycin A	Sigma-Aldrich	A8674
Poly(DL)ornithine	Sigma-Aldrich	P8638
Collagenase Type IV	Life Technologies	17104-019
Laminin	Corning	354232
Percoll	Sigma-Aldrich	P1644

mitoTracker Deep red	Fisher	M22426
mitoSOX	Fisher	M36008
mito-TEMPO-H	Enzo Life Sciences	ALX-430-171-M005

Synthesis of KU-596

KU-596, N-(2-(5-(((3R,4S,5R)-3,4-Dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)oxy)-3'-fluoro-[1,1'-biphenyl]-2-yl)ethyl)-acetamide, was synthesized and structural purity (>95%) verified as described previously [297].

2.2.2 Methods

2.2.2.1 Induction of diabetes

Eight-week-old male and female WT and Hsp70 KO mice were randomly assigned to either the non-diabetic or diabetic group. Mice were rendered diabetic with an intraperitoneal injection of 100 mg/kg streptozotocin (STZ) freshly prepared in 50 mM sodium citrate, pH 4.5 and given over two consecutive days. Control mice were treated similarly but received only the vehicle. One week after the second injection, mice were fasted for 6 hrs and those with a fasting blood glucose ≥ 290 mg/dl were deemed diabetic [299].

2.2.2.2 Mechanical and Thermal Sensitivity Assessments

To monitor the development of diabetic neuropathy, the onset of a mechanical and thermal hypoalgesia was determined over 14 weeks after the induction of diabetes. This time frame was chosen as our previous work found that this duration of diabetes was sufficient to promote mitochondrial dysfunction in sensory neurons [298, 299]. Mechanical

sensitivity was assessed using a Dynamic Plantar Aesthesiometer (Stoelting Inc.) fitted with a stiff monofilament that was applied to the plantar surface at an upward force of 8g. Thermal sensitivity was assessed by paw withdrawal latency to a ramping, focal heat using a Hargreaves Analgesiometer (Stoelting Inc.) [299]. Responses from each animal were measured four times on alternate feet and averaged.

2.2.2.3 Adult DRG Sensory Neuron Isolation

After 12-14 weeks of diabetes, adult sensory neurons were isolated from the non-diabetic and diabetic WT or Hsp70 KO mice as previously described [226, 299]. Mice were euthanized via gradual CO_2 asphyxiation, the spinal column dissected and the L4–L6 DRG collected from 6 - 8 mice in each group. Connective tissue was removed and the ganglia were placed in 1 ml serum-free Ham's F10 medium (Corning, #10-070-CV). The trimmed ganglia were dissociated by incubating in 1 ml of 1.25% collagenase for 45 min at 37 ℃, and subsequent addition of 1 ml 2% trypsin for 30 min at 37 ℃. Digested cells were centrifuged at 1000 ×g for 5 min at 4ºC and the pellet was further dissociated by trituration with a fire polished glass pipette in F10 medium. The cell suspension was overlain onto a 10 ml gradient of sterile iso-osmotic Percoll (0.9 ml of 10×PBS, 6.5 ml H_2O, 2.6 ml Percoll) and centrifuged at 800 ×g for 20 min at 4ºC to remove all cell debris and myelin fragments. The cell pellet was re-suspended in fresh F10 medium with N2 supplement (Invitrogen, Carlsbad CA). Isolated neurons were plated at a density of 5×10^3 cells/well in F10 medium with N2 supplement on 96 well plates that had been coated overnight with 0.1 mg/ml poly-DL-ornithine and 2 μg/ml laminin (3 hrs) [299, 339]. After plating, the adult sensory neurons were treated with 1μM KU-596 or 0.01% DMSO overnight (~16 hrs) and subsequently incubated in F10 medium containing basal glucose (6.1mM) or 26.1 mM

glucose for an additional 24 hrs. The cells were then used to assess mtBE or superoxide production as shown in Fig. 2.2.2.1.

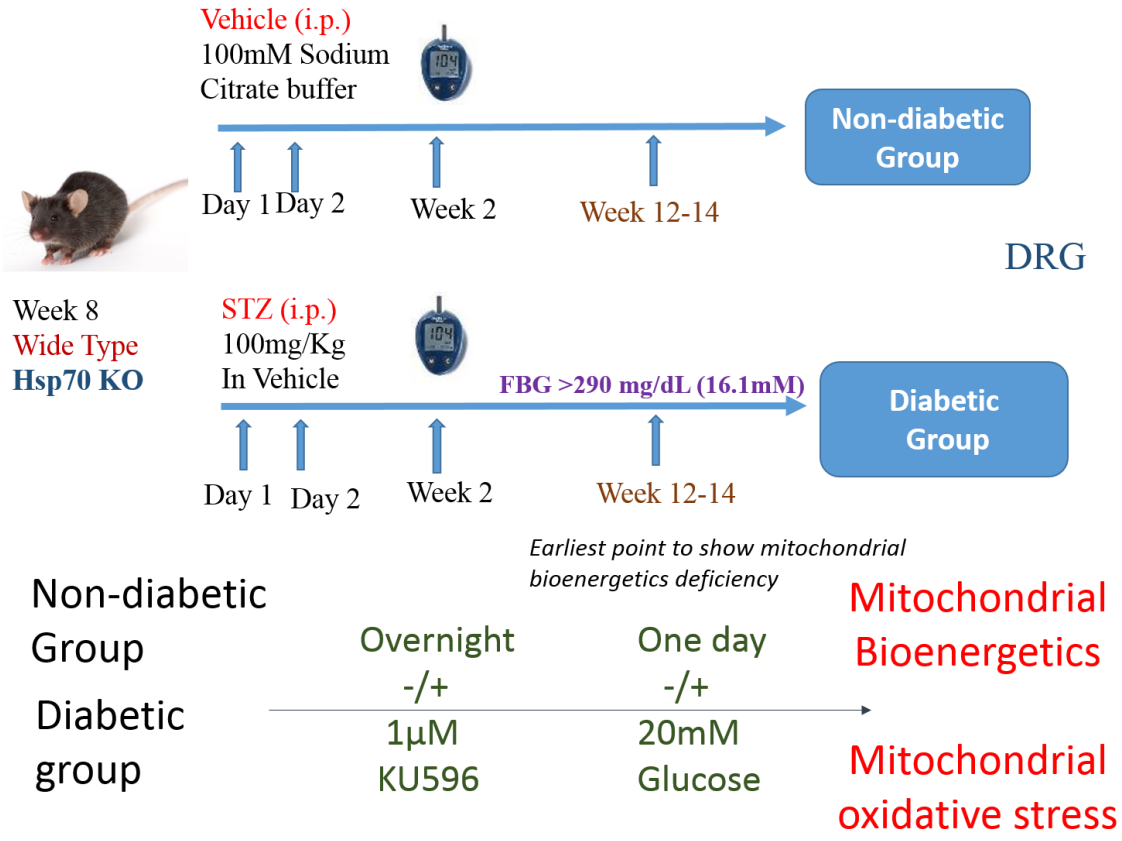


Figure 2.2.2.1: Time-course of this current study

2.2.2.4 Mitochondrial Bioenergetics (mtBE) Assessment

Sensory neuron mtBE were analyzed using a Seahorse XF96 Analyzer (Seahorse Biosciences, Billerica, MA). After incubation in basal or hyperglycemic medium, the cells were placed in bicarbonate free DMEM containing 5.5mM glucose plus 1 mM pyruvate and incubated for 1 hr at 37°C. The plate was then introduced into the Seahorse Analyzer using a 3 minute mix cycle to oxygenate the medium followed by a 4 min measurement of the Oxygen Consumption Rate (OCR). The initial four OCR rates provide a measure of basal respiration prior to assessing mitochondrial dysfunction using various respiratory

chain inhibitors. The portion of basal OCR that is coupled to ATP synthesis was estimated by the decrease in OCR followed by the addition of 1 μg/ml oligomycin, an ATP synthase inhibitor. Residual OCR remaining after oligomycin treatment is from uncoupled respiration (proton leak). Next, Maximal Respiratory Capacity (MRC) was assessed following dissipation of the proton gradient across the inner mitochondrial membrane with 1 μM of the protonophore FCCP. Non-mitochondrial respiration was then assessed by co-injection of 1μM rotenone + 1μM antimycin A [298, 299].

2.2.2.5 MitoSOX Staining

Superoxide levels were measured in some experiments by Mito SOX Red staining in black 96-well plates. After incubation in basal or hyperglycemic medium, MitoTracker Deep Red (300 nM) and MitoSOX Red (400 nM) were added to each well and the cells incubated for 15 min. After washing, the cells were imaged using an Olympus 3I Spinning Disk confocal microscope using excitation/emission wavelengths of 488/573 nm (MitoSox Red) and 644/665 nm (MitoTracker Deep Red). Fluorescence density of the red and deep red signals of 35 - 50 cells per treatment was quantified using Image J analysis software [228].

2.2.2.6 Knockdown of MnSOD Expression by shRNA and Superoxide Assessment by Electron Paramagnetic Resonance (EPR) Spectroscopy

Recombinant adenoviruses expressing shRNA targeting mouse MnSOD (Ad-GFP-U6-mSOD2-shRNA, #shADV-272843) or scrambled shRNA with GFP (Ad-GFP-U6-scrambled-shRNA, #1122) were purchased from Vector BioLabs (Malvern, PA).

The sequence of scrambled shRNA is:

GATCC-AGTACTGCTTACGATACGG-TTCAAGAGA-CCGTATCGTAAGCAGTAC-TTTTTT

The sequence of mouse MnSOD shRNA is:

CCGG-GAGGCTATCAAGCGTGACTTTCTCGAGAAAGTCACGCTTGATAGCCTC-TTTTTTG

To evaluate the silencing efficiency, sensory neurons were infected immediately after plating with either the scrambled shRNA or the mouse MnSOD shRNA for 48 h. Cell lysates were prepared and protein expression was evaluated by immunoblotting with a MnSOD polyclonal antibody (Millipore-Sigma, #06-984).

Immediately after plating, the isolated sensory neurons were treated with vehicle or 1μM KU-596 and infected with the scrambled shRNA or MnSOD shRNA adenoviruses (1:50) for 36 h. The cells were then incubated in basal glucose or 26.1 mM glucose for an additional 24 hr. During the final 4 hrs, the cells were incubated with 100 μM mitoTEMPO-H (1-hydroxy-4-[2-triphenylphosphonio-acetamido]-2,2,6,6-tetramethylpiperidine). mitoTEMPO-H is a mitochondria-targeted antioxidant and is a specific spin trap for detecting mitochondrial superoxide [340]. The cells from 8 wells were pooled together, pelleted by brief centrifugation, re-suspended in 0.2 ml of fresh F-10 medium and frozen at -80 ℃ until use. When performing the EPR measurements, cells were introduced into a quartz tube and EPR was performed with a Bruker EMX plus EPR spectrometer (Billerica, MA, USA) using Bruker Xenon software. Settings for the spectrometer were the following: center field: 3480 G; Sweep width: 100 G; modulation amplitude: 1 G; and time constant, 0.01 s [341, 342]. The cells were recovered from the cuvette and total protein determined using the microBCA assay with bovine serum albumin as the standard.

2.2.2.7 Statistical Analysis:

Student's t test, one-way ANOVA, and multivariate-ANOVA were conducted for between-group comparison by Minitab Software and SAS. Post-hoc analysis using

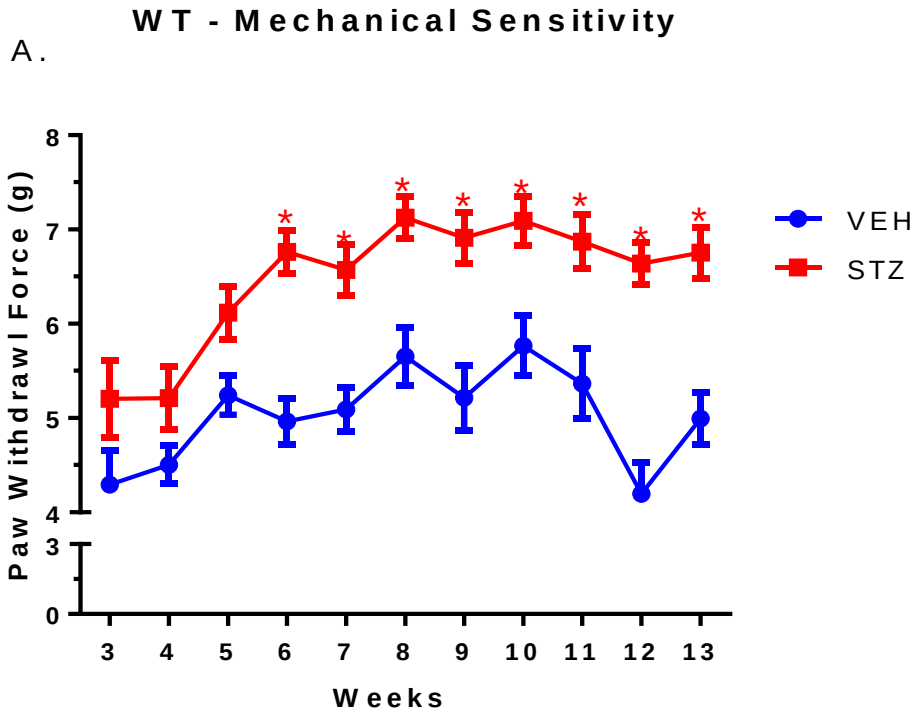
Tukey's test and Wilks' lambda test was applied to determine differences between treatments. All data are presented as mean ± SEM.

2.3 Result

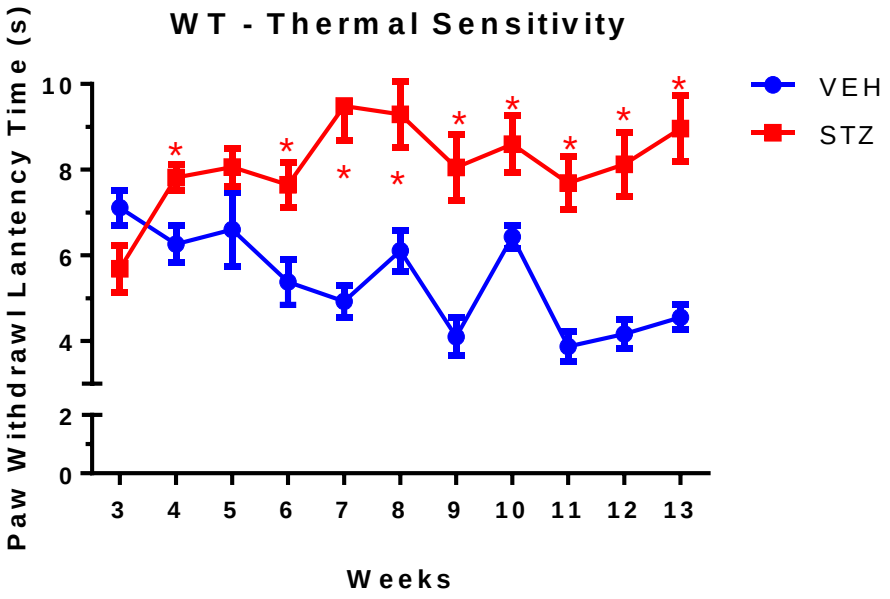
2.3.1 *Ex vivo* Treatment of KU-596 Improves mtBE in Diabetic Sensory Neurons in an Hsp70 Dependent Manner

2.3.1.1 Mouse Models of Diabetic Peripheral Neuropathy

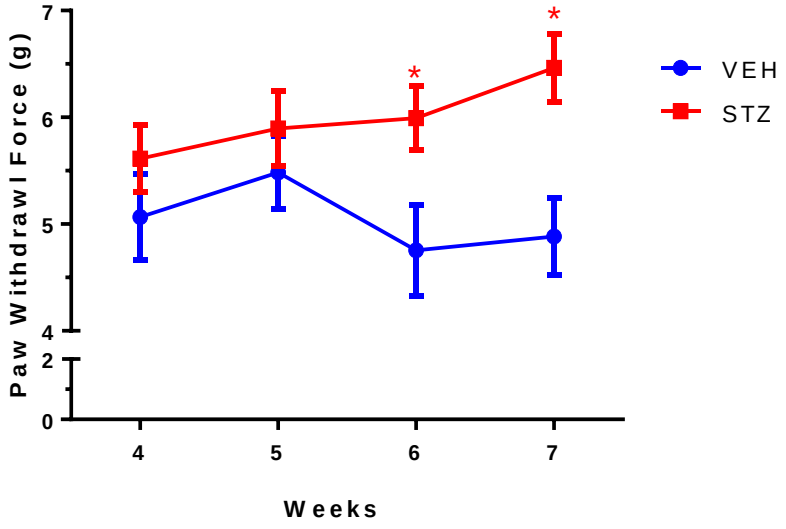
Diabetic mice were monitored for the development of DPN by assessing the onset of mechanical and thermal hypoalgesia. After 6 weeks of diabetes, both WT and Hsp70 KO mice developed mechanical and sensory hypoalgesia by requiring more force or longer time to withdraw their hind paw in response to mechanical pain or thermal stimulation (Figs. 2.3.1.1.A-D). As we reported previously, the development of insensate DPN is consistent throughout the whole course of diabetes.



B.



C. Hsp70 KO - Mechanical Sensitivity



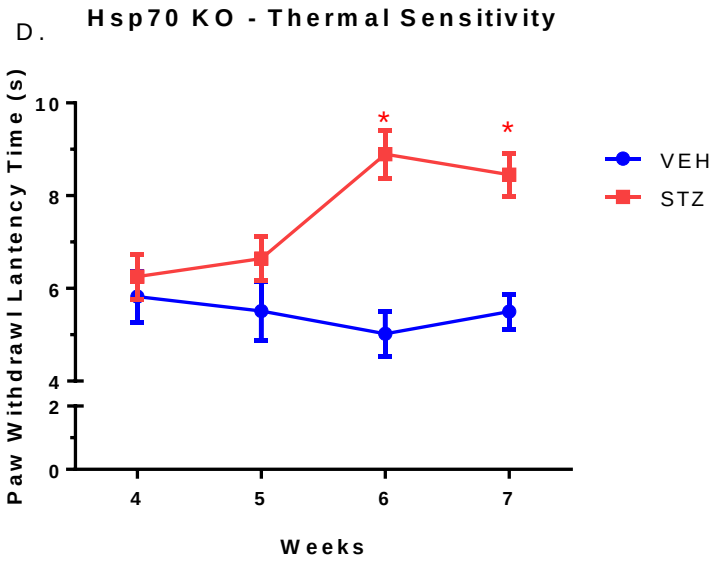


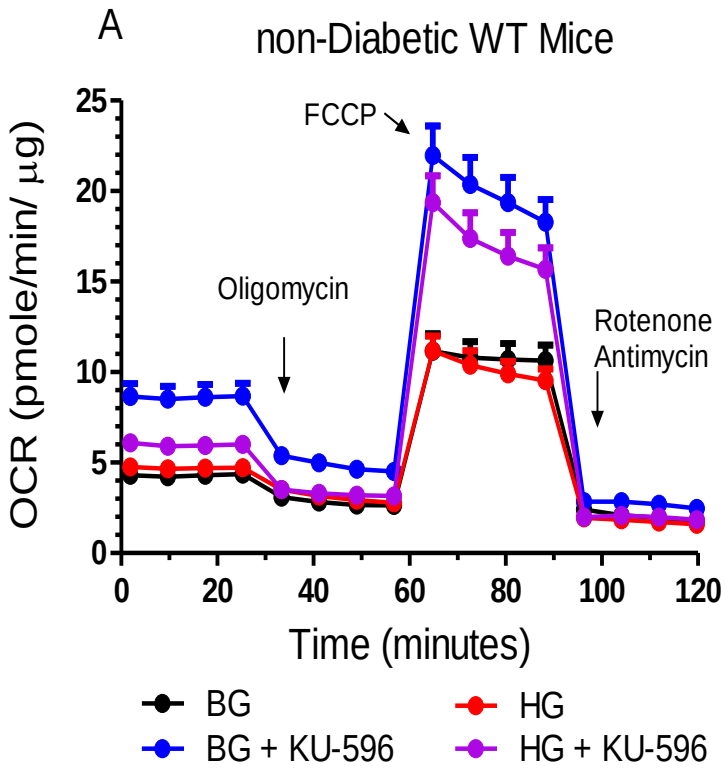
Figure 2.3.1.1 Mechanical and thermal hypoalgesia developed in C57Bl/6 mice over 6 weeks of Type-1 Diabetes.
Diabetes induced significant mechanical (A, C) and thermal hypoalgesia (B, D) in both WT (A, B) and Hsp70 KO (C, D) mice (*:$p<0.05$, vs time-matched non-diabetic mice with VEH).

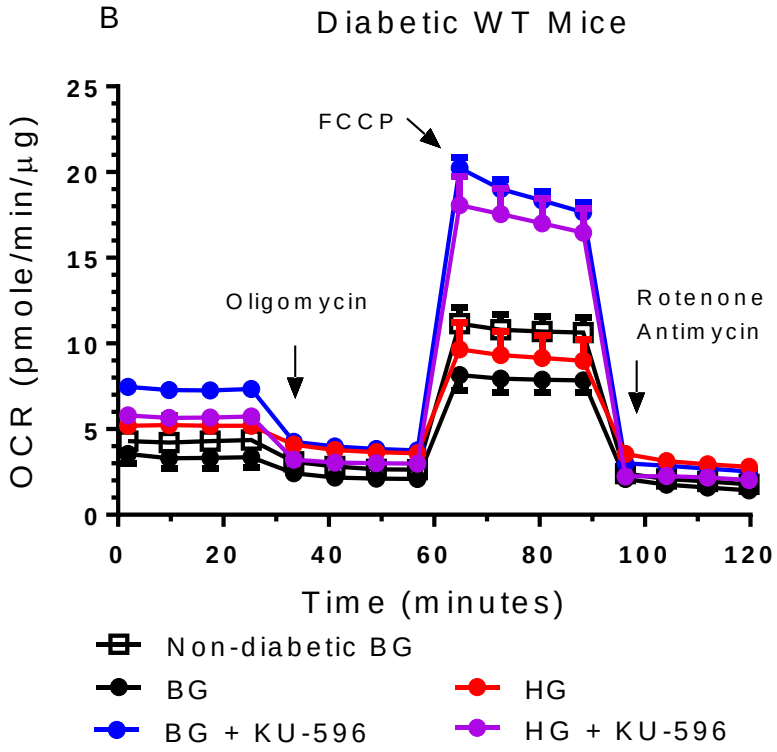
2.3.1.2 KU-596 Improves mtBE in WT Sensory Neurons under Both Normal and Hyperglycemic Conditions

After 14 weeks, adult sensory neurons were isolated from L4 to L6 DRG of non-diabetic and diabetic WT and Hsp70 KO mice since these ganglia provide the sensory axons of the peripheral nerves that are affected in DPN. The isolated neurons were maintained in F-10 medium which has a basal glucose (BG) concentration of 6.1mM. After isolation, the neurons were treated with vehicle (0.01% DMSO final concentration) or 1μM KU-596 overnight (~16 hr). The cells were then maintained for an additional 24 hrs in medium with BG or subjected to hyperglycemic (HG) stress by incubation in F-10 medium containing 26.1 mM glucose. The cells were then prepared for analysis of mtBE by Seahorse XF96 Analyzer as described in the Methods.

One advantage of this *ex vivo* cell culture model is that neurons from diabetic animals have developed metabolic changes due to chronic *in vivo* diabetes that cannot be adequately recapitulated using adult neurons that are only exposed to hyperglycemia and KU-596 *in vitro*. As shown previously [185], this *ex vivo* culture model may more accurately reflect the *in vivo* response of the neurons to further episodes of glucotoxic stress.

Neurons isolated from the diabetic mice and maintained in BG had a significantly lower OCR, MRC and spare respiratory capacity (SRC) than neurons obtained from the non-diabetic mice (Figs. 2.3.1.2.A-D). Though 14 weeks of diabetes was sufficient to significantly impair mtBE (black stripe vs solid black), HG did not significantly decrease MRC in either the non-diabetic or diabetic neurons. Consistent with the ability of *in vivo* therapy with KU-596 to improve mtBE [298], addition of 1μM KU-596 significantly improved OCR, MRC and SRC of non-diabetic and diabetic neurons regardless of the glucose concentration. MRC reflects the rate of maximal electron transport and substrate oxidation achievable in the absence of limits imposed by the inner mitochondrial membrane proton gradient, while SRC indicates the energy reserve available to respond to further environmental demands. The decrease in MRC and SRC suggests that electron transport in the diabetic mitochondria was impaired, and this deficit was clearly improved by KU-596, which suggests that modulating molecular chaperones is efficient at improving mtBE.


A
non-Diabetic WT Mice
FCCP
Oligomycin
Rotenone
Antimycin
OCR (pmole/min/ µg)
25
20
15
10
5
0
0
20
40
60
80
100
120
Time (minutes)
BG
HG
BG + KU-596
HG + KU-596


B
Diabetic WT Mice
FCCP
Oligomycin
Rotenone
Antimycin
OCR (pmole/min/µg)
25
20
15
10
5
0
0
20
40
60
80
100
120
Time (minutes)
Non-diabetic BG
BG
HG
BG + KU-596
HG + KU-596

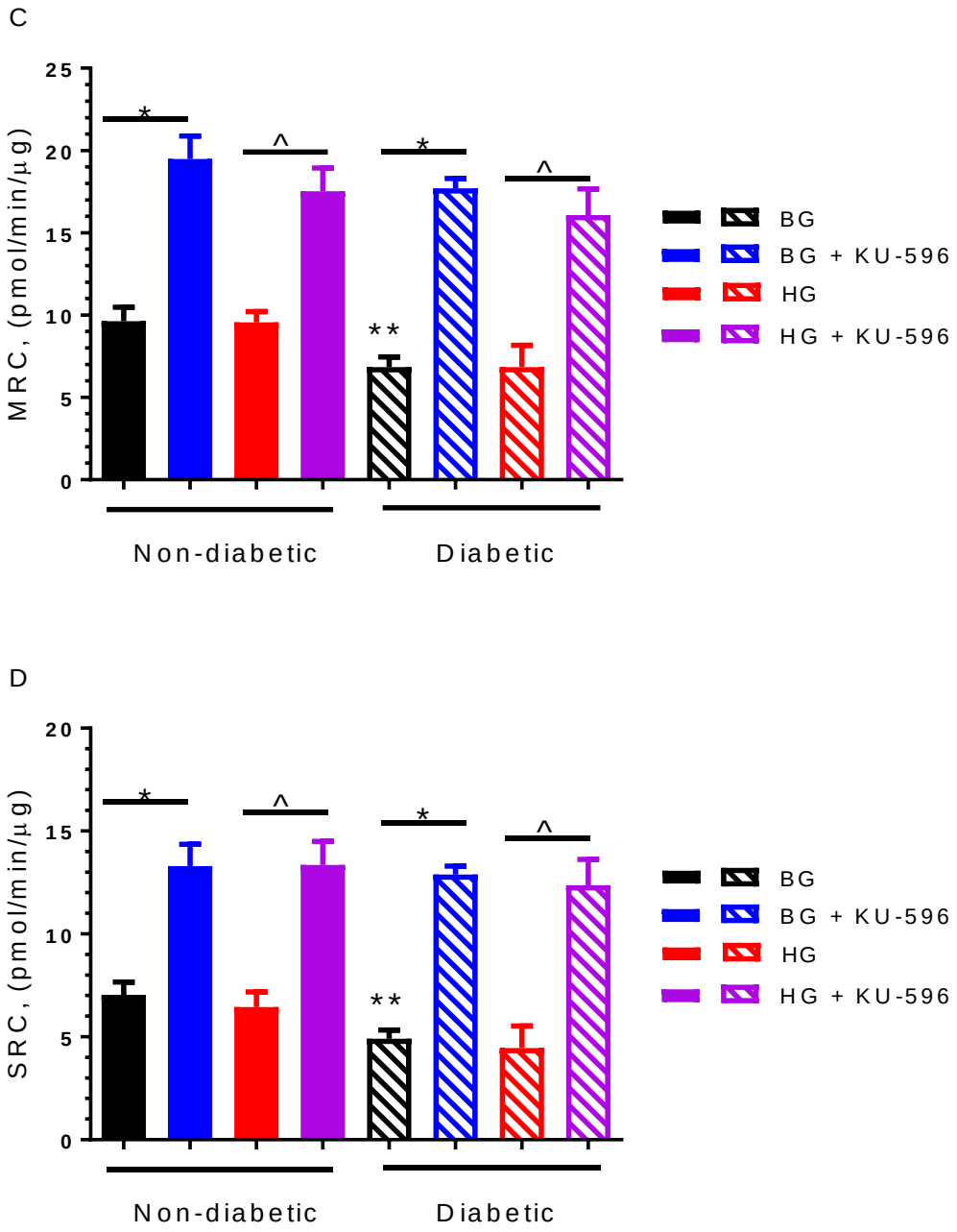


Figure 2.3.1.2 KU-596 improves mBE in hyperglycemically stressed WT diabetic neurons.

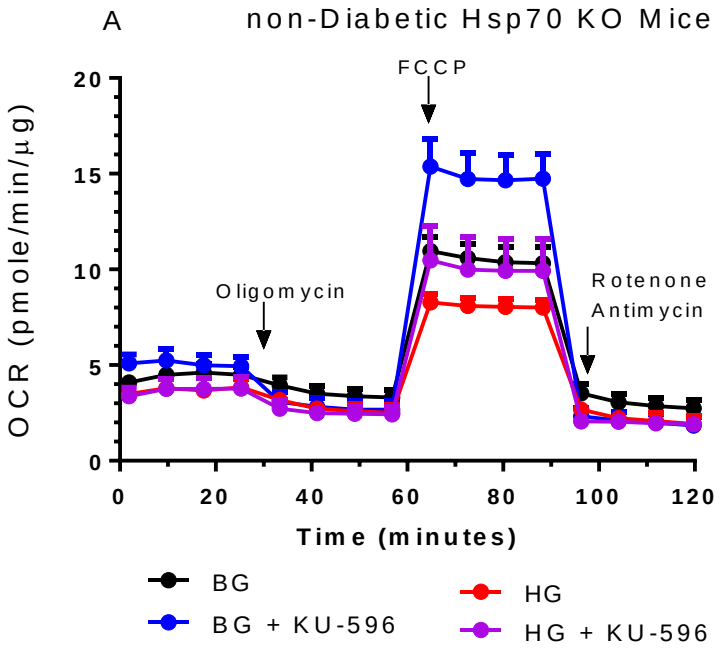
Adult sensory neurons were isolated from non-diabetic or diabetic WT mice and treated +/- 1µM KU-596 overnight in medium containing 6.1 mM basal glucose (BG). Hyperglycemia (HG) was then induced in some cultures for 24 hrs by raising the total glucose concentration to 26.1 mM. A) KU-596 improves OCR in WT non-diabetic sensory neurons under BG and HG conditions. B) KU-596 improves OCR in WT diabetic sensory neurons under BG and HG conditions. C) Quantification of MRC. D) Quantification of

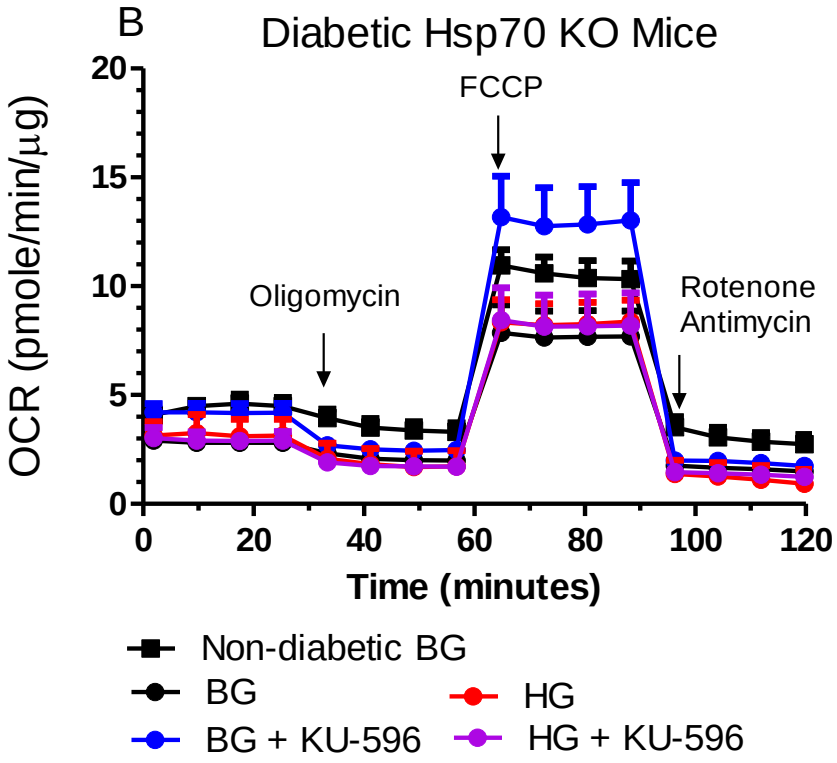
SRC.*, p<0.05 vs BG; ^, p<0.05 vs HG; **, p<0.05 vs non-diabetic BG. Results are from 4-5 wells of neurons per group that were obtained from 8 non-diabetic or diabetic mice.

2.3.1.3 Hsp70 is Necessary for KU-596 to Improve mtBE in Hyperglycemically Stressed Sensory Neurons.

Mechanistically, previous data supported that Hsp70 was necessary for the drug to improve DPN when administered *in vivo* [298]. However, whether Hsp70 may have directly or indirectly contributed to improved nerve function was unclear. To determine if neuronal Hsp70 was needed to improve mtBE, the above studies were repeated using sensory neurons isolated from non-diabetic or diabetic Hsp70 KO mice.

In contrast to the response in WT mice, diabetes suppressed, but did not sufficiently decrease MRC and SRC to a level that was significantly different from the non-diabetic Hsp70 KO mice (black stripe vs black bar) (Figs. 2.3.1.3.A, C and D). More unexpected was that KU-596 treatment improved MRC and SRC in non-diabetic and diabetic Hsp70 KO neurons that were incubated in BG. On the other hand, when subjected to HG stress, KU-596 treatment could not improve MRC in either non-diabetic or diabetic neurons (Figs. 2.3.1.3.B, C and D). These data suggest that KU-596 may have some Hsp70-independent effects under certain cellular conditions to improve mtBE, but this response is lost in the presence of HG stress. Thus, hyperglycemia is shifting efficacy to improve mtBE to become more Hsp70-dependent, possibly due to the role of Hsp70 in regulating mitophagy [343].


A
non-Diabetic Hsp70 KO Mice
FCCP
Oligomycin
Rotenone
Antimycin
OCR (pmole/min/µg)
Time (minutes)
BG
BG + KU-596
HG
HG + KU-596


B
Diabetic Hsp70 KO Mice
FCCP
Oligomycin
Rotenone
Antimycin
OCR (pmole/min/µg)
Time (minutes)
Non-diabetic BG
BG
BG + KU-596
HG
HG + KU-596

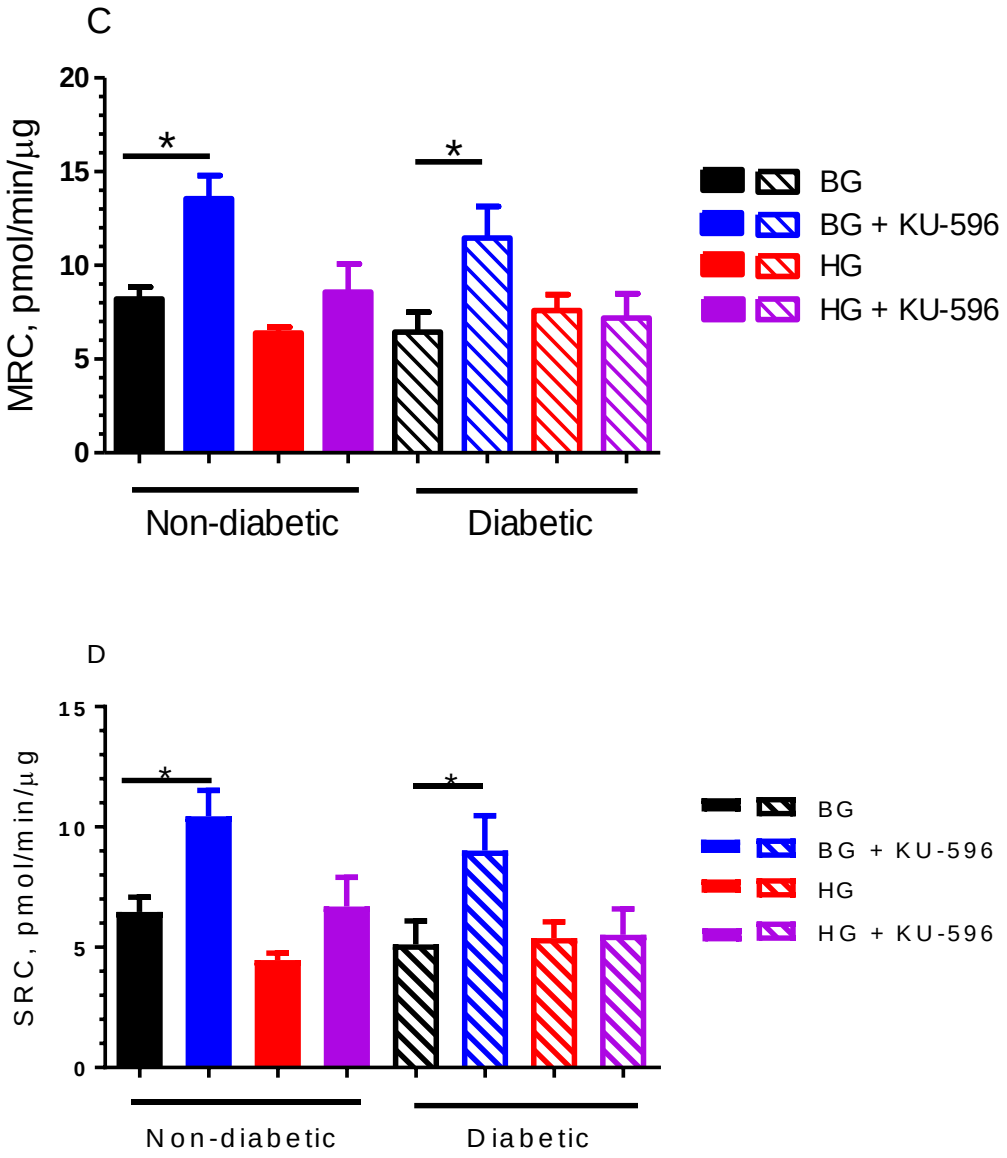


Figure 2.3.1.3 KU-596 does not improve mBE in hyperglycemically stressed Hsp70 KO neurons.

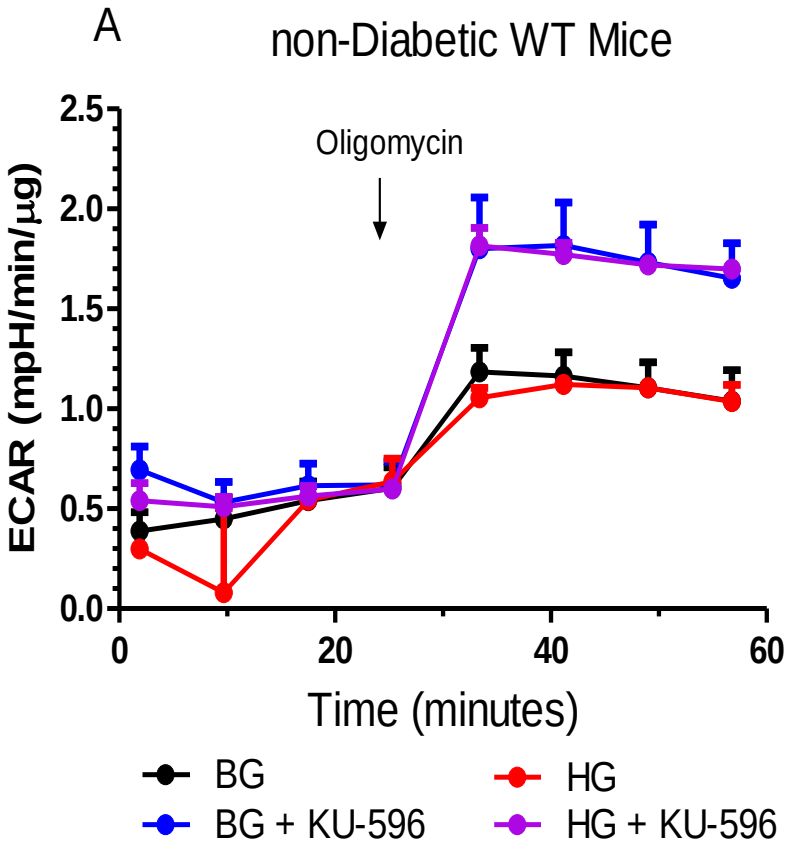
Adult sensory neurons were isolated from non-diabetic or diabetic Hsp70 KO mice and treated +/- 1µM KU-596 overnight in medium containing 6.1 mM basal glucose (BG). Hyperglycemia (HG) was then induced in some cultures for 24 hrs by raising the total glucose concentration to 26.1 mM. KU-596 improved OCR in non-diabetic (A) and diabetic (B) sensory neurons under BG condition but not after the cells were subjected to HG stress. C) Quantification of MRC. D) Quantification of SRC.*, $p<0.05$ vs BG. Results are from 3 - 5 wells of neurons per group that were obtained from 6 non-diabetic or diabetic mice.

2.3.1.4 KU-596 Improves Glycolytic Capacity in WT Sensory Neurons under Both Normal and Hyperglycemic Conditions

Glycolysis and oxidative phosphorylation are the two-major energy producing pathways in the cell. Glycolysis takes place in the cytosol and converts one molecule of glucose into two molecules of pyruvate. When oxygen is present, the mitochondria will undergo aerobic respiration to reduce pyruvate into acetyl-CoA which enters the citric acid cycle (Krebs cycle) inside the mitochondrial matrix. However, if oxygen is not present, fermentation of the pyruvate into lactate will occur [344, 345]. Most cells possess the ability to shift dynamically between these two processes, adapting to changes in their environment. Even in the presence of oxygen, a high rate of glycolysis followed by pyruvate and lactate accumulation can supply sufficient energy as compensation. In glycolysis, the conversion of glucose to pyruvate and subsequently lactate, results in a production and extrusion of protons into the extracellular medium. The Seahorse instrument can also measure the acidification rate as Extracellular Acidification Rate (ECAR) level along with OCR to indicate the rate of glycolysis. When oxidative phosphorylation is disrupted by the ATP synthase inhibitor, Oligomycin shifts the energy production to glycolysis to maintain homeostasis, subsequently increasing ECAR. This elevated rate of glycolysis is referred to as the Glycolytic Capacity of the cell.

The trend of ECAR change is similar with OCR level in WT mice. In the WT non-diabetic mice, KU-596 significantly increased the ECAR regardless of glucose concentration (Fig. 2.3.1.4.A). Though diabetes can cause mitochondrial deficiency, glycolytic capacity was also decreased. KU-596 again increased the ECAR level under normal and hyperglycemia conditions (Fig. 2.3.1.4.B). The ability of *ex vivo* KU-596

treatment to increase glycolytic capacity suggests that modulating molecular chaperones is efficient at improving overall bioenergy supply (Fig. 2.3.1.4.C).



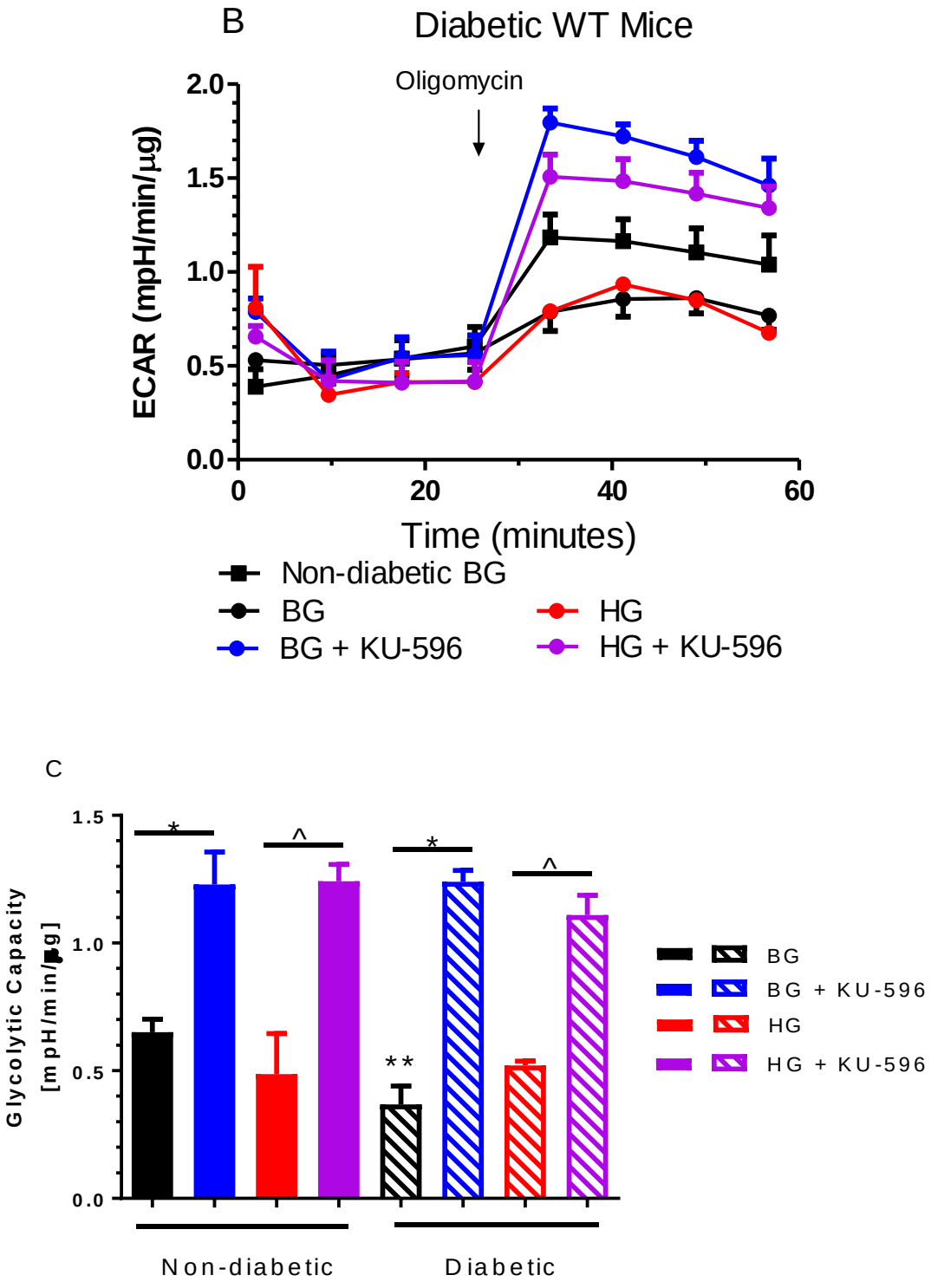
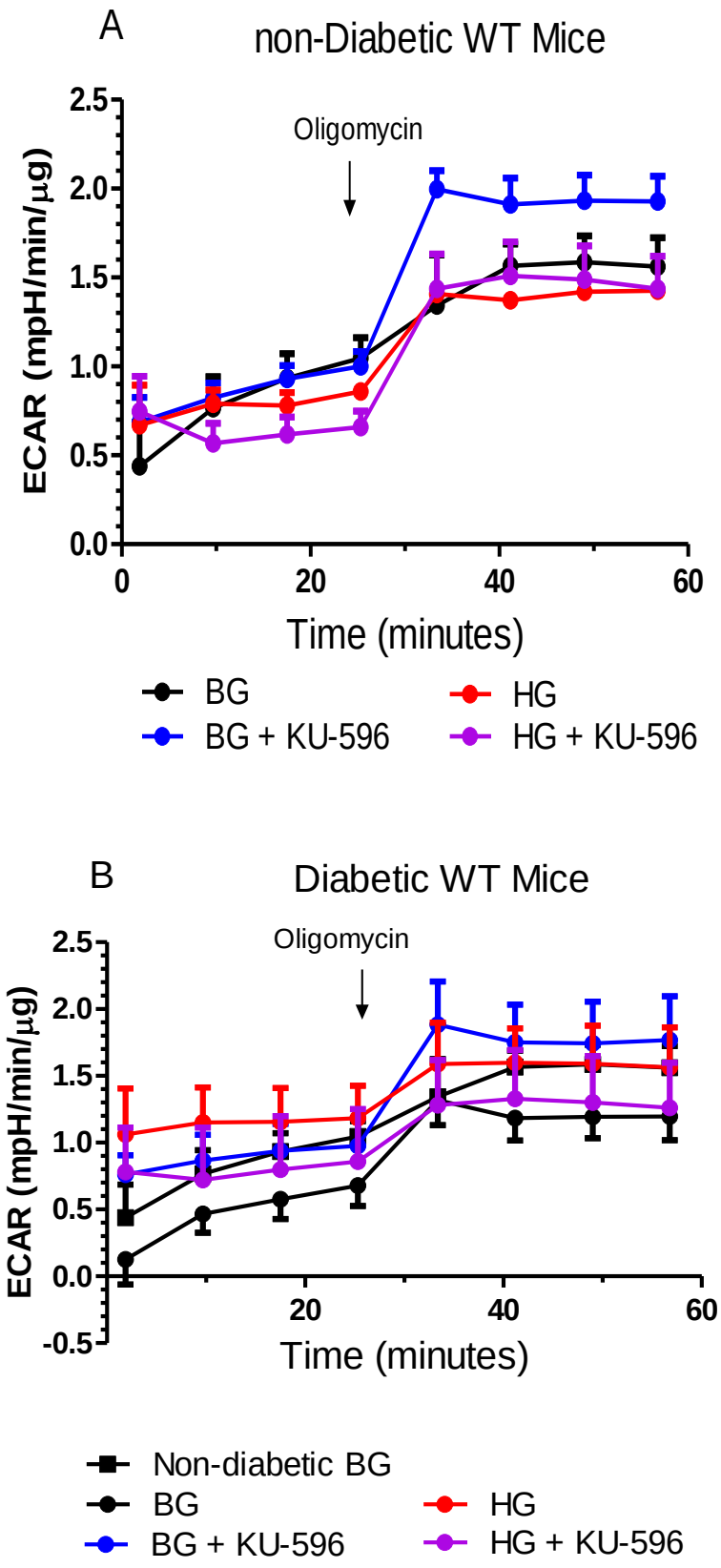


Figure 2.3.1.4 KU-596 treatment improves glycolysis in WT sensory neurons. Sensory neurons were isolated from C57Bl/6 WT non-diabetic and diabetic mice under the same condition as on mtBE measurement. We then studied the drug effect on glycolysis by injecting ATP synthesis inhibitor, Oligomycin, to shift the energy production to

glycolysis. A) KU-596 improved ECAR in WT non-diabetic sensory neurons under both normal condition and hyperglycemic condition. B) ECAR level was also increased in WT diabetic sensory neurons by KU-596. C) Quantification of Glycolytic Capacity. KU-596 significantly improved glycolysis in non-diabetic and diabetic sensory neurons under both normal and hyperglycemic conditions. *, $p<0.05$ vs BG; ^, $p<0.05$ vs HG; **, $p<0.05$ vs non-diabetic BG. Results are from 4-5 wells of neurons per group that were obtained from 8 non-diabetic or diabetic mice.

2.3.1.5 Hsp70 is Necessary for KU-596 to Improve Glycolysis in Diabetic Sensory Neurons under Hyperglycemic Condition.

In non-diabetic Hsp70 KO mice, KU-596 treatment improved the glycolytic capacity, to an extent that was similar to non-diabetic WT mice (Fig. 2.3.1.5.A). In diabetic Hsp70 KO sensory neurons, KU-596 also increased the ECAR level under basal glucose condition. However, this improvement of ECAR level was completely blocked under hyperglycemic stress (Fig. 2.3.1.5.B). This indicates that in diabetic sensory neurons, hyperglycemia shifted the improvement in glycolytic capacity by KU-596 to become Hsp70 dependent (Fig. 2.3.1.5.C).


A
non-Diabetic WT Mice
Oligomycin
ECAR (mpH/min/µg)
2.5
2.0
1.5
1.0
0.5
0.0
0
20
40
60
Time (minutes)
BG
HG
BG + KU-596
HG + KU-596
B
Diabetic WT Mice
Oligomycin
ECAR (mpH/min/µg)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
20
40
60
Time (minutes)
Non-diabetic BG
BG
HG
BG + KU-596
HG + KU-596

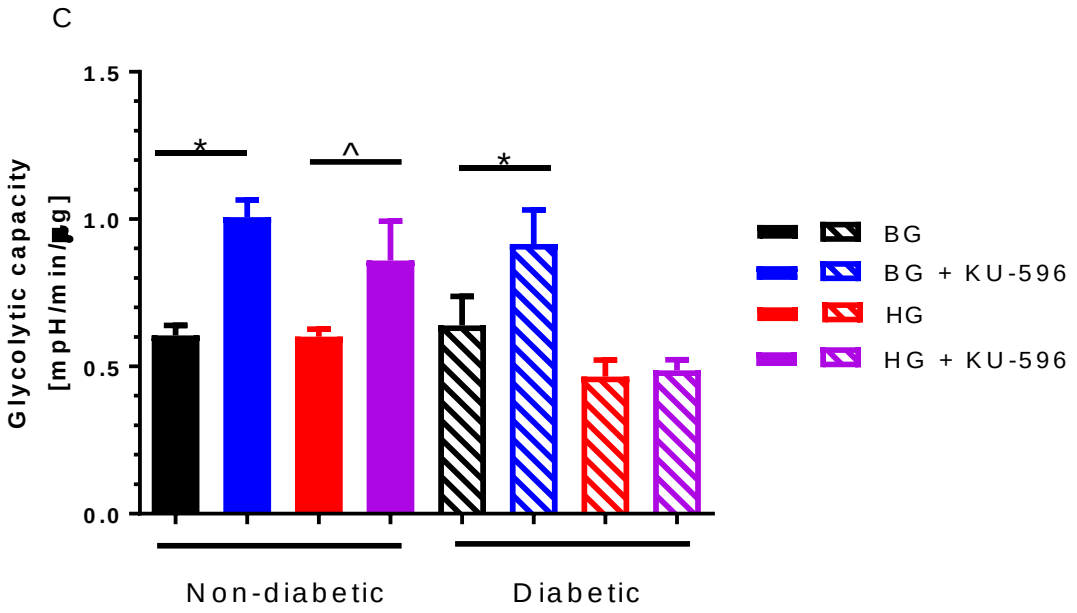


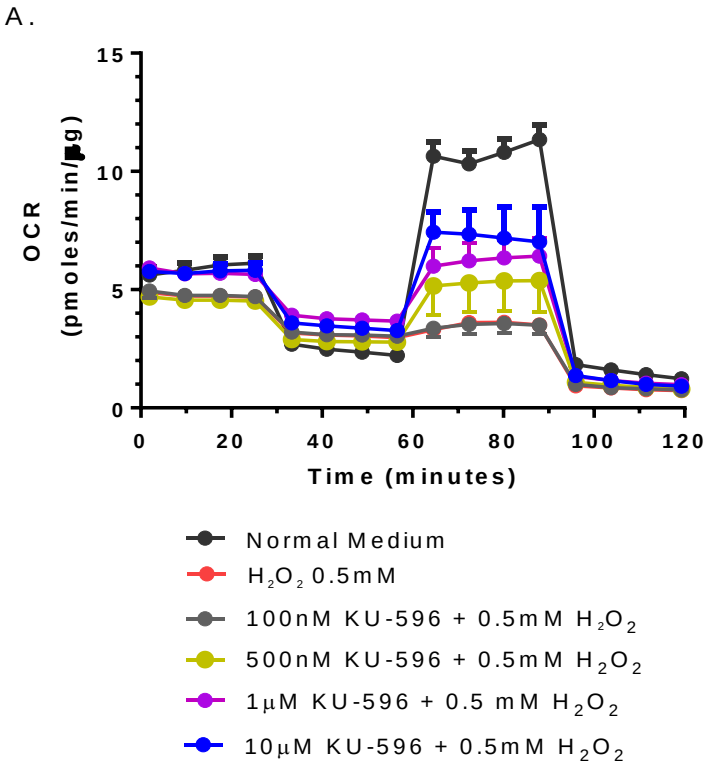
Figure 2.3.1.5 Hsp70 is necessary for KU-596 to improve glycolysis in diabetic sensory neurons under hyperglycemic condition.
Sensory neurons were isolated from Hsp70 KO mice the same way as described previously. We then studied the drug effect on glycolysis. **A)** KU-596 improved glycolysis in Hsp70KO non-diabetic sensory neurons under both normal and hyperglycemic condition. **B)** Though KU-596 has the trend to improve glycolysis in Hsp70 KO diabetic sensory neurons under normal condition. However, the drug effect was blocked by 20mm extra glucose. **C)** Quantification of glycolytic capacity. *, $p<0.05$ vs BG; ^, $p<0.05$ vs HG; Results are from 3-5 wells of neurons per group that were obtained from 6 non-diabetic or diabetic mice.

2.3.2 Decreasing Mitochondrial Oxidative Stress is One Mechanism for KU-596 to Improve Mitochondrial Bioenergetics in Diabetic Sensory Neurons

2.3.2.1 KU-596 Improves mtBE Deficits Caused by Oxidative Damage of H_2O_2.

One mechanism for KU-596 to improve cellular bioenergy supply might be through decreasing oxidative stress. We first determined if KU-596 can improve a deficiency in respiratory capacity that was caused by oxidative stress. We used an immortalized rat sensory neuron cell line 50B11 since this cell line has a very low basal level of Hsp70

expression like primary sensory neurons. 50B11 cells were seeded into each well of a 96-well plate at the density of 4×10^4 and maintained in DMEM medium with 10% FBS. After 24h, the medium was changed into DMEM with 1% FBS and the cells were treated with 100nM, 500nM, 1µM and 10µM KU-596 overnight. On the next day, the cells were stressed with 0.5mM H_2O_2 for 2 hours and transferred to the Seahorse XF96 analyzer to measure mtBE. H_2O_2 largely decreased the OCR elevation in response to FCCP, however, KU-596 increased the FCCP response in a dose-dependent manner (Figs. 2.3.2.1.A and B). This result shows that modulating chaperones can protect the mtBE deficiency caused by oxidative stress. Glycolytic capacity was also measured and though KU-596 trended to increase glycolysis, it was not statistically significant (Fig. 2.3.2.1.C).



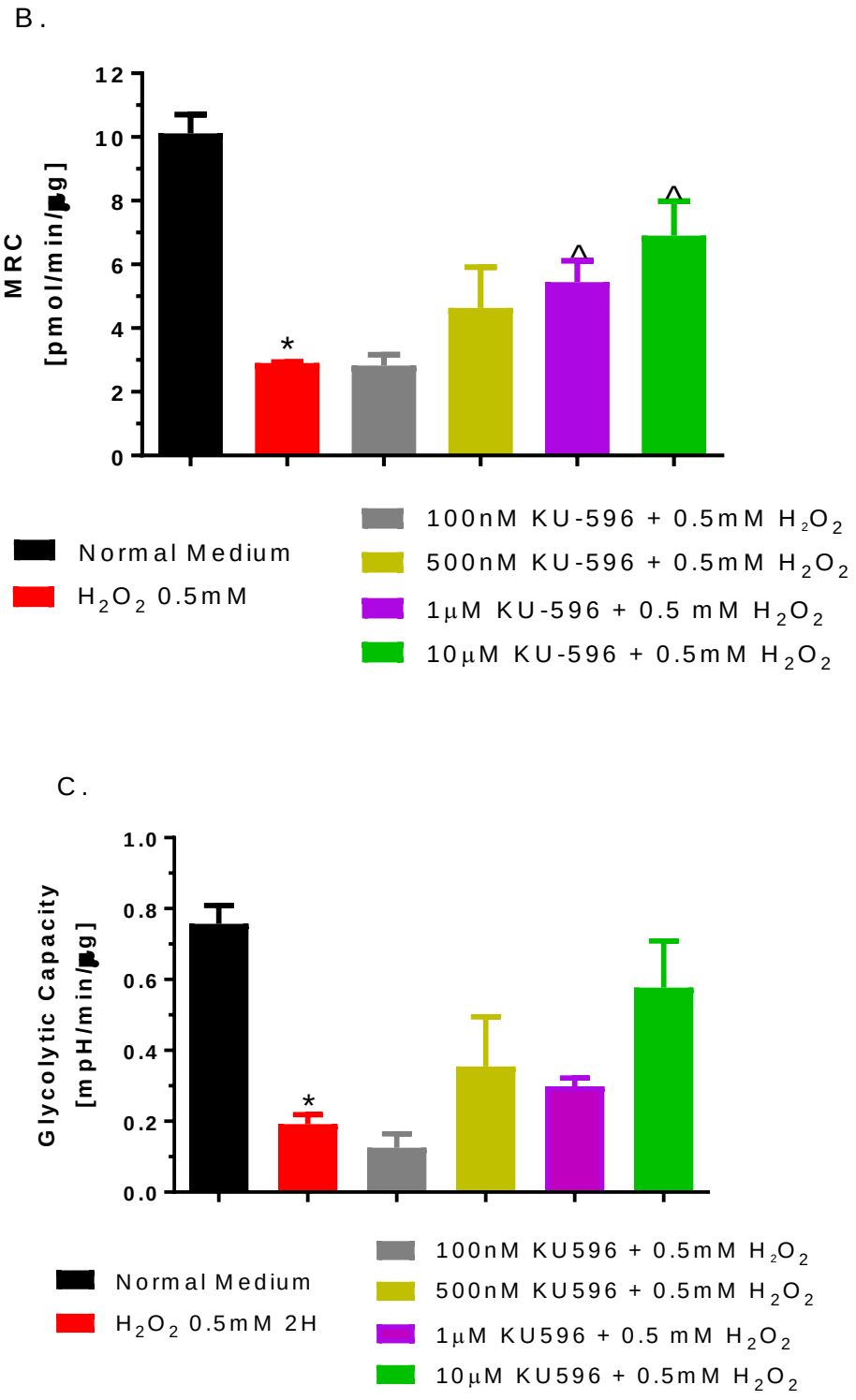
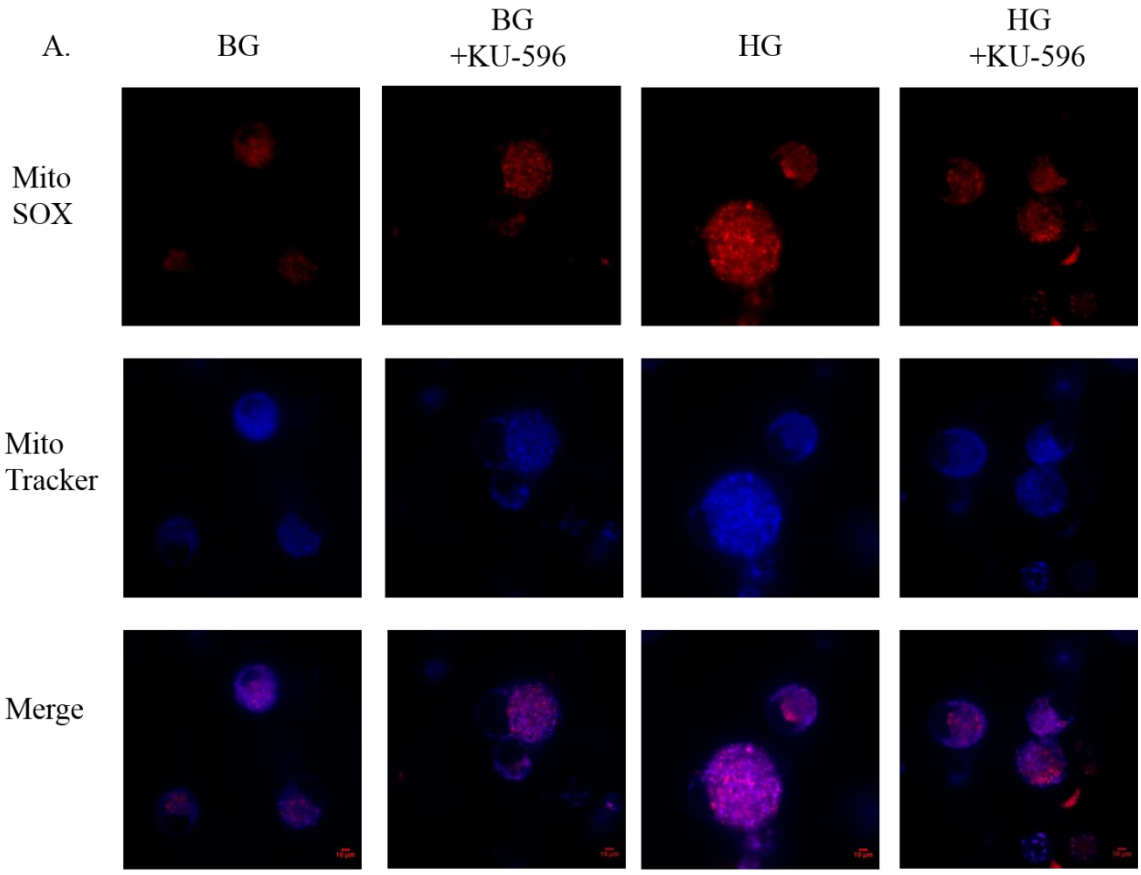


Figure 2.3.2.1 KU-596 improves mtBE deficits cause by H_2O_2.
A) Treatment of 0.5mM H_2O_2 for 2 hours significantly decreased FCCP response in 50B11 cells. KU-596 improved it in dose-dependent manner. B) Quantification of MRC indicates

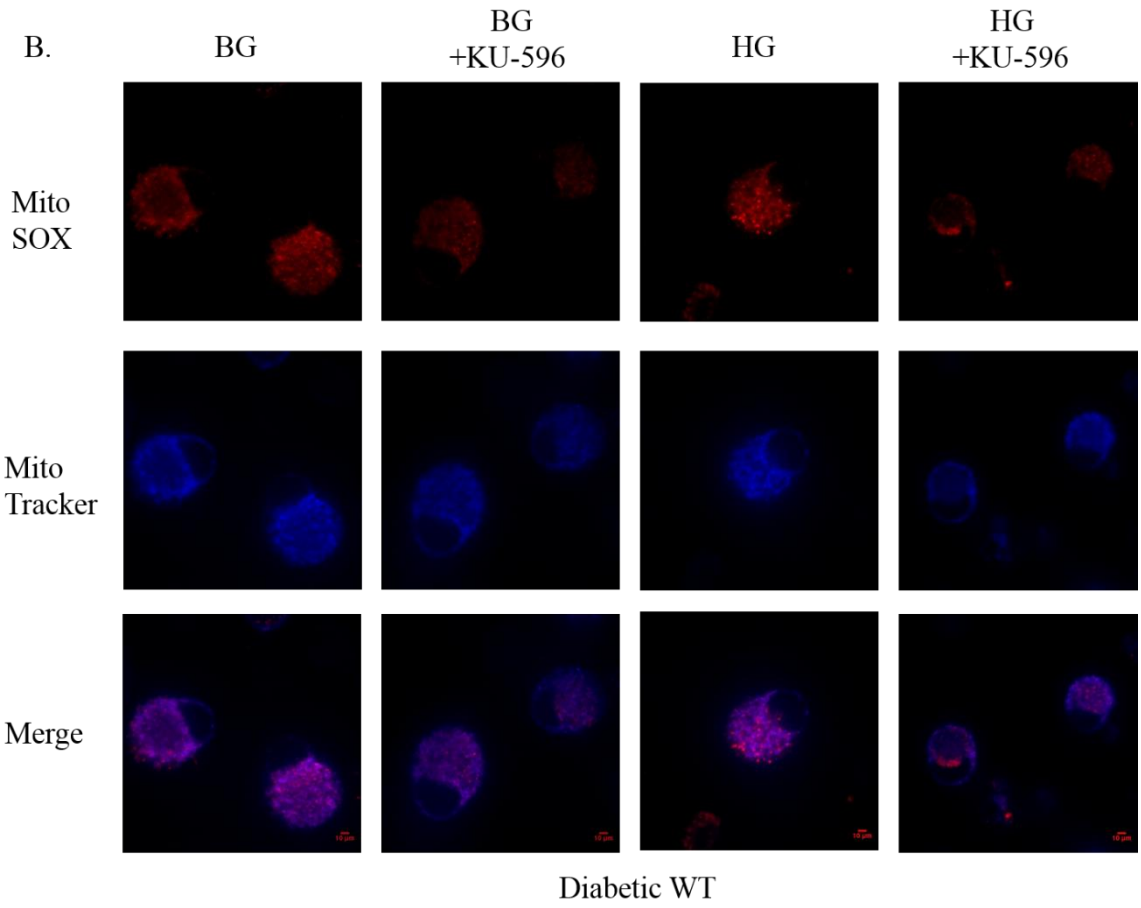
that KU-596 improved mtBE significantly, starting from 1μM. C) Quantification of Glycolytic Capacity. *, $p<0.05$ vs normal medium; ^, $p<0.05$ vs H_2O_2 treatment; Results are from 5 or 6 wells of neurons per group

2.3.2.2 KU-596 Decreases Oxidative Stress in WT Sensory Neurons

In diabetes, glucose induced superoxide production within mitochondria is a key feature of glucotoxicity. To determine the *ex vivo* effect of KU-596 treatment on mitochondrial superoxide levels, the sensory neurons were stained with MitoSOX Red and mitoTracker Deep Red (blue signal) to localize the signal to mitochondria. The intensity of the superoxide signal was normalized with the signal from mitoTracker Deep Red to rule out absorption differences between different cells. As anticipated, the superoxide signal intensity in diabetic WT neurons was much higher than in non-diabetic neurons regardless of glucose concentration (Figs. 2.3.2.2.A - C). Subjecting both the non-diabetic and diabetic neurons to HG stress significantly increased superoxide levels in both groups (Figs. 2.3.2.2.A - C) and this was normalized by KU-596 treatment. Since superoxide was low in non-diabetic neurons that were maintained in BG, KU-596 showed minimal efficacy in decreasing these levels. In contrast, KU-596 decreased the significantly higher level of superoxide present in diabetic neurons maintained in BG (blue vs black stripe). Together, these data support that modulating chaperones with KU-596 can directly improve mtBE and decrease oxidative stress in diabetic neurons.



Non-diabetic WT



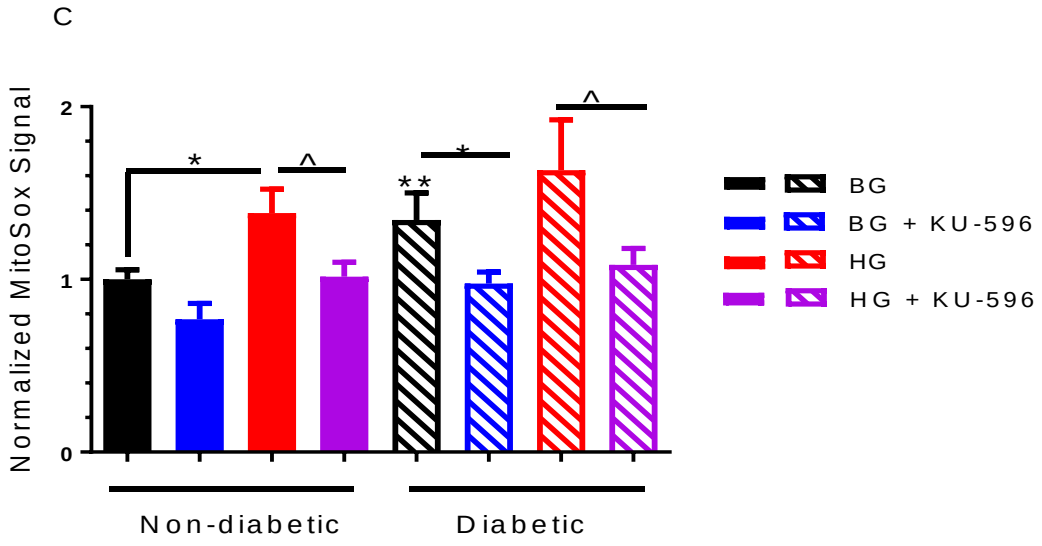


Figure 2.3.2.2 KU-596 decreases mitochondrial superoxide levels in hyperglycemically stressed WT neurons.

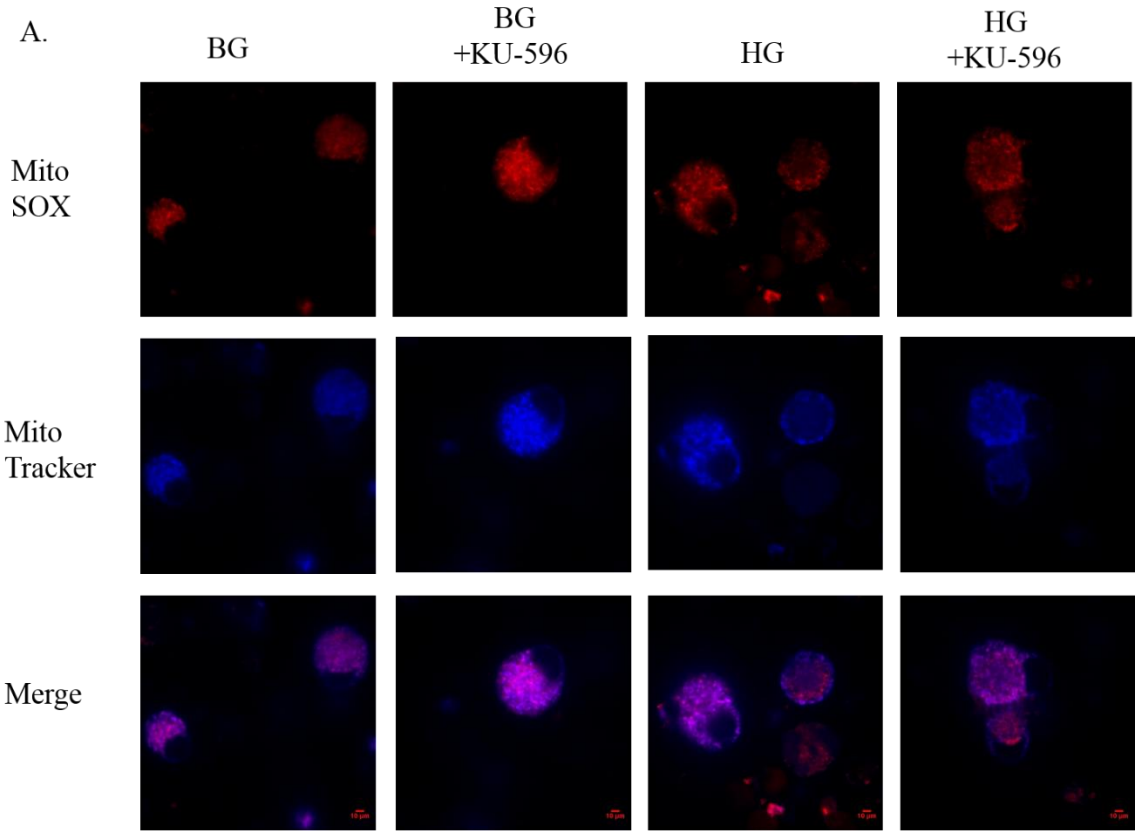
Cells were treated as described in Fig. 2.3.1.2 and mitochondrial superoxide assessed by confocal microscopy (A and B) after staining the cells with MitoSox red and MitoTracker Deep Red. A) Oxidative stress was increased in non-diabetic sensory neurons subjected to HG stress and this was decreased by KU-596 treatment. B) Diabetes increased the level of superoxide production regardless of glucose concentration and this was significantly decreased by KU-596. C) Quantification of MitoSOX staining intensity normalized to MitoTracker Deep Red staining intensity. *, $p<0.05$ vs BG; ^, $p<0.05$ vs HG; **, $p<0.05$ vs non-diabetic BG. Results are from 3 - 4 wells of neurons per group that were obtained from 6 non-diabetic or diabetic mice.

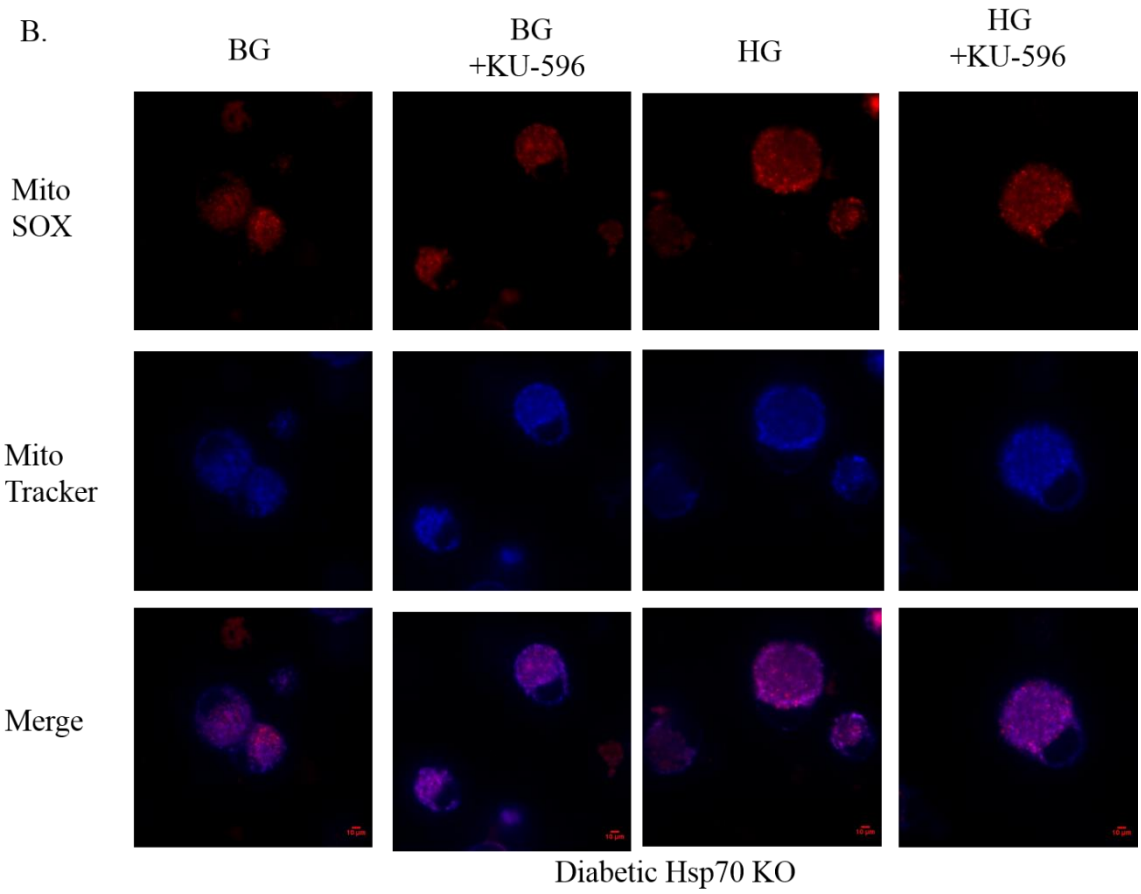
2.3.2.3 Hsp70 is Necessary for KU-596 to Decrease Oxidative Stress in Diabetic Sensory Neurons.

To determine if Hsp70 is necessary for the drug to decrease oxidative stress, sensory neurons were isolated from non-diabetic and diabetic Hsp70 KO mice and treated as described above. When examining superoxide, neither HG stress nor KU-596 caused any significant change in superoxide levels in neurons from non-diabetic Hsp70 KO mice (Figs. 2.3.2.3.A and C). Although neurons from diabetic WT mice maintained in BG showed a significant increase in superoxide, this response was not recapitulated in diabetic Hsp70 KO neurons maintained in BG. Exposing the diabetic Hsp70 KO neurons to HG stress significantly increased superoxide levels and KU-596 was unable to decrease this response (Fig. 2.3.2.3.C, purple vs red stripes), in contrast to what was observed in similarly treated diabetic WT neurons (Fig. 2.3.2.2.C, purple vs red stripes). The lack of efficacy in decreasing superoxide in the diabetic Hsp70 KO neurons supports that neuronal Hsp70 is a key component in the mechanism of action of KU-596.

The results on mtBE and oxidative stress from WT diabetic sensory neurons, suggest that decreased mtBE correlated with increased mitochondrial oxidative stress, which could both be improved by KU-596 treatment. However, in diabetic sensory neurons under hyperglycemic condition, KU-596 can neither improve the MRC nor mitochondrial

superoxide levels. These data support that KU-596 can enhance mitochondrial respiration and decrease oxidative stress in diabetic sensory neurons in an Hsp70-dependent manner. However, what remains unclear is the cause and effect relationship between improved respiration and decreased oxidative stress. Thus, this raises the question: Does KU-596 improve mitochondrial bioenergetics through reducing oxidative stress, or vice versa? Since MnSOD is the main mechanism to detoxify mitochondrial superoxide radicals [346, 347], we sought to knock down MnSOD by shRNA to increase oxidative stress in mitochondria and determine if KU-596 can decrease the oxidative stress and improve mitochondrial functions.




B.
BG
BG
+KU-596
HG
HG
+KU-596
Mito
SOX
Mito
Tracker
Merge
Diabetic Hsp70 KO

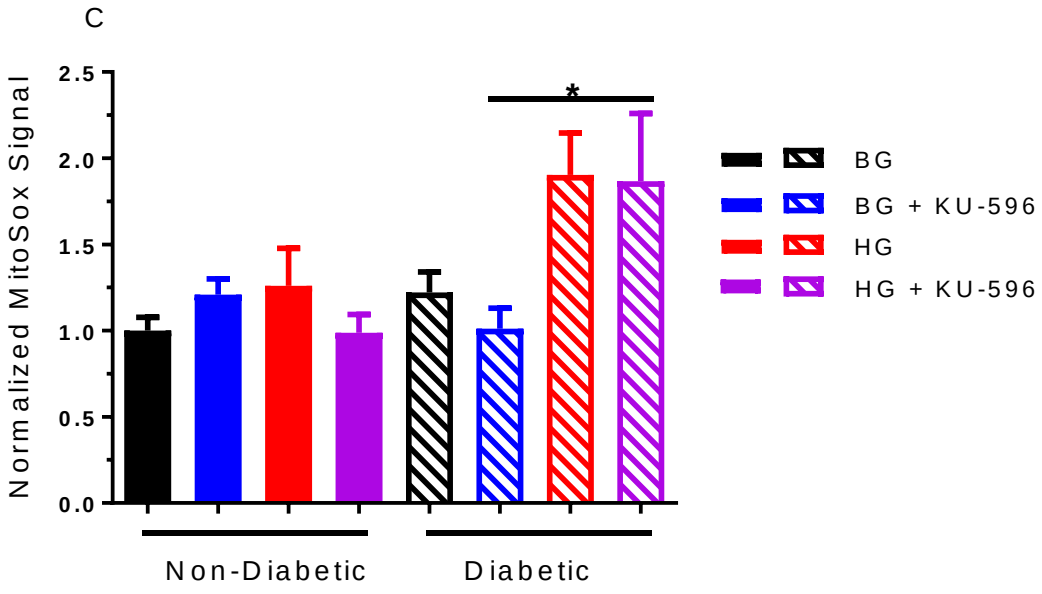

C
Normalized MitoSox Signal
2.5
2.0
1.5
1.0
0.5
0.0
*
BG
BG + KU-596
HG
HG + KU-596
Non-Diabetic
Diabetic

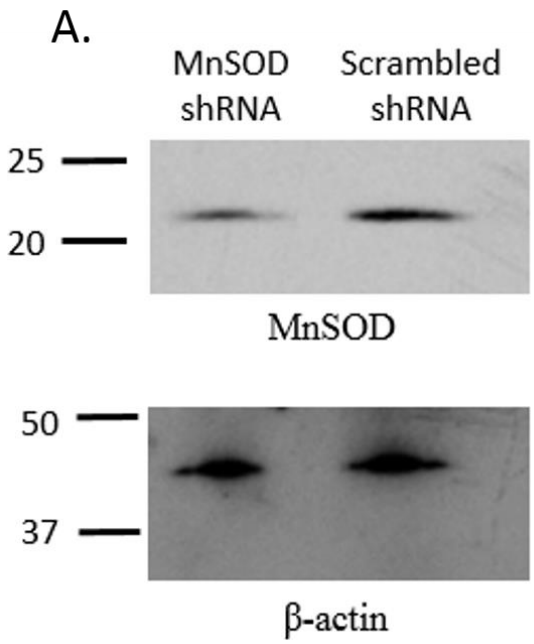
Figure 2.3.2.3 KU-596 does not decrease mitochondrial superoxide levels in hyperglycemically stressed Hsp70 KO neurons.
Cells were treated as described in Fig. 2.3.1.3 and mitochondrial superoxide was assessed by confocal microscopy (A and B) after staining the cells with MitoSox red and MitoTracker Deep Red. **A)** Oxidative stress was not increased in non-diabetic Hsp70 KO sensory neurons subjected to HG stress. **B)** HG stress increased superoxide production id diabetic Hsp70 KO neurons and KU-596 treatment was unable to attenuate this response. **C)** Quantification of MitoSOX staining intensity normalized to MitoTracker Deep Red staining intensity. *, $p<0.05$ vs BG. Results are from 3 - 4 wells of neurons per group that were obtained from 6 non-diabetic or diabetic mice.

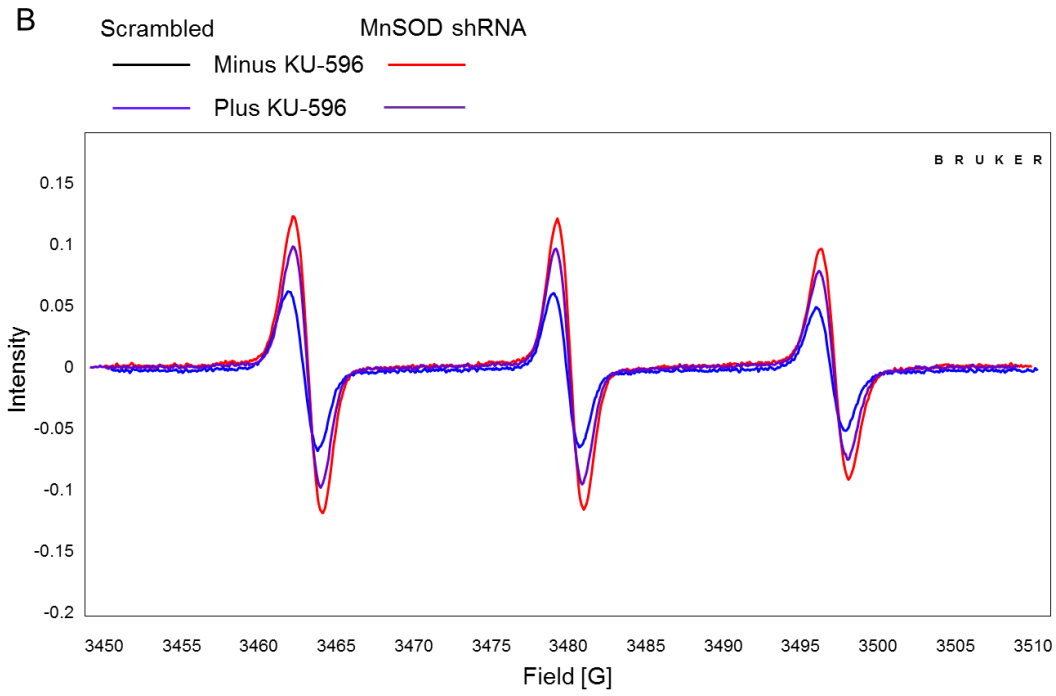
2.3.2.4 KU-596 Decreases Mitochondrial Oxidative Stress with MnSOD knock down.

To determine if KU-596 improved mtBE by reducing superoxide production, WT neurons were treated with recombinant adenoviruses expressing shRNA against murine MnSOD or a scrambled shRNA. Infection of adult neurons with the MnSOD shRNA adenovirus decreased the expression of MnSOD by about 60% (Fig. 2.3.2.4.A). To determine the effect of decreasing MnSOD expression on the production of superoxide radicals, we employed electron paramagnetic resonance (EPR) spectroscopy using mito-TEMPO-H, a mitochondria selective spin trap. This approach was necessary since the MnSOD shRNA virus co-expressed EGFP for visualizing the efficiency of viral infection. When using MitoSox Red to detect superoxide, this resulted in an overlap between the green and red fluorescence signals.

Neurons from non-diabetic and diabetic WT mice were prepared as described above and maintained in BG medium for the knockdown experiments. Relative changes in the superoxide signal were quantified by integration of the first EPR peak and normalized to total protein content. Knockdown of MnSOD increased the mitochondrial superoxide signal about two-fold in non-diabetic sensory neurons (Figs. 2.3.2.4.B and D). However, KU-596

did not significantly decrease superoxide levels in non-diabetic neurons following knockdown of MnSOD. Consistent with the MitoSox data, diabetes increased the superoxide signal relative to that present in non-diabetic neurons treated with the scrambled shRNA (Figs. 2.3.2.4.C and D). Downregulating MnSOD expression in the diabetic neurons further increased the superoxide signal about 2- fold. KU-596 significantly decreased the magnitude of the superoxide signal by 60% in diabetic cells infected with the scrambled and by 50% in diabetic neurons infected with the MnSOD shRNA. These data suggest that MnSOD may not be necessary for the drug to decrease mitochondrial superoxide, though the incomplete knockdown may leave a sufficient reservoir of enzyme to account for this effect.




B
Scrambled
MnSOD shRNA
Minus KU-596
Plus KU-596
BRUKER
Intensity
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
3450
3455
3460
3465
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3475
3480
3485
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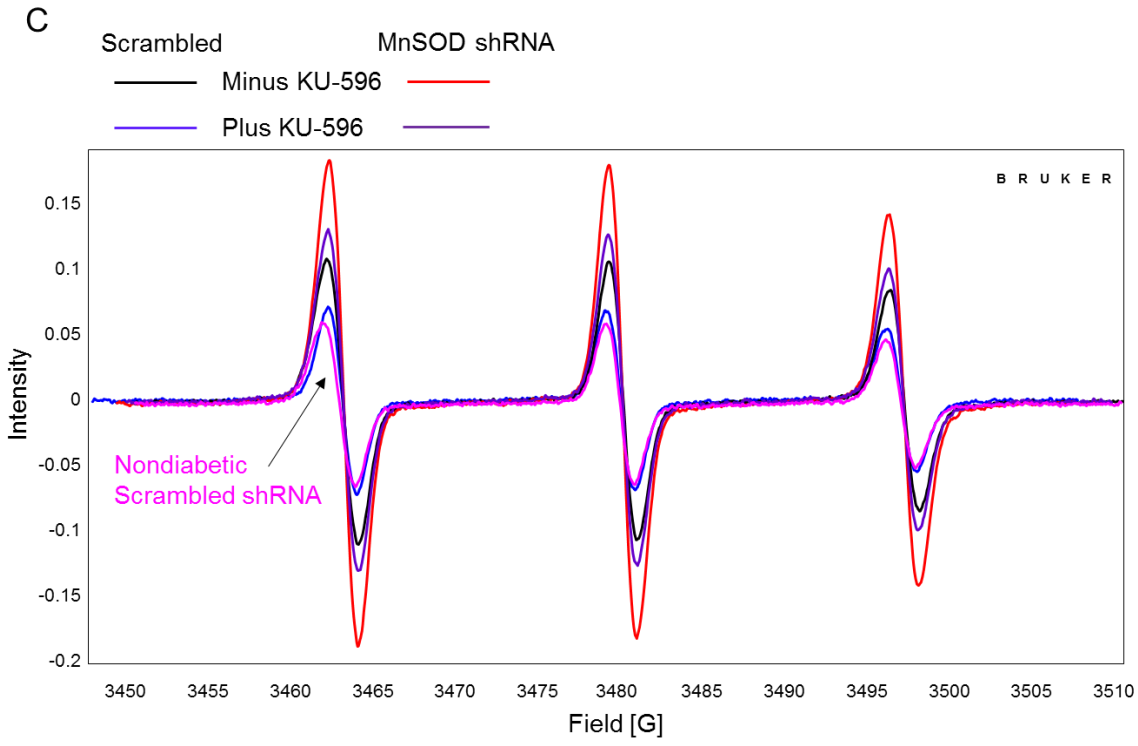
Field [G]


C
Scrambled
MnSOD shRNA
Minus KU-596
Plus KU-596
BRUKER
Intensity
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
Nondiabetic
Scrambled shRNA
3450
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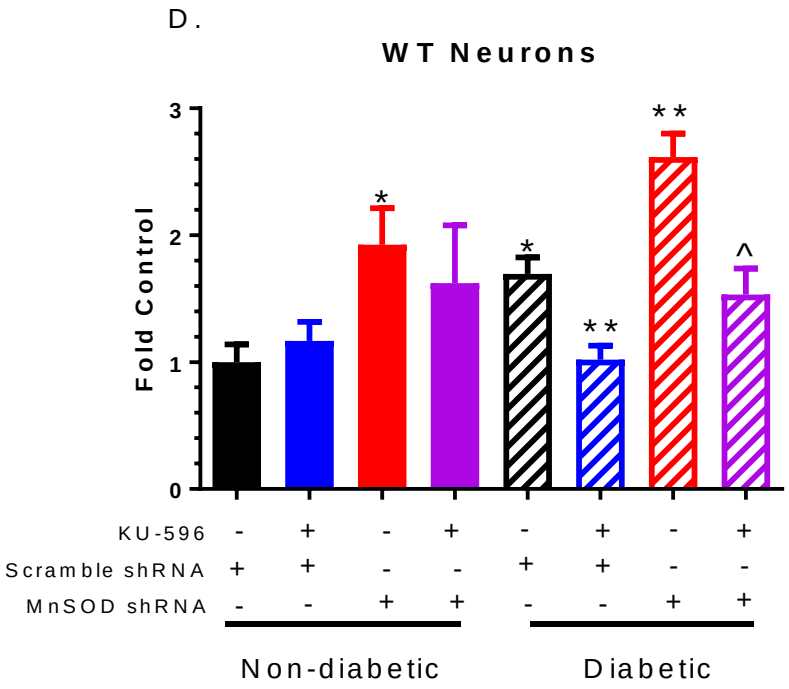


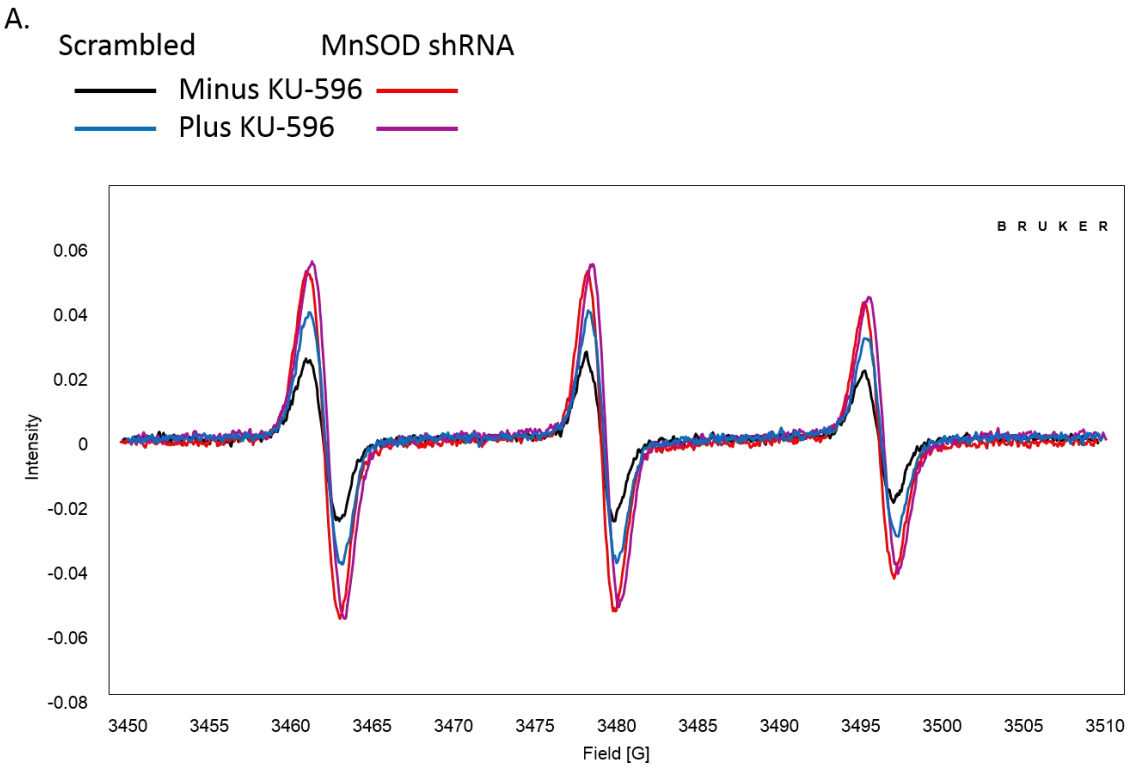
Figure 2.3.2.4 KU-596 decreased superoxide levels in diabetic neurons following downregulation of MnSOD.
A) MnSOD expression in sensory neurons after infection with MnSOD shRNA adenovirus. (B & C) EPR assessment of the effect of MnSOD downregulation and KU-596 treatment on the superoxide signal in **(B)** non-diabetic and **(C)** diabetic neurons from WT mice. **D)** Quantification of the area under the curve of the first superoxide signal peak, normalized to total protein and expressed as the fold of the non-diabetic plus scrambled shRNA control. *, $p < 0.05$ versus non-diabetic plus scrambled shRNA; **, $p < 0.05$ versus diabetic neurons plus scrambled shRNA; ^, $p < 0.05$, vs diabetic neurons plus MnSOD shRNA. Results are from 6 separate experiments in which 3 - 4 wells of neurons per group were obtained from 6 non-diabetic or diabetic mice.

2.3.2.5 KU-596 Decreases Mitochondrial Oxidative Stress in Hsp70 Dependent Manner with MnSOD knock down.

We also detected superoxide radicals in Hsp70 KO sensory neurons by EPR following knock down of MnSOD. In non-diabetic Hsp70 KO sensory neurons, MnSOD down-regulation increased mitochondrial superoxide radicals. However, after knocking down MnSOD, KU-596 did not have much effect on decreasing superoxide levels (Figs.

2.3.2.5.A and C). In diabetic sensory neurons treated with scrambled shRNA, superoxide radical levels were slightly increased compared to non-diabetic sensory neurons and KU-596 did not have much effect. Knocking down MnSOD in Hsp70 KO diabetic sensory neurons increased superoxide level compared to neurons that received the scrambled shRNA, but KU-596 was unable to decrease the superoxide signal (2.3.2.5.B and C).

Compared to the WT group, the lack of drug efficacy in diabetic sensory neurons from Hsp70 KO mice treated with scrambled shRNA means that Hsp70 is necessary for KU-596 to decrease mitochondrial oxidative stress. Since KU-596 still decreased superoxide levels in diabetic neurons following MnSOD knock down, there may be other mechanisms that contribute to this decline, however, this effect is still Hsp70 dependent (compare purple and red stripe bars in Figs.2.3.2.4.C and 2.3.2.5.C).



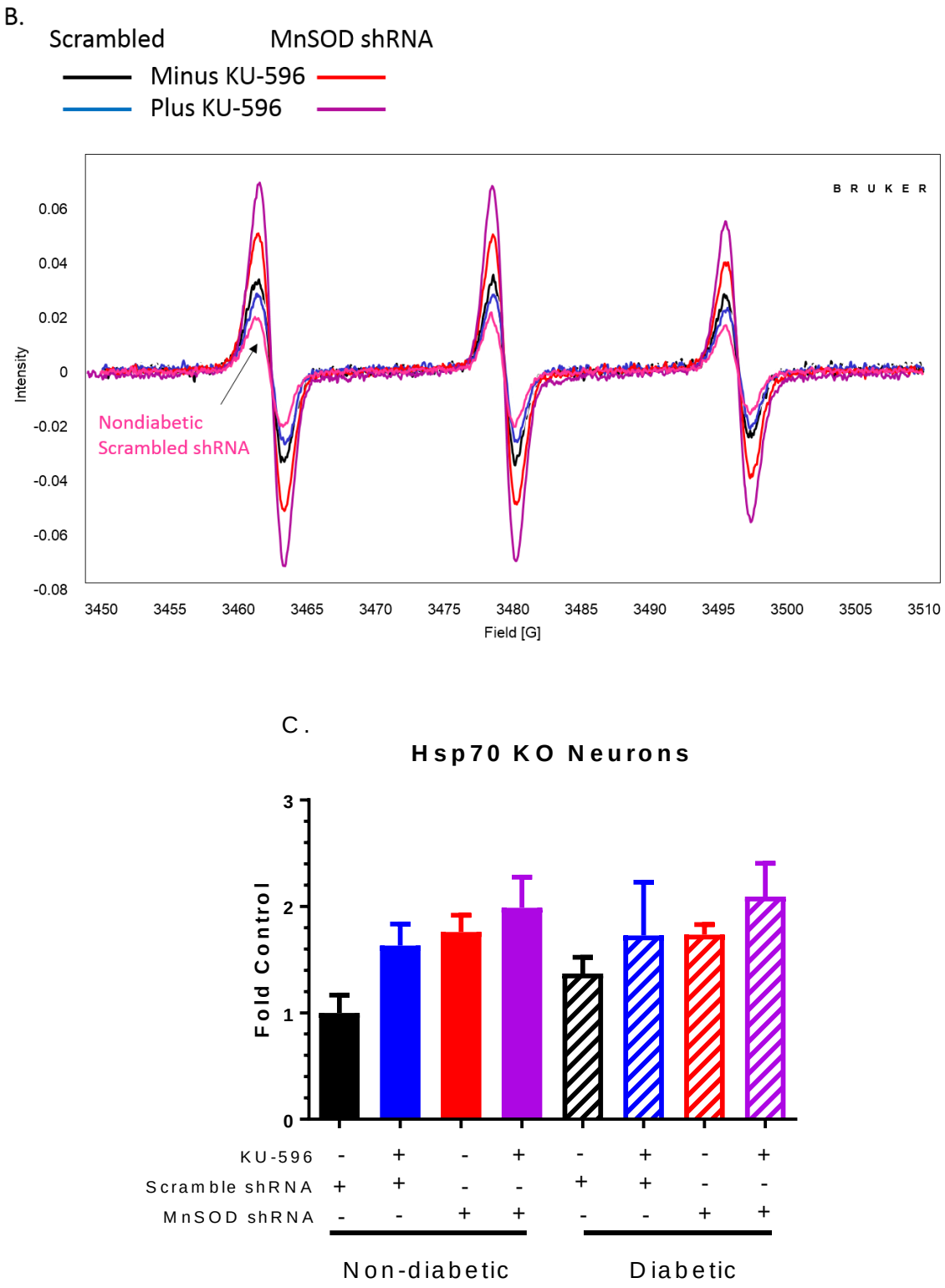


Figure 2.3.2.5 KU-596 decreases mitochondrial oxidative stress in Hsp70 dependent manner with MnSOD knock down.

Mitochondrial superoxide levels after knocking down MnSOD were also determined by EPR. **A)** KU-596 effect on Hsp70 KO non-diabetic neurons +/- MnSOD shRNA. **B)** KU-596 effect on Hsp70 KO diabetic neurons +/- MnSOD shRNA. **C)** Quantification of superoxide radical level. Unlike in WT sensory neurons. KU-596 cannot decrease mitochondrial oxidative stressed in Hsp70 KO neurons.

2.3.2.6 KU-596 Improves mtBE through Reducing Mitochondrial Oxidative Stress.

Next, the effect of MnSOD downregulation and KU-596 on mtBE in neurons from non-diabetic and diabetic mice was assessed. In non-diabetic neurons treated with scrambled shRNA, KU-596 didn't improve mitochondrial respiration and downregulating MnSOD in the absence or presence of KU-596 had little effect on mtBE (Fig. 2.3.2.6). As expected, diabetic sensory neurons treated with scrambled shRNA showed a markedly diminished MRC compared to similarly treated non-diabetic neurons, and this deficit was significantly improved by KU-596 (Figs. 2.3.2.6 black and orange). However, MRC was also significantly increased following MnSOD downregulation in diabetic neurons and treating these neurons with KU-596 did not increase MRC any further (Figs. 2.3.2.6 green and blue). Since KU-596 significantly decreased the superoxide signal in diabetic neurons treated with MnSOD shRNA (Fig. 2.3.2.4.D), these data suggest that the ability of KU-596 to improve MRC is not necessarily dependent on decreasing mitochondrial superoxide.

To determine if the increase in MRC following downregulation of MnSOD in diabetic neurons was mainly driven by the increase in superoxide, cells were treated with mito-TEMPO-H to scavenge superoxide. In non-diabetic neurons, downregulating MnSOD did not increase MRC and neither KU-596 nor mito-TEMPO-H significantly improved MRC (Figs. 2.3.2.6). In diabetic neurons, knockdown of MnSOD increased MRC and this was significantly decreased by mito-TEMPO-H. This data suggests that an increase in superoxide levels is associated with the elevation in MRC following

knockdown of MnSOD. KU-596 was unable to improve MRC following MnSOD knockdown in either the presence or absence of mito-TEMPO-H (Figs. 2.3.2.6).

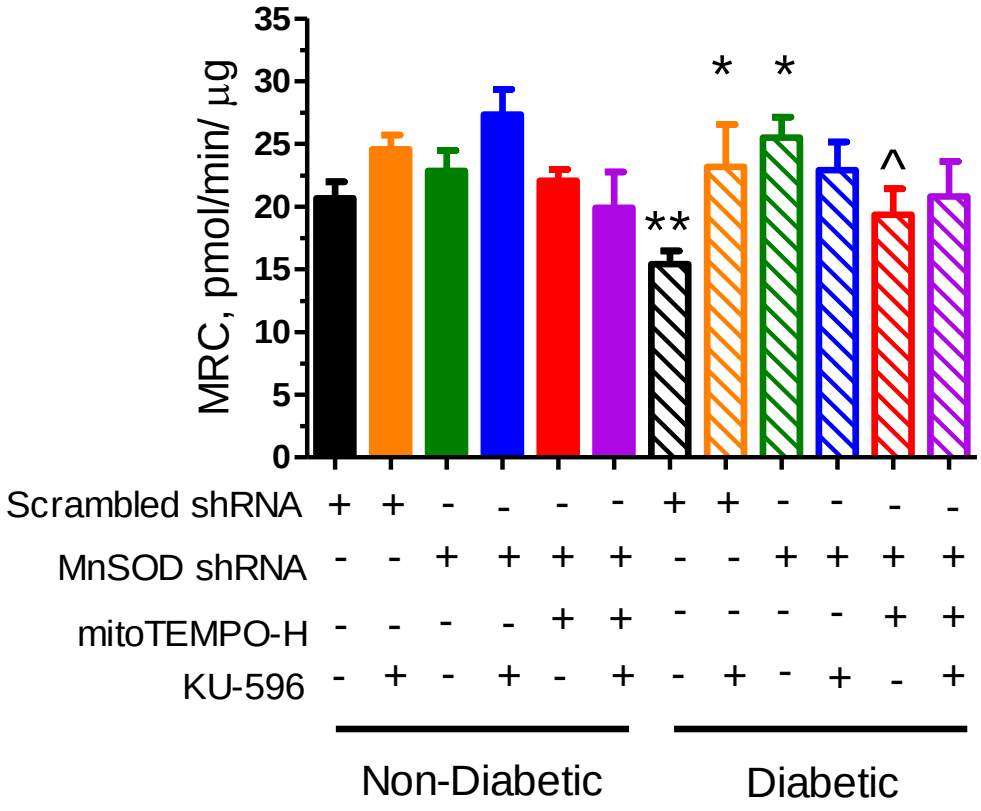


Figure 2.3.2.6 KU-596 does not improve MRC following downregulation of MnSOD in diabetic neurons.
Quantification of MRC in WT non-diabetic and diabetic neurons in the presence and absence of mito-TEMPO-H and KU-596 following MnSOD downregulation. MnSOD down-regulation, KU596 and mito TEMPOH didn't have much effect on mtBE in non-diabetic sensory neurons. KU-596 improved OCR in WT diabetic sensory neurons with scrambled shRNA and this drug effect was blocked with MnSOD down-regulation. With mito TEMPOH treatment, the increase of mtBE caused by MnSOD shRNA was decreased and KU-596 didn't have effect on improving mtBE. **, $p < 0.05$ versus non-diabetic neurons treated with scrambled shRNA; *, $p < 0.05$, versus diabetic neurons treated with scrambled shRNA; ^, $p < 0.05$, versus diabetic neurons treated with MnSOD shRNA. For each figure the results are from 4 separate experiments in which 3 - 4 wells of neurons per group were obtained from 6 non-diabetic or diabetic mice**.**

Chapter 3. Conclusion

Mitochondrial dysfunction is a major pathogenic contributor to DPN since neurons highly depend on mitochondria for their energetic supply. Reduced respiratory chain activity is observed in sensory neurons and these bioenergetic abnormalities can contribute to the degeneration of peripheral axons [348]. Our previous study has shown that sensory neuron mitochondrial bioenergetic defects induced by diabetes can be rescued by novologue therapy [299]. In addition to aerobic respiration in mitochondria, glycolysis in the cytosol is the other mechanism to produce ATP [344, 345]. Most cells possess the ability to shift dynamically between glycolysis and oxidative phosphorylation to help adapt to changes in their environment. In this work, we identified that KU-596 improved both glycolysis and mitochondrial respiration in sensory neurons. SCs are highly glycolytic, delivering lactate to axons, but mitochondrial dysfunction can also cause SC degeneration and lead to demyelination [87]. Many cells have the ability to shift energy production between oxidative phosphorylation and glycolysis, and many factors can regulate the respiration process. In the future, we also need to determine if Hsp70 can reprogram SCs from glycolytic to oxidative metabolism to promote remyelination and improve nerve functions in DPN.

In vivo, neurons are polarized cells consisting of relatively small cell bodies, dendrites with multiple branches and a thin axon that can extend for meters in peripheral nerves [349]. It is important to understand the KU-596 effects both on the cell body and axons. Since axons are generated from neurons and can extend for meters in peripheral nerve, neurons require specialized mechanisms to efficiently distribute mitochondria to far axonal branches where energy is in high demand [349, 350]. Axons are important in action

potential generation and synaptic transmission while constant ATP supply supports nerve functions, thereby maintaining mitochondrial trafficking and distribution is important in axonal physiology [351]. Besides identifying KU-596 can improve overall mitochondrial energy production in peripheral nerves, it also deserves further effort to understand if KU-596 can advance the distribution of mitochondria in peripheral nerve to improve nerve functions.

It has been well known that reducing MnSOD can increase mitochondrial superoxide and reactive oxygen species (ROS) in both mitochondria and cytoplasm. In our results, MnSOD shRNA decreased the protein expression of MnSOD by about 60% and increased mitochondrial superoxide levels. Curiously, this was associated with an increase in mitochondrial respiration in diabetic sensory neurons. The increase in superoxide levels following downregulation of MnSOD seemed responsible for the increased respiration since scavenging mitochondrial ROS with mitoTEMPO blocked the increase in cellular respiration in diabetic sensory neurons. This phenomenon was also observed in normal kidney cells [352]. Though unexpected, excessive superoxide may be activating redox-sensitive survival and metabolic pathways, including Akt, ERK, PKC, and MAPK [353, 354]. Exogenous H_2O_2 can lead to the activation of MAPK pathways [355, 356]. Antioxidants can prevent ROS accumulation and MAPK activation [357, 358], indicating ROS is involved in activating MAPK pathways. Though the exact mechanism of how ROS regulate MAPK pathway is not known, this might be due to oxidative modifications of MAPK signaling proteins and inactivation or degradation of MKPs (MAPK phosphatase) [359].

We clearly show that KU-596 decreased mitochondrial superoxide before and after knocking down MnSOD using two independent measures. However, after downregulating MnSOD, KU-596 didn't have any further effect on improving MRC. Since KU-596 can decrease the superoxide radical levels after MnSOD knocking down, these data suggest that the ability of KU-596 to improve MRC is not necessarily dependent on decreasing mitochondrial superoxide. There are other mechanisms can be modulated by Hsp70 to improve mtBE.

Increased mitochondrial oxidative stress can also increase the autophagy response, which is a lysosome-mediated degradation process for damaged cellular components [360, 361]. H_2O_2 and increased O_2^- can mediate autophagy. For example in HeLa cells, MnSOD overexpression decreased superoxide radical and autophagy. Knocking down MnSOD with siRNA increased mitochondrial superoxide levels and autophagy [362]. Autophagy is emerging as an important mediator of pathological responses and engaging in cross-talk with ROS in both cell signaling and protein damage. Mitochondrial turnover can be accelerated by an autophagic process called mitophagy [363]. Mitophagy removes mitochondria via a double membrane autophagolysosome to degrade for recycling [364]. After knocking down MnSOD, the increased superoxide radical may induce mitophagy to increase mtBE. Though KU-596 can still decrease the superoxide levels, the drug may still induce mitophagy to compensate for mtBE. After scavenging superoxide with mitoTEMPO, KU-596 did not improve mtBE in the absence of superoxide radicals. This means superoxide radical might be important to induce mitophagy to protect mtBE. However, the crosstalk between mitophagy, redox signaling and mitochondrial dysfunction is still not well understood.

The selectivity of mitophagy is controlled by a variety of proteins, including PTEN-induced putative kinase 1 (PINK1) and Parkin. PINK1 is a mitochondria-targeted serine–threonine kinase that regulates mitochondrial homeostasis. Initially, PINK1 is stabilized by mitochondrial depolarization, and direct or indirect PINK1 activation leads to the phosphorylation of parkin and its recruitment into mitochondria [365]. The basal parkin expression is low in mitochondrial cristae in proliferating cells [366], but parkin can be largely transported to mitochondria when their mitochondrial membrane potential is disrupted by uncoupling agents or ROS [367, 368]. Parkin then stimulates the perinuclear accumulation of mitochondria, via a microtubule dependent mechanism, before the recruitment of LC3 and the remaining autophagic machinery to stimulate mitochondrial degradation [369-372]. Parkin functions to reduce cytochrome c release from damaged mitochondria, thereby preventing subsequent apoptosis [373]. PINK1 and Parkin knockout mice have a reduced lifespan, locomotor deficits, mitochondrial defects and increased oxidative stress [374, 375]. Mutations in parkin are unable to stimulate the degradation of damaged mitochondria [371, 372]. Hsp70 is a substrate of parkin and is necessary to transport parkin into mitochondrial matrix to facilitate mitochondrial clearance of damaged proteins [376]. In Hsp70 KO mice, parkin was unable to translocate, ubiquitinate and mediate mitophagy, leading to abnormal changes in mitochondrial morphology, reduced muscle respiratory capacity, lipid accumulation, and muscle insulin resistance [343, 377]. Parkin overexpression rescued mitochondrial membrane potential loss, abnormal ROS accumulation and mitochondrial DNA synthesis under H_2O_2-induced stress conditions [378]. Since KU-596 improved mtBE via Hsp70 in diabetic sensory neurons, it is important

to determine if novologue therapy to ameliorate DPN is through reducing neuronal mitophagy in a parkin-dependent manner.

Besides superoxide, there are other factors that can be modulated by KU-596 to decrease oxidative stress. KU-596 can also regulate non-mitochondrial ROS producing NADPH oxidases (NOX). In diabetic sensory neurons, KU-596 decreased NOX mRNA overexpression in an Hsp70 dependent manner. Thioredoxin reductase inhibitory protein (TXNIP) can interfere with the antioxidant activity of thioredoxin (Trx) and drive oxidative stress. TXNIP is one of the most inducible genes by high glucose in diabetic sensory neurons, retina, pancreatic islets and kidneys [379-381]. In mitochondria, TXNIP inhibits Trx2 causing oxidative stress, bioenergetics imbalance, mitophagy, and apoptosis. TXNIP mRNA level was increased 2 fold in Type 1 diabetic mice and KU-596 therapy decreased it to control levels. Moreover, its expression levels can be affected by Hsp70 [380] since KU-596 could not decrease its expression in diabetic Hsp70 KO mice [298, 382]. Though our results have identified that Hsp70 can attenuate intracellular oxidative stress induced by diabetes, it still remains to be identified whether KU-596 inhibits TXNIP overexpression directly or due to the alleviation of hyperglycemic/oxidative stress [382].

As a cellular protective mechanism, oxidative stress can activate several cytoplasmic factors, including NFκB, p53, mTOR, ERK and MAPK, which can translocate into the nucleus and activate transcription of MnSOD. MnSOD is encoded by a nuclear gene, synthesized in the cytosol and imported into the mitochondrial matrix through translocases on the outer and the inner membrane. The precursor form can be cleaved into the mature form and to detoxify only mitochondrial ROS [383, 384]. Before transporting into mitochondria, newly synthesized proteins can interact with chaperones in the cytosol,

including Hsp90 and Hsp70 [257, 385, 386]. Both MnSOD and Cu/ZnSOD activities were significantly decreased in HSP70.1 KO mice compared to WT mice [387, 388]. Intracellular Hsp70 helps to transport MnSOD into mitochondria, while mitochondrial Hsp70 facilitates MnSOD import into the mitochondrial matrix [388]. This might account for higher basal superoxide levels in non-diabetic Hsp70 KO sensory neurons.

In non-diabetic and diabetic Hsp70 KO sensory neurons incubated in BG, KU-596 treatment improved mtBE. When subjected to HG stress, KU-596 treatment could not improve MRC in either non-diabetic or diabetic neurons. This suggests KU-596 may have some Hsp70-independent effects under certain cellular conditions to improve mtBE. Our previous RNA-seq study has suggested KU-596 reversed transcriptomic changes associated with the inflammatory pathways in an Hsp70-independent manner [298]. Though hyperglycemia is shifting KU-596 efficacy to improve mtBE to become more Hsp70-dependent, it is unlikely that Hsp70 works alone to promote efficacy against DPN. Overexpression of Hsp27 protected diabetic sensory neuropathy development, and this correlated with decreased NFκB activities [389]. Hsp40 interacts with Hsp70 to suppress amylin misfolding and aggregation to ameliorate Type 2 diabetes [390]. Over-expression of Hsp70 alone in motor neurons of SOD1 mutant mouse model of ALS was not sufficient to decrease neuronal cell death, while induction of multiple chaperones in SOD1 mice was cytoprotective [391, 392]. Thus, induction of other chaperones may also contribute to KU-596's ability to improve mtBE. The drug may promote other chaperones that work in conjunction with Hsp70 under hyperglycemic stress.

In summary, this dissertation provides evidence to show novologue therapy improved mtBE and decreased mitochondrial oxidative stress of diabetic sensory neuron in an

Hsp70-dependent manner. After knocking down MnSOD, KU-596 can still decrease mitochondrial oxidative stress but had no further effect on mtBE. This indicates besides decreasing mitochondrial oxidative, there are other mechanisms that can be modulated by novologues to improve mtBE. This study elucidates the protective mechanism of modulating molecular chaperones in the therapeutic management of DPN in patients with type 1 and type 2 diabetes.

Up to now, the drug development strategies targeting DPN have mainly centered on inhibiting specific pathogenic pathways factors linking to hyperglycemia, but with limited success. The results of this study advance our knowledge about how chaperones antagonize the pathogenesis of DPN and extend our assessment of the translational value of modulating molecular chaperones to treat DPN. Our data continue to support that novologue therapy may provide an innovative and ground breaking approach to treat DPN. Though we are not claiming that modulating chaperones is the only universal mechanism to treat DPN, the results of this study extend our understanding about the neuroprotective role of molecular chaperones to improve nerve function in DPN.

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