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MECHANISMS OF SCHWANN CELL INJURY IN DIABETES: ROLE OF THE CYTOCHROMES P450 PATHWAYS

by

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AN ABSTRACT OF THE THESIS OF

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Title: Mechanisms of Schwann Cell Injury in Diabetes: Role of the Cytochromes P450 Pathway

Background: Among the microvascular complications associated with diabetes, diabetic neuropathy (DN) is one of most commonly reported affecting 50% - 70% of diabetic subjects. Clinically, DN is characterized by reduced electrophysiological recordings, sensorimotor loss, and paresthesia. Research has correlated these observations with nerve fiber injury leading to degeneration, axonal atrophy, demyelination with limited regenerative potential. The pathogenesis of DN remains to be elucidated; however, ROS production is now considered the final common key mediator. Cytochrome P450 (CYPs) enzymes are known to be major sources of ROS and have been shown to mediate other diabetic complications. Yet, no studies have investigated their role in DN.

Aim: The following study aims to investigate CYP4A enzymes as a source of ROS in Schwann cells (SCs) and sciatic nerves. Alteration in CYP4A expression is studied in addition to SC apoptosis and myelin protein level alterations. The effects of hyperglycemia and CYP4A inhibition via HET0016 are further investigated *in vivo* to study behavioral changes, peripheral nerve injury and alterations in AKT signaling, a key pathway involved in myelination.

Methods: Mouse Schwann Cells (MSC80) cultured in a hyperglycemic milieu and 10-week Type II Diabetic MKR adult male mice were used to model a diabetic phenotype for this study. Western blot analyses were performed to assess alterations in CYP4A, phosphorylated-AKT and myelin protein levels in SCs and Sciatic nerves. The Cellular DNA Fragmentation Assay was used to assess SC apoptosis. Dihydroethidium (DHE) staining and NADPH oxidase activity assay were used for the detection of intracellular ROS in sciatic nerves. Sensorimotor function was assessed via three behavioral tests: The Raised Beam Walking test, Hind paw Withdrawal test and Grip Strength test.

Results: Hyperglycemia resulted in CYP4A upregulation at the level of SCs and Sciatic nerves which correlated with alterations in myelin protein levels, increased SCs apoptosis and abnormal sensorimotor function. Diabetes-induced CYP4A levels were associated with increased ROS production and injury at the molecular and behavioral levels. The deleterious biological output was shown to be mediated by AKT hyperphosphorylation. HET0016 treatment was shown to normalize myelin protein and AKT levels, and restore pain and thermal perception in diabetic mice.

Conclusion: The results of this study suggest that hyperglycemia leads to ROS production by altering CYP4A expression which affects the normal physiology of SCs. This further leads to AKT hyperphosphorylation and abnormal expression of myelin proteins; both of which are pivotal for maintaining myelin and SC integrity and peripheral nerve fiber functionality. The resulting injury was further corroborated at the behavioral level where sensorimotor deficits were observed.

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FIGURES

ABBREVIATIONS

DN/ DPN:	Diabetic Neuropathy/ Peripheral Neuropathy
ROS:	Reactive Oxygen Species
CYP/CYP P450	Cytochrome P450 enzyme
20-HETE	20-Hydroxyeicosatetraenoic acid
MSC80:	Mouse Schwann Cells 80
SC:	Schwann cell
NG/ HG:	Normal/High Glucose
P0 or MPZ	Myelin Protein Zero
PMP22:	Peripheral Myelin Protein 22
HET0016	N-Hydroxy-N'-(4-butyl-2-methylphenyl) formamidine
DHE	Dihydroethidium
mTOR:	Mammalian Target of Rapamycin
NADPH:	Nicotinamide Adenine Dinucleotide Phosphate

CHAPTER I

INTRODUCTION

Diabetes Mellitus (DM) is a chronic, systemic malfunction characterized by a failure in glucose metabolism that can be classified into two types. Type I DM is marked by a deficiency in insulin production due to an autoimmune destruction of pancreatic β-cells. Type II DM is the more prevalent form, and is characterized by insulin resistance or a reduced overall insulin secretion (International Diabetes Federation, 2015). Persistent hyperglycemia is implicated in macrovascular and microvascular complications provoking cardiovascular, renal, and nervous system injuries (American Diabetes Association, 2015). Cardiovascular disorders, heart failure, dyslipidemia, hypertension in addition to skin ulcers and infections arise from damage to the macrovasculature. As for complications contributing largely to the morbidity of DM, microvascular injury manifests as nephropathy, retinopathy, and neuropathy (DCCT, 1993; Ali et al., 2013). Diabetic neuropathy (DN) is the most common complication among diabetic individuals. It is estimated that approximately 50-70% of diabetic subjects will develop DN through the course of their disease, and that risk rises with prolonged duration (World Health Organization, 2016). The clinical manifestations of diabetes-induced nerve dysfunction are heterogeneous and have been reported to include tingling, numbness, sensorimotor wasting, loss of proprioception, pain, and bone structure alterations. The manifestations also include phenotypes of a number of disorders affecting the viscera such as neurogenic bladder, orthostatic hypotension and gastrointestinal disturbances. Subsequently, DN can be classified as peripheral, autonomic, proximal and focal neuropathy (Tesfaye et al., 2010; Callaghan et al., 2012).

The most common type of neuropathy among diabetic patients is Diabetic Peripheral Neuropathy (DPN) which is characterized by paresthesia, pain, loss of sensation and motor function that are often asymptomatic until complications are severe at late stages of diabetes (Genuth, S. 2006; Ziegler et al., 2014; Zhang et al., 2014). Therefore, rigorous clinical examinations are critical for the early detection of DPN.

DPN affects small and large myelinated and unmyelinated fibers in a length-dependent, symmetric or asymmetric manner and progresses from the extremities proximally. The predominant form of DPN is Distal Symmetric Polyneuropathy (DSPN) which is associated with a reduced intraepidermal nerve fiber density, axonal degeneration, reduced nerve conduction velocity, hyperalgesia, allodynia and abnormal neurotropism. DSPN is a disabling complication that increases the risk of ulcers, infections and calluses with subsiding nociception and eventually leads to gangrene and limb amputation. Although the pathophysiology of DSPN is poorly understood, an increasing body of data has shown the effect of diabetes on metabolic and vascular interactions. The long-standing consensus for DPN management involves rigorous glycemic control and symptomatic relief (Rizza et al., 1985; Genuth, S. 2006). Nevertheless, the risk of microvascular complications persists even after optimal management (DCCT 1995; UKPDS 1998). Thus, further investigations are sine qua non for a better understanding of the cellular mechanisms involved in the pathogenesis of diabetic neuropathy.

A. Myelinating Schwann Cells and Injury in the Peripheral Nervous System

1. Myelin Proteins and the Myelination Process

Neural crest-derived Schwann cells (SCs) are the myelin-producing cells in the PNS. SCs play a prominent role in nerve - axon interactions, and in the regeneration, support and growth of nerve fibers. During PNS development SC division is halted in response to neuregulin, which then triggers myelination and the spiral enwrapment of SCs around axons (Michailov et al., 2004; Schulz et al., 2014). The myelin sheath is a specialized, multi-layered and insulating extension of SC membranes that engulfs a nerve axon to produce a myelinated fiber (Sherman, D. L., & Brophy, P. J. 2005). The Node of Ranvier, an unmyelinated segment where two SCs meet, facilitates the conductance of electric potentials along nerve fibers via "saltatory conduction" (Garbay et al., 2000). Myelin integrity is critical for SC and nerve function. Myelin is composed of lipids (71%) and proteins (29%) that are critical for the physiology of neurons (Norton, W. T., & Cammer, W. 1984; Inouye, H., & Kirschner, D. A. 1988). Myelin Protein Zero (P0), peripheral myelin protein 22 (PMP22), P1, P2 and myelin-associated glycoprotein (MAG) constitute the protein component and function at different stages during the myelination process and contribute to maintaining the physiology of SCs and their myelin sheaths (Bilbao, J. M., & Schmidt, R. E. 2015).

Myelin Protein Zero (P0) is the predominant protein (60%) of the PNS produced solely by SCs. P0 is described to be a 30KDa transmembrane, adhesion protein involved in the compaction of myelin through interactions between its extracellular and cytoplasmic domains (Giese et al., 1992; Suter, U., 1997). Defects in P0 expression have been implicated in disorders due to loss of compaction such as in dysmyelinating neuropathies (Hayasaka et al., 1993; Latour et al., 1995; Shy, ME., 2006). However, it has been reported that altered P0 expression does not lead to complete dysmyelination which suggests that P0 works in collaboration with other myelin proteins to maintain the myelin sheath's molecular architecture (Giese et al., 1992).

Peripheral Myelin Protein (PMP22) is another myelin protein exclusive to the PNS whose expression is concomitant with myelin production by SCs (Snipes et al., 1992; Jetten, A. M., & Suter, U. 2000). PMP22 mutations in humans have been associated with genetic disorders such as Charcot-Marie-Tooth and inherited neuropathies (Katona et al., 2009). Studies conducted in transgenic animal models have reported that mutations in the PMP22 gene during development affect SC differentiation, myelination, and may lead to SC apoptosis (Robertson et al., 1999; Niemann et al., 2000; Sahenk et al., 2003; Jun et al., 2013). The importance of PMP22 in initiating the myelination process was investigated in PMP22 $^{-/-}$ mice models. Motor nerves obtained from the PMP22 deficient mice showed immature SCs with failed myelin formation (Adlkofer et al., 1995).

More importantly, PMP22 is involved in heterophillic interactions with P0 (Hasse, B., & Bosse, F. 2004). Thus, P0 and PMP22 interactions determine the precise arrangement and function of myelin. Together, any alteration in expression of either protein would have direct and detrimental effects on SCs, neuronal function, as well as signal exchange to and from the PNS (D'Urso, D., Ehrhardt, P., & Müller, H. W. 1999).

2. Diabetes and Schwann Cell Dysfunction

Neural networks in the periphery are complex structures characterized by interactions with surrounding vascular endothelia. The primary source of energy for the PNS is glucose which is processed by SCs and is largely attributed to axonal repolarization (Bradbury, M.W.B., & Crowder, J. 1976). The key pathogenic factor triggering DPN is elevated blood glucose levels (Green et al., 1999). Hyperglycemia provokes injury at the level of blood vessels and capillaries through metabolic abnormalities and oxidative stress due to excessive glucose metabolism

(Cameron, N. E., & Cotter, M. A. 1997; Gao et al., 2014, Cinci et al., 2015). Consequently, pathophysiology that affects the vasa nervorum around the endoneurium may affect nerve fibers (King et al., 1989). The PNS tissue has been reported to be insulin-independent and highly vulnerable to chronic hyperglycemia due to the inefficiency in regulating glucose uptake in contrast to endothelial cells (Leinninger et al., 2006; Hinder et al., 2013). Indeed, mounting evidence suggests that SCs are primarily targeted in DPN (Magnani et al., 1998; Askwith et al., 2009; Chan et al., 2011).

In vitro studies on cultured rat primary SCs reported that hyperglycemia significantly increased apoptosis (Wu et al., 2012; Liu et al., 2016) and reduced proliferative potential (Gumy et al., 2008). Furthermore, recent investigations showed the implications of chronic hyperglycemia in SC de-differentiation due to underproduction of neurotrophic factors (Dey et al.,2013; Hao et al., 2015) showing a state of severe SC dysfunction. Similarly, morphological abnormalities were reported in nerve biopsies from diabetic animal models. Electron microscopy of sciatic nerves from diabetic rats showed disordered myelin sheaths and SCs with shrunken vacuoles corroborating previously reported SC dysfunction (Li et al., 2016). Also, glucose-mediated neuronal injury was observed where neurons were described to be hypertrophic with giant vacuoles lining axons of neurons in dorsal root ganglia (DRG) of diabetic rats. SCs surrounding DRG neurons were reported to be loosely bound and misarranged relative to their controls.

Moreover, electrophysiological studies showed biphasic nerve malfunction in diabetic subjects. During the early stages of DPN, small myelinated and unmyelinated fibers are affected which manifests as hyperalgesia (Courteix et al., 1993; Ohsawa et al 2008). By the end stages of DPN, nociception is diminished and marked by reduced nerve conduction velocities (Ishii, D. N.

(1995), dysmyelination, impaired and slow axonal transport (Juranek et al., 2013), axonal atrophy and degeneration (Fross, R. D., & Daube, J. R. 1987) in addition to altered sensory and motor potentials (Becker et al., 2014).

B. Pathophysiology of Reactive Oxygen Species in Diabetes

1. Endogenous Reactive Oxygen Species in Diabetes

Reactive Oxygen Species (ROS) are oxygen-containing biologically active molecules that are byproducts of ongoing cellular reactions. ROS are crucial entities for cellular physiology and are involved in gene expression, signal transduction, and homeostatic maintenance (Turpaev, K. T. 2002). Intracellular ROS signaling is well regulated via antioxidant defense mechanisms that neutralize the bioactive radicals (Bursell et al., 1999; Haak et al., 2000; Packer et al., 2001; Xu et al., 2014). Hyperglycemia has been shown to disrupt the oxidant-antioxidant balance by triggering additional ROS production (Cameron, N. E., & Cotter, M. A., 1999; King, G. L., & Loeken, M. R. 2004; Singh et al., 2014). Several studies showed that blockade of ROS can be beneficial but also in some cases injurious (De Zeeuw et al., 2013). Subsequently, the identification of cellular sources of ROS is central to understanding the pathobiology of diabetes.

Intracellular glucose metabolism is associated with ROS production via glucose autoxidation, mitochondrial oxidative phosphorylation, and the production of advanced glycation end products. Additionally, a number of enzymes have been implicated in hyperglycemia-induced ROS production and are reported to include nicotinamide adenine dinucleotide phosphate oxidase (NOX), cytochrome P450 monoxygenase, nitric oxide synthase, lipoxygenase, cyclooxygenase, and xanthine oxidase (Niedowicz, D. M., & Daleke, D. L. 2005).

When ROS overwhelm cellular defense responses, injury ensues through lipid and protein oxidation, altered metabolism, activation of intracellular signaling and transport pathways, and ultimately programmed cell death (Vincent et al., 2004).

2. Oxidative Stress in Diabetic Complications

Extensive research has shown that ROS overproduction poses a pathogenic state in multiple organs and is the common feature to the pathogenesis of diabetic complications (Lambeth Krause, K. H., & Clark, R. A., 2008; Maalouf et al., 2012; Naziroglu et al., 2012; Eid et al., 2013; Nayernia et al., 2014; Kowluru, A. R., & Mishra, M. 2015; Filla, A. L., & Edwards, L. J. 2016). Numerous studies have shown that inhibition of ROS sources ameliorated oxidative stress in diabetes-induced renal, cardiovascular and other system injuries (Lambeth, J. D., Krause, K. H., & Clark, R. A. 2008; Eid et al., 2009; Eid et al., 2010; Wu et al., 2012). However, the role of, as well as knowledge of the sources, of ROS and their inhibition in the nervous system are still under investigation (Nayernia et al., 2014; Li et al., 2016). Recent studies described several mechanisms leading to ROS-induced organ damage and in some cases nerve damage. Yet, a lot of work still needs to be done to elucidate the mechanisms of injury. Increased superoxide dismutase activity (a superoxide radical scavenger) via puerarin was shown to reduce oxidative stress and apoptosis in SCs cultured in high-glucose (Wu, Y., Xue, B., Li, X., & Liu, H. 2012). Inhibition of aldose reductase of the polyol pathway, known to be upregulated in diabetes, in both diabetic rats treated with epalrestat (Li et al., 2016) and human sural nerves treated with zenalrestat (Greene et al., 1999) showed reduced myelinated nerve fiber loss and SC injury by decreasing oxidative stress.

C. Cytochrome P450 Enzymes in Diabetes

1. Cytochromes and Arachidonic Acid Metabolism

Cytochromes P450 (CYP450s) are a large family of hemoproteins responsible for metabolism of endogenous (hormones, cholesterol, fats, steroids, acids, vitamins), and exogenous molecules (toxic compounds and drugs). CYPs have been described to be located bound to membranes of either the mitochondria or endoplasmic reticulum and to be associated with redox reactions (Rendic, S., & Carlo, F. J. D. 1997). Additionally, CYPs are reported to be major sources of ROS (Figure 1) in numerous tissues (Bondy, S. C., & Naderi, S. 1994; Puntarulo, S., & Cederbaum, A. I. 1998; Fleming et al., 2001; Dunn et al., 2008; Medhora et al., 2008) with implications in diabetic complications (Eid et al., 2009; Eid et al., 2013). In humans, approximately 60 CYP genes have been identified; any polymorphism associated with a gene is associated with enzyme dysfunction and a range of disorders (Panda et al., 2001; Nebert, D. W., & Russell, D. W. 2002; Fan et al., 2015).

One of the physiologically relevant reactions catalyzed by CYP450 enzymes is arachidonic acid metabolism. Arachidonic acid release is induced by the activation of phospholipase A2 from membrane phospholipids. Free arachidonic acid may be metabolized by the cyclooxygenase, lipooxygenase and moonoxygenase pathways. CYP isoforms involved in the moonoxygenase pathway may be classified as hydroxylases or epoxygenases and yield numerous eicosanoid products. The ω-hydroxylation (addition of a hydroxyl moiety at the terminal ω-carbon) of arachidonic acids produces 20-hydroxyeicosatetraenoic acid (20-HETE) and is catalyzed by ω -hydroxylases of the CYP4A and CYP4F subfamilies (Figure 2). The most extensively studied subfamily is CYP4A and is largely expressed in liver and kidney tissue, although CYP4A expression has also been observed in the brain, vascular smooth muscle, prostate, lungs and

intestine (Strömstedt et al., 1990; Zhou et al., 1996; Zhu et al., 1998; Strömstedt, M., Warner, M., & Gustafsson, J. Å. 1994; Koike et al., 1997). Yet, the cellular distribution of CYP4A, in addition to the physiological and pathophysiological role, in the PNS has not been described yet. Moreover, 20-HETE has been reported to have various and antagonistic functions depending on the synthesis site and target tissue (Zhu et al., 2000; McCarthy et al., 2005; Dahly-Vernon et al., 2005; Nilakantan et al., 2008; Dhanasekaran et al., 2009; Zeng et al., 2010). 20-HETE plays a major role in circulation hemodynamics (Wang et al., 2011), vascular remodeling (Ding et al., 2013), angiogenesis (Carmeliet, P., & Jain, R. K. 2011), in addition to blood flow and pressure regulation via the renin-angiotensin axis (Alonso-Galicia et al., 2002). However, recent studies have attributed a role of 20-HETE in organ damage. Inhibition of 20-HETE production reduces abnormal cellular growth (Guo et al., 2008), vascular inflammation (Hoff et al., 2011) and diabetic nephropathy (Eid et al., 2013) suggesting a pathogenic feature of 20-HETE. However, the role of 20-HETE in DPN is not yet elucidated.

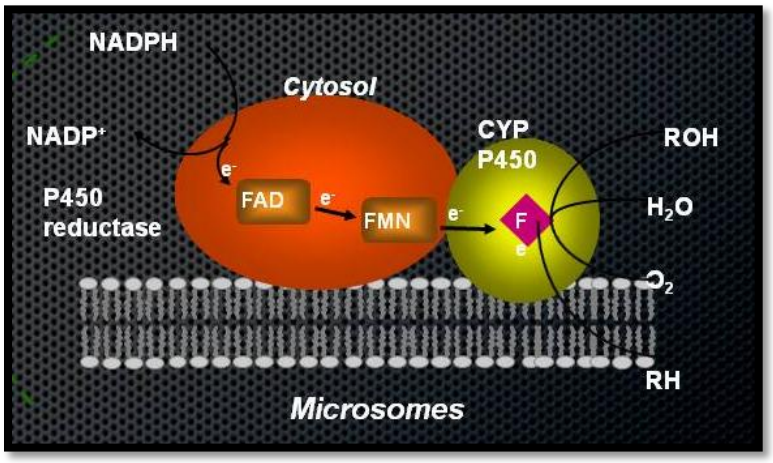


Figure 1. Reactive oxygen species production and redox cycling by CYP monooxygenases. CYPs of the monooxygenase subfamily contain a heme-based catalytic center, an NADPH reductase subunit and an NADH/NADPH oxidase as cofactors. CYP450s are present in ferric and ferrous forms and the initial chemical reaction that takes place is the binding of a substrate to the ferric form. CYP450 reductase donates an electron to reduce the ferric form to a ferrous

intermediate which then binds to an oxygen molecule to form a complex. The complex is reduced by the addition of a proton which is concomitant with H_20 production and an oxenoid complex whereby activated oxygen bound to the heme moiety incorporates into the substrate to yield an oxidized form (Johnston et al., 2011; Adapted from Eid et al., 2014).

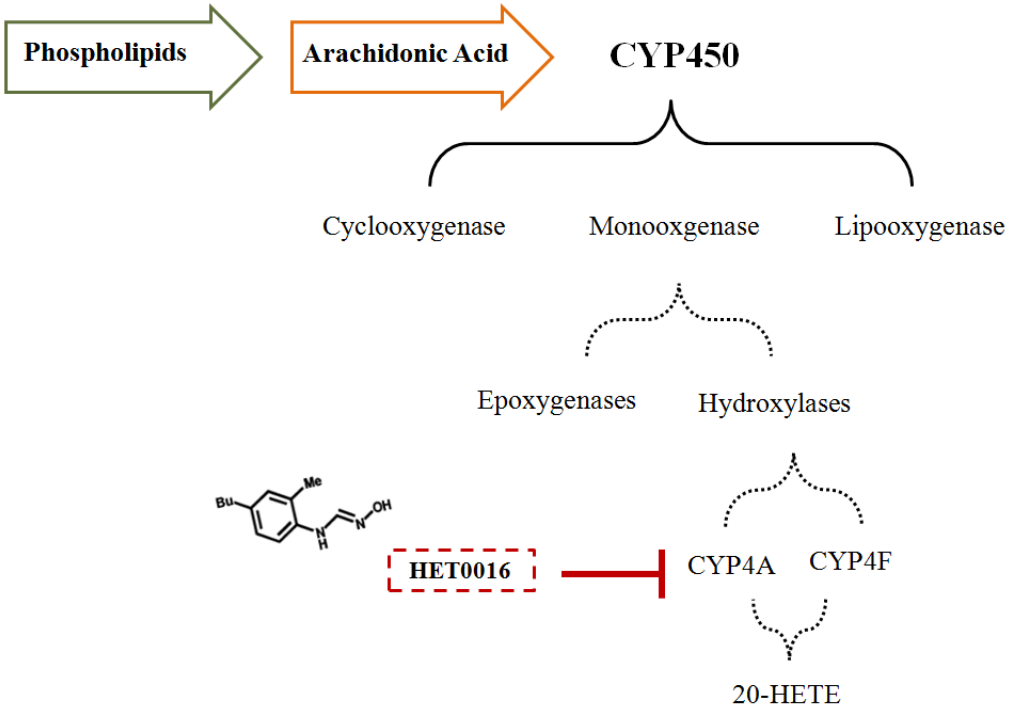


Figure 2. Pathways of Arachidonic Acid Metabolism.
Phospholipids catabolized by phospholipase to produce free arachidonic acid which is further metabolized via CYP450 enzymes through three different pathways.

2. Oxidative Stress in Diabetic Complications

The physiological significance of cytochrome proteins and their metabolites is under intense investigations in diabetic complications. In the past decade, 20-HETE has been increasingly shown to play a role in oxidative stress (Guo et al., 2007; Cheng et al., 2008; Medhora et al., 2008; Zeng et al., 2010) and in diabetic nephropathy, cardiomyopathy and retinopathy with no description of its role in neuropathy. It has been reported that high glucose upregulates CYP4A, 20-HETE and ROS production in a sequential manner with NOX enzymes which leads to kidney

glomerular epithelia apoptosis, tubular and glomerular hypertrophy, in addition to ischemia, leading to renal dysfunction and kidney injury. But, the inhibition of 20-HETE synthesis via HET0016, a specific CYP4A inhibitor, was shown to reverse renal injury (Eid et al., 2014). Moreover, 20-HETE is known to be a vasoconstrictor that may induce cardiac hypertrophy and dysfunction (Jenkins, C. M., Cedars, A., & Gross, R. W. 2009; Alsaad et al., 2013). Similarly, 20-HETE in diabetic retinopathy was shown to influence retinal microvascular hemodynamics. Retinal blood flow measurements in diabetic mice recorded a decreased blood flow, velocity and shear rate which may be related to clinical observations of capillary occlusion due to endothelial damage. The inhibition of 20-HETE synthesis was reported to significantly ameliorate the decreases in retinal circulatory parameters suggesting an injurious role for 20-HETE in diabetic retinopathy (Wang et al., 2011). However, other studies discussed a beneficial role of 20-HETE in the kidney and lungs (McCarthy et al., 2005; Regner et al., 2009; Dhanasekaran et al., 2009). Although the physiology of 20-HETE is beginning to unravel in various organs, the role of CYP4A and 20-HETE in the central nervous system are limited. However, to our knowledge, no investigations have been conducted in the PNS and diabetic neuropathy.

3. 20-HETE and the Neurovascular Unit in the Central Nervous System

With limited reserves, the energy and oxygen requirements of the brain are supplied via the neurovascular unit; a complex meshwork of neurons, astrocytes and an endothelium lined by myocytes and pericytes (Lecrux, C., & Hamel, E. 2011). The unit is vital for monitoring and adjusting cerebral blood flow and perfusion according to neuronal activity through cerebral autoregulation mechanisms (ensuring constant pressure and flow) and neurovascular coupling (ensuring a spatial and temporal distribution between neurons) (Zonta et al., 2003; Hamel, E. 2006). It has been reported that cells of the neurovascular unit express CYP450 enzymes (Köhler

et al., 1988) and are capable of 20-HETE biosynthesis (Nithipatikom et al., 2001). The vasoactive agent, 20-HETE, has been increasingly shown to play a critical role in the central nervous system (Filosa, J. A., & Iddings, J. A. 2013).

CYP4A expression is reported to be localized in arteriolar muscle tissue and astrocytes while 20-HETE to be involved in cerebral vascular tone and autoregulation (Gebremedhin et al., 2000; Kim et al., 2015; MacVicar, B. A., & Newman, E. A. 2015). Elevated 20-HETE levels have been shown to reduce cerebral blood flow (Fordsmann et al., 2013). However, the vasoconstrictive actions of 20-HETE in the brain are dynamic and are influenced by nitric oxide levels and other CYP metabolites and prostaglandins (Yu et al., 2004; Liu et al., 2008). The importance of 20-HETE in the myogenic response and blood brain barrier physiology was investigated in CYP4A1 transgenic animal models. The data showed that a genetic deficiency in 20-HETE production was detrimental and may lead to cerebral injury in hypertensive rats (Fan et al., 2015). Additionally, 20-HETE has been shown to be of significance in brain development (Anwar-Mohamed et al., 2014).

In contrast, various studies investigated the protective role of 20-HETE inhibition via HET0016. *In vitro* studies on cultured endothelial cells have shown that 20-HETE may be involved in inflammation (Cheng et al., 2008; 2010) and oxidative stress (Toth et al., 2013). HET0016 treatments in hypertensive rats were capable of reducing superoxide production, oxidative stress, inflammation, and restoring vasomotor function (Toth et al., 2013). A study on hippocampal slices subjected to oxygen-glucose deprivation followed by reoxygenation showed a rise in 20-HETE production in addition to ROS and caspase-dependent neuronal death. However, HET0016 was shown to be neuroprotective against ischemia (Renic et al., 2012) in

addition to brain edema and blood brain barrier dysfunction following reperfusion injury (Liu et al., 2014).

The debate as to whether 20-HETE insults or compliments the central nervous system is ongoing and extensive *in vivo* studies are pivotal to progress further. However, with regards to the periphery, the bulk of investigations on 20-HETE were limited to the cardiovascular and renal systems. They were conducted within a pathophysiological, vascular endothelial context such as in hypertension, acute kidney injury and diabetic complications with the exception of DPN. Indeed, vascular alterations have been shown to mediate DPN although the underlying factors influencing the pathogenesis have not yet been identified (Cameron et al., 2001).

D. The PI3K/Akt/mTOR Signaling Pathway

The mammalian target of rapamycin (mTOR) pathway is a nutrient, energy and stress sensor and a modulator of cellular growth and division. mTOR plays a role in a panoply of injurious pathways and has been shown to be involved in diabetes onset and progression (Zoncu, R., Efeyan, A., & Sabatini, D. M. 2011). mTOR is a large protein association consisting of two complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2). The downstream effectors of mTORC1 are p70S6 kinase and 4E-binding protein 1 which interact in response to rapamycin. By contrast, mTORC2 is rapamycin insensitive and phosphorylates Akt (a serine/threonine kinase and a key effector) at its Serine473 residue fully activating it (Sarbassov et al., 2005).

With regards to the PNS, mTORC1 deficiency is described to be associated with influencing lipid-synthesizing enzyme expression, hypomyelination and reduced nerve conduction velocities (Hall, M. N., & Suter, U. 2014). Also, mTORC1 pathway has been shown to mediate diabetic nephropathy and neurocardiac injury (Gödel et al., 2011; Maiese, K. 2015). Ongoing studies in

our laboratory investigate the role of mTORC1 in DPN. By contrast, studies on mTORC2 are very limited. Eid S et al. showed the implications of mTORC2 through an Akt dependent pathway to play a key role in diabetic kidney injury, thus suggesting a therapeutic potential of mTORC2 inhibition (Eid et al., 2016).

In the same spirit, there is evidence suggesting that Akt activation in dorsal root ganglia and spinal cord may play a role in the development of neuropathic pain (Xu et al., 2007). It was also demonstrated that an increase in ROS and activated Akt may contribute to the development of neuropathic pain, further suggesting a crosstalk between the two pathways (Guedes et al., 2008). In contrast, Akt was described to be neuroprotective in diabetic enteric neuropathy and that neuronal loss may be mediated by reduced Akt activation (Anitha et al., 2006; Du et al., 2009). Additionally, emerging literature reveal the role of Akt in myelination. In rat SCs, Akt is critical to the survival and progression of the cell cycle (Li et al., 2001; Monje, P. V., Bartlett Bunge, M., & Wood, P. M. 2006) and regulation of the migratory potential following peripheral nerve injury (Yu et al., 2015). A study conducted in the central nervous system of zebra fish suggested that mTOR activity in oligodendrocytic cells requires cholesterol for the expression of myelin genes yet axon enwrapment with myelin is facilitated through the Akt pathway independent of mTOR (Mathews, E. S., & Appel, B. 2016). In the PNS, Akt was shown to play a role in myelin thickness regulation, axonal wrapping, and membrane production. However, prolonged Akt activation may lead to dysmyelination (Domènech-Estévez et al., 2016). Yet, the roles of Akt and mTORC2 in DPN have yet to be characterized.

E. Hypothesis and Aim of the Study

Diabetes targets numerous systems among which is the nervous system and particularly the PNS. Diabetic complications may lead to organ damage but could be ameliorated with rigid glycemic control. Yet despite that, DPN onset is autonomous. The comprehension of the mechanistic progression of DPN is central to identify novel therapeutic targets. There is a clear coalition between CYP-induced oxidative stress and diabetic complications, but the implication of CYPs as sources of oxidative stress in SC injury is a theory subject to investigations. The hypothesis for this study is that diabetes induces oxidative stress through altering CYP4A expression and activity affecting the Akt signaling pathway. These insults eventually lead to SC injury, which could be assessed by measuring alterations in myelin protein levels. It is also hypothesized that selectively inhibiting CYP4A may reverse damage at the molecular and behavioral levels.

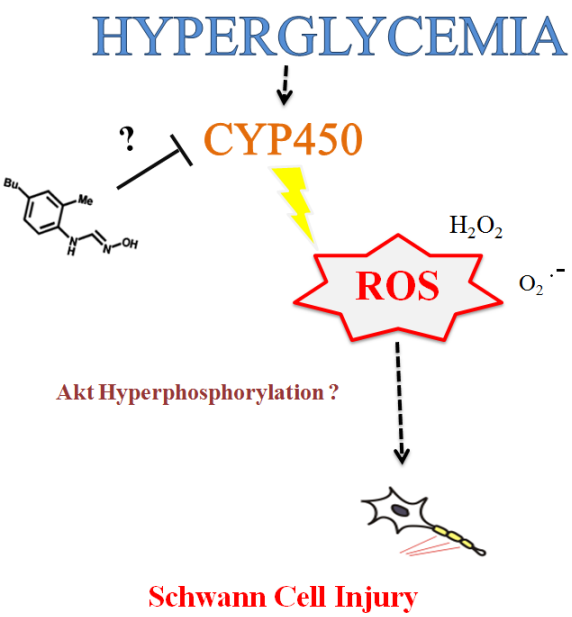


Figure 3: Hypothesis. Hyperglycemia leads to oxidative stress through activation of CYP4A ω-hydroxylase. Increased ROS production causes Schwann cell injury, myelin protein alterations and induces apoptosis through Akt hyperphosphorylation**.**

CHAPTER II

MATERIALS AND METHODS

A. Cell Culture

Mouse Schwann cells (MSC80) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Steinheim, Germany) containing 10% Fetal Bovine Serum (FBS) (Sigma-Aldrich) and 1% Penicillin/Streptomycin (P/S) (Sigma-Aldrich). Cells were incubated at 37ºC with 5% CO2 until confluency was reached. For experimental work, ells were serum deprived overnight then treated with high glucose (25mM) in the presence or absence of CYP4A inhibitor (HET0016). In parallel experiments, cells were treated with 1.5 μM 20-Hydroxyeicosatetraenoic acid (20-HETE, Cayman Chemicals) in the presence of normal glucose (5mM). Cells incubated with 5mM glucose are euglycemic and considered controls.

B. Microsome Isolation

MSC80 cells were incubated either in normal glucose (NG), high glucose (HG), or 20-HETE and then homogenized, dounced, and sonicated in a 250 mM sucrose, 1mM EDTA, 2μg/ml aprotinin, 2μM leupeptin and 1mM PMSF. The homogenates were then centrifuged at 10,000g for 15 minutes at 4ºC. The supernatant was collected and differentially centrifuged 65000 rpm for 30 min at 4ºC. The microsomal pellet was then resuspended in a buffer consisting of 50 mM potassium phosphate of pH 7.5, containing 0.2mM EDTA and 20% glycerol. The microsomal pellet was then used for Western blotting (Eid et al., 2009).

C. Animal Models

All animal work was conducted according to the institutional guidelines and approved by the Institutional Animal Care and Use Committee. Animal models exhibiting the genotypic and phenotypic profiles of type II diabetes were used for this study. Male 6-week-old MKR mice were used. FVB/NJ males of the same age were used as a control. The animals were grouped into three subsets 1) non-diabetic controls 2) untreated diabetics 3) diabetics treated with HET0016, a specific CYP4A inhibitor (Miyata et al., 2001) for 10 weeks. HET0016 was administered subcutaneously at a 2.5 $mg \cdot kg^{-1} \cdot day^{-1}$ dose (Eid et al., 2009). All animals were kept in a temperature-controlled room and on a 12/12-dark/light cycle and had standard chow and water access. Blood glucose levels were monitored twice a week via tail vein punctures and a glucometer and were significantly different in diabetic animals relative to control littermates. Prior to sacrifice, sensory motor dysfunction was assessed using the Raised Beam Walking Test and Hind paw Withdrawal Test. Neuromuscular interaction was assessed using the Grip Strength Test.

D. Raised Beam Walking Test

Motor coordination and balance assessment is performed via the Raised beam walking test (Luong et al., 2011). Animals were placed on a platform with a rod of 1.2 cm diameter, 70 cm length and around 50cm above a flat surface. At one end of the rod we set a secure platform to house the animal. First, the mouse was allowed to adapt and then trained to cross after which the time taken to cross, the speed, the number of stops and the number of faults/slips were recorded for analysis.

E. Plantar Analgesia Test

Diabetic animals are reported to have reduced sensation. Thermal analgesia and pain perception were assessed for this study using the Hind paw withdrawal test (Dirig et al.,

1997). The IITC plantar Analgesia meter was used and set up according to the manufacturer's protocol. The test is characterized by a heating beam set at an idle intensity of 2% and active intensity of 25% with a cut-off time set at 20 seconds. The platform onto which animals were placed for acclimation was set at 32 °C. The heating beam was targeted at the hind paw of animals and the time to sense the heat and withdraw their paws was recorded for analysis.

F. Grip Strength Test

Muscle tone and neuromuscular strength was assessed using the grip strength test (Brooks, S. P., & Dunnett, S. B. 2009). Mice were trained to hang using their forelimbs from a stand approximately 10cm long and 50cm high until their grip fails. The time spent hanging was recorded over 3 consecutive days. The animals with a reduced capacity to grip unhandled the bar faster that animals with greater muscle strength, which is typical of diabetic vs. non-diabetic animals.

G. Western Blot

Cultured MSC80 cells and mouse sciatic nerves were lysed using RIPA buffer containing 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxylate, 150 mM sodium chloride, 100 mM EDTA, 50 mM Tris-hydrochloride, 1% Tergitol (NP40), 1% of the protease and phosphatase inhibitors and 1mM phenylmethylsulfonyl fluoride. The lysates were centrifuged at 13,600 rpm for 30 minutes at 4°C. Protein concentration in the supernatants was measured using the Bradford Protein Assay. For immunoblotting, 20-40 μg of proteins were separated on 12-15% polyacrylamide gel Electrophoresis (Bio-Rad Laboratory, CA, USA) and transferred to nitrocellulose membranes (Bio-Rad Laboratory, CA, USA). The blots were blocked with 5% BSA in Tris-buffered saline and then incubated overnight with

rabbit polyclonal anti-CYP4A (1:2000, Abcam), rabbit polyclonal anti-P0 (1:1000, Abcam), rabbit polyclonal anti-PMP22 antibody (1:1000, Abcam) and rabbit polyclonal anti-pAKT^{Ser473} (1:1000, Cell Signaling). The primary antibodies were detected using horseradish peroxidase–conjugated IgG (1:1000, Bio-Rad). Bands were visualized by enhanced chemiluminescence. Densitometric analysis was performed using Image J software (Eid et al., 2009)

H. Cellular DNA Fragmentation / Apoptosis Assay

Schwann cells apoptosis was investigated *in vitro* using the cellular DNA fragmentation ELISA kit (Roche Diagnostics GmbH, Mannheim, Germany). BrdU-labeled DNA fragments in. MSC80 cells were grown in 12-well tissue culture plates until 60-70% confluence and serum deprived overnight and then treated for 48 hours according to the experimental conditions. A microplate was prepared using an anti-DNA coating solution containing anti-DNA antibody adsorptively fixed to the wells of the plate. Then, BrdU-labeled DNA fragments (10 μM), which bind to the immobilized anti-DNA antibody, were added to the cultured MCT cells 12 hours before stopping the treatment. In the third step, the immunocomplexed BrdU-labeled DNA fragments were denatured and fixed on the surface of the microplate by microwave irradiation, in order to improve the accessibility of the antigen BrdU for detection by the antibody. As a final step, anti-BrdU-peroxidase conjugate reacted with the BrdU incorporated into the DNA. The amount of peroxidase bound in the immune complex was photometrically determined, after the removal of unbound peroxidase conjugates and the addition of the substrate solution. Absorbance was measured at 450 nm against a reference wavelength of 650 nm using a microplate reader (Multiskan Ex).The mean of triplicate experiments was used +/- SD.

I. NADPH Oxidase Activity Assay

NADPH oxidase activity was measured in sciatic nerves and in Schwann cells grown in complete medium (10% FBS, 1% P/S). Proteins were extracted from sciatic nerves using cooled mortar and pestle by smashing the frozen nerve and suspending the remnants in the lysis buffer (20 mM KH2PO4 (pH 7.0), 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 0.5 μg/ml leupeptin). Cultured MSC80 cells were washed twice with ice-cold phosphate-buffered saline and scraped from the plate on ice using the lysis buffer as well. To start the assay, 25 μg of homogenates were added to 50 mM phosphate buffer (pH 7.0) containing 1 mM EGTA, 150 mM sucrose, 5 μM lucigenin (behaving as the electron acceptor), and 100 μM NADPH (acting as the substrate for the NADPH oxidase). Photon emission expressed as relative light units (RLU) was measured every 30 s for 5 minutes in a luminometer. Superoxide production was expressed as relative light units/min/mg of protein. Protein content was measured using the Bio-Rad protein assay reagent.

J. ROS Assessment in Sciatic Nerves

Dihydroethidium (DHE), which is relatively specific for superoxide anion measurement, is an oxidative fluorescent dye that undergoes a two-electron oxidation to form the DNA-binding fluorophoreethidium bromide. DHE staining for superoxide was carried out as previously described (Maalouf et al., 2012). Briefly, frozen sciatic nerves were cut into 4 μm thick sections and placed on glass slides. DHE (20 μmol/l) was applied to each tissue section, and the slides were incubated in a light-protected humidified chamber at 37°C for 30 min. Fluorescent images of ethidium-stained tissue were obtained with a laser-scanning confocal microscope (Zeiss, LSM 710) at t=30 mins. Fluorescence was detected at 561 nm long-pass filter. Superoxide generation was demonstrated by red fluorescent labeling. The average of

four areas per section stained with DHE was taken as the value for each animal. Quantification was done using Zen light Software.

K. Statistical Analysis

Results are expressed as mean ± SE from multiple independent experiments. Statistical significance is assessed by one-way ANOVA and student's unpaired t-test. *p*-value <0.05 is considered as statistically significant. When comparing control and diabetic, one asterisk (*) is used when $p<0.05$ and ** when $p<0.01$; The (#) symbol is used when comparing untreated diabetic groups vs. treated diabetics at $p<0.05$.

CHAPTER III

RESULTS

A. High Glucose alters CYP4A expression in cultured Schwann cells.

Reactive oxygen species (ROS) production has been well documented to be associated with diabetic complications. Cytochromes P450 enzymes (CYPs) in particular, have been identified as a source of these chemical species (Eid et al., 2009; Eid et al., 2013a,b). Enzymes of the CYP4A subfamily have been extensively studied in our lab and shown to be involved in nephropathy and cardiomyopathy (Eid et al., 2009; Abou Salem et al., unpublished data). However, CYP expression has not been investigated nor reported in the peripheral nervous system (PNS) and diabetic neuropathy. Subsequently, we first examined whether CYP4A is expressed in Mouse Schwann cells (MSC80). Microsomes were isolated from SCs cultured with 5 mM (normal glucose) and 25mM (high glucose). The present study is the first to demonstrate the expression of CYP4A in SCs . This expression was significantly increased 6 hours after treatment with high glucose (25mM) and sustained until 48 hours (Figure. 4) implicating its role in diabetes.

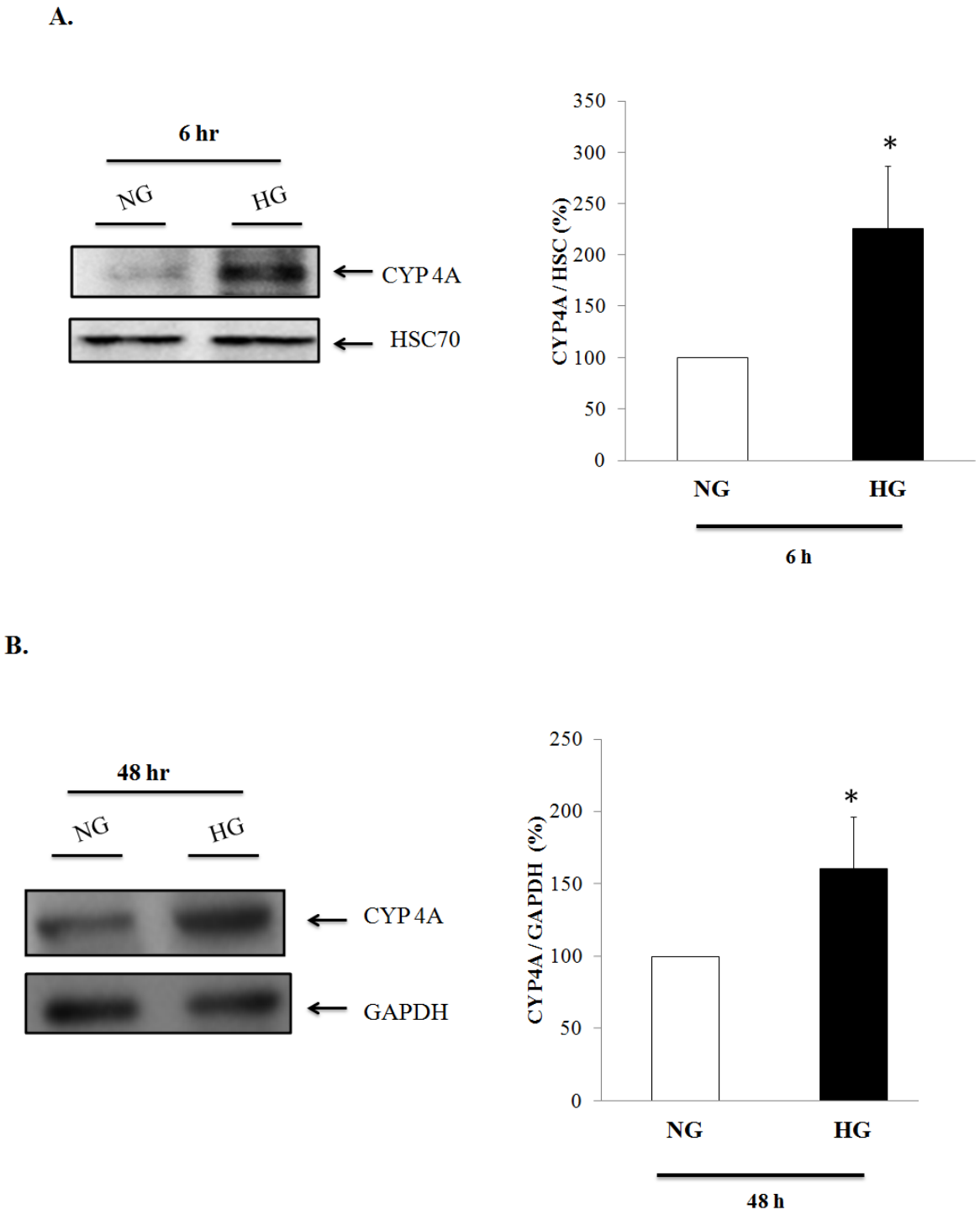
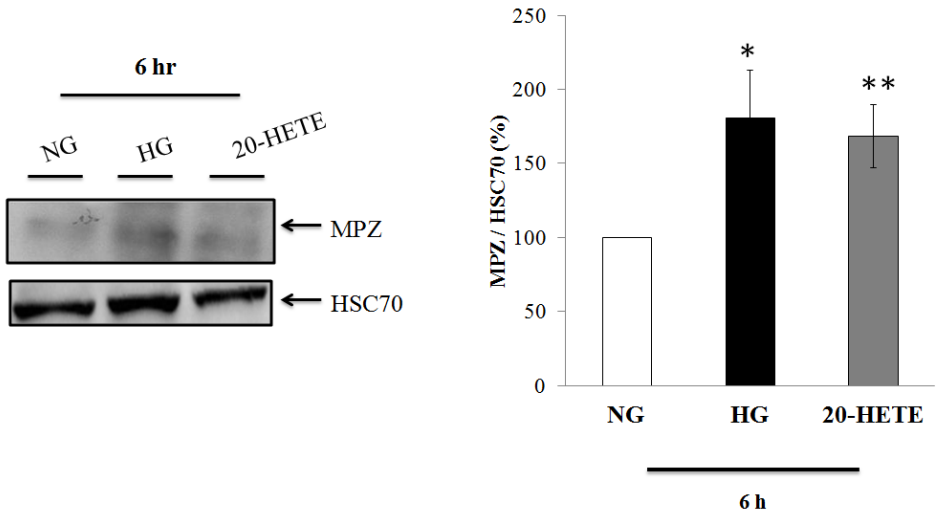


Figure 4. HG induces CYP4A protein expression in mouse Schwann cells.
Representative western blot analyses of microsomal proteins obtained from MSC80 cells in the presence of NG (5mM) or HG (25mM) showing CYP4A expression and a histogram showing the protein levels after 6 hours (A) and 48 hours (B) of HG treatment. Values of six independent experiments (n=6) are the mean ± SE. *P<0.05, high glucose vs. normal glucose.

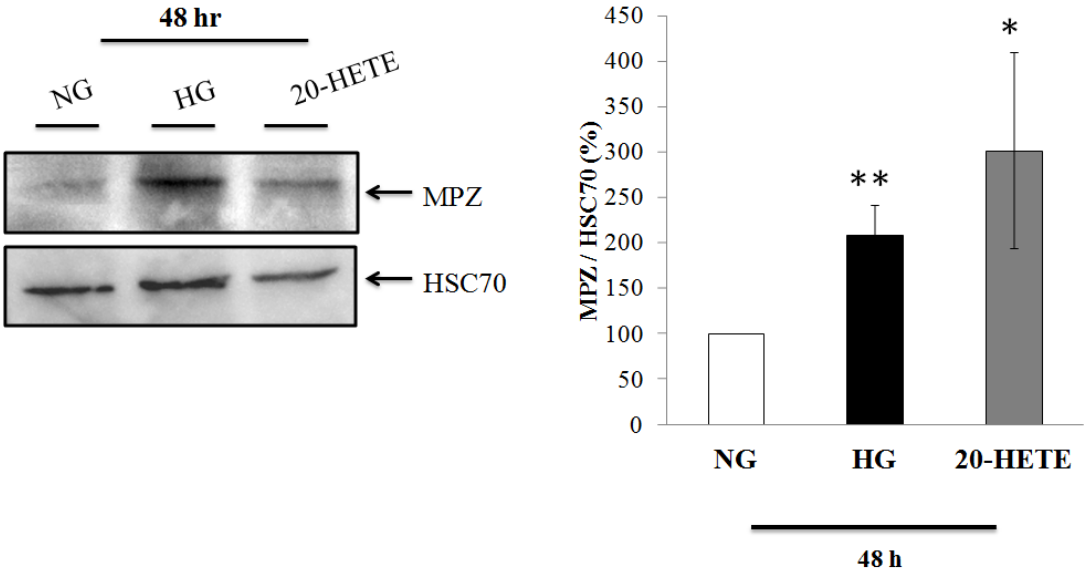
B. HG induces Schwann cell hypermyelination through a Cytochrome P450-dependent pathway.

The role of CYP4A alteration as well as 20-HETE production on myelin protein expression and myelin injury has also been determined in this study. Our findings provided evidence that SC injury could be triggered by HG treatments through an increased expression of MPZ and PMP22. Concomitantly, 20-HETE treatments mimicked the effect of HG on SCs where MPZ expression was significantly elevated at 6 and 48 hours of treatment (Figure 5 A-B). Similarly, PMP22 expression was increased slightly at 6 hours, and significantly at 48 hours (Figure 5 C-D).

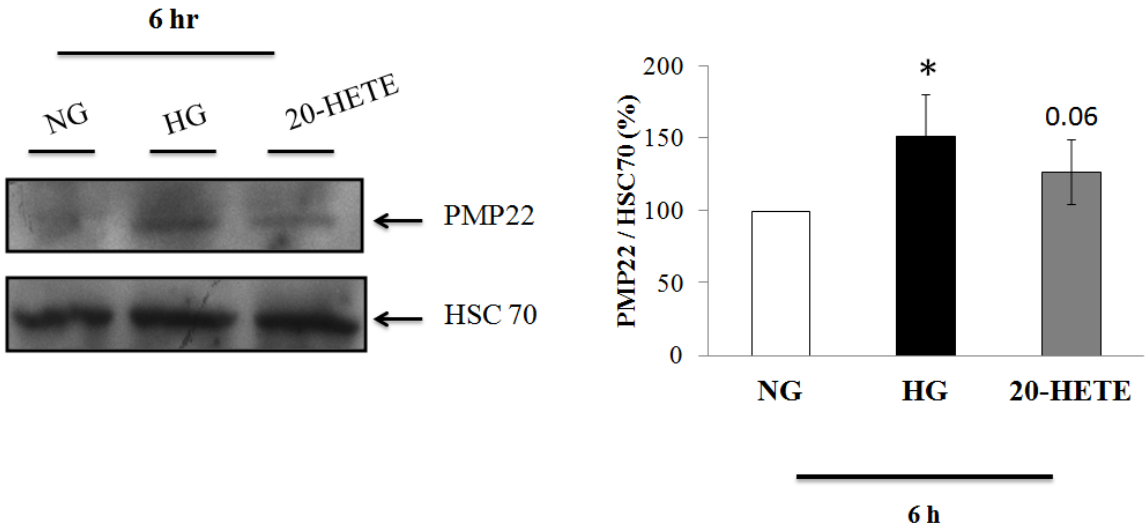
A.



B.


48 hr
NG
HG
20-HETE
MPZ
HSC70
MPZ / HSC70 (%)
450
400
350
300
250
200
150
100
50
0
**
*
NG
HG
20-HETE
48 h

C.


6 hr
NG
HG
20-HETE
PMP22
HSC 70
PMP22 / HSC70 (%)
200
150
100
50
0
*
0.06
NG
HG
20-HETE
6 h

D.

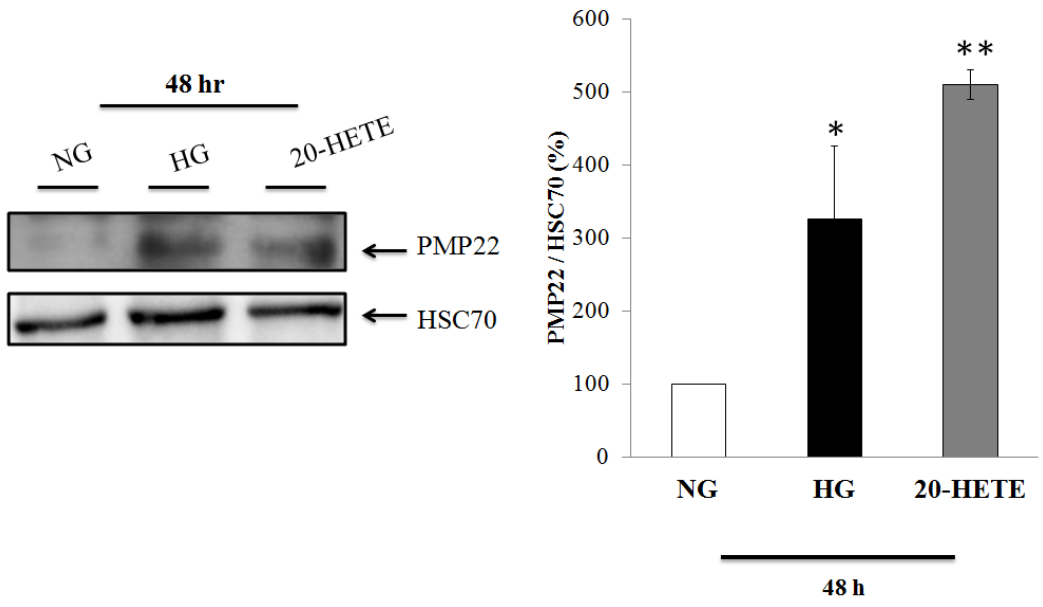


Figure 5. HG and 20-HETE lead to myelin protein alterations
Western blot analysis of MPZ and PMP22 myelin proteins from cells exposed to HG and 20-HETE for 6 and 48 hours. Representative western blots of MPZ (n=3) (A-B) and PMP22 (C n=4, D n=3) and their respective densitometric analysis at 6 and 48 hours. Values are the mean ± SE. *P<0.05, high glucose or 20-HETE vs. normal glucose. **P<0.01, high glucose or 20-HETE vs. normal glucose.

C. High glucose and exogenous 20-HETE levels induce Schwann cell apoptosis

MSC80 cells were treated with HG (25mM) and 20-HETE (1.5 μM) for 48 hours. Cellular DNA Fragmentation ELISA Assay was used to assess apoptosis by measuring BrdU-labeled DNA fragments. The data showed a significant increase in cellular apoptosis in hyperglycemic SCs (Figure 6). Interestingly, a significant induction of apoptosis was observed upon exogenous 20-HETE treatment suggesting that the effect of 20-HETE mimics that of HG. This data is indicative of a CYP4A-mediated injurious effect and that the blockade of CYP4A may be a promising therapeutic approach.

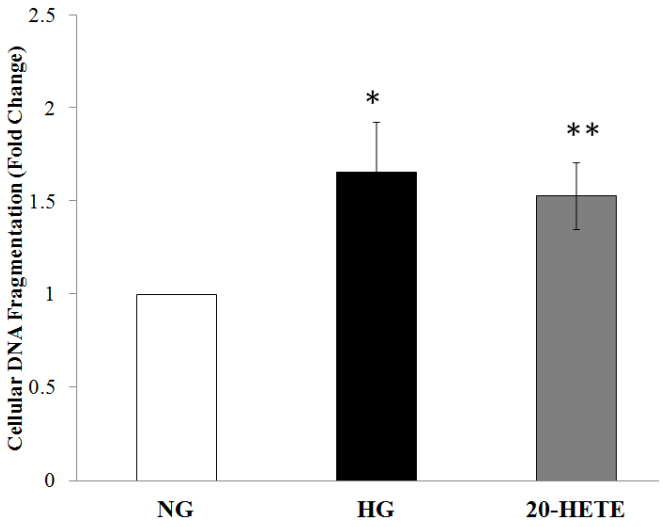


Figure 6. CYP4A-dependent 20-HETE production mediates HG-induced SC apoptosis
MSC80 cells were serum-deprived overnight and treated with either 25mM HG or 1.5 μM of 20-HETE for 48 hours (n=4). Values are the mean ± SE. *P<0.05, ** P<0.01, High glucose or 20-HETE vs. Normal glucose.

D. CYP4A alteration mediates hyperglycemia-induced sensorimotor coordination defects in Type II diabetic mice.

Diabetes is described to be one of the major causes of reduced sensory and motor coordination in patients with peripheral nerve injury. It presents as a coalition of heightened sensitivity (hyperalgesia and allodynia) in the early stages followed by a nearly complete loss of sensation and motor function in the late stages. Six weeks, nonobese type II diabetic (MKR) mice were treated with CYP4A inhibitor, HET0016, for 10 weeks. Sensorimotor coordination and neuromuscular interaction were assessed by the raised beam walking test, hind paw withdrawal test, and grip strength test.

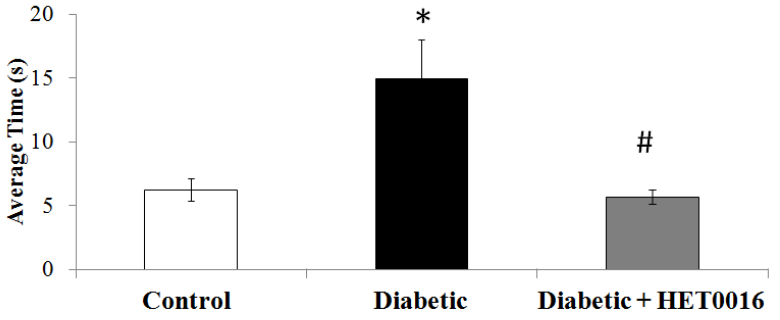
The data from the raised beam walking test showed a relatively longer period of time for the diabetic mice to cross the beam with an increased tendency to slip and stop in comparison

to their FVB controls that seemed to cross with minimal setbacks. Importantly, MKR type II diabetic mice treated with HET0016 behaved similar to the control FVB mice (Figure 7).

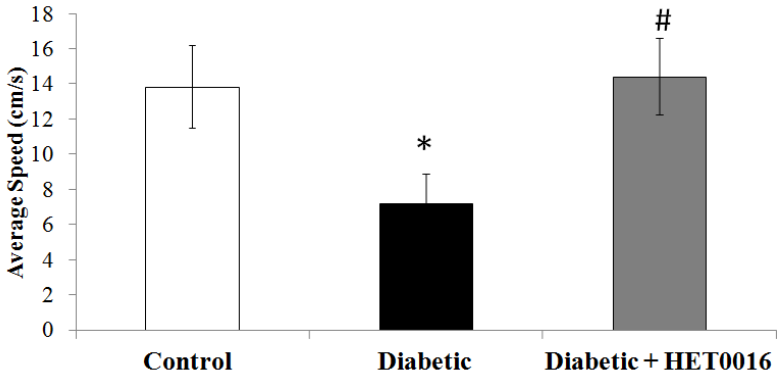
It was predicted that mice with no peripheral nerve injury are capable of thermal sensation and withdraw their hind paw prior to tissue damage, while those with peripheral nerve injury to lack sensation. Indeed, the results of the hind paw withdrawal test show that diabetic mice took a significantly longer time to sense the heat of the beam and withdraw their paws by contrast to the controls. HET0016 treated diabetic mice had a significantly lower latency suggesting that the treatment restored thermal algesia (Figure 8 A).

The third test conducted was the grip strength test which is used to assess neuromuscular strength. Our data show an overall decrease in grip strength of diabetic mice relative to their controls with the least time spent hanging suggesting muscle weakness. Treatment with HET0016 restored strength in diabetic mice, similar to the controls, where the mice spent a significantly longer time suspended (Figure 8 B).

A.


Average Time (s)
20
15
10
5
0
*
#
Control
Diabetic
Diabetic + HET0016

B.


Average Speed (cm/s)
18
16
14
12
10
8
6
4
2
0
*
#
Control
Diabetic
Diabetic + HET0016

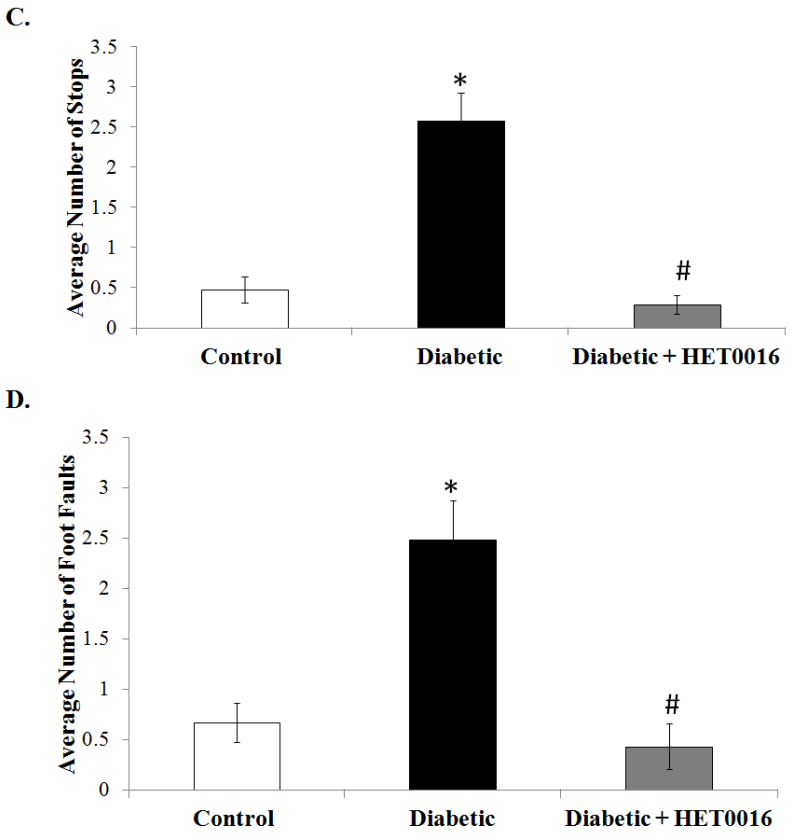


Figure 7. The effect of diabetes and HET0016 administration on motor coordination of type II diabetic mice.

Assessment of motor coordination by the raised beam walking test in 3.5 months old FVB and MKR mice (n=7). The histograms represent the average time, speed, faults, and stops. Values are the means ± SE. *P<0.05, Diabetic vs. Control. #P<0.05, Diabetic + HET0016 vs. Diabetic

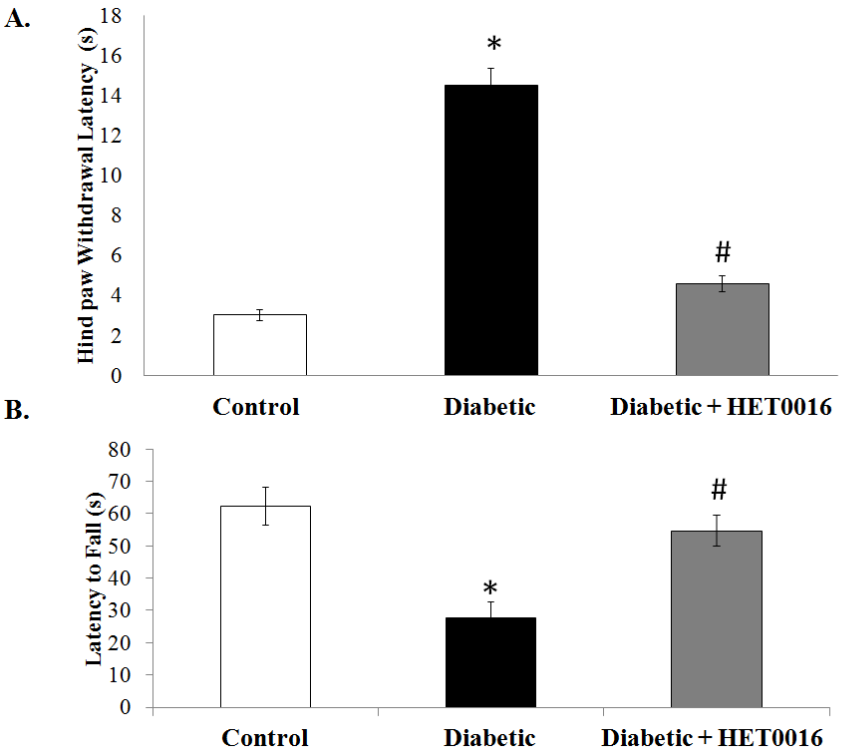


Figure 8. The effect of diabetes and HET0016 administration on grip strength and nociception.
FVB controls and MKR diabetic and diabetic treated with HET0016 mice (n=7) were tested using the grip strength test and hind paw withdrawal test. The graphs represent the time it took to withdrawal their paw in response to a heating stimulus (A) and the time the mice spent hanging (B). Values are the means ± SE. *P<0.05, Diabetic vs. Control. #P<0.05, Diabetic + HET0016 vs. Diabetic

E. Diabetes alters CYP4A and MPZ protein expression in MKR mice.

In order to correlate the behavioral results and the *in vitro* findings, further molecular tests were conducted in mice. Our data show a significant upregulation of CYP4A expression in diabetic mice sciatic nerves relative to the controls, and a significant reduction in CYP4A protein levels upon HET0016 treatment (Figure 9 A). In parallel to CYP4A upregulation, a significant reduction of MPZ was observed in sciatic nerves isolated from the diabetic mice. By contrast,

HET0016 treatment reversed this effect and restored MPZ protein to levels compared to that of the control littermates (Figure 9 B).

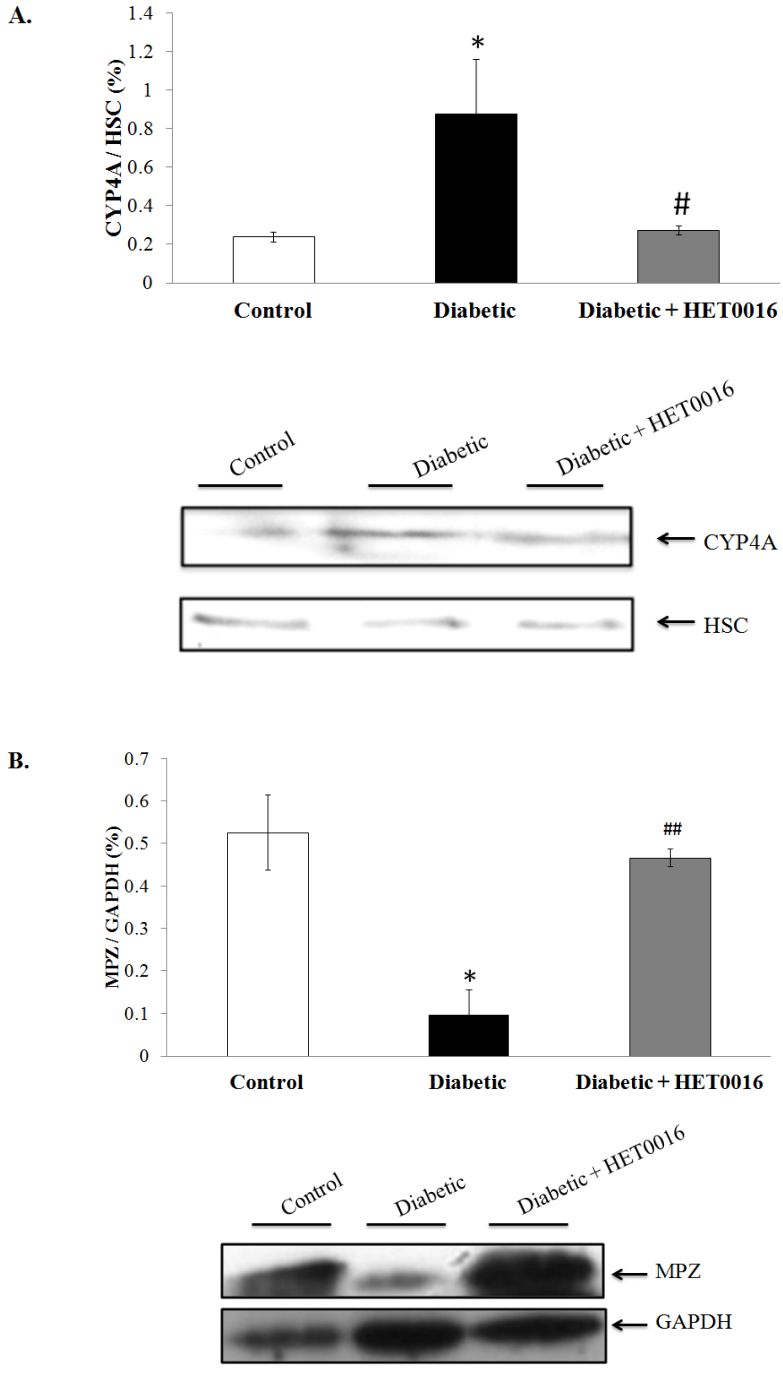


Figure 9. Diabetes alters CYP4A and MPZ protein expression.
An illustration of western blots of CYP4A (A) and MPZ (B) expression in sciatic nerves of diabetic mice and HET0016 treated mice with the respective densitometric quantification. Values

are the means ± SE. *P<0.05, Diabetic vs. Control. #P<0.05, Diabetic + HET0016 vs. Diabetic. ##P<0.01, Diabetic + HET0016 vs. Diabetic.

F. CYP4A-induced ROS production *in vivo* is attenuated upon HET0016 administration in diabetic animals.

Diabetes has been described and shown by our group to be associated with oxidative stress. CYP4A as a major source of ROS was examined in sciatic nerves of diabetic mice using DHE staining. The data reveal a significant increase in ROS production in sciatic nerves of diabetic animals and attenuation upon HET0016 administration (Figure 10). These results show that CYP4A enzymes are a source for ROS production in the sciatic nerves.

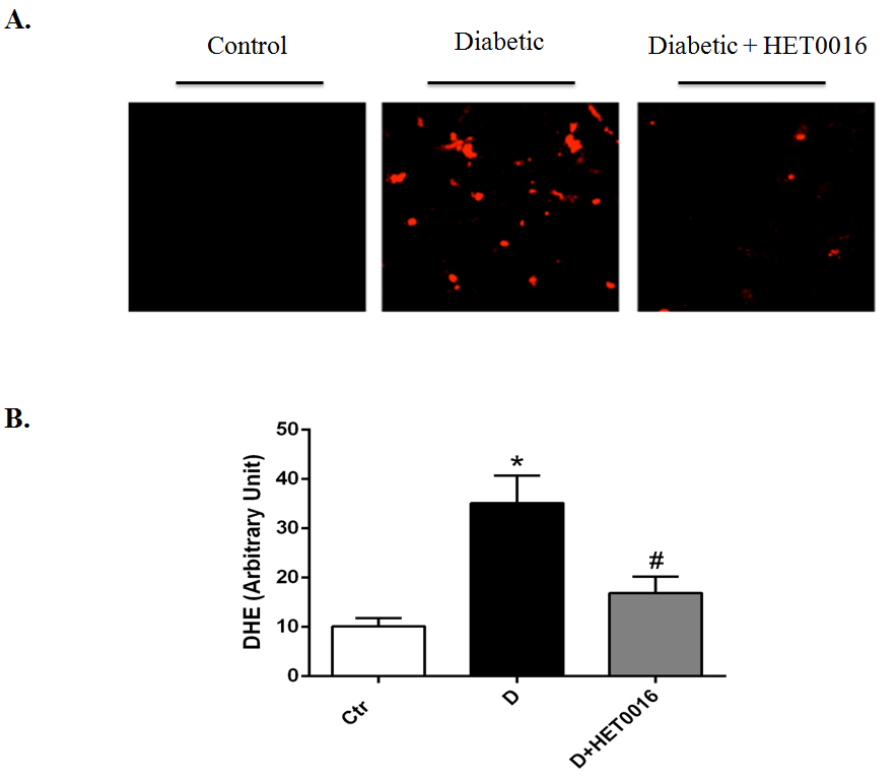


Figure 10. HET0016 administration ameliorates CYP4A-induced oxidative stress within the sciatic nerves of diabetic mice.
A) Representative images of ROS production assessed by DHE staining. B) Histograms

representing the quantification of ROS production in the sciatic nerve of diabetic mice (n=4) using the Image-Pro Plus 4.5 software. Values are the means ± SE of four different areas taken from four individual mice in each group. *P<0.05, Diabetic vs. Control. #P<0.05, Diabetic + HET0016 vs. Diabetic.

G. **HET0016 treatment reverses CYP4A mediated HG-induced ROS production.**

CYP450 enzymes have been reported to be associated with an NADPH subunit. NADPH oxidase activity correlates with a direct measure of superoxide production. Subsequently, NADPH dependent ROS production was assessed in sciatic nerves of mice. The data show that superoxide production significantly increased in diabetic animals in comparison to their controls and is significantly reduced upon HET0016 treatment (Figure 11). These results may suggest that SC nerve injury is correlated with an increase in ROS production through an NADPH-dependent pathway.

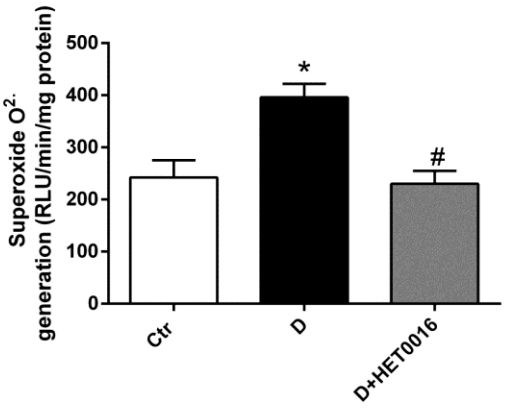
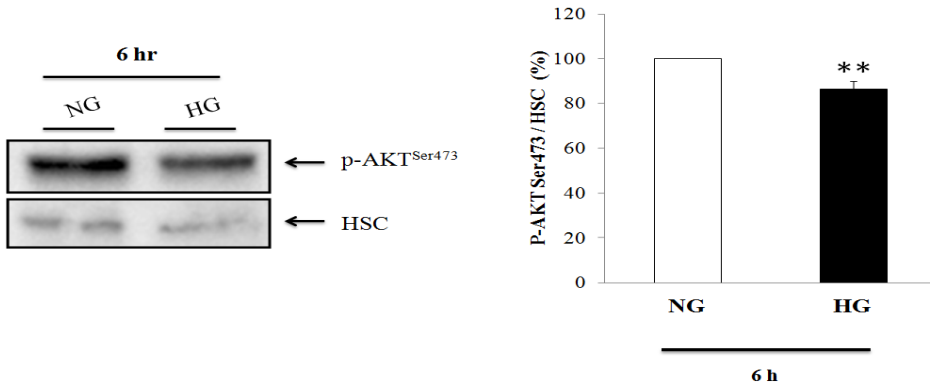


Figure 11. Diabetes induced NADPH-dependent ROS generation which is attenuated by HET0016.
NADPH-induced ROS generation is increased in diabetic mice and reduced in HET0016 treated mice (n=4). Values are the means ± SE. *P<0.05, Diabetic vs. Control. #P<0.05, Diabetic + HET0016 vs. Diabetic.

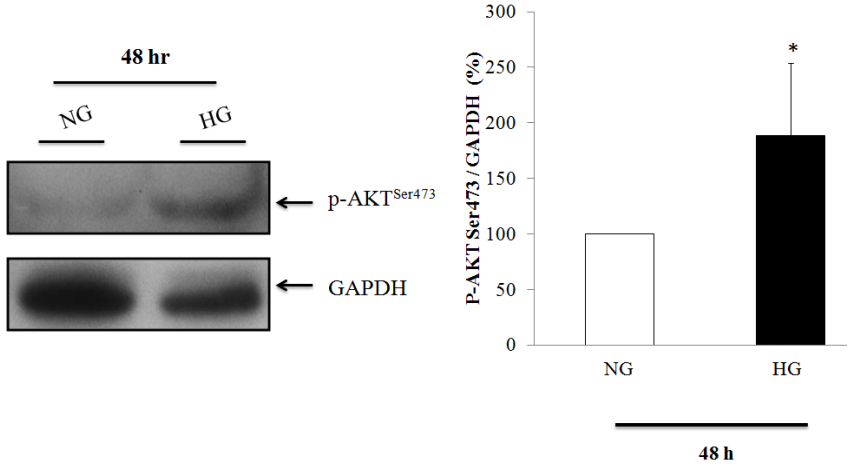
H. Diabetes induces Akt phosphorylation at the Ser473 site in MSC80 cells and in sciatic nerves of diabetic mice

The Akt pathway has been shown to be a major pathway involved in the myelination process and physiology of SCs. However, the mechanisms leading to Akt alterations are still unknown. Our data show that HG treatment for 48 hours induces Akt phosphorylation at its activation site Serine473 in MSC80 cells (Figure 12 A-B). These *in vitro* findings correlated with the increase of Akt phosphorylation in diabetic sciatic nerves (Figure 12 C). Blockade of CYP4A using HET0016 reversed the effect of hyperglycemia-induced Akt hyperphosphorylation. These data suggest that phosphorylated Akt may play an injurious role in type II diabetes.

A.


6 hr
NG
HG
p-AKT Ser473
HSC
P-AKT Ser473 / HSC (%)
120
100
80
60
40
20
0
**
NG
HG
6 h

B.


48 hr
NG
HG
p-AKT Ser473
GAPDH
P-AKT Ser473 / GAPDH (%)
300
250
200
150
100
50
0
*
NG
HG
48 h

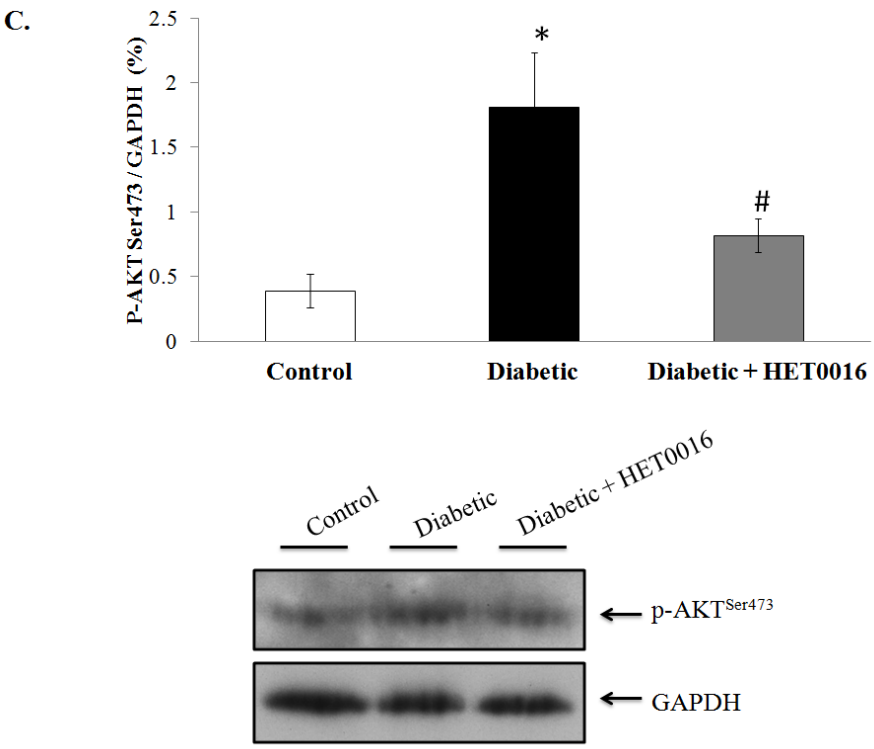


Figure 12. Diabetes triggers Akt hyperphosphorylation at its Serine 473 residue
Representative western blot of p-Akt^{Ser473} in MSC80 cells at 6 hr (n=3) (A) 48 hr (n=4) (B) and sciatic nerves (n=4). Values are the means ± SE. *P<0.05, High glucose vs. Normal glucose or Diabetic vs. Control. **P<0.01 High glucose vs. Normal glucose. #P<0.05, Diabetic + HET0016 vs. Diabetic

CHAPTER IV

DISCUSSION

Neural tissue outside the central nervous system comprises the peripheral nervous system (PNS) which is an elaborate meshwork that has been shown to be vulnerable to homeostatic alterations. Diabetes is now recognized to be among the leading insults to the PNS. Diabetic Peripheral Neuropathy (DPN) is one of the complications triggered by diabetes with clinical manifestations including sensorimotor dysfunction, reduced nerve conduction velocities, slowed axonal transport, nerve morphological abnormalities, and physiological faults (Johnson, P. C., Doll, S. C., & Cromey, D. W. 1986; Fross, R. D., & Daube, J. R. 1987; Ishii, D. N. 1995; Zochodne, D. W. 1999; Hill, R. E., & Williams, P. E. 2002; Ohsawa et al 2008; Juranek et al., 2013; Becker et al., 2014). The myelinating cells of the PNS, or Schwann cells (SCs), in particular are implicated in DPN pathogenesis (Askwith et al., 2009; Cinci et al., 2015). Studies have shown that SCs undergo high-glucose induced loss of axonal association (Dey et al., 2013), reduced regenerative potential (Gumy et al., 2008), apoptosis (Wu et al., 2012) in addition to de-myelination and de-differentiation (Hao et al., 2015). SC injury has been described to be associated with myelin dysfunction and alterations in myelin protein levels central to the proper physiology and survival of SCs (Niemann et al., 2000; Niemann, A., Berger, P., & Suter, U. 2006). However, the mechanisms through which hyperglycemia leads to SC alteration and dysfunction are poorly characterized.

This study provides evidence that hyperglycemia has a deleterious effect on SCs and sciatic nerve integrity through the generation of a state of oxidative stress by CYP4A enzymes. CYP4A protein levels are shown to be significantly upregulated in hyperglycemic SCs leading to

the alteration in MPZ and PMP22 protein levels. MPZ and PMP22 are myelin proteins which are crucial for maintaining myelin sheath functionality; therefore any variation in their expression may lead to SC dysfunction (D'Urso, D., Ehrhardt, P., & Müller, H. W. 1999. High glucose and 20-HETE treatments *in vitro* induced SC apoptosis demonstrated that CYP4A upregulation and 20-HETE production is deleterious. These data lend support to the hypothesis that hyperglycemia-induced injury commenced through a CYP4A-dependent pathway. A significant increase in CYP4A protein expression was also observed in diabetic sciatic nerves of type II diabetic mice; however, in contrast to the *in vitro* results, MPZ levels were significantly reduced. To speculate, although the data show an upregulation in myelin protein expression at the cellular level, these proteins may be non-functional and aggregated as some studies reported (Fortun et al., 2007). The consequence of CYP4A-induced peripheral nerve injury was also contemplated at the behavioral level of diabetic mice.

Numerous biochemical pathways are affected by dysmetabolism caused by hyperglycemia. Our group as well as others' research focuses on oxidative stress as a major mediator of diabetic complications (Eid et al., 2009; Ceriello, A. 2003). The sources of ROS have been intensively studied and their inhibition is shown to reduce pathogenesis of microvascular complications (Eid et al., 2009; Eid et al., 2010; Eid et al., 2013[a,b]; Eid, A. et al., 2013; Eid, S. et al., 2013; Eid et al., 2014). Specifically in DPN, it has been shown that the reduction of oxidative stress may be of therapeutic potential (Cotter, M. A., & Cameron, N. E. 2003; Zhao et al., 2014). In fact, antioxidant treatments have been shown to attenuate progression of peripheral nerve injury (Cameron, N. E., & Cotter, M. A. 1999; Reljanovic 1999). Recent efforts that attempted to inhibit ROS production indicated that high-glucose induced SC injury was ameliorated (Yang et al., 2016) in addition to the partial restoration of the

perineurium morphology, nerve conduction velocity, and pain and thermal perception (Greene et al., 1999; Cotter, M. A., & Cameron, N. E. 2003; Zhao et al., 2014; Li et al., 2016).

Our findings are in agreement with previous studies where ROS inhibition ameliorated SC injury. Our results showed that myelin protein expression and ROS production were normalized close to control levels upon CYP4A inhibition via HET0016. More importantly, this study has, in addition, determined the source of ROS production seen in DPN. Although CYP450 enzymes are well known to be major sources of ROS, their role in DPN has not been investigated yet. Previous work in our lab has correlated CYP4A upregulation induced by hyperglycemia with increased oxidative stress through 20-HETE production leading to injury in kidney tubules podocytes (Eid et al., 2009; Eid et al., 2013[a,b]) and in the lung endothelia (Medhora et al., 2008). Our findings show increased CYP4A protein levels in hyperglycemic SCs. In response to exogenous 20-HETE *in vitro*, SCs injury was shown to be induced through apoptosis, MPZ and PMP22 upregulation. The increased expression of CYP4A implies that 20-HETE synthesis may be increased. The peripheral nerves are vulnerable to any homeostatic alterations that take place, unlike the central nervous system, which is protected by a blood barrier (Cameron, N. E., & Cotter, M. A. 1994; Morris, S. J., Shore, A. C., & Tooke, J. E. 1995; Stys, P. K. 2005). Extensive research has shown that DPN may be in part further exacerbated by peripheral vascular disease (Cameron, N. E., Eaton, S. E. M., Cotter, M. A., & Tesfaye, S. 2001) and that SCs may be the mediators (King et al., 1989). Furthermore, it has been reported that there is a diabetes-associated tendency for vasoconstriction in peripheral vasculature (Pieper, G. M.1998). In particular, *in vivo* studies in diabetic animals showed that a reduction in sciatic nerve endoneurial blood flow within a hypoxic milieu was evident in the early stages of diabetes (Tuck, R. R., Schmelzer, J. D., & Low, P. A. 1984; Cameron, N. E., Cotter, M. A., & Low, P. A. 1991). Similarly, sural

nerve angiography in diabetic patients with chronic motor and sensory neuropathy reported an impaired blood flow and abnormal epineural vessel morphology (Tesfaye et al., 1993). This observation may be correlated with a dysfunctional neurovascular response by C-fibers which was evident in the diabetic foot and is concurrent with abnormal pain perception and ischemia (Ibrahim et al., 1999; Dinh, T., & Veves, A.2005). Accordingly, and given our findings, further studies would likely examine the role of 20-HETE and its correlation with vascular injury in DPN.

In vivo studies on CYP450 enzymes in DPN have not been conducted previously. To our knowledge, this study is the first to examine CYP4A expression in SCs as well as type II diabetic mice within the context of DPN. Molecular tests in sciatic nerve tissues showed increased CYP4A protein levels concurrent with decreased MPZ protein levels in diabetic mice. By contrast, the inhibition of CYP4A via HET0016 showed upregulated MPZ levels indicating a possible therapeutic benefit. The observed decrease in MPZ levels upon diabetes induction *in vivo* is consistent with other studies (Kawashima et al., 2007; Cermenati et al., 2012). Moreover, CYP enzymes are known to be associated with a NADPH subunit. It was shown in this study that NADPH activity was elevated in sciatic nerves of diabetic animals, but HET0016 treatment reduced this activity further verifying the association of CYP4A with increased ROS production through an NADPH-dependent pathway.

CYP4A overexpression in diabetic animals implies increased 20-HETE levels. It has been shown that 20-HETE mimics TRPV1 channel agonists of sensory neurons, and that it was capable of sensitizing and activating these channels in both humans and mice. TRPV1 channels have been described to be associated with thermal hyperalgesia (Wen et al., 2012). Therefore, it was of particular interest, to further examine these findings via behavioral testing as well as

through a study that could correlate CYP4A/20-HETE production to TRPV1 channels. Type II diabetes is reported to affect sensorimotor coordination leading to loss of proprioception and balance (Palma et al., 2013; Toosizadeh et al., 2015). Allodynia and hyperalgesia are two additional phenotypes commonly occurring in diabetes (Ohsawa, M., & Kamei, J. 1999; Kuhad, et al., 2008). These phenotypes were assessed in the following study via three different tests. The elevated beam walking test is used to assess motor function and balance. It was shown that in diabetic mice, the performance was poor relative to control mice and HET0016 treated mice. Likewise, the grip strength test, used to assess muscular tone and neuromuscular strength, showed a reduced ability to maintain a grip in diabetic animals relative to controls and treated animals. The third test conducted to assess thermal and pain perception showed a relatively larger tolerance to pain in diabetic animals in comparison to controls and treated animals suggesting an almost complete loss of sensation. The findings of these behavioral tests lend support to the notion that myelin protein alterations have deleterious effects on the functional intergrity of peripheral nerves (Niemann et al., 2000; Wrabetz et al., 2000; Yin et al., 2000; Niemann et al., 2006) and suggest a clear correlation between CYP4A-induced peripheral nerve injury in diabetes and dysmyelination.

The current findings have, in addition, established a link between CYP4A-induced ROS production and one pivotal pathway for cellular survival: the mTOR/Akt pathway. It has been shown that increased mTORC1 activity leads to increased myelin thickness and myelin protein levels, while the loss of mTORC1 activity is associated with hypomyelination in the central nervous system (Flores et al., 2008; Narayanan et al., 2009). Similarly, in the PNS, sciatic nerves from mTOR mouse mutants have been shown to be associated with thin myelin sheaths with abnormal elongation and SC growth in addition to reduced MPZ levels (Sherman et al., 2012).

However, the involvement of mTORC1 or mTORC2 was not specified. A more recent study has set the distinction between mTORC1 and mTORC2 associations in PNS myelination and showed that mTORC1deficiency mediates hypomyelination in addition to reduction in nerve conduction velocities and not mTORC2; mTORC2 deficiency solely does not lead to hypomyelination (Norrmen et al., 2014). Nevertheless, the role of mTORC1 and mTORC2 has yet to be understood. The downstream effector of mTORC2, Akt, has been described in the literature to enhance myelination in the central nervous system, and not PNS, when active (Flores et al., 2008). Further studies have showed that Akt plays a role in regulating myelin sheath thickness in the PNS in collaboration with basic regulatory proteins that determine cellular polarization and protein trafficking (Cotter et al., 2010). A disruption in one of the collaborative proteins that interact with Akt, such as PTEN, has been shown to increase Akt activation which leads to hypermyelination resulting in myelin pathologies such as outfoldings and tomacula (Goebbel et al., 2012; De Paula et al., 2014). Previous studies reported a decrease in phosphorylated Akt levels in SCs upon HG treatments (Ii et al., 2005). However, preliminary data from this study in SCs cultured in a hyperglycemic milieu suggested an increased phosphorylation of Akt at its Serine473 active site. Similarly, hyperphosphorylation of Akt was significantly increased in sciatic nerves from diabetic animals in parallel to CYP4A upregulation. HET0016 administration was shown to reduce both CYP4A and Akt upregulation, indicating the possibility of CYP4A-induced peripheral nerve injury through the Akt pathway.

Taken together, these findings showed that hyperglycemia elevated ROS production through CYP4A upregulation. Hyperglycemia-induced ROS generation and 20-HETE production resulted in PNS injury. The progression of injury was shown to involve the Akt pathway that can be due to alleviation in mTOR signaling. Akt hyperphosphorylation was proven to be injurious

and was observed in both early (in SCs) and late stages of diabetes (in animals), consistent with our findings. In summary, CYP4A-induced ROS generation correlated with alterations in myelin proteins which are fundamental for demyelination and eventual peripheral nerve injury observed at the molecular and behavioral levels.

This study opens future perspectives that include rigorous additional *in vitro* testing. Firstly, the effect of HET0016 on MSC80 cell physiology and apoptosis is a critical step to progress further in this research. A measurement of 20-HETE synthesis in SCs and sciatic nerves is pivotal to verify diabetes-mediated 20-HETE-induced injury. An examination of cellular death upon HG, 20-HETE and HET0016 treatments using additional techniques such as flow cytometry and caspase cleavage is of major importance. Myelin proteins are described to be dosage-sensitive. Therefore, to assess the extent to which HET0016 may normalize myelin protein levels in hyperglycemic SCs, a microgradient is essential to determine the appropriate concentration to achieve a positive physiological outcome. These tests pave the way for further *in vitro* assessment of ROS upon HET0016 treatment in addition to a NADPH-activity assaying once the appropriate dosage is determined. It would also be of interest to investigate the short term effect of HET0016 *in vivo* mimicking early stage diabetes to contemplate its therapeutic potential and the possibility of a complication- preventative benefit. Additionally, CYP4A silencing in hyperglycemic MSC80 cells or CYP4A knockout in diabetic mice treated, as well as treatments with Akt inhibitors would be an investigation worth pursuing to fully grasp how hyperglycemia works through CYP4A. This study targets one contributor to the meshwork of pathways involved in myelination and SC physiology/pathophysiology. However future studies must comprehend possible crosstalk among mTORC1, mTORC2, and Akt pathways. Thus the listed initiatives are essential to fully fathom DPN.

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