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Peripheral nerve function and structure in experimental models of diabetic neuropathy

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Molecular Pathology

by

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2008
The dissertation of Joshua A. Gregory is approved and it is acceptable in quality and form for publication on microfilm:

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Chair

University of California, San Diego

2008
This dissertation is dedicated to all the animals involved in scientific research around the world. Their sacrifice makes possible the pursuit of a better human life.

What is natural is the microbe. All the rest-health, integrity, purity (if you like)-is a product of the human will, of a vigilance that must never falter. The good man, the man who infects hardly anyone, is the man who has the fewest lapses of attention.

*Albert Camus*
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Publications


Abstracts


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Despite extensive research, the etiology of diabetic neuropathy remains unclear. Several key metabolic abnormalities such as increased polyol pathway flux and non-enzymatic glycation have been implicated in the pathogenesis of diabetic neuropathy, as have vascular factors. Both metabolic and vascular aberrations caused by chronic hyperglycemia lead to increased free radical production and oxidative stress, which in turn exacerbate the nerve injury caused by diabetes.

A number of antioxidant therapies have been successful at preventing or reversing indices of experimental diabetes. Studies were designed to assess the ability of antioxidants with previously demonstrated therapeutic benefits; as well as several previously untested antioxidant compounds, in preventing the development of experimental diabetic neuropathy. α-lipoic acid, an antioxidant
previously shown to ameliorate aspects of diabetic neuropathy was not beneficial to diabetic rats in these studies. However, taurine, another compound with previously demonstrated therapeutic benefit, as well as ellagic acid had positive effects on nerve conduction velocity and were successful in preventing the development of tactile allodynia and oxidative damage in the sciatic nerve.

Vascular damage and nerve ischemia are considered important aspects of diabetes-induced nerve injury. Exercise, near-infrared therapy and ultrasound therapy, three non-pharmacologic approaches to improve nerve blood flow, were studied in STZ-diabetic rats. There was no clear evidence that these methods were beneficial to nerve function or structure in these studies.

The role of hypertension as a risk factor for the development of diabetic neuropathy has been recently established. However, little is known about how the combination of hypertension and diabetes affects the peripheral nervous system. The effects of concurrent hypertension on the development of diabetic peripheral neuropathy were studied in this dissertation. Both diabetes and hypertension alone led to nerve conduction slowing and reductions in nerve blood flow. The combination of diabetes and hypertension resulted in a worsening of the nerve conduction and nerve blood flow deficits. Also, Schwann cell damage was observed in hypertensive animals and also, to a greater extent, in animals with both hypertension and STZ-diabetes. Hypertension exacerbates the nerve injury associated with experimental diabetes, and this experimental model, combining both hypertension and diabetes, may provide an important tool to study the pathogenesis of diabetic neuropathy.
CHAPTER 1 – INTRODUCTION

1.1 Diabetes mellitus

1.1.1 Diabetes

Diabetes mellitus is the term used to describe a group of metabolic disorders in which there is impaired glucose utilization resulting in high blood sugar. The underutilization of glucose in diabetes mellitus is caused by absolute or relative insulin deficiency. Though hyperglycemia is the most commonly considered biochemical marker in diabetes, carbohydrate, protein, lipid, and electrolyte metabolism are also affected, as are a multitude of tissues and organs, and their respective functions. Type I and type II diabetes are the most common forms of the disease, however other forms of the disease such as gestational diabetes also occur. More than 20 million Americans currently suffer from diabetes, and more than 40 million individuals are pre-diabetic with impaired glucose tolerance and will likely develop diabetes at some point in their lifetime (Ioannou et al., 2007).

Type I diabetes, also referred to as insulin-dependant diabetes mellitus (IDDM), accounts for approximately 10% of diabetes cases and is characterized by impaired insulin production by pancreatic ß-cells. Inherited susceptibility, autoimmune disease and environmental factors have all been linked to the pathogenesis of type I diabetes (Eisenbarth, 1986).

Type II or non-insulin-dependant diabetes (NIDDM) accounts for approximately 90% of diabetes cases and is by far the most common form of the disease. In most cases of type II diabetes, insulin resistance is the key metabolic
disorder arising from failure of the insulin receptor to function normally. Insulin resistance results in increased insulin production, and the increased demand on pancreatic β-cells to produce insulin can lead to β-cell failure and ultimately to insulinopenia (Crawford and Cotran, 1994). Though the precise details of the pathogenesis of type II diabetes remain unclear, genetic predisposition, obesity and lifestyle are strongly linked to type II diabetes (Yki-Järvinen, 1994). Genetic inheritance is thought to be of even greater importance in type II than in type I diabetes, with a concordance rate close to 100% among identical twins (Crawford and Cotran, 1994).

1.1.2 Secondary complications of diabetes

There are a number of complications that develop in patients who suffer from chronic diabetes. The most common complications associated with long-term diabetes are macro and microangiopathy, retinopathy, nephropathy and neuropathy. These complications lead to the increased morbidity and mortality of diabetic patients. A tremendous amount of research has focused on the pathogenesis, prevention and potential treatment of diabetic complications. At present, hyperglycemia is widely accepted as the primary pathogenic factor for the various complications associated with diabetes.

The vasculature is severely impacted by diabetes, which injures the largest vessels (macroangiopathy) as well as the smaller arteries and arterioles and the capillary beds (microangiopathy). The larger vessels, such as the aorta and coronary arteries, undergo accelerated and advanced atherosclerosis in diabetes (Clare-Salzer et al., 2003). Hypertension, a co-morbidity affecting up to
75% of diabetic patients, accelerates the development of athero and arteriosclerosis, and is a risk factor for the development of diabetic neuropathy (Tesfaye et al., 2005). Microvascular complications are a predominant feature of diabetic peripheral neuropathy (Zochodne, 2007). The vascular pathologic changes associated with diabetes will be discussed in more detail in section 1.2.9.

Retinopathy is a disease of the retinal capillary bed, and thus is closely related to microangiopathy. In patients with diabetic retinopathy, retinal capillaries develop a thickened basement membrane and become more permeable. As the disease progresses, retinal capillaries begin to close, possibly due to procoagulatory events occurring as a result of endothelial disturbances (Gries et al., 2003). Occlusion of retinal capillaries leads to retinal ischemia, which triggers angiogenesis in an attempt to compensate for the loss of retinal blood flow. The abnormal growth of blood vessels in the retina, (proliferative retinopathy), results in new vessel formation in the preretinal space and loss of vision due to bleeding from the new vessels (neovascular glaucoma), or to retinal detachment caused by fibrotic structures developing in the retina (ETDRS_RG, 1991).

Nephropathy is another complication associated with diabetes. As with other diabetic complications, hyperglycemia is a primary contributing factor to the development of nephropathy. However, hyperglycemia by itself may not be sufficient to cause nephropathy. More than 50% of diabetes sufferers never develop diagnosable nephropathy. Although normal kidneys transplanted into
diabetics develop pathology typical of diabetic nephropathy, the development may be independent of glycemic control and varies widely among patients (Mauer et al., 1989). Hypertension is also a risk factor for development of diabetic nephropathy (Parving et al., 1987). In the case of diabetic nephropathy, glomerular filtration rate is increased. Microalbuminuria follows as a result of increasing glomerular capillary pressure and a loss of negative charge in the glomerular basal lamina. Over time, glomerular filtration rate decreases due to deposition of abnormally glycosylated proteins in the renal vasculature and a thickening of the endothelial layer (Morgensen, 1989). In patients with chronic diabetes, nephropathy can lead to increases in systemic blood pressure, dramatic reduction in glomerular filtration rate and ultimately, kidney failure.

The central and peripheral nervous systems are also damaged by diabetes mellitus. In the central nervous system, generalized degeneration can occur in the brain, particularly in the white matter (Zochodne, 2007). The development of cerebral microangiopathy greatly increases the risk of an infarct or hemorrhage in the brain. Microangiopathy is increased in diabetics due to atherosclerosis, commonly compounded by hypertension (Clare-Salzer et al., 2003). Diabetes also leads to significant damage in the peripheral nervous system. The most common presentation of diabetic peripheral neuropathy consists of a symmetric neuropathy in the distal extremities. Motor, sensory and autonomic neurons are all affected; however, sensory neuropathy is typically the predominant clinical feature.
1.2 Diabetic peripheral neuropathy

1.2.1 Incidence of diabetic neuropathy

Nerve injuries associated with diabetes can be categorized as focal, multifocal or symmetric neuropathies (Thomas, 1973). Distal symmetrical polyneuropathy is the most common diabetes-induced disorder of the peripheral nervous system. Distal symmetrical polyneuropathy is an axonal length-dependent disorder that primarily affects the distal extremities (i.e. hands and feet), and involves motor, sensory, and autonomic nerves. Clinically, distal symmetric polyneuropathy tends to present with abnormalities such as nerve conduction velocity slowing, neuropathic pain and progressive sensory loss. The development of peripheral neuropathy is a major contributing factor to the development of lower limb ulceration in diabetic patients and often leads to amputation (McNeely et al., 1995).

In the developed world, diabetic neuropathy is the most common form of nerve injury (Zochodne, 2007). More than 50% of patients with a 20-year history of diabetes will develop neuropathy (Pirart et al., 1978). It is unclear if patients with type I or type II diabetes are more or less likely to develop neuropathy. Studies have argued that both type I diabetes poses a greater risk for development of diabetic neuropathy (Dyck et al., 1999) and that both type I and type II diabetes place an individual at equal risk for neuropathy (Vinik et al., 2000).

1.2.2 Hyperglycemia-induced structural changes to the peripheral nerve
Pathologic changes in the peripheral nerve occur in diabetic patients and in experimental models of diabetic neuropathy. In human diabetic neuropathy, axonal loss in peripheral nerve is perhaps the most obvious and severe morphologic change. The loss of axons is most pronounced distally, with severe and diffuse fiber loss in the tibial, sural, and peroneal branches of the sciatic nerve (Sugimura and Dyck, 1982; Dyck et al., 1988; Llewelyn et al., 1991). The presence of endoneurial edema has also been observed in diabetic patients with either symptomatic or asymptomatic neuropathy (Eaton et al., 1996). Axonal atrophy does not always occur, despite the occurrence of edema and axonal loss observed in diabetic patients (Engelstad et al., 1997). In addition to axonal changes caused by diabetic neuropathy, Schwann cell responses have also been examined. In patients with type II diabetes, reactive, proliferative, and degenerative changes occur in myelinating Schwann cells of the sural nerve. These changes include: accumulation of lipid droplets, pi granules of Reich, enlarged mitochondria, demyelination, and onion bulb formation (Kalichman et al., 1998). These pathologic alterations were only observed in myelinating Schwann cells, the same population of Schwann cells where the enzyme aldose reductase is found (Powell et al., 1991). This finding points to increased flux through the polyol pathway as a possible mechanism for the degeneration of these cells (see section 1.2.4).

Due to the potential for loss of function, nerve biopsies are no longer commonly taken from patients. However, animal models provide another means by which to assess correlations between the progression of sensory perception
and physiologic parameters of diabetic neuropathy and the degree of concurrent structural pathology in the nerve. A number of studies have been conducted on STZ-diabetic rats to determine the morphologic changes that occur in the peripheral nerve as a result of hyperglycemia.

Although structural changes in the peripheral nerve that have been observed in STZ-diabetic rats have not been shown to recapitulate the severe damage observed in diabetic patients, such as axon loss and degenerative Schwann cell changes, some more subtle structural alterations to the peripheral nerve have been reported. Cross-sectional nerve fiber area, axon area and myelin area are all reduced in the peroneal nerve in rats with 4 weeks of STZ-diabetes compared to control rats (Jakobsen, 1976). Axon caliber changes are more pronounced than myelin sheath changes, and small fibers are more affected than large fibers (Jakobsen, 1976). The axon caliber changes observed following 4 weeks of STZ-diabetes are prevented by insulin treatment, thus implicating hyperglycemia and the primary cause for the structural damage (Jakobsen, 1979). Diminution of fiber caliber, observed by Jakobsen, in the peripheral nerve of rats with 4 weeks of STZ-diabetes has also been observed following 8 (Ochodnicka et al., 1995), 12 (Thomas et al., 1990), 24 (Thomas et al., 1990), 28 weeks (Chokroverty et al., 1988) and 1 year of STZ-diabetes (Thomas et al., 1990). There is some conflict in the scientific literature regarding the morphometric changes in the peripheral nerve of diabetic rats, with some authors reporting no changes in fiber or axon caliber (Sharma et al., 1981; Wright and Nukada, 1994; Walker et al., 1999). Although the weight of evidence
supports a diabetes-induced reduction in peripheral nerve axon caliber, the finding of no axon caliber changes by some investigators highlights the lack of severe structural damage in the peripheral nerve of diabetic rodents compared to that seen in patients with diabetic neuropathy. There is general agreement that fiber loss does not occur in the peripheral nerves of STZ-diabetic rats. This finding has been documented in the sural (Sharma and Thomas, 1974), tibial (Sharma et al., 1981), peroneal (Jakobsen, 1976) and sciatic nerve (Wright and Nukada, 1994).

Dorsal roots and dorsal root ganglia are also affected by STZ-diabetes. Following 4 weeks (Sidenius and Jakobsen, 1980) or 1 year (Sasaki et al., 1997) of experimental diabetes, cell-body volume is reduced. However, there does not appear to be any loss of neurons associated with the decrease in perikaryal size. Dorsal roots become edematous and suffer from myelin splitting and ballooning in rats with 28 weeks (Tamura and Parry, 1994) or 1 year of STZ-diabetes (Sasaki et al., 1997).

The galactose-fed rat, a model in which rats are fed a 40% galactose diet, is a model of hyperglycemia without hypoinsulinemia. The advantage to this model is that more severe structural pathology occurs in the peripheral nerve than that observed in the streptozotocin-diabetic rat. Following 2, 4, or 24 months of galactose intoxication, the rat sciatic nerve has increased interstitial space and endoneurial and subperineurial edema. After 24 months of galactose-induced hyperglycemia, axonal degeneration, fiber loss, and severe Schwann cell pathology, including demyelination and remyelination, and onion-bulb
formation occur (Kalichman et al., 1998). Although the galactose model is useful in that it generates structural changes in the peripheral nerve, it does not necessarily reflect type I diabetes. Galactose-fed rats have normal insulin secretion, do not model complete flux through the polyol pathway and generate excess polyols in nerve tissue (Dines et al., 1995b). Studies utilizing galactose-fed rats must be carefully interpreted when considered in the context of diabetic neuropathy.

1.2.3 Etiology of diabetic neuropathy

Irrespective of the type of diabetes, the complications associated with diabetes mellitus are considered a result of the primary metabolic disturbance, hyperglycemia. Careful regulation of blood sugar has been shown to delay the progression of diabetic complications and in a large-scale, long-term trial, the onset of neuropathy was reduced by 57% in patients employing strict glycemic control (Diabetes Control and Complications Trial Research Group, 1993). Understanding the neurobiology underlying the development of diabetic neuropathy has proven challenging. Neurons in diabetic patients are subjected to a number of interdependent stresses that collectively lead to a perpetuating cycle of nerve injury (see Figure 1.1). Among the most important mechanisms responsible for the development of diabetic neuropathy are: increased flux through the polyol pathway, non-enzymatic glycation, oxidative stress, protein kinase C activation (PKC), loss of neurotrophic support and macro- and microangiopathy.

1.2.4 Polyol pathway
In certain tissues, such as lens, kidney, blood vessels and nerve, insulin is not required for glucose uptake (Gries et al., 2003). This makes these tissues particularly susceptible to damage caused by hyperglycemia. Increased blood sugar as a result of diabetes leads to an increase in intracellular glucose in these tissues and the subsequent activation of the polyol pathway. The polyol pathway involves aldehyde metabolism, in which intracellular glucose is metabolized first into sorbitol and then fructose, by the enzymes aldose reductase and sorbitol dehydrogenase (Hers, 1956). Under normal conditions, glucose metabolism via the polyol pathway is limited. However, in conditions of chronic hyperglycemia, the polyol pathway becomes hyperactive. Exaggerated flux through the polyol pathway can lead to accumulation of intracellular sorbitol resulting in myoinositol depletion with associated decrease in phosphoinositide metabolism, PKC activation and Na\(^+\)-K\(^+\)-ATPase channel inactivation (Greene et al., 1987; Oates, 2002). It is not entirely clear if polyol accumulation or simply increased flux through the polyol pathway is responsible for the nerve dysfunction linked to the polyol pathway (Calcutt et al., 1988). There is general agreement that increased flux through the polyol pathway is involved in the generation and maintenance of several secondary mechanisms that also contribute to diabetic neuropathy, such as increased oxidative stress, nerve ischemia, reduction in neurotrophic support and increased non-enzymatic glycation.

1.2.5 Non-enzymatic glycation

In the nerves of diabetic patients, as well as in experimental animals, there is an increase in non-enzymatic glycation of proteins (Monnier and Cerami,
1182). This process occurs when monosaccharides bind to proteins. Fructose, the glucose metabolite generated via the polyol pathway, has ten times the potency as a glycating agent compared to glucose. Diabetic patients are therefore particularly susceptible to non-enzymatic glycation (Suarez et al., 1989). Non-enzymatic glycation of certain molecules may be reversible and essentially harmless, but in some instances, molecules such as DNA and matrix basement membrane proteins undergo non-reversible reactions to form advanced glycation endproducts (AGEs) (Gries et al., 2003). Advanced glycation endproducts lead to diabetic complications in several ways. AGEs aberrantly trap non-glycosylated proteins, are resistant to proteolytic destruction, induce lipid peroxidation, and quench nitric oxide release (Brownlee et al., 1988). In addition, AGEs glycate axonal neurofilaments, tubulin, actin and the myelin protein P₀ (Vlassara et al., 1983, 1985, 1994). AGE binding to neuronal proteins may contribute to impairments in axonal transport with subsequent axonal atrophy and nerve degeneration (Vlassara et al., 1985). AGEs can stimulate macrophages to express receptors to advanced glycation endproducts (RAGEs) (Schmidt et al., 1996). Binding of AGEs to their receptors on macrophages stimulates production of free radicals, the release of inflammatory cytokines such as TNF-α and IL-1 and can lead to neuronal dysfunction (Brownlee et al., 1988; Vlassara et al., 1994; Gries et al., 2003).

1.2.6 Oxidative stress

Oxidative stress can lead to impaired biological function (Vincent et al., 2004). Free radical species, such as nitric oxide (NO), hydrogen peroxide (H₂O₂)
and superoxide ($O_2^-$) are produced under healthy conditions in the body and are critical for normal physiologic function. However, when the production of free radicals exceeds the antioxidant capacity, as can occur in diabetes, tissue damage may ensue. Reactive oxygen species (ROS) in diabetes can come from a variety of sources including: AGE binding to RAGE, increased flux through the polyol pathway, MAPK activity and mitochondria (Obrosova et al., 2000, 2002; Vincent et al., 2004).

AGE/RAGE binding initiates an intracellular MAPK-dependant signaling cascade that induces ROS production through a mechanism involving NADPH oxidase and the cell surface localization of prooxidant molecules (Vincent et al., 2004). Oxidative stress resulting from AGE/RAGE interaction has been implicated in the endothelial cell dysfunction and vascular complications of diabetes (Cameron and Cotter, 1999).

The enzymatic activity of aldose reductase in converting glucose to sorbitol as the first step in the polyol pathway is dependant on NADPH as a cofactor (Vincent et al., 2004). A potent cellular antioxidant, glutathione (GSH), also depends on NADPH as a cofactor. Thus, increased polyol pathway flux caused by hyperglycemia depletes intracellular NADPH and, as a result, GSH levels are depleted. Reduced GSH levels makes the cell vulnerable to oxidative damage from free radicals produced during normal cellular function, such as electron transfer (Vincent et al., 2004). The overproduction of sorbitol by excess polyol pathway flux also creates intracellular osmotic stress and reduces the
levels of taurine and ascorbate, two other endogenous antioxidants, which can lead to further ROS production (Stevens et al., 1993; Obrosova et al., 2002).

PKC activity is increased in endothelial cells of the microvasculature in diabetic rats, and PKC activation in turn activates MAPKs (Ishii et al., 1998). PKC activation leads to reduced nerve blood flow in diabetic rats and inhibition of PKC reduces oxidative stress and normalizes blood flow and nerve conduction velocity deficits (Ishii et al., 1998; see section 1.2.7).

Reactive oxygen species are also produced at the level of the mitochondria. Superoxide is produced under normal conditions in the mitochondria as a by-product of metabolism. Hyperglycemia can overload the metabolic capacity of the electron transport chain and oxidative phosphorylation leading to excess superoxide production (Vincent et al., 2004). Mitochondrial oxidative stress alters cellular energy regulation through NO-cytochrome c interaction, protein dysregulation and altered permeability of the inner membrane of the mitochondria (Vincent et al., 2004).

Cellular injury mediated by oxidative stress occurs in a number of ways. Superoxide and nitric oxide are relatively benign as reactive oxygen species. When produced in excess however, these molecules can combine to form the highly reactive peroxynitrite, which readily attacks and impairs function of proteins and lipids (Vincent et al., 2004). Lipids found in the plasma membrane, mitochondria and endoplasmic reticulum are the major sites of peroxidation by reactive oxygen species. Proteins and nucleic acids are also targets of peroxidation and nitrosylation by reactive oxygen species. Modified proteins
have impaired function and can induce neuronal dysfunction, such as slowed axonal transport and reduced neurotrophic support (Metodiewa and Kiosk, 2000). Excess accumulation of modified proteins and DNA damage can ultimately lead to cellular apoptosis.

1.2.7 PKC activation

Another putative mechanism for the development of diabetic neuropathy is increased PKC activation. Diacylglycerol (DAG) is a physiologic activator of PKC enzymes and hyperglycemia increases intracellular DAG content, primarily through de novo synthesis (Ishii et al., 1998). Increased intracellular DAG content leads to PKC activation, especially of the β and δ-isoforms (Brownlee, 2001). PKC activation has been implicated in blood flow abnormalities associated with diabetes, possibly through NO-dependant mechanisms or by increasing endothelin-1 activity (Ishii et al., 1996). PKC activation increases VEGF expression in smooth muscle cells and can lead to vascular permeability and angiogenesis (Williams et al., 1997). PKC activation has also been linked to increased microvascular collagen and fibronectin deposition and increased synthesis of fibrinolytic inhibitor PAI-1 resulting in capillary occlusion and reduced blood flow (Brownlee, 2001). Vitamin E therapy, which reduces DAG levels in diabetic rats, prevents blood flow changes in the retina and kidney (Ishii et al., 1998). PKC β inhibition also restores hemodynamic changes in animals with experimental hyperglycemia (Ishii et al., 1998). In patients with mild neuropathy PKC inhibition improves indices of diabetic neuropathy (Vinik et al., 2005). Increases in PKC activity have been localized to the microvasculature of diabetic
rats, but there is no direct evidence for increased PKC activity in peripheral neurons (Lee et al., 1989). Thus, the effects of PKC and subsequent MAPK activation on the development of diabetic peripheral neuropathy are mediated by microvascular disease and reduction in nerve blood flow rather than direct ROS damage to neurons.

1.2.8 Impaired neurotrophic support in diabetic neuropathy

Reduced neurotrophic support to peripheral neurons is another mechanism involved in etiology diabetic neuropathy. Synthesis of neurotrophic factors, such as nerve growth factor (NGF), neurotrophin-3 (NT-3), ciliary neurotrophic factor, glial cell derived neurotrophic factor (GDNF) and insulin-like growth factors (IGF) is impaired by diabetes (Hellweg and Hartung, 1990; Calcutt et al., 1992; Rodriguez-Pena et al., 1995; Zhuang et al., 1997, Mizisin et al., 1999a,b). The reduction of these growth factors, as well as the slowed axonal transport characteristic of nerves subjected to hyperglycemia, results in diminished neurotrophic support in the diabetic nervous system. A number of experimental studies have shown efficacy in reversing or preventing the development of diabetic neuropathy with neurotrophic factor therapy (Apfel et al., 1994; Zhuang et al., 1997; Mizisin et al., 1999b; Christianson et al., 2003; Calcutt et al., 2004). These studies provide evidence of a role for impaired neurotrophic support in the pathogenesis of diabetic neuropathy.

1.2.9 Vascular complications associated with diabetic neuropathy

There has been difficulty attempting to establish the relative contribution of metabolic and vascular factors in the pathogenesis of diabetic neuropathy.
Currently, it is considered that both metabolic and vascular factors are involved in a complex system of interactions that together conspire to generate nerve injury in diabetic patients. However, the appearance of vascular perturbations such as impairment of endothelium-dependent vasodilation in pre-diabetic subjects, prior to the development of hyperglycemia, suggests a key role of vascular factors in the development of diabetic neuropathy (Jaap et al., 1994; Caballera et al., 1999).

Macroangiopathy is a well-established complication of diabetes. Large blood vessels, such as the aorta and coronary arteries, undergo accelerated and advanced atherosclerosis in diabetes. Macroangiopathy can lead to myocardial infarction, which is the number one cause of death among diabetic patients (Clare-Salzer et al., 2003). Hyaline arteriosclerosis occurs in the smaller, muscular blood vessels and is characterized by a hyaline thickening of arteriole vessel walls, resulting in a decreased lumen size and restricted blood flow. Hypertension, a co-morbidity affecting up to 75% of diabetic patients, accelerates the development of athero and arteriosclerosis and is a risk factor for the development of diabetic neuropathy (Clare-Salzer et al., 2003; Tesfaye et al., 2005). Control of hypertension has been shown to mitigate the macrovascular changes associated with diabetes (UKPDS, 1998).

Microangiopathy is also known to occur in diabetic subjects and is an important consideration in understanding the etiology of diabetic neuropathy. Endothelial cell damage appears to be a primary insult in diabetes-associated microvascular damage. Vasodilation of capillaries in response to nitric oxide is
impaired by diabetes-induced hyperglycemia (Calver et al., 1994; Kihara and Low, 1995). Advanced glycation end products also lead to vasodilatory impairment of the capillaries by quenching endothelium-derived nitric oxide (Bucala et al., 1991). Oxidative stress and polyol pathway flux have also been implicated in impaired vasodilation of the microcirculation of diabetic subjects (Zochodne, 2007). The alterations in the ability of the capillaries to relax under chronic hyperglycemic conditions results in a functional vasoconstriction of these blood vessels and a diminution of blood flow.

Changes in capillary lumen area and occlusion of capillary vessels may also play a role in microangiopathy associated with diabetes and the subsequent reduction in blood flow. The number of capillaries closed in human sural nerve has been shown to correlate with severity of neuropathy (Dyck et al., 1985), although other investigators have not reported this observation. Proliferation of the intima has also been observed in epineurial arterioles of diabetic patients (Korthals et al., 1988). Other hemodynamic changes observed in diabetic subjects, such as abnormal fibrin deposition, erythrocyte aggregation and platelet activation could also play a role in blood vessel occlusion and impaired nerve blood flow (Cameron et al., 2001a). Interestingly, increased endoneurial capillary lumen area has also been observed in the sural nerve of diabetic patients and may represent an angiogenic response in the vasa nervorum to diabetes-induced nerve ischemia (Malik, 1997).

It is clear that metabolic perturbations associated with diabetes play a key role in the development of diabetic neuropathy. These metabolic changes are
accompanied by vascular changes both in large and small vessels and within the vasa nervorum. The impairment and alteration of large and small blood vessels and of endothelial cells in the microvasculature, leads to a functional deficit of nerve blood flow in diabetic subjects. Reduced nerve blood flow can ultimately lead to ischemic damage to neurons and subsequent structural damage and neuropathy. Changes in nerve blood flow in diabetic patients, as well as in experimental models of diabetes, and the potential implications for nerve function and structure will be considered in the following sections.

1.3 Peripheral nerve blood flow

1.3.1 Peripheral nerve vasculature

The vasculature of the peripheral nerve is comprised of two distinct blood supplies: the extrinsic and intrinsic. The extrinsic blood flow supplies the epineurial and perineurial regions of the nerve, while the intrinsic blood supply, also referred to as the vasa nervorum, supplies blood to the inner, endoneurial nerve compartment. Differences between the extrinsic and intrinsic systems have important implications on how the peripheral nerve is perfused and how pathologic alterations of blood flow can affect the ability of the vasculature to meet the metabolic demands of nerve tissue.

The extrinsic circulation to the peripheral nerve is derived from large arteries and veins in addition to branches of blood vessels nourishing nearby muscle and bone (Cameron, 2003). These blood vessels are primarily arranged longitudinally along the surface of the nerve and form a highly anastomosing network with prominent arteriovenous shunts. Arising from the extrinsic
circulation is a large number of connections to the inner intrinsic blood supply (Bell and Weddell, 1984). Regulation of peripheral nerve blood flow is mediated primarily by the extrinsic vasculature, which is self-innervated by sympathetic nerves arising from the nerve trunk itself (Appenzeller et al., 1984; Zochodne, 2002). The nerve fibers innervating blood vessels of the vasa nervorum contain neurotransmitters including: norepinepherin, serotonin, substance P, vasoreactive intestinal peptide, and calcitonin gene related peptide (CGRP) (Appenzeller et al., 1984). Axons that innervate the epineurial vasculature are unmyelinated and are among the first to be injured by diabetic neuropathy, thus compromising the ability of the peripheral nerve to regulate blood flow via the extrinsic vasculature (Beggs et al., 1992).

The intrinsic blood supply on the other hand does not appear to autoregulate (Smith et al., 1977) and there is at best a curvilinear relationship between mean arterial pressure (MAP) and endoneurial blood flow (Low and Tuck, 1984). Intrinsic nerve blood flow is passive and is controlled by upstream regulatory events of the vasoresponsive arterioles in the extrinsic vasculature. The lack of autoregulation in the vasa nervorum makes the nerve susceptible to ischemia. Endoneurial blood flow originates from branches of the extrinsic supply that penetrate into the endoneurium. Intrafascicular vessels are primarily capillaries that run parallel to the axons. These capillaries are lined by an endothelium bound together by tight junctions to form part of the blood-nerve barrier. Capillaries of the vasa nervorum are of a larger diameter and are more spaced apart than in other tissues, another factor subjecting the nerve to risk of
ischemia (Low et al. 1989). The inability of the vasa nervorum to respond to ischemic events, due to its inability to autoregulate, can lead to nerve hypoxia and damage in instances when nerve blood flow is compromised. Peripheral nerves are able to lose up to 50% of their blood supply prior to suffering ischemia; thus in normal, healthy animals, a transient drop in nerve blood flow would not be considered harmful (Conn and Dyck, 1975). However, when nerve blood flow is already compromised, as occurs with diabetes, further decreases in nerve blood flow can cause nerve hypoxia and lead to nerve injury (Nukada, 1992). Within the dorsal root ganglia (DRG), blood flow is three to five times greater than that of the peripheral nerve itself, reflective of the increased metabolic demand in this region of the nerve (Cameron, 2003). The DRG can autoregulate in response to changes in blood pressure, thus protecting the cell bodies of sensory neurons from ischemic damage (McManis et al., 1997).

Nerve blood flow changes occur as a result of diabetes-induced hyperglycemia and have been measured both clinically and in experimental models of hyperglycemia using a variety of techniques (Boulton et al., 1982; Tuck et al., 1984; Newrick et al., 1986; Cameron et al., 1991; Tesfaye et al., 1993; Sasaki et al., 1997; Ibrahim et al., 1999).

1.3.2 Techniques for measurement of nerve blood flow

There are four primary means of assessing nerve blood flow in the laboratory: microelectrode hydrogen clearance polarography, $[^{14}\text{C}]$iodoantipyrine distribution, microsphere embolization and laser Doppler flowmetry.
Hydrogen clearance polarography is performed by inserting a platinum microelectrode directly into the nerve. Nerve tissue is saturated with hydrogen by the addition of hydrogen to the inspiratory gas of the animal. Following the removal of hydrogen from the gas mixture, clearance of hydrogen from the nerve, as detected by the microelectrode, is measured as a representation of local nerve blood flow. The washout curve from a typical nerve blood flow study using this technique is biexponential, with the fast portion of the curve representing the epineurial blood flow, and the slow portion of the curve representing the intrinsic blood flow (Day et al. 1989).

Autoradiography can be used as a measure of nerve blood flow by assessing the distribution of $[^{14}\text{C}]$ iodoantipyrine, an isotope with high tissue penetrance. As with the hydrogen clearance method, this technique has the advantage of being able to distinguish epineurial from endoneurial blood flow. Measures of local endoneurial blood flow using this technique have been shown to be comparable to those using hydrogen clearance or laser Doppler flowmetry (Rundquist et al., 1985).

The distribution of injected, radiolabeled microspheres in the peripheral nerve has also been used a measure of nerve blood flow. This technique requires the removal of the entire nerve trunk following injection of microspheres, and does not distinguish between epineurial and endoneurial blood flow. Typically this method yields lower values of nerve blood flow than other methods, and this is thought to be due to microspheres not being trapped in the larger epineurial vessels (Zochodne, 2002).
Laser Doppler flowmetry utilizes a fiber optics transducer that can both emit and receive a laser signal. With the use of a micromanipulator, the laser probe is positioned at the surface of the exposed nerve and the laser signal emitted by the probe is reflected by the erythrocytes in the nerve vasculature. The moving blood cells scatter the laser signal, and based upon the speed and number of red cells moving past the probe, the wavelength emitted from probe undergoes a Doppler shift that is interpreted by the transducer as a measure of flow. The flow value calculated by laser Doppler can be converted to vascular conductance when normalized to arterial blood pressure. The laser Doppler signal transmits and receives through both the epineurium and endoneurium, thus it is a measure of both intrinsic and extrinsic nerve blood flow. However, blood vessels of the extrinsic vasculature that can be visualized by the experimenter can be avoided by the laser Doppler probe, providing a blood flow measure more representative of intrinsic circulation.

1.3.3 Alterations in nerve blood flow in human diabetes

Due to the invasive nature of blood flow measurements, the number of studies examining peripheral nerve blood flow in patients has been limited. However, existing reports consistently show nerve blood flow to be reduced in diabetic patients (Boulton et al., 1982; Newrick et al., 1986; Tesfaye et al., 1993; Theriault et al., 1997; Ibrahim et al., 1999). Fluorescence videoangiography and laser Doppler flowmetry have been used to confirm deficits in sural nerve blood flow (Tesfaye et al., 1993; Theriault et al., 1997). Using microlightguide spectrophotometry and microelectrodes, sural nerve oxygen tension is also
Endoneurial oxygen tension can be even lower than that in downstream veins due to epineurial shunting and alterations in circulation at the skin (Boulton et al., 1982). This phenomenon could be a result of the early damage to the sympathetic nerve fibers that innervate the epineurial vessels of the peripheral nerve, leading to changes in flow between endoneurial and shunt vessels.

1.3.4 Nerve blood flow changes in experimental models of diabetes

The majority of studies examining experimental models of diabetes have shown a reduction of nerve blood flow in diabetic animals (Tuck et al., 1984; Kalichman et al., 1998; Cameron et al., 1991; Sasaki et al., 1997). The hydrogen clearance (Tuck et al., 1984), autoradiography (Sasaki et al., 1997) and laser Doppler (Kalichman et al., 1998) techniques have all consistently reported nerve blood flow deficits in diabetic rodents. Several reports using the microspheres method to measure nerve blood flow have found increased nerve blood flow in diabetic rats (Pugliese et al., 1989; Tilton et al., 1989; Sutera et al., 1992). It has been suggested that changes in biochemical or structural components of the microvasculature affect microsphere capture and do not provide an accurate representation of blood flow dynamics (Cameron and Cotter, 1994; Chang et al., 1997). Other studies have reported no decline in nerve blood flow in experimental diabetes, despite the observation of nerve conduction slowing and reduced oxygen tension in the nerve vasculature (Zochodne and Ho, 1992; Kennedy and Zochodne, 2002). Kihara et al. (1994) found an increase in total blood flow, but a decrease in nutritive blood flow in rats that had been on strict
glycemic control with insulin therapy. The reasons for the discrepancies in nerve blood flow are unclear and may stem from the method used to acquire the blood flow measurements. The balance of evidence however, suggests a reduction in nerve blood flow as a result of experimental diabetes. Nerve blood flow deficits have been reported as early as one week following induction of streptozotocin-diabetes (Cameron et al., 1991) and persist beyond one year (Sasaki et al., 1997). In conjunction with decreases in blood flow, decreases in nerve O$_2$ tension and increases in nerve vascular resistance have been reported (Zochodne and Ho, 1992; Wright and Nukada, 1994). The severity of diabetes impacts nerve blood flow. Pugliese et al. (1989) showed that overt hyperglycemia led to a significant reduction in sciatic nerve blood flow compared to animals with mild diabetes.

1.3.5 Attempts to restore nerve blood flow in experimental diabetes

Vascular perturbations and changes in nerve blood flow are at least partially responsible for the development of diabetic neuropathy. At this time there are no FDA approved drugs to treat the underlying cause of diabetic neuropathy and only aldose reductase inhibitors in Japan are approved for clinical use to treat the disease (Yagihashi et al., 2007). Although animal models of diabetic neuropathy are imperfect they do recapitulate a number of metabolic, neurochemical, and functional defects that occur in patients, including nerve blood flow deficits. Therefore, in an attempt to develop treatments to prevent or slow the progression and manifestation of diabetic neuropathy, it is important to show efficacy of potential treatments in animal models before these therapies
can be tested in clinical trials. To this end, a large number of studies have been conducted to assess the ability of therapeutic interventions to restore peripheral nerve blood flow in animal models of diabetes. Multiple pathologic mechanisms have been proposed for the observed diminution in nerve blood flow and have been targeted for treatment in experimental diabetes.

Impaired nitric oxide-mediated endothelium-dependant relaxation has been observed in the vasculature of diabetic animals and has been implicated in the development of peripheral neuropathy. Increased polyol pathway flux is also known to be an important metabolic disturbance induced by diabetes. The presence of the polyol pathway in endothelial cells within epineurial blood vessels of the peripheral nerve has led to the hypothesis that hyperactivity of the polyol pathway could be responsible for NO-related vascular dysfunction (Jiang et al., 2006). Indeed, the use of aldose reductase inhibitors (ARIs) partially or completely restores nerve blood flow deficits and impaired vascular relaxation caused by experimental diabetes (Calcutt et al., 1994; Cameron et al., 1997; Cotter et al., 1998). Interestingly ARIs, but not a sorbitol dehydrogenase inhibitor, correct blood flow in streptozotocin-induced diabetes, suggesting that the first step of the polyol pathway is more important in the development of vascular complications (Cameron et al., 1997). Other studies targeted at NO-mediated vasodilation in the peripheral nerve have also shown efficacy in restoring nerve blood flow in diabetic rats (Cameron and Cotter, 1995; Cotter and Cameron, 1998; Cotter et al., 2003).
Oxidative stress is another contributor to the vascular and neural complication of diabetes. Antioxidant protection is impaired in the nerve, as is glutathione content, and markers of lipid peroxidation are increased. The NO system in endothelial cells is also affected by oxidative stress in the peripheral nerve. In experimental diabetes, free radical scavengers improve nerve blood flow (Cotter et al., 1995; Cameron et al., 2001b,c; Ford et al., 2001). α-lipoic acid (LA) is one of the most effective antioxidants due to its metal chelator properties, its ability to scavenge free radicals, as well as regenerate levels of endogenous antioxidants, such as GSH and vitamins C and E. LA therapy has been shown to reverse blood flow deficits in diabetic rats and also to ameliorate diabetes-induced thermal and mechanical hyperalgesia (Cameron et al., 2001b). Upregulation of NAD(P)H oxidase is considered an important mechanism responsible for the generation of free radicals. Nerve blood flow was corrected by 50% in streptozotocin-diabetic rats treated with apocynin, an inhibitor of NAD(P)H oxidase, suggesting that NAD(P)H oxidase is indeed involved in the generation of reactive oxygen species (Cotter and Cameron, 2003).

In addition to impaired NO activity and oxidative stress, defective fatty acid metabolism has also been implicated in the development of impaired nerve blood flow and nerve function. To address this potential pathogenic mechanism, rats with experimental diabetes have been treated with one of a number of a number of treatments, such as gamma-linolenic acid, evening primrose oil and omega-6 fatty acids (Dines et al., 1995a; Cameron et al., 1996; Cotter and Cameron, 1997; Ford et al., 2001). In each of these studies both nerve blood flow and
nerve conduction velocity were at least partially restored. The use of an ARI in conjunction with evening primrose oil has a synergistic effect on protecting nerve blood flow (Cameron et al., 1996).

Chronic hyperglycemia results in increased PKC activity, particularly of the β isoform of PKC. PKCβ inhibits the synthesis of NO synthase (NOS), which results in impaired NO availability in vascular tissues. Thus, PKC inhibition, especially the PKCβ isoform, could protect microvascular dysfunction caused by diabetes. This hypothesis has been supported by a number of studies in which streptozotocin-diabetic rats treated with PKC inhibitors were protected or rescued from reductions in nerve blood flow (Cameron et al., 1999; Nakamura et al., 1999; Cameron and Cotter, 2002; Cotter et al., 2002).

The renin-angiotensin system has also been the target of therapeutic intervention to restore blood flow in diabetic animals. Angiotensin II antagonists and angiotensin converting enzyme (ACE) inhibitors have been used successfully in streptozotocin-diabetic rats to protect or reverse nerve blood flow deficits (Maxfield et al., 1993; Cameron and Cotter, 1996; Cotter et al., 2001). ACE inhibition prevents the conversion of angiotensin I into angiotensin II and thus promotes vasorelaxation and increased blood flow. Sciatic nerve capillary density has also been shown to increase in diabetic rats following treatment with ZD 8731, an angiotensin II receptor antagonist (Maxfield et al., 1993).

A variety of other strategies to maintain or restore nerve blood flow in experimental diabetes have been attempted with some efficacy. Kihara et al. (1995) normalized nerve blood flow in the diabetic rat with use of the anti-platelet
agent cilostazol. The vasoconstrictive effects of 5HT2A were prevented with a 5HT2A inhibitor and this effect was beneficial to nerve blood flow in the rat sciatic nerve (Cameron and Cotter, 2003). Intramuscular gene transfer of plasmid DNA encoding VEGF-1 or VEGF-2 restored nerved blood flow in streptozotocin-diabetic rats and alloxan-diabetic rabbits (Schratzberger et al., 2001). The wide range of therapeutic strategies that have proven efficacious in preventing or reversing blood flow reductions in the peripheral nerve in animal models has led to clinical trials investigating putative nerve blood flow modulators on the development of neuropathy in diabetic subjects.

1.3.6 Attempts to restore blood flow deficits in clinical diabetes

As mentioned earlier, due to the invasiveness of nerve blood flow measurements, relatively few studies have been conducted in humans. In order to assess the effect of a particular therapeutic intervention on nerve blood flow in a patient, repeated measurements are required, making these studies even more rare. However, there are some human studies that have addressed, if indirectly, the efficacy of various treatments to restore nerve blood flow and function.

PKCβ inhibition was shown to be effective in preventing forearm blood flow attenuation following the administration of a hyperglycemic clamp. In this study, healthy subjects were given either an oral or intravenous PKCβ inhibitor and blood flow was measured in the forearm during euglycemia and clamp-induced hyperglycemia. The subjects who received the inhibitor were protected from hyperglycemia-induced reduction in blood flow (Beckman et al., 2002). In patients treated with an ACE inhibitor, Malik et al. (1998) showed improvement of
peroneal motor nerve conduction. Based on animal studies, it is possible that this effect was at least partially mediated by increased nerve blood flow.

1.3.7 Relationship of blood flow deficits to nerve structure

Reductions in nerve blood flow can potentially lead to endoneurial ischemia and consequent structural damage to peripheral nerves. However, the relationship between blood flow and the development of overt pathology in the peripheral nerve due to diabetes remains unsolved. In experimental models of diabetes, nerve perfusion measurements generally report reduced nerve blood flow. However, morphometric analysis of the nerve does not typically reveal any fiber loss, Schwann cell changes, edema, or other pathologic features typically found in peripheral nerve biopsies from patients with long-term diabetes. The relatively short-term duration of diabetes in experimental studies is a plausible explanation for the lack of structural change in animal models. It is therefore difficult to assess the ability of treatments that target decreases in nerve blood flow to prevent structural damage in the peripheral nerve in current animal models of diabetes. Although structural pathology is quite obvious in the peripheral nerves of patients with moderate to severe symptoms of diabetic neuropathy, the limited number of studies that have measured nerve blood flow in diabetic patients makes it difficult to determine the relationship between blood flow and structure in human diabetic neuropathy. Nevertheless, the presence of nerve pathology in patients has generated hypotheses about how nerve blood flow may be affecting nerve structure in these patients.
Biopsies of peripheral nerve taken from diabetic patients exhibit fiber loss, diminution in axon caliber, endoneurial edema and axonal dystrophy. This type of structural pathology is comparable to that seen in experimental models of ischemic injury, suggesting that reductions in nerve blood flow and resultant ischemia are responsible for the nerve damage (Dyck et al., 1986a,b). The severity of the progression of diabetic neuropathy has been correlated with structural abnormalities in nerve biopsies. Endothelial cell basement membrane thickening and pericyte degeneration were both shown to precede any clinical manifestations of diabetic neuropathy and to be directly correlated to severity of clinical neuropathy, suggesting an ischemic origin of the disease (Giannini and Dyck, 1995). It was also reported that, although there was no reduction in capillary number in biopsies from diabetic patients compared to non-diabetic subjects, the nerves of diabetic patients had a significantly greater number of closed capillaries (Dyck et al., 1985). Malik et al. (1989), however, did report a reduction in capillary number in patients with severe diabetes as compared to mild or non-diabetic patients. Basement membrane thickness and diffusion barrier was also increased in patients with severe diabetic neuropathy. Thus, although there is limited direct evidence, the balance of data supports a reduction in blood flow being associated with structural changes seen in nerves from diabetic patients.

1.4 Hypertension

The etiology of diabetic neuropathy has proven elusive. Diabetes-induced hyperglycemia leads to the occurrence of a multitude of pathologic mechanisms
that may contribute to the progression of neuropathy. Because each of these mechanisms is complex and intertwined, it is a challenge to sort out which mechanism triggers the perpetuating cycle of events that ultimately lead to diabetic polyneuropathy. It has also become increasingly appreciated that several potentially modifiable co-morbidities of diabetes can increase the risk of development of diabetic neuropathy. These include increased triglyceride levels, body-mass index and hypertension (Tesfaye et al., 2005). Hypertension, a disease present in more than 75% of diabetic patients has recently been identified a risk factor that can double the likelihood a diabetic patient will develop neuropathy (Tesfaye et al., 2005).

1.4.1 Essential hypertension

Hypertension or elevated arterial blood pressure has been studied for thousands of years. The link between kidney disease and heart disease and the risk of excessive salt intake were described by the Chinese Yellow Emperor, Hsuang Ti over 4,000 years ago (Veith, 1966). However, the importance of early observations of hypertension could not be fully understood until the description of the circulation of blood by William Harvey in 1628. Harvey described the circulation with tremendous accuracy and though he could not visualize the capillary connection of the arterial and venous vasculature he postulated that it existed. Shortly after Harvey’s death, the Italian scientist Malpighi observed the presence of the capillary system with the use of a microscope (Beevers and Robertson, 2007). Essential or primary hypertension implies that no specific cause for the elevation in blood pressure can be found. Hypertension is defined
as a systolic blood pressure in excess of 140 mmHg with a diastolic blood pressure consistently over 90 mmHg. The pathogenesis of primary hypertension remains poorly understood. The genetic basis of essential hypertension has become less emphasized as a pathogenic factor in recent years and the pathophysiology of the arterial wall as well as inappropriately high sympathetic nervous system activity have been implicated as having causative roles in the basis of hypertension (Beevers and Robertson, 2007).

Macroangiopathy is a common complication of hypertension. In medium and large size blood vessels (>1mm diameter), vessel walls become rigid and dilated with thickening of the tunica media and prevalence of atheroma (Beevers and Robertson, 2007). In smaller blood vessels both the tunica media and tunica intima undergo hyperplasia and thickening such that luminal narrowing is common.

There is also microangiopathy and endothelial cell dysfunction associated with hypertension. Endothelial cells are involved in regulation of vascular tone via nitric oxide and endothelium derived hyperpolarizing factor (EDHF), regulation of vascular permeability, regulation of the blood clotting, and in the inflammatory response (Cines et al., 1998; Blann, 2000). Oxidative stress induced by hypertension can cause endothelial cell dysfunction and an increase in blood vessel contractility, leading to hypoperfusion (Touyz and Schiffrin, 2004). Oxidative stress can also result in lipid peroxidation, increased deposition of extracellular matrix proteins, vascular smooth muscle proliferation and death in the microvasculature (Touyz, 2004). Endothelial cell dysfunction, caused by
hypertension, in turn exacerbates the existing hypertension because the normal compensatory responses of endothelial cells to increases in systemic blood pressure are impaired. Physical inactivity is also a contributor to the generation and maintenance of hypertension. An inverse relationship exists between the amount of physical inactivity in an individual and their systolic blood pressure (Davy and Gentile, 2007).

The prevalence of diabetes mellitus, especially type II diabetes, is rising rapidly and it is estimated that within the next several decades over 300 million individuals will be affected (Mugo et al., 2007). Hypertension occurs in approximately 30% of type I diabetics and up to 80% of type II diabetic patients (Landsberg and Molitch, 2004). The co-existence of hypertension and diabetes greatly increases the risk of co-morbidities associated with diabetes, including diabetic neuropathy.

1.4.2 Experimental models of hypertension

Currently, a variety of animal models exist to study the etiology and effects of hypertension in vivo. These animal models can broadly be considered as either non-genetic or genetic models. Non-genetic hypertension can be induced experimentally via surgical intervention, such as renal artery or aortic constriction, or through pharmacologic, dietary or endocrine manipulation. The most common surgical model of hypertension is the 2K1C model developed by Goldblatt et al. (1934) and involves the surgical constriction of one of the renal arteries. The DOCA (deoxycorticosterone-acetate) model introduced over 60 years ago is still the most common dietary/endocrine model (Selye, 1942).
Genetic models of hypertension are considered as either genotype- or phenotype-driven. Genotype-driven models rely on genetic-based manipulations and involved the mutation, addition or removal of certain genes responsible for blood pressure regulation. Mice are the most common species utilized in genotype-driven models. Typical genes altered in genotype-driven models include those involved in renin-angiotensin regulation, sympathetic nervous system, nitric oxide production, endothelin regulation and vasoactive regulatory activity (Lerman et al., 2005). Phenotype-driven models make use of natural physiologic variations between inbred animal strains. The phenotype-driven rat model is the most commonly used animal model to study the basis and effects of hypertension (Lerman et al., 2005). Phenotype-driven inbred strains are created through selective breeding of animals that display a phenotype of interest, such as hypertension. Breeding of a fixed trait for 20 or more generations can lead to a colony of animals with genetic homogeneity, such that the phenotype of interest will be reliably reproduced in successive generations (Lerman et al., 2005). Examples of phenotype-driven animal models of hypertension include the Dahl salt-sensitive rat, the obese Zucker rat and the spontaneously hypertensive rat (SHR). SHR rats are among the most common animals used to study hypertension and were developed in Japan in the early 1960’s (Okamoto and Akoi, 1963). Both neural and vascular alterations have been implicated in the genetic basis of hypertension in these animals (Lerman et al., 2005), but recently oxidative stress has also been shown to play a role in hypertension in SHR rats (Chan et al., 2006).
1.4.3 Vascular risk factors for the development of diabetic neuropathy

As discussed previously, the etiology of diabetic neuropathy is complex and not completely understood. It is clear, however, that vascular changes such as endothelial cell dysfunction and reduced nerve blood flow contribute to the nerve injury associated with diabetes. Hypertension, present in the vast majority of diabetic patients, exerts deleterious effects on the peripheral nerve vasculature. A recent study examined the risk of certain co-factors on the development of neuropathy in approximately 1,200 patients over a 7-10 year period (Tesfaye et al., 2005). Risk factors found to increase the likelihood of neuropathy in this study included cholesterol level, body mass index, smoking, and hypertension. Cardiovascular disease present at the start of the study led to a doubling of the risk of development of neuropathy.

Despite the common coexistence of diabetes and hypertension and the high rate of neuropathy among diabetic patients, relatively few experimental studies have examined the effects of hypertension on the development of diabetes-induced injury in the peripheral nerve. The effects of hypertension or the combination of hypertension and STZ-diabetes have been studied in the central nervous system. Hypertension led to a decrease in neuron cell number and volume and an increase in astrocyte density in the cortex of SHR rats (Mignini et al., 2004). The combination of STZ-diabetes and hypertension also led to decreased cortical volume and cell number as well as blood vessel narrowing and wall thickening in small cerebral arteries (Tomassoni et al., 2004a). The effects of STZ-diabetes and hypertension together led to more
pronounced neuronal and vascular changes than hypertension alone. Peripheral nerve vasculature is also affected by hypertension in SHR rats. Intrafascicular arteries in the sciatic nerve of SHR rats have increased wall thickness and decreased luminal area compared to non-hypertensive wistar Kyoto rats (Sabbatini et al., 2001; Tomassoni et al., 2004b). Nerve conduction velocity slowing was associated with alterations of the vasa nervorum (Tomassoni et al., 2004b). Antihypertensive treatment was able to reverse the vascular changes in the sciatic nerve of the SHR rats (Sabbatini et al., 2001). The changes observed in the CNS in SHR rats, as well as the vascular modifications in the vasa nervorum of the peripheral nerve in SHR rats suggest that hypertension can potentially increase the progression of peripheral nerve injury in experimental diabetes. There is a need to further study the combined effects of hypertension and diabetes to better understand how these disease processes collectively affect nerve structure and function.

1.5 Conclusion

Based on the myriad studies investigating the pathogenic mechanisms involved in the onset and maintenance of diabetic neuropathy, it is clear that the disease is extraordinarily complex. Hyperglycemia is the primary insult to the peripheral nervous system, but a multitude of secondary events occur that each exert stress on the nervous system and often perpetuate other pathologic processes (Figure 1.1). Polyol pathway hyperactivity and non-enzymatic glycosylation resulting from diabetes-induced hyperglycemia both generate reactive oxygen species and oxidative stress. All of these processes result in
vascular damage with concomitant nerve ischemia in diabetes and together ultimately lead to structural changes in the nerve, axonal loss and irreversible nerve damage. Recent research has also identified certain risk factors for neuropathy that may potentially be modifiable. Hypertension, a co-morbidity found in more than 75% of diabetics, more than doubles the risk of neuropathy among diabetes suffers. The work detailed in this dissertation investigates a variety of means by which to protect nerve function though antioxidant therapy and non-pharmacologic approaches to increase nerve blood flow in experimental diabetes. In addition, the effects of concurrent hypertension on physiologic nerve function and nerve structure are described.
1.6 Specific aims

The following specific aims will be addressed in this dissertation:

1. To determine if indices of experimental diabetic neuropathy can be ameliorated by treatment with novel antioxidant compounds.

2. To determine if non-pharmacologic manipulation of peripheral nerve blood flow can prevent the development and progression of STZ-mediated peripheral nerve injury.

3. To describe the effects of concurrent hypertension on peripheral nerve function and structure in a model of experimental diabetes.
Figure 1.1 Proposed etiologic cascade of key pathogenic factors of diabetic peripheral neuropathy in the context of this dissertation.
CHAPTER 2 – GENERAL METHODS

2.1 Streptozotocin diabetic rat model

The studies described in this dissertation were performed on female rats and tissues isolated from those rats. Generally, these experiments were designed such that non-diabetic control rats were compared to age and sex-matched diabetic rats. Any treatment or experimental intervention was typically applied to both control and diabetic groups. Harlan Sprague-Dawley rats were used in Chapters 3 and 4 for studies involving attempts to modulate nerve blood flow and antioxidant treatment. For the experiments described in Chapter 5 Spontaneously Hypertensive Rats (SHR) rats were used to model hypertension with Wistar Kyoto (WKY) rats serving as normotensive genetic controls.

In all studies, diabetes was induced by injection of the pancreatic β-cell specific toxin, streptozotocin (STZ). STZ has been used to model type I diabetes in the rat since the mid-1960's (Junod et al., 1967). STZ, an alkylating agent, is a glucosamine-nitrosouria compound that causes damage and destruction of β-cells primarily through nitric oxide mediated DNA damage (Kaneto et al. 1995). STZ enters β-cells via the GLUT-2 transporter, to which it binds readily due to its similarity in structure to glucose. The destruction of β-cells in the pancreas in STZ-injected rats eliminates the endogenous source of insulin and generates an insulinenic and severely hyperglycemic type I diabetes-like syndrome in these animals.

STZ injections were performed on animals included in diabetic groups following a period of overnight fasting. Fasting prior to STZ injection is required
in order to reduce the blood glucose level of the animals to a level that circulating glucose does not out-compete STZ for GLUT-2 binding and transport into \( \beta \)-cells. STZ was dissolved in 0.9% saline and administered by intraperitoneal (IP) injection at a dose of 50-55 mg/kg. Smaller rats (175-225 g) received a slightly higher dose of STZ (55 mg/kg) than larger rats (225-275 g; 50 mg/kg) based on the pancreatic cell mass/body mass ratio. The smaller animals used in these studies have less fat than the larger rats, but a larger beta-cell mass/body mass ratio, thus they need a slightly higher dose of STZ to be rendered hyperglycemic following STZ injection. Successful injection of STZ in rats generates a disease state similar to type I diabetes. STZ-injected rats develop hyperglycemia due to insulinopenia and become polyphagic, polydipsic, with polyuria and diarrhea. Weight loss also occurs in STZ injected diabetic rats, although in some instances diabetic rats do not lose weight. It is thought that in these instances the STZ injection was successful in ablating enough pancreatic \( \beta \)-cells to result in hyperglycemia, but that some \( \beta \)-cells remain intact and provide a low-level of circulating insulin. STZ injected diabetic animals can be maintained for weeks to months with close observation, during which time indices of diabetic neuropathy start to become apparent. Animals that become severely ill, cachetic or lose more that 20% of their body weight from the time of STZ injection were treated with small amounts of exogenous insulin (Linplant, Linshin Canada Inc., Scarborough, Ontario). This type of insulin treatment does not correct hyperglycemia, but does allow for a low level of glucose utilization and the prevention of fatal ketoacidosis.
2-3 days following STZ injection, blood sugar was measured using a strip-operated reflectance meter (One Touch Ultra, Lifescan Co., Milipitas, CA) to confirm the development of hyperglycemia. Blood sugar levels greater than 270 mg/dL (15 mM) were considered diabetic, although typically the animals had blood sugar levels well in excess of 300 mg/dL and often above the upper limit of detection of the device (>600 mg/dL).

All animals used in these studies were housed in a UCSD vivarium with controlled temperature, airflow and a 12:12 hour light-dark cycle. Control animals were maintained in cages with standard bedding, while diabetic animals were provided Tek-Fresh bedding due to the polyuria and diarrhea associated with STZ diabetes in these animals. Water and food (LabDiet 5001, Purina Mills, St. Louis, MO) were provided ad libitum to the animals at all times except on days prior to STZ injection.

All animal studies were performed with the approval of the UCSD Animal Subjects Committee.

2.2 In vivo methods

2.2.1 Measurement of tactile response threshold

The response to light touch was performed based on the aesthesiometer developed by Maximillian von Frey in 1896 and further characterized for the assessment of tactile allodynia in rodents (Chaplan et al., 1994). Rats were placed on a testing surface consisting of wire mesh, partitioned individually in plexiglass cubicles and allowed to acclimate for 15-20 minutes. von Frey filaments (Stoelting, Wood Dale, IL, USA) were used to determine the 50%
threshold for hind paw withdrawal. A series of filaments, each with a specified buckling force, were applied to the center of the plantar surface of the rat paw. Each filament was applied for 5 seconds and resulted in either lifting of the paw (positive response) or no reaction from the animal (negative response). Following a positive response, the next lightest filament was applied or, following a negative response, the next heaviest filament was applied. This sequence was continued until four measurements had been made after an initial change in response (positive to negative or negative to positive) or until 5 consecutive negative responses or three consecutive positive responses had been obtained. 50% response thresholds were determined, as described by Chaplan et al. (1994), for both left and right hind paws.

2.2.2 Measurement of thermal response latency

This test is used to assess thermal nociception, which is transmitted via small-caliber unmyelinated C fibers (Yeomans and Proudfit, 1996). Thermal response latencies and thresholds in these studies were determined using a modification of the method developed by Hargreaves et al. (1988; Dirig et al., 1997). Rats were placed on a glass surface maintained at 30°C, housed individually in plexiglass cubicles and allowed to acclimate for 15-20 minutes. A movable heat-emitting light bulb was located directly below the glass surface and positioned beneath the plantar surface of one hind paw of the rat. Activation of the light bulb heats the glass surface below the rat paw and also starts a digital timer. The light bulb is connected to a motion detector, and any movement of the rat hind paw in response to the heat produced by the light bulb immediately stops
the timer and inactivates the light bulb. If there is no response from the animal after a 20 second period, the light bulb automatically shuts off in order to prevent tissue injury to the paw. Time from light bulb activation to foot removal was recorded as thermal withdrawal latency. Following the 15-minute acclimation time, a single measurement is taken on the hind paw to allow the animal to adjust to the testing conditions. This initial measurement was discarded, and the median of three subsequent measurements taken as the thermal response latency for a given rat paw. Both left and right paw response latencies were measured.

Day-to-day variations in the amount of heat produced by the light bulb have been noted and therefore a temperature calibration was performed immediately following each experiment. To do this, the light bulb was positioned below a thermister probe and the light bulb turned on. Over the 20 second heating time, the temperature produced by the light bulb was recorded at 5-second intervals. In this way, a time-versus-temperature curve is generated and thermal response latency can be converted to threshold temperature.

2.2.3 Measurement of sciatic nerve conduction velocity

In all studies, motor and sensory sciatic nerve conduction velocities were measured at baseline prior to the induction of diabetes and at 4-week intervals following STZ-injection until the study was concluded. Rats were anesthetized in an induction box with 5% isoflurane. Once the animals were unconscious the left hind flank was shaved and cleaned with 95% ethanol. Nerve conduction measurements were made with head and forelimbs of the animal placed within a
nose cone supplying 2% isoflurane anesthesia. The sciatic nerve was exposed with a skin incision on the hind left flank, followed by blunt dissection of the connective tissue between the biceps femoris and the gluteus maximus muscles. The nerve temperature of the rats was maintained at a constant 37°C by placing a thermister probe with an attached temperature controller and heat lamp adjacent to the sciatic nerve. A heating pad was placed under the rats to maintain body temperature throughout the experiment.

Conduction velocity was measured using sciatic nerve motor (M) and sensory (H) waves. A bipolar stimulating electrode was inserted percutaneously at the ankle and the reference electrode inserted subcutaneously into the dorsal musculature. A second bipolar stimulating electrode was inserted at the sciatic notch. Supramaximal square wave stimulations (5–10 V, 50 s pulse width) were applied to the nerve and the resulting muscle compound action potentials recorded from the interosseous muscles of the ipsilateral foot with two needle electrodes (Grass Instruments, Quincy, MA). Signals were amplified (P15 AC amplifier, Grass Instruments, Quincy, MA) and displayed on a 5110 Storage Oscilloscope (Tektronix Inc., Beaverton, OR). The median latency difference between three pairs of ankle- and notch-evoked M wave responses was taken as the time for motor nerve conduction to travel the inter-electrode distance, measured with calipers in the fully extended hind limb. MNCV (m/s) was calculated by dividing the inter-electrode distance (mm) by the latency difference (ms). Similar procedures were followed for calculation of sciatic SNCV, using the latency difference of the H wave, a monosynaptically evoked reflex resulting from
stimulation of afferent proprioceptive Ia fibers that excite spinal cord motor neurons.

2.2.4 Measurement of femoral artery blood pressure

Prior to measurement of sciatic nerve blood flow, a cannula was surgically implanted in the right femoral artery to monitor arterial blood pressure. Animals were anesthetized in an induction box with 5% isoflurane until they were unconscious. Once the animals were adequately anesthetized, the ventral surface of the abdomen and right hind limb were shaved and cleaned with 95% ethanol. Animals were maintained on 2% isoflurane for the remainder of the femoral artery cannula implantation and subsequent nerve blood flow measurement. A 3-4 cm incision was made on the skin of the rat directly above the femoral artery. Using blunt dissection, fascia and muscle tissue were dissected away and the femoral artery isolated. A suture was placed around the distal artery and tied off. A loose ligature was placed proximally around the femoral artery. Using a 25-gauge needle as a catheter introducer, a saline-filled PE-10 (Becton Dickinson and Co., Sparks, MD) cannula was inserted into the femoral artery and secured with the proximal suture. The cannula was connected to a disposable pressure transducer (Deltran II, Utah Medical Products, Midvale, UT) and mean arterial pressure (MAP) measured with a MacLab/8s system (AD Instruments, Castle Hill, Australia).

2.2.5 Measurement of conscious blood pressure

In order to assess resting blood pressure in rat not under anesthesia for the SHR studies detailed in Chapter 5, conscious blood pressure was also...
measured. In this case, a femoral artery catheter was initially implanted in the same fashion as described in section 2.2.4. A small incision was then made at the base of the skull of the animal and a trocar inserted subcutaneously at the incision site and pushed through the subcutaneous fascia along the right side of the animal until it emerged at the site of femoral artery catheter insertion. The catheter was then guided through the lumen of the trocar and the trocar was then retracted, leaving the femoral artery catheter externalized at the base of the skull. The wound sites at the site of the femoral artery and the skull were closed with sutures and lidocaine was injected at the wounds to alleviate pain in the animals upon regaining consciousness. The cannulated rats were then placed in a small cylindrical confinement chamber, the externalized cannula was connected to a pressure transducer and MAP was measured as described in section 2.2.4. MAP was monitored until the animals had fully regained consciousness and blood pressure had stabilized for at least 10 minutes. Prior to the measurement of conscious blood pressure in these studies, animals were habituated to waking up from anesthesia within the cylindrical confinements for 5 days prior to the actual blood pressure recording.

2.2.6 Measurement of sciatic nerve blood flow

Laser Doppler measurements were performed immediately following femoral artery cannulation. Rats were maintained on 2% isoflurane throughout the experiment. Core temperature of the animals were maintained at 37°C using a heating pad. The sciatic nerve was exposed with a skin incision on the hind left flank, followed by blunt dissection of the connective tissue between the biceps
femoris and the gluteus maximus muscles. All nerve laser Doppler flow (LDF) measurements were made from the left hind limb. Nerve temperature at the site of LDF recordings was monitored with a thermister probe. Once the sciatic nerve was exposed the 0.85 mm diameter laser Doppler probe (Vasamedics Inc., St. Paul, MN) attached to a Laserflo® Blood Perfusion Monitor 403A (TSI Inc., St. Paul, MN) was positioned with a micromanipulator just above the surface of the mid-sciatic nerve. Once the probe flow measurements were stabilized, five measurements were recorded at 1 mm increments along the sciatic nerve. The median value of the five measurements was used as the representative nerve blood flow for any given animal. At the conclusion of the experiment an arterial blood sample was collected for determination of plasma glucose and/or insulin concentration and the rats were sacrificed by decapitation.

2.2.7 Measurement of muscle blood flow

Muscle blood flow was measured in the biceps femoris muscle immediately following nerve blood flow measurements. Using blunt dissection, skin was dissected away from the underlying biceps femoris in the same region where nerve blood flow was assessed. A laser Doppler disc probe (Model SN PR90929, Oxford Optronix, Oxford, UK) was inserted between the skin and biceps femoris muscle blood flow was recorded continuously recorded using Chart 5 software (AD Instruments, NSW, Australia) and saved for offline analysis. 30-second duration muscle blood flow recordings were averaged to give a mean value for muscle blood flow during the acquisition time.
2.2.8 Vascular perfusion

In order to preserve certain tissues for histologic processing, animals were perfused with 2.5% glutaraldehyde at the conclusion of certain studies. Prior to perfusion, animals were sedated and anesthetized with an intraperitoneal injection of solution containing pentobarbital and diazepam. Anesthetized animals were then placed in a restraining device to prevent excess muscle fiber contraction during perfusion. The thoracic and peritoneal cavities were exposed and the liver was lacerated to allow blood drainage. An 18-gauge needle, connected to a pressurized reservoir of 2.5% glutaraldehyde solution, was then inserted into the left ventricle of the rat. The glutaraldehyde solution was circulated throughout the vasculature of the animal at a pressure within the range of physiologic blood pressure and the animal's blood supply was replaced with 2.5% glutaraldehyde. Perfusion is a useful technique in that tissues are fixed in place at the same time the animal is sacrificed.

2.3 Histologic methods

2.3.1 Tissue collection and fixation

Immediately following sacrifice of an animal, nerve and foot skin were collected for histologic, morphometric and biochemical analysis. The sciatic, tibial and sural nerves were collected by careful dissection of the hind limb. Care was taken not to compress or distort the nerves during the dissection to prevent structural damage to the nerve that could compromise morphometric analysis. Once the peripheral nerves were removed from the animal they were placed lengthwise on a wood applicator and submerged in 2.5% glutaraldehyde solution.
The nerves remained in glutaraldehyde for 24 hours for fixation of proteins and then were transferred to a 0.1 M sodium phosphate buffer solution. Following 24 hours in buffer solution the nerves were osmicated for myelin and lipid fixation and embedded in resin blocks for sectioning and placement on microscope slides.

Once the nerve tissue was collected, plantar surface foot skin was removed from both hind paws. The foot skin was laid out on a flat porous surface and submerged in 4 % paraformaldehyde. The foot skin remained in the paraformaldehyde for 24 hours for fixation and was then transferred to a 0.1M sodium phosphate buffer solution. At least 24 hours following transfer to buffer solution the foot skin was embedded in paraffin blocks for sectioning and placement on microscope slides. A small piece of sciatic nerve and one of the two foot-skin pads from each animal was snap frozen in liquid N\textsubscript{2} for future biochemical analysis.

2.3.2 Tissue processing and embedding in resin and paraffin

Following osmication, nerves were processed and embedded in resin. Nerves were first subjected to a series of dehydration rinses for 10-15 minutes in 30, 50, 70, 95 and 100% ethanol. These rinses were followed by a 15-minute rinse in 100% propylene oxide and then a 2-hour rinse in 50% propylene oxide/50% resin solution. Samples were then placed in 100% resin and left in a vacuum hood overnight. The following day samples were removed from resin, placed in histologic molds, embedded in fresh araldite resin and placed in an oven for 2-4 hours to facilitate the polymerization of the resin. Once tissue was
in resin blocks, thick sections were cut using an MT-1 microtome, sections were then placed on glass slides (Superfrost Plus, Fisher Scientific, Tusin, CA) and stained with P-phenylene diamine. Samples prepared for teasing were processed in the same manner, with the exception that hardener was not added to the resin and the nerve samples were left in the unpolymerized resin until they were teased and placed on microscope slides.

Footpads, collected from animals and fixed as described above, were processed and embedded in paraffin. Footpads were placed into individual cassettes and then placed in an automated tissue processor (Autotechnicon Mono Model 2A, Technicon Corporation, Tarrytown, NY) for a 24-hour dehydration rinse cycle and paraffin infiltration. During dehydration, samples were exposed to a series of rinses consisting of 70, 95 and 100% ethanol, as well as HistoClear, xylene and paraffin. Following automated processing, the samples were embedded in paraffin blocks, cut with a paraffin microtome into 6 µm thick sections, which were then placed on glass slides. Footpad slides were then stained with the antibody PGP 9.5 to reveal nerve fibers present in the foot skin.

2.3.3 Footpad immunohistochemistry

Glass slides with footpad sections were incubated with a polyclonal rabbit antibody against the pan-neuronal marker PGP9.5 (1:1000)(Chemicon International, Temecula, CA or Biogenesis Ltd., UK), followed by incubation with a secondary biotinylated antibody. The slides were then incubated with a peroxidase-conjugated avidin-biotin enzyme complex (Vector Laboratories,
Specificity was confirmed by omitting the primary antibody on certain sections.

2.3.4 Quantification of intra-epidermal nerve fibers

PGP 9.5 immunoreactive profiles were then viewed and quantified using a light microscope at 400x magnification by an observer unaware of the treatment groups. The total number of immunoreactive profiles throughout the epidermis was then normalized to the length and/or area of the section. Subepidermal nerve plexuses, immunoreactive profiles in the immediately subjacent papillary dermis, were also quantified. Length and area measurements of skin sections were determined using the point-counting method. A grid reticle (Microscope Depot, Tracy, California) consisting of 100 25x25 µm squares was used to determine both the length and area of the sections analyzed. For measurement of length, the number of intersections between the epidermis, measured along the stratum granulosum, and the grid lines was quantified. Length was calculated using a previously derived formula (Kalichman et al. 1995): Length = no. of intersections x 1/2 x (Π/2 x 25 micrometers). The epidermal area of each section was quantified by counting the number of grid intersections contained within the epidermis, excluding the stratum lucidum and the stratum corneum. The total number of points was then multiplied by the area of each individual square within the grid to calculate the total area of the section analyzed.
2.3.5 Morphometric analysis of nerve tissue

Morphometric analysis of thick sections (1 µm) of sciatic, sural and tibial nerves was performed by light microscopy. Digital images of nerve cross sections were captured with BH-2 light microscope (Olympus, Melville, NY), a U-PMTVC video camera (Olympus, Melville, NY) and an LX-450 system digitizer (Optronics Engineering, Goleta, CA). Imaging and analysis were done with a 40x objective such that the digitization resulted in an image with 4.05 pixels equaling one micrometer. Each nerve section was sampled in a back and forth serpentine pattern such that the entire nerve section was analyzed with no overlapping fields. Artifacts and non-ideal axonal cross sections (longitudinal cuts or paranodal profiles) were excluded from the analysis. Using the NIH Image program 1.55, axonal areas and perimeters were measured for each nerve section. These measurements did not include the myelin sheath or g-ratio. The resolution of this analysis was 1 µm.

In later studies, the morphometric analysis was conducted using the HistoQuant software developed by Jared Goor, a former graduate student in the bioengineering department at UCSD. For these analyses, digital photos of sural, tibial and sciatic nerve sections were captured (Retiga 2000R, QImaging, BC, Canada) at 60x magnification using an Olympus BX51 microscope (Olympus, Center Valley, PA). Digital photos were then analyzed using HistoQuant to measure axon caliber (diameter), axon area, myelin thickness and myelin area of nerve fibers. An observer unaware of the experimental groups or nerves being analyzed performed all morphometric measurements.
2.3.6 Pathologic assessment of myelinated nerve fibers

Thick sections (1 µm) of sciatic, sural and tibial nerves underwent a qualitative pathologic assessment. Each section was scanned in a back and forth serpentine pattern with no overlapping fields using a light microscope with a 40x objective. Sections were analyzed for presence of fibers with a disproportionately thin myelin sheath relative to the caliber of the axon (see Figure 5.2A). Once the total number of thinly myelinated fibers was determined, the area of the nerve section was measured and number of thinly myelinated fibers per mm$^2$ was calculated. An observer unaware of the identity of the nerve or experimental groups being analyzed performed this analysis.

2.3.7 Measurement of endoneurial blood vessel density

Endoneurial blood vessel density was determined in the sciatic, sural and tibial nerves by light microscopy. Using a 40x objective, the number of blood vessels present within the nerve fascicle was counted using a back and forth serpentine pattern. Once the total number of blood vessels was determined, the area of the nerve section was measured and blood vessels density expressed as #/mm$^2$. As with previous histologic analyses, the nerve and experimental group of each sample was not known until after the analysis was completed.

2.4 Statistical methods

Data in this dissertation are reported as mean ± sem unless otherwise noted. For studies involving three or more experimental groups, data were analyzed by one-way ANOVA followed by a Newman-Keuls post hoc test to
assess differences between groups. In studies involving only two experimental groups, a Student’s unpaired t-test was used to assess statistical differences.
CHAPTER 3 – ANTIOXIDANT TREATMENT OF EXPERIMENTAL DIABETIC NEUROPATHY

3.1 Introduction

3.1.1 Diabetic neuropathy

As discussing in chapter 1, nerve injury is one of the most common and severe complications of diabetes mellitus, affecting more than 50% of patients with diabetes. The leading hypotheses regarding the pathogenesis of diabetic neuropathy include: microangiopathy and nerve ischemia, non-enzymatic glycosylation of proteins and AGE formation, and increased glucose metabolism by aldose reductase leading to increased flux through the polyol pathway (Brownlee, 2005). A common underlying consequence of each of the aforementioned pathologic mechanisms is the formation of reactive oxygen species and the generation of oxidative stress in the nerve of diabetic patients (Vincent et al., 2004).

3.1.2 Oxidative stress in the pathogenesis of diabetic neuropathy

Oxidative stress occurs in a biological system when the production of reactive oxygen species exceeds the antioxidant capacity of the system (Vincent et al., 2004). Oxidized or nitrosylated byproducts of free radicals damage proteins, lipids and nucleic acids. Modified proteins have impaired function and cause nerve dysfunction through mechanisms such as slowed axonal transport and reduced neurotrophic support (Metodiewa and Koska, 2000). Plasma membrane phospholipids as well as lipids found in the endoplasmic reticulum and mitochondria are sites of peroxidation and damage by free radicals.
(Vincent et al., 2004). Oxidative-stress-mediated DNA damage can lead to cellular apoptosis (Vincent et al., 2004). Essential fatty acids are also a target of free radical attack and fatty acid damage can lead to endothelial dysfunction and vascular abnormalities that ultimately reduce blood flow in the nerve (Cameron and Cotter, 2002). Diabetes also leads to reductions in the antioxidant defense capacity of the peripheral nerve. Taurine and ascorbate, two endogenous antioxidants, are reduced in the sciatic nerve of diabetic rodents (Stevens et al., 1993; Obrosova et al., 2002). Glutathione, another potent antioxidant, is depleted by glucose metabolism through the polyol pathway under hyperglycemic conditions (Vincent et al., 2004).

3.1.3 Antioxidant therapy to treat diabetic neuropathy

Based on the data supporting the role of oxidative stress in the pathogenesis of diabetic neuropathy, a variety of antioxidants have been used experimentally to treat the nerve injury caused by diabetes. α-lipoic acid (LA) is likely the most widely used antioxidant treatment studied in diabetic neuropathy (Vincent et al., 2004). LA can be delivered in the diet, or by intravenous or intraperitoneal injection, and can cross the blood-nerve and blood-brain barrier (Packer et al., 1997). LA is considered one of the more potent antioxidants based its ability to scavenge free radicals, to act as a metal chelator and to regenerate levels of endogenous antioxidants such as glutathione (Obrosova, 2002). In experimental studies, LA has been shown to prevent STZ-diabetes induced slowing of nerve conduction velocity and nerve blood flow deficits (Cameron et al., 1998; Stevens et al., 2000; Coppey et al., 2001). LA
administration has also been shown to reduce markers of oxidative stress, such as hepatic catalase activity, in STZ-diabetic rats (Maritim et al., 2003).

Although not approved for use in the United States, LA is currently licensed for use in Germany to treat diabetic patients. Evidence from this population suggests that, even in patients with poor glycemic control, LA supplementation improves antioxidant defenses and reduces oxidative stress (Borcea et al., 1999). Results from the SYDNEY phase III clinical trial also reported beneficial effect on nerve function in patients with diabetic neuropathy receiving intravenous LA treatment (Ametov et al., 2003). These data suggest a possible role for LA in the treatment of diabetic neuropathy.

Another antioxidant that has been shown to impart therapeutic benefits in models of experimental diabetic neuropathy is the amino acid taurine. In the peripheral nerve, taurine is localized in the endothelial cells and Schwann cells and functions as an antioxidant, calcium modulator and a vasodilator (Pop-Busui et al., 2001). Given its cellular localization and range of physiologic functions, taurine may be a link between the metabolic, vascular and functional changes observed in nerves exposed to chronic hyperglycemia (Pop-Busui et al., 2001). In the sciatic nerve of STZ-diabetic rats, both taurine and ascorbate levels are depleted (Obrosova et al., 2001; Pop-Busui et al., 2001). Taurine supplementation has been shown to improve or restore nerve conduction velocity and nerve blood flow deficits in STZ and Zucker diabetic fatty rats (Pop-Busui et al., 2001; Li et al., 2006). Diabetes-induced neurotrophic factor depletion is also partially restored by taurine treatment in STZ-diabetic rats and Zucker diabetic
fatty rats (Obrosova et al., 2001; Li et al., 2006). Indices of oxidative stress have also been reduced in rats treated with taurine. Lipid peroxidation in the sciatic nerve is increased in diabetic rats and this effect is reduced or reversed in rats treated with taurine (You and Chang, 1998; Obrosova et al., 2001). mRNA of heme oxygenase 1 (HO-1), an oxidative-stress-induced enzyme upregulated by hyperglycemia, is reduced in diabetic rats treated with taurine (Patriarca et al., 2005). 5% taurine supplementation in the diet has also been shown to extend survival rates in STZ-diabetic rats (Di Leo et al., 2004). This is an interesting finding, as drugs currently approved to treat diabetic patients do not prolong life.

Two other compounds with known antioxidant properties that have not yet been studied in the context of diabetic neuropathy are ellagic acid and the carboxyfullerene C3. Ellagic acid is a polyphenol antioxidant that is found in a variety of fruits, vegetables and nuts. The highest levels of ellagic acid are found in raspberries, strawberries and pomegranates. In neuronal PC12 cells exposed to oxidative stress, ellagic acid treatment reduced lipid peroxidation and glutathione depletion, limited reactive oxygen species formation and prevented cell death (Pavlica and Gebhardt, 2005). In an animal model of oxidative stress and hyperlipidemia, rabbits on a high cholesterol diet also receiving ellagic acid treatment had reduced lipids, reactive oxygen species and lipid peroxidation products in plasma compared to rabbits on the high cholesterol diet alone (Yu et al., 2005). In addition, ellagic acid treatment dramatically reduced the percentage of atherosclerotic lesions found in the thoracic aorta of the cholesterol-fed rabbits.
Fullerenes are a family of carbon allotropes that can take on the form of a plane, tube, ellipsoid or a hollow sphere. The spherical form, often referred to as a buckyball, was named after the architect, Richard Buckminster Fuller, who made famous the geodesic dome. The $C_{60}$ buckminsterfullerene is a spherical fullerene composed of 60 carbon atoms and its molecular structure resembles that of a soccer ball. The antioxidant C3 is derived from the $C_{60}$ buckminsterfullerene and has 3 malonic acid residues per carbon molecule on the surface of the sphere (Figure 3.1) (Dugan et al., 1997). C3 has been shown to scavenge free radicals in solution, inhibit excitotoxic death of cultured neurons, and delay death and functional impairment in transgenic mice with a mutant superoxide dismutase gene (Dugan et al., 1997). Also, in an Sod2 $^{−/−}$ mouse model, C3 treatment extended lifespan of the transgenic mice by 300% (Ali et al., 2004).

This chapter describes several approaches to prevent experimental nerve injury associated with hyperglycemia using antioxidant therapy. α-lipoic acid, a well-studied and potent antioxidant, was used to treat hyperglycemia induced by STZ-diabetes or by galactose feeding. The galactose-fed rat is a model of hyperglycemia without insulinopenia that is known to cause nerve dysfunction and structural nerve damage in rats (Dines et al., 1995b; Kalichman et al., 1998). In addition, rats were treated with the carboxyfullerene C3 and the antioxidants taurine and ellagic acid to assess their therapeutic potential in the treatment of diabetic peripheral neuropathy. Given the previously shown efficacy of α-lipoic acid and taurine as a treatment for experimental diabetic neuropathy, it is
expected that $\alpha$-lipoic acid and taurine will again be successful in preventing indices of nerve injury in these studies. In addition, the based on the putative antioxidant properties of C3 and ellagic acid, these treatments should also be effective as in reducing diabetes induced nerve injury in these studies.

3.2 Methods

3.2.1 Experimental design

Four studies were performed to assess the role of oxidative stress and antioxidant treatment in nerve injury associated with STZ-diabetes. The first two studies utilized the potent antioxidant $\alpha$-lipoic acid and are termed LA studies I and II. The third study treated STZ-diabetic rats with the carboxyfullerene, C3 and is referred to as the C3 study. The fourth study used the antioxidants taurine and ellagic acid and is called the EA study. LA studies I and II and the C3 study were 8 weeks in duration and the EA study was 11 weeks long. In LA studies I and II and the EA study, rats were made hyperglycemic by intraperitoneal injection of streptozotocin (50 mg/kg) and hyperglycemia was confirmed 2-3 days post STZ injection with blood collected by a tail-prick and a strip-operated reflectance meter (see section 2.1). Galactose-fed rats were also used as a model of hyperglycemia in LA study I. Mice were used in the C3 study and were made diabetic with STZ injection at a dose of 180 mg/kg.

Rats used in LA study I were assigned to one of 6 treatment groups: control, diabetic, diabetic IP LA, diabetic OG LA, galactose and galactose LA. Diabetic IP LA animals were designed to serve as a positive control for the efficacy of LA as a neuroprotective antioxidant, and received IP injections of $\alpha$-
lipoic acid (100 mg/kg) 6 days/week for 8 weeks (as described by Stevens et al., 2000). Diabetic OG LA animals were treated 6 days/week by oral gavage with a slow release $\alpha$-lipoic acid (100 mg/kg) pellet. Galactose animals were not made diabetic, but were fed a high-galactose diet (40% D-galactose weight/weight). Galactose LA animals were fed a high-galactose diet and were also treated 6 days/week with 100 mg/kg IP $\alpha$-lipoic acid injections. Blood glucose and body weight was monitored at weekly intervals during LA study I. Motor and sensory nerve conduction velocity was measured at onset and at weeks 4 and 8. At the conclusion of LA study I, mean arterial blood pressure and laser Doppler nerve blood flow were also measured.

LA study II was designed to address issues arising from LA study I. Rats were assigned to one of three groups in LA study II: diabetic, diabetic LA 10 and diabetic LA 50. Rats in the diabetic group were untreated STZ-diabetic animals. Diabetic LA 10 and diabetic LA 50 rats were treated by IP injection with either 10 or 50 mg/kg $\alpha$-lipoic acid, 6 days/week. As with the LA study I, blood glucose and body weight were monitored weekly throughout the study, and motor and sensory nerve conduction velocity were assessed at onset and at weeks 4 and 8. Mean arterial pressure and laser Doppler blood flow were also measured at the conclusion of LA study II.

Swiss Webster mice were used in the C3 study and were assigned to one of four treatment groups: control placebo, control C3, diabetic placebo and diabetic C3. Mice were used in this study because C3 has been previously shown to exert neuroprotective effects in mice (Dugan et al., 1997). Diabetic
mice were made hyperglycemic by a single injection of STZ (180 mg/kg). Mice receiving C3 were treated with 100 mg/kg/day C3 in drinking water. C3 in solution turns drinking water a reddish color; so placebo-treated mice were fed drinking water dyed red with food coloring to enable treatments to be delivered without knowledge of which animals were receiving placebo or C3. Body weight and blood sugar were monitored weekly in these mice, and motor nerve conduction velocity was measured at onset and at weeks 4 and 8 of the study.

Rats involved in the EA study were assigned to one of four treatment groups: control, diabetic, diabetic taurine or diabetic EA. Control and diabetic rats were untreated. Diabetic taurine rats were treated with 1% taurine in drinking water and diabetic EA rats were treated daily by oral gavage with ellagic acid at a dose of 75 mg/kg. Blood glucose and body weight were monitored at weekly intervals, and motor and sensory nerve conduction velocity measured at onset and at 4-week intervals, as well as, at the conclusion of the study (week 11). Hind paw tactile sensitivity was assessed by von Frey filaments every four weeks and at the conclusion of the study. Nerve tissue was collected from the animals at the time of sacrifice, and lipid peroxidation in the sciatic nerve was measured by ELISA for all animals in the study.

3.2.2 Nerve conduction velocity measurement

Motor and sensory nerve conduction was measured in the sciatic nerve of the left hind leg for all animals in this chapter (see section 2.2.3).

3.2.3 Tactile allodynia
50% paw withdrawal latency was measured on the plantar surface of the left and right hind paw using von Frey filaments (see section 2.2.1).

### 3.2.4 Nerve blood flow

Laser Doppler flowmetry was used to assess nerve blood flow. The right femoral artery was cannulated to monitor arterial blood flow and laser Doppler flow was measured in the mid-sciatic nerve on the left hind limb (see section 2.2.4 and 2.2.6).

### 3.2.5 Tissue extraction, homogenization and lipid peroxidation measurement

At the end of each study, rats were anaesthetized and killed by decapitation. Sciatic nerves were quickly removed, immediately frozen in liquid nitrogen and stored at -70°C. For analysis, sciatic nerve segments (~2 cm) were weighed and homogenized in 400 µl of PBS buffer, pH 7.4, containing 5 mM butylated hydroxytoluene (Sigma-Aldrich) and 0.4% protease inhibitor cocktail (Sigma-Aldrich). The samples were centrifuged at 10,000g for 15 minutes at 4°C, and the supernatants were collected to assay protein concentration (BCA™ Protein Assay Kit; Pierce, Rockford, IL, USA), lipid peroxidation and protein oxidation (see below).

Measurement of total MDA and HAE levels was performed using a commercial kit (LPO-586 assay, Oxis International Inc., CA, USA). The method is based on the reaction of the chromogenic reagent, N-methyl-2-phenylindole, with MDA and HAE at 45°C. Two hundred microliters of homogenate were used for measurements of total MDA+HAE, according to the manufacturer’s instructions. The absorbance of chromogenic product was measured at 586 nm
with a Uvikon 930 spectrophotometer (Kontron Instruments, USA) and compared
to the absorbance in corresponding MDA standards. The total MDA+HAE
concentration was expressed as micromoles per milligram of protein.

3.3 Results

3.3.1 LA studies I and II

In LA studies I and II, diabetic rats became hyperglycemic within 2-3 days
of STZ injection and hyperglycemia persisted until the conclusion of the study,
regardless of treatment (Table 3.1 and 3.3). Galactose-fed animals had a non-
significant increase in blood glucose compared to control rats (Table 3.1).
Diabetic animals in LA study I experienced a reduction in body weight compared
to control animals, though this result was only significant for the diabetic IP LA
group (p<0.05; Table 3.1). Galactose-fed animals had body weights comparable
to diabetic rats (Table 3.1). Blood glucose and body weights were similar
between all 3 experimental groups in LA study II (Table 3.3).

Despite a trend towards MNCV slowing in diabetic and especially in
galactose-fed rats compared to control animals in LA study I, there were no
significant differences between any of these groups (Table 3.2). There was no
apparent effect of LA treatment on MNCV in LA study I. SNCV was significantly
slower in all diabetic and galactose-fed animals compared to control in LA study I
(p<0.05; Table 3.2). There was no effect of LA treatment on SNCV in any group
in this study. Diabetic rats treated with lower doses of α-lipoic acid in LA study II
did not have significantly different MNCVs or SNCVs compared to untreated
diabetic animals (Table 3.4).
Sciatic nerve vascular conductance was measured in both LA studies I and II (Figure 3.2A and B). Although the difference was not statistically significant, diabetic animals had a reduced nerve vascular conductance compared to control animals in LA study I. Daily IP α-lipoic acid treatment (100 mg/kg) resulted in a reduction in nerve vascular conductance compared to controls (p<0.05; Figure 3.2A). Daily treatment of diabetic animals with slow release oral α-lipoic acid pellets (100 mg/kg) resulted in a partial protection in these animals from a reduction in nerve vascular conductance (Figure 3.2A). Untreated galactose-fed rats had nerve vascular conductance similar to that of control animals, but daily IP α-lipoic acid treatment resulted in a significant reduction of conductance compared to both control and untreated galactose-fed rats (p<0.05; Figure 3.2A). MAP was significantly lower in all three diabetic groups compared to control animals (p<0.05) indicating that sciatic nerve blood flow, as measured by laser Doppler, was even more substantially reduced in diabetic animals compared to controls (Table 3.2). In LA study II low-dose α-lipoic acid treatment resulted in a trend toward a reduction in nerve vascular conductance in both the 10 and 50 mg/kg treated rats compared to untreated diabetic animals (Figure 3.2B). There was no difference in MAP between any of the diabetic groups in LA study II (Table 3.4).

3.3.2 C3 study

Diabetic mice in the C3 study were hyperglycemic (Table 3.5). STZ-diabetes led to a significant reduction in body weight (p<0.001) and this was not affected by treatment (Table 3.5). Four weeks into the study there was a
significant reduction in MNCV (p<0.05) in diabetic animals compared to control animals (Table 3.5). Following 8 weeks of experimental diabetes, MNCV was reduced even further in diabetic as well as C3 treated animals compared to control mice (p<0.001; Table 3.5). C3 treatment did not alter MNCVs at 4 or 8 weeks in control mice, compared to placebo-treated control mice.

3.3.3 Taurine and ellagic acid

Diabetic rats in the EA study became severely hyperglycemic following STZ injection and this effect was not altered by taurine or EA treatment (Table 3.6). Body weight of diabetic animals, including the taurine and EA treated rats, were significantly lower than controls (p<0.001) at the conclusion of the study (Table 3.6).

Motor nerve conduction velocity was significantly reduced in all 3 diabetic groups compared to control animals (p<0.001). Although there was no clear effect of treatment on MNCV in this study, the EA treated diabetic animals did have a slight trend towards an improvement in MNCV (Table 3.7). There was also a significant reduction in SNCV in diabetic animals compared to controls in the EA study (p<0.001; Table 3.7). Although taurine treatment led to a modest improvement in SNCV in diabetic animals compared to untreated diabetic rats, SNCV of diabetic taurine treated rats was still significantly slower than that measured in controls (p<0.01). However, in diabetic rats treated with ellagic acid, SNCV slowing was prevented and diabetic EA animals had a significantly faster SNCV than untreated diabetic rats (p<0.01; Table 3.7).
Antioxidant protection against experimental diabetic neuropathy was also evident in measures of tactile response. The 50% paw withdrawal threshold in response to von Frey filament stimulation was markedly reduced in untreated diabetic animals compared to controls (p<0.001), indicating the development of tactile allodynia in diabetic rats (Table 3.7). Both taurine and EA treatment prevented the development of tactile allodynia and led to significantly higher paw withdrawal threshold compared to untreated diabetic rats (p<0.01; Table 3.7).

Lipid peroxidation, considered a measure of oxidative stress, was measured in the sciatic nerve in the EA study. Normalized to either mg of tissue or mg of protein, lipid peroxidation was significantly higher in diabetic sciatic nerve compared to control sciatic nerve (p<0.001; Figure 3.3A, B). Both taurine and EA treatment significantly reduced lipid peroxidation in the sciatic nerve of diabetic animals (Figure 3.3A, B). These findings demonstrate the efficacy of the antioxidants, taurine and ellagic acid.

3.4 Discussion

Four different antioxidants were used in the studies described in this chapter to treat nerve injury associated with experimental hyperglycemia. With the exception of a partial protective effect on the diabetes-induced nerve blood flow deficit, α-lipoic acid was not effective in preventing nerve dysfunction in LA study I. In fact in most instances, LA treatment worsened nerve function. Based on the negative results of LA study and anecdotal reports that LA dosage must be carefully titrated in order to confer a therapeutic benefit, a second LA study was conducted. At the lower doses of 10 and 50 mg/kg/day, LA treatment was
ineffective and, in fact, was again deleterious to nerve function in STZ-diabetic rats. Similarly, C3 treatment did not improve MNCV in treated diabetic mice. On the other hand, both taurine and ellagic acid treatment provided protection against nerve conduction slowing, the development of tactile allodynia and lipid peroxidation in the sciatic nerve of diabetic rats.

Given the existing reports in the literature regarding the beneficial effects of LA on the treatment of diabetic neuropathy, it was not expected that LA therapy in STZ- and galactose-fed rats in these studies would further exacerbate the nerve injury associated with hyperglycemia. Indeed, other research has shown LA treatment to be effective at preventing nerve conduction velocity slowing and reductions in nerve blood flow in diabetic rats (Nagamatsu et al., 1995; Cameron et al., 1998; Stevens et al., 2000; Coppey et al., 2001). A number of clinical trials also support the role of LA as a viable therapeutic option to treat diabetic neuropathy (Borcea et al., 1999; Haak et al., 2000; Ametov et al., 2003). Despite the weight of evidence in support of LA as an antioxidant treatment for diabetic neuropathy, some data suggest LA may have no effect or may even be harmful. In a large (> 500 patients) multicenter, randomized, double-blind and placebo-controlled clinical trial designed to test the effects on LA on symptomatic diabetic polyneuropathy, LA treatment did not alleviate neuropathic symptoms (Ziegler et al., 1999). Maritim et al. (2003) reported deaths in all STZ-diabetic rats receiving 50 mg/kg daily IP injections of LA within 3-6 days of onset of treatment.
In some instances the presence of reactive oxygen species are critical for normal physiologic function. For example, pain perception transduced by the heat-sensing TRPV1 relies on free radicals. Nerve growth factor upregulation induces NADPH oxidase production. NAPDH oxidase upregulation results in increased generation of reaction oxygen species and this has been shown to be important in normal expression of TRPV1 (Puntambekar et al., 2005). Excess scavenging of free radicals can therefore lead to loss of function in certain situations. Because LA is such a potent antioxidant, it is possible the deleterious effects of LA treatment reported in this chapter are a result of excess free radical scavenging by LA.

Lipoic acid has also been shown to stimulate glucose metabolism in patients with type II diabetes (Ziegler et al., 1999), improve insulin-stimulated glucose uptake in skeletal muscle of obese Zucker rats (Jacob et al., 1996) and stimulate uptake of glucose by adiposites in culture via the GLUT1 and GLUT4 glucose transporters (Estrada et al., 1996). LA-stimulated glucose uptake in cultured adiposites has been shown to be dependant on activation of the mitogen-activated kinase p38 (Konrad et al., 2001). The phenomenon could also be a potential explanation of the adverse effect of LA treatment on nerve function reported in this chapter. Price et al. (2004) demonstrated that p38 activation is increased in both the DRG and cell bodies of motor neurons in the ventral horn of the spinal cord in rats with 12 weeks of STZ-induced diabetes. The increase in p38 activation was correlated with a reduction in both MNCV and SNCV, and treatment with a p38 inhibitor restored conduction velocity to normal levels.
Thus, despite the antioxidant capabilities of LA, it is possible that through LA-mediated excess free radical scavenging, p38 activation, or some other unidentified mechanism, LA can be ineffective or deleterious as a treatment for diabetic neuropathy.

Fullerenes have been shown to exhibit a very potent antioxidant effect. A single $C_{60}$ molecule can scavenge up to 34 free radicals (Krusic et al., 1991). However, $C_{60}$ is only soluble in a limited number of organic solvents and lot-to-lot variations in synthesis of $C_{60}$ can result in significant differences in solubility and biological action (Dugan et al., 1997). Due to the inconsistencies in the production of $C_{60}$, a more consistently produced derivative, C3, was developed. C3 utilizes 3 malonic acid residues on its surface to mediate its antioxidant and neuroprotective effects (Dugan et al., 1997). In the study described in this chapter, C3 did not confer any observable neuroprotection to STZ-diabetic mice. At the conclusion of the study, it was discovered that the synthesis of C3 was compromised by a contaminant (Dugan, personal communication). It is plausible that the contaminated synthesis prevented the drug from providing any benefit to nerve function in the diabetic mice treated with C3.

An important consideration regarding the lack of therapeutic efficacy in LA studies I and II and the C3 study is the antioxidant effects of the treatment compounds. Indices of oxidative stress were not measured in these studies and thus it is not known if LA or C3 treatment was successful in reducing oxidative stress. To address this issue, lipid peroxidation was used as a measure of oxidative stress in the EA study. Taurine has previously been shown to improve
nerve conduction velocity and nerve blood flow, restore neurotrophic factor depletion and reduce lipid peroxidation in the sciatic nerve of diabetic rats (You and Chang, 1998; Obrosova et al., 2001; Pop-Busui et al., 2001; Li et al., 2006).

Thus, taurine was used as a positive control in this study to show efficacy of the model in improving indices of nerve function in experimental diabetes. Ellagic acid, an antioxidant found in a variety of fruit species, also has properties as an aldose reductase inhibitor (Ueda et al., 2004). Given the importance of both the polyol pathway and oxidative stress in the development of neuropathy, it was of interest to test the efficacy of ellagic acid as a treatment of experimental nerve injury.

Taurine treatment of diabetic animals partially restored the diabetes-induced SNCV deficit and completely protected STZ-diabetic rats from developing tactile allodynia and from lipid peroxidation in the sciatic nerve. Taurine depletion in nerve tissue contributes to both vascular and metabolic defects in nerve function. Given the complex cycle of metabolic and vascular changes that occur and are implicated in the pathogenesis of diabetic neuropathy, it is reasonable to consider taurine treatment as a potential therapy for diabetic neuropathy. Taurine treatment has also been shown to reduce oxidative damage in other tissues susceptible to hyperglycemia-induced damage such as lens, retina and kidney (Obrosova and Stevens, 1999; Pop-Busui et al., 2001). Taurine exerts its neuroprotective effects through the prevention of free radial generation and by scavenging reactive oxygen species. The localization and diabetes-induced depletion of taurine in the nerve vascular endothelium also
highlights the importance of taurine in maintaining nerve blood flow and the potential for taurine depletion to cause vascular impairment and nerve hypoxia in experimental diabetes.

The effects of ellagic on nerve function and oxidative damage were similar to that of taurine treatment. Ellagic acid was even more effective as therapy for experimental diabetic neuropathy, in that it partially restored the diabetes-induced MNCV slowing and completely restored the SNCV deficit. The utility of ellagic as a treatment for diabetic-induced nerve injury has not been previously reported. However, in cell culture and in animal models, ellagic acid has been shown to convey antioxidant effects (Pavlica and Gebhardt, 2005; Yu et al., 2005). The antioxidant properties of ellagic acid are mediated by scavenging of H$_2$O$_2$ and through stimulation of glutathione-S-transferase (Ratnam et al., 2006). In addition, it has recently been shown that ellagic acid acts as an ARI, and prevents sorbitol accumulation in the sciatic nerve of STZ-diabetic rats (Ueda et al., 2004). The data presented in this chapter suggest a potential therapeutic role for ellagic acid in the treatment of diabetic neuropathy. Drug delivery of ellagic acid in diabetic patients may prove difficult. Ellagic acid has poor solubility, poor membrane permeability, is relatively unstable and is highly metabolized in the liver prior to reaching systemic circulation (Ratnam et al., 2006). However, based on the paucity of options to treat diabetic neuropathy and the promising effects of ellagic on nerve function in diabetic rats, further study of ellagic acid as a treatment for diabetic neuropathy is warranted.
There are a number of experiments that would be of interest to further investigate the effects of ellagic acid on experimental nerve injury. In order to determine if the beneficial effects observed on nerve function in this study were due to the antioxidant effects of ellagic acid, a compound with similar molecular structure, but without either the antioxidant or ARI effects, could be administered to STZ-diabetic rats. This experiment would provide insight in the mechanism of action of ellagic acid in mediating neuroprotective effects. The experiments described in chapter 5 of this dissertation describe a model of experimental diabetes (SHR/STZ-diabetic rat) that induces more structural damage to the peripheral nerve than that observed in previous STZ-diabetic rat models. It would be of interest to test the efficacy of ellagic acid in preventing or reversing structural changes to the peripheral nerve using the SHR/STZ-diabetic rat model.

I gratefully acknowledge Dr. Joice Cunha for contributing figure 3.3 to this chapter.
Table 3.1

Blood sugar and body weight measurements of rats used in LA study I.

<table>
<thead>
<tr>
<th></th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(n= 5-10)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>173 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>281 ± 6</td>
</tr>
<tr>
<td>Diabetic</td>
<td>530 ± 18</td>
<td>243 ± 10</td>
</tr>
<tr>
<td>Diabetic IP LA</td>
<td>591 ± 9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>233 ± 13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic OG LA</td>
<td>503 ± 25</td>
<td>252 ± 15</td>
</tr>
<tr>
<td>Galactose</td>
<td>238 ± 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>250 ± 4</td>
</tr>
<tr>
<td>Galactose IP LA</td>
<td>233 ± 14&lt;sup&gt;a&lt;/sup&gt;</td>
<td>245 ± 5</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. 
<sup>a</sup>, p<0.001 versus all diabetic groups; 
<sup>b</sup>, p<0.05 versus control; IP, intraperitoneal; OG, oral gavage; LA, α-lipoic acid.
Table 3.2

Physiologic measurements of rats used in LA study I.

<table>
<thead>
<tr>
<th>(n= 5-10)</th>
<th>MAP (mmHg)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>94 ± 2</td>
<td>67.1 ± 3.6</td>
<td>55.4 ± 2.3</td>
</tr>
<tr>
<td>Diabetic</td>
<td>78 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.2 ± 2.4</td>
<td>47.0 ± 1.7&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic IP LA</td>
<td>74 ± 8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>62.2 ± 3.1</td>
<td>47.5 ± 2.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic OG LA</td>
<td>78 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>61.4 ± 3.2</td>
<td>49.0 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Galactose</td>
<td>89 ± 2</td>
<td>55.3 ± 2.1</td>
<td>45.8 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Galactose IP LA</td>
<td>83 ± 4</td>
<td>55.5 ± 2.4</td>
<td>44.4 ± 2.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a p<0.05 versus control; b, p<0.01 versus control. MAP, mean arterial blood pressure; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; IP, intraperitoneal; OG, oral gavage; LA, α-lipoic acid.
Table 3.3

Blood glucose and body weight of rats used in LA study II.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>566 ± 21</td>
<td>222 ± 11</td>
</tr>
<tr>
<td>Diabetic LA 10</td>
<td>585 ± 7</td>
<td>238 ± 7</td>
</tr>
<tr>
<td>Diabetic LA 50</td>
<td>586 ± 8</td>
<td>230 ± 13</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. LA, α-lipoic acid.
Table 3.4

Physiologic measurements of rats used in LA study II.

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP (mmHg)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic</td>
<td>70 ± 5</td>
<td>69.2 ± 2.5</td>
<td>45.4 ± 1.5</td>
</tr>
<tr>
<td>Diabetic LA 10</td>
<td>75 ± 4</td>
<td>65.2 ± 1.5</td>
<td>47.6 ± 2.6</td>
</tr>
<tr>
<td>Diabetic LA 50</td>
<td>77 ± 4</td>
<td>68.2 ± 3.9</td>
<td>44.4 ± 1.3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. There were no significant differences between any of the groups in the LA II study. MAP, mean arterial blood pressure; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; LA, α-lipoic acid.
Table 3.5
Physiologic measurements of mice used in the C3 study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
<th>MNCV week 4 (m/s)</th>
<th>MNCV week 8 (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control placebo</td>
<td>ND</td>
<td>30.9 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.0 ± 1.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.2 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control C3</td>
<td>ND</td>
<td>31.2 ± 0.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44.8 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>42.7 ± 0.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic placebo</td>
<td>458 ± 19</td>
<td>25.9 ± 0.9</td>
<td>40.5 ± 1.6</td>
<td>36.3 ± 1.5</td>
</tr>
<tr>
<td>Diabetic C3</td>
<td>404 ± 30</td>
<td>25.5 ± 1.6</td>
<td>37.4 ± 1.8</td>
<td>35.0 ± 0.8</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.001 versus both diabetic groups; b, p<0.05 versus diabetic C3. MNCV, motor nerve conduction velocity; ND, no data available.
Table 3.6

Blood sugar and body weight measurements of rats used in the EA study.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n= 6-12)</td>
<td>ND</td>
<td>276 ± 4</td>
</tr>
<tr>
<td>Diabetic</td>
<td>524 ± 39</td>
<td>243 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic taurine</td>
<td>581 ± 12</td>
<td>240 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic EA</td>
<td>562 ± 15</td>
<td>238 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. <sup>a</sup>, p<0.001 versus control. EA, ellagic acid; ND, no data available.
Table 3.7

Physiologic measurements of rats used in the EA study.

<table>
<thead>
<tr>
<th>Group</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
<th>50% PWT (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n= 6-12)</td>
<td>54.3 ± 1.3</td>
<td>49.8 ± 1.1</td>
<td>12.4 ± 0.9</td>
</tr>
<tr>
<td>Diabetic</td>
<td>43.8 ± 1.0a</td>
<td>41.5 ± 0.7a</td>
<td>4.8 ± 1.3a</td>
</tr>
<tr>
<td>Diabetic taurine</td>
<td>43.6 ± 1.0a</td>
<td>44.2 ± 0.9b</td>
<td>10.4 ± 1.2c</td>
</tr>
<tr>
<td>Diabetic EA</td>
<td>46.8 ± 1.1a</td>
<td>47.0 ± 1.1c</td>
<td>11.2 ± 1.1c</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.001 versus control; b, p<0.01 versus control; c, p<0.01 versus diabetic. MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; PWT, paw withdrawal threshold; EA, ellagic acid.
Figure 3.1 The molecular structure of the carboxyfullerene C3 (Ali et al., 2004).
Figure 3.2 Nerve vascular conductance for LA studies I (A) and II (B). Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. *, p<0.05 versus control; #, p<0.05 versus galactose. n=5-10. AUF, arbitrary units of flow; MAP, mean arterial blood pressure; IP, intraperitoneal; OG, oral gavage; LA, α-lipoic acid.
Figure 3.3 Lipid peroxidation in the sciatic nerves of rats used in the EA study. A. Lipid peroxidation normalized to µg of sciatic nerve tissue. B. Lipid peroxidation normalized to µg of protein in the sciatic nerve. Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. *, p<0.001 versus control; #, p<0.01 versus diabetic. n=8-10. MDA, malondialdehyde; HAE, 4-hydroxyalkenal; EA, ellagic acid.
CHAPTER 4 – EFFECTS OF EXERCISE, NEAR INFRARED ENERGY AND ULTRASOUND ON INDICES OF DIABETIC PERIPHERAL NEUROPATHY IN STZ-DIABETIC RATS

4.1 Introduction

4.1.1 Diabetic neuropathy

Based on evidence that diminished nerve blood flow and consequent ischemia is a contributor to the development of nerve disorders in experimental diabetes (Tuck et al., 1984), there is interest in developing therapeutic strategies designed to increase nerve blood flow in diabetic patients. Vasoactive agents such as vasodilators are an obvious possibility to increase nerve blood flow and their use has shown efficacy in preventing the development of nerve conduction velocity slowing in experimental models of diabetic neuropathy (Cameron and Cotter, 1997). However, systemic delivery of vasodilators may not be practical in a clinical setting due to the potential risk of increasing blood flow in regions of the body where this effect would not be desirable. Because there is currently no vasoactive drug available that selectively targets the vasa nervorum, there is a need to develop other means to locally increase nerve blood flow. Three non-pharmacologic approaches (exercise, near infrared energy and low-intensity ultrasound) to increase nerve blood flow locally and the concomitant effects on nerve function and structure and are considered in this chapter.

4.1.2 Exercise physiology

As early as the 5th century B.C. the health benefits of exercise have been
appreciated. The Greek physician Herodicus was the first to prescribe exercise, as well as good diet and massage, to patients as a therapeutic option to treat disease. Since that time, the physiology and underlying molecular mechanisms involved in exercise and how it may be useful in the prevention and treatment of disease has been studied in depth. Herodicus’ message of proper diet and exercise is still as valid today as it was 2,500 years ago.

Aerobic exercise is known to elicit a variety of acute physiologic changes in the human body. Heart rate and stroke volume both increase rapidly to increase overall cardiac output. Recruitment of additional muscle fibers occurs to match the demands of the workload. Local blood flow is shunted to working skeletal muscle to optimize performance. Respiratory rate increases to eliminate excess CO₂ that results from increased metabolic activity. Increased ventilation also results in O₂ saturation of circulating hemoglobin. Lactate is produced in active muscles by glycolysis and transferred to adjacent resting muscles or into the circulation to be converted to glucose in the liver. All the while, the body is trying to reach a balance between ATP production and consumption (Bacharach, 2004).

If aerobic exercise is performed routinely over a period of weeks to months, chronic changes occur in the cardiovascular system as a response to the constant demands placed on the body. Chronic cardiovascular adaptations associated with long-term exercise include: increased plasma and blood volume, increased red cell mass, increased venous return of blood to the heart and increased stroke volume and cardiac output. In addition, capillary density,
muscle blood flow and overall $O_2$ delivery increases. Heart rate and sympathetic stimulation of the heart decrease with chronic exercise (Bacharach, 2004).

In addition to cardiovascular modifications induced by aerobic exercise, peripheral tissues, primarily muscle, also undergo chronic adaptations in order to utilize the increased $O_2$ delivery from the cardiovascular system. Fast-twitch muscle fibers develop enhanced oxidative potential and slow-twitch fibers increase in cross-sectional size. Aerobic enzymes associated with the Krebs cycle also increase in skeletal muscle (Bacharach, 2004). The benefits associated with chronic aerobic exercise are reversible and are lost in less time than is required to develop them.

### 4.1.3 Exercise as a treatment for diabetic neuropathy

Exercise as a means to treat or prevent diabetes has been studied in the clinical setting. Although there is some debate on the efficacy of exercise in the prevention and treatment of diabetes, several studies suggest that physical exercise can reduce HbA1c levels and increase insulin sensitivity in type II diabetic patients (Rönnevmaa et al., 1986; Schneider et al., 1992; Dela et al., 1995). There are also studies that argue that exercise can prevent the development of type II diabetes (Pan et al., 1997; James et al., 1998).

Recent clinical studies have reported beneficial effects of exercise on the development or progression of diabetic neuropathy, including delayed onset of neuropathy and improvement of nerve conduction velocity (Balducci et al., 2006; Fisher et al., 2007). Decreased metabolic capacity, associated with poor fitness in patients, has been shown to increase the risk for neuropathic complications in
diabetic patients (Estacio et al., 1998). Walking exercise in patients with diabetic neuropathy, although beneficial in many respects, has been shown to increase risk of plantar foot injury in these subjects and the use of non-weight-bearing exercise may be a lower risk therapy compared to walking or running (Kanade et al., 2006).

Experimental data detailing the effects of exercise on diabetic neuropathy is limited and has not yet provided a clear understanding of how exercise affects the progression of experimental diabetic neuropathy. A 12-week treadmill training protocol used in Sprague Dawley rats with diabetic neuropathy did not show any changes in myosin heavy chain expression or cross-sectional area of soleus muscle fibers compared to non-exercised diabetic or control animals (Snow et al., 2005). However, this treatment regime resulted in an improvement in motor nerve conduction velocity in exercised diabetic animals compared to sedentary diabetic rats. A 10-week, 2 hours/day, 5 days/week, treadmill training protocol improved bradycardia and hypotension induced by STZ-diabetes and improved arterial baro- and chemoreflex (De Angelis et al., 2000; Harthman et al., 2007). Swimming exercise in diabetic rats for 1 hour/day for 5 weeks improved glucose tolerance and increased β-cell mass in type II Zucker diabetic fatty rats compared to non-exercised animals (Kiraly et al., 2007). In non-diabetic animals, swimming exercise is associated with increased angiogenesis in cardiac tissue compared to non-exercised control animals or animals that received intramuscular VEGF gene transfer (Efthimiadou et al., 2004). Swimming exercise has also been shown to reduce inflammation in formalin-
injected rats and to reduce neuropathic pain in rodents with partial sciatic nerve ligation (Kuphal et al., 2007). Taken together, these data suggest a possible benefit of exercise on the development and progression of diabetic neuropathy.

4.1.4 Near infrared energy (NIRE) therapy

Infrared light is electromagnetic radiation of a wavelength longer than can be seen by the human eye and shorter than microwave radiation. Visible light has a wavelength within the range of 380 nm and 750 nm. The range of wavelengths of infrared light is 750 nm to 1 mm, spanning five orders of magnitude. As classified by the German Institute for Standardization, near infrared light has a wavelength between 750 nm and 1.4 µm. NIRE emitting devices are FDA-approved for use in a variety of medical applications, primarily to relieve pain and stiffness and to improve circulation.

The mechanism of action for NIRE-mediated physiologic changes has been proposed to be increased local blood flow at the site of irradiation mediated by endothelium-derived nitric oxide. Maegawa et al. (2000) reported significant vasodilation in NIRE-treated blood vessels and a concomitant increase in blood flow in rat mesenteric arterioles. These effects were attenuated by treatment with L-NAME, implicating nitric oxide as the laser-treatment-induced vasodilatory agent. Ultraviolet light, light of a shorter wavelength than the visible light spectrum, has also been shown to mediate vasorelaxation. Ultraviolet light, between a range of 310-350 nm, produces relaxation of vascular smooth muscle cells in vitro, independent of endothelial cells (Matsunaga and Furchgott, 1989).
Wound healing is accelerated by photo energy. Fibroblasts in vitro (when irradiated by low-intensity laser therapy) show increased growth factor release and cell proliferation (Yu et al., 1997). There is also increased epithelialization, granulation tissue and collagen deposition in a wound-healing model in diabetic db/db mice (Yu et al., 1997). In patients with diabetes, athermic laser irradiation improves skin microcirculation and accelerates wound healing of ulcers (Schindl et al., 1998, 1999). In healthy subjects, treatment with NIRE increases nerve conduction velocity of the median nerve (Baxter et al., 1994; Noble et al., 2001). NIRE therapy has also had beneficial effects on the nervous system by reducing the incidence of migraine episodes in women following 10, 25-minute treatments (Allais et al., 2003).

Several reports have indicated that NIRE treatment, in patients suffering from diabetic peripheral neuropathy, has therapeutic benefit. In an unblinded study, 48 out of 49 patients treated with NIRE exhibited improved sensation on the plantar surface of their feet following 6, 30-minute treatments. Following 12 treatments, all 49 patients reported improved sensation to their foot skin as measured by the Semmes-Weinstein monofilament test (Kochman et al., 2002). In a blinded study, using the Anodyne Therapy System (ATS) NIRE devices, patients had improved Semmes-Weinstein scores, improved Michigan neuropathy scores, less incidence of pain and improved balance following 12 treatments with NIRE (Leonard et al., 2004). Another study utilizing ATS NIRE treatment found improvement of sensory nerve current perception threshold in the peroneal nerves of all 27 patients receiving NIRE therapy and complete
normalization of sensory responses in the peroneal nerve in 16 of 27 patients following 10, 40-minute NIRE treatments (Prendergast et al., 2004). Another study that evaluated the effect of infrared therapy on patients with long-standing and severe diabetic neuropathy found that 24, 30-minute treatments over 8 weeks improved Semmes-Weinstein scores in patients, even those with no response to a 200-g Semmes-Weinstein monofilament prior to treatment (Arnall et al., 2006).

There are studies utilizing NIRE therapy that report no improvement in peripheral nerve function following treatment. In healthy patients, no difference was found in skin temperature or nerve conduction velocity of the superficial radial nerve between laser-treated and placebo-treated subjects (Walsh et al., 2000). A clinical trial assessing the effect of NIRE treatment on patients with diabetic sensorimotor neuropathy found no difference between sham or NIRE treated subject in nerve conduction velocity, Toronto Clinical Score, sympathetic skin response or quantitative sensory testing. There was a modest improvement in pain scores as measured by the visual analog scale, but the study concluded there was insufficient evidence to recommend NIRE treatment to treat diabetic neuropathy (Zinman et al., 2004).

4.1.5 Low-intensity ultrasound therapy

Ultrasound energy is high frequency, cyclic sound pressure that produces mechanical vibrations. The frequency of ultrasound is greater than the upper limit of human hearing (>20 kHz). The most well known use of ultrasound is in sonography for visualization of tissues and structures within the body, such as a
developing fetus. High-intensity ultrasound administered in a range between 5-300 W/cm² has the capacity to dissect tissues and induce blood clot dissolution. Ultrasound within the range of 5-1000 mW/cm² is considered low-intensity and is not damaging to body tissues (e.g. sonography).

Clinical data suggests the ultrasound is beneficial in wound healing. In a study investigating the effect of low-intensity ultrasound on bone healing in patients with nonunion fractures with no sign of healing for at least 3 months, 25 of 29 patients healed within an average of 22 weeks following daily, 20 minute ultrasound treatments. Interestingly, smoking was found to inhibit the therapeutic effect of ultrasound treatment in these patients (Nolte et al., 2001). Although low-intensity ultrasound devices are approved by the FDA, the mechanism behind the potential therapeutic uses of ultrasound remains poorly understood.

A number of experimental studies have attempted to assess the therapeutic potential of ultrasound therapy and to elucidate the therapeutic mechanism of action. In adult rats with flank skin lesions, daily ultrasound treatment for 5 days resulted in more granulation tissue and fewer polymorphonuclear leukocytes at the site of injury compared to sham-treated animals (Young and Dyson, 1990a). The acceleration of wound healing produced by ultrasound treatment may be due an increase in blood flow. Ultrasound treatment resulted in increased blood vessel density in equivalent regions of granulation tissue at the site of skin lesion in adult rats following 5 days of treatment (Young and Dyson, 1990b). A bone-fracture study showed that dogs treated with low-intensity ultrasound had increased vascularity at the
site of injury (at days 7 and 11 post injury; Rawool et al., 2003). An angiogenic response to ultrasound therapy was also reported in a rat hind-limb ischemia model. Following femoral or iliac artery ligation, 3 sessions of ultrasound treatment led to angiogenesis, improved perfusion and an increase in VEGF mRNA in ischemic thigh muscle in treated compared to untreated animals (Barzelai et al., 2006). In addition to angiogenesis-mediated increased blood flow to injury sites, ultrasound mediates vasodilation in existing blood vessels, likely through a nitric oxide dependant mechanism (Suchkova et al., 2002; Miyamoto et al., 2003).

Low-intensity ultrasound treatment also exerts beneficial effects on the peripheral nerve. Rats with sciatic nerve axotomy and subsequent ultrasound treatment to the proximal stump displayed rapid nerve regeneration compared to sham-treated animals (Crisci and Ferreira, 2002). Three weeks post nerve crush, functional and morphologic indices were improved in the sciatic nerve after a 10-day ultrasound treatment (Raso et al., 2005). In addition to angiogenesis and increased perfusion, ultrasound may also help mediate nerve regeneration through direct activation of Schwann cells and macrophages (Young and Dyson, 1990c; Chang and Hsu, 2004).

The work presented in this chapter was conducted to gain a more complete understanding of the effects of non-pharmacologic approaches to mediate increases in peripheral nerve blood flow and their effects on the development and progression of experimental diabetic neuropathy. Based on the encouraging data regarding the potential benefits of swimming exercise in
diabetic rats and because of the potential damage to the plantar surface of the foot from weight-bearing exercise, swimming was chosen as the method of exercise in the following experiments. Reports regarding the use of NIRE and ultrasound as a therapy for diabetic neuropathy have also shown promise but have been inconclusive, thus there is interest in further examining the efficacy and mechanisms involved in NIRE and ultrasound to treat diabetic neuropathy. Exercise, NIRE and ultrasound therapy are safe, non-invasive and are cost effective when used to treat chronic conditions and are therefore appealing options as potential therapies to treat diabetic neuropathy.

Exercise, NIRE and ultrasound can all affect changes in blood flow. Increases in muscle blood flow can potentially lead to decreases in endoneurial blood flow of proximal nerve due to the lack of autoregulation in the endoneurium of peripheral nerves. It is possible that by increasing muscle blood through exercise, NIRE or ultrasound treatment, that nerve blood flow may be even further compromised. This chapter investigates the hypothesis that by increasing blood flow in skin and muscle in the hind limb of diabetic rodents, enough blood is drawn away from the sciatic nerve to initiate an angiogenic response in the nerve. Thus the temporary hypoxic stress placed on the nerve by treatment may ultimately be beneficial to the health of the nerve by increasing chronic nerve blood flow due to the neovascularization.
4.2 Methods

4.2.1 Experimental design: exercise study

Two exercise studies were conducted and are referred to as exercise studies I and II. Both experiments were 4 weeks in duration and designed to study the effects of swimming exercise on the development of nerve injury in STZ-diabetic rats. Animals were made diabetic in both exercise studies by intraperitoneal injection of streptozotocin (50 mg/kg) and hyperglycemia was confirmed 2-3 days post STZ injection using blood taken by tail-prick and a strip-operated reflectance meter (see section 2.1).

For exercise study I, rats were randomly assigned one of four experimental groups: control no exercise, control exercise, diabetic no exercise, and diabetic exercise. Swimming exercise was initiated immediately upon confirmation of high blood glucose levels in the diabetic groups. The exercise regime consisted of 90 minutes per day, 5 days per week of swimming in large plastic containers. Water temperature was controlled at 37°C and water depth was set at a level to prevent animals from wading in the water or avoiding swimming by balancing on the tips of their extended tails. In an effort to maintain consistent exercise among all animals, an observer monitored the swimming containers and encouraged the rats to swim continuously. At the end of the study, motor and sensory nerve conduction velocity and nerve blood flow were measured in all rats. Additionally, sciatic nerve tissue was collected to assess endoneurial blood vessel density and soleus muscle was dissected and weighed to look for differences in muscle growth as a result of the exercise protocol.
Exercise study II was conducted to include additional controls to the design used in exercise study I. In exercise study II, rats were randomly assigned into 6 treatment groups: control no exercise, control 30° exercise, control 37° exercise, diabetic no exercise, diabetic 30° exercise, and diabetic 37° exercise. Animals that were exercised were placed in plastic water containers at either 30° or 37°C for 90 minutes per day, 5 days per week for 4 weeks. In this study, animals that were not subjected to exercise were placed in shallow water at 37°C that allowed the animals to wade about freely without the need to exercise. An observer watched all animals during exercise to ensure the animals were continually exercising and not in distress. At the conclusion of the study, motor and sensory nerve conduction velocity and nerve blood flow were assessed in all animals.

4.2.2 Experimental design: NIRE study

The NIRE study was 12 weeks in duration. Animals were made diabetic by intraperitoneal injection of streptozotocin (50 mg/kg) and hyperglycemia was confirmed 2-3 days post STZ injection using blood taken by tail-prick and a strip-operated reflectance meter (see section 2.1).

To study the effects of NIRE on nerve function, rats used were assigned to one of six treatment groups: control untreated, control heat, control NIRE, diabetic untreated, diabetic heat, and diabetic NIRE. NIRE treatment was administered for 15 minutes per day, 6 days per week over the 12-week study period. Near infrared energy was delivered at 890 nm via diode array pads (Anodyne Inc., Tampa, FL). Each diode pad is composed of an array of 60
superluminous gallium aluminum arsenide diodes emitting 9 mW/cm$^2$. Individual rat treatment devices were composed of two diode array pads that were fitted at a 90° angle from each other within a short (10 inches) section of cylindrical plastic tubing. One diode pad functioned as the floor of the tube focusing NIRE on the plantar surface of the left hind paw, while the other pad was positioned as a sidewall for left hind flank NIRE exposure to target the mid-thigh sciatic nerve. For NIRE treatment, rats were placed within the plastic tubing fitted with NIRE diode pads (Figure 4.3A). Animals were shaved as needed to maintain a smooth skin surface for NIRE treatment and to minimize the possibility of NIRE being diffracted by fur. Because the NIRE treatment also produced heat, animals in the heat-treated groups were exposed to an equivalent amount of heat as produced by the NIRE treatment, but without NIRE.

Blood glucose levels and body weight were measured on a regular basis throughout the 12-week study period. Motor and sensory nerve conduction velocities were measured at the beginning of the study and at 4-week intervals thereafter. At the conclusion of the study, nerve blood flow and mean arterial blood pressure were measured. Sciatic nerve tissue was collected from the ipsilateral sciatic nerve once the animals were sacrificed and used for histologic analysis of axon caliber and blood vessel density and also for measurement of sciatic nerve VEGF protein content.

4.2.3 Experimental design: ultrasound (US) study

The US study was also 12 weeks in duration. Animals were made diabetic by intraperitoneal injection of streptozotocin (50 mg/kg) and
hyperglycemia was confirmed 2-3 days post STZ injection using blood taken by
tail-prick and a strip-operated reflectance meter (see section 2.1).

Rats used to study the effects ultrasound treatment on nerve function
were assigned to one of four groups: control placebo, control ultrasound, diabetic
placebo, and diabetic ultrasound. Ultrasound treatment was administered to the
mid-thigh sciatic region of the left hind flank for 20 minutes per day, 5 days per
week throughout the 12-week study. Ultrasound treatment was delivered using
an Exogen disc probe (Smith and Nephew, York, UK) at 30 mW/cm² with a
frequency of 1.5 MHz and pulse period of 200 µs.

Animals receiving ultrasound treatment or placebo treatment were placed
in cylindrical confinements similar to those used in the NIRE study (Figure 4.3B).
As with the NIRE study, the left hind limbs of the rats were shaved on a regular
basis to enable maximal penetration and efficacy of the low-intensity ultrasound
treatment. Contact gel was also applied to flank skin at the site of ultrasound
treatment to facilitate penetrance of the low-intensity ultrasound into the tissue.

Blood glucose and body weight were monitored weekly during the course
of the study. Motor and sensory nerve conduction velocity measurements were
made at the onset of the study and then every 4 weeks until the animals were
sacrificed at week 12. Mean arterial pressure, biceps femoris muscle blood flow
and sciatic nerve blood flow were measured at the conclusion of the study. Left
sciatic nerve tissue (treated side) was collected to assess VEGF protein content.
4.2.4 Nerve conduction velocity measurement

Motor and sensory nerve conduction velocities were measured in the sciatic nerve of the left hind leg in the exercise, NIRE and US studies (see section 2.2.3). In both the NIRE and US studies, nerve conduction velocity measurements were also taken on the contralateral (right) side of treatment to determine if any effects of NIRE or ultrasound treatment were local or systemic.

4.2.5 Nerve blood flow

Laser Doppler flowmetry was used to assess nerve blood flow. The right femoral artery was cannulated to monitor arterial blood pressure and laser Doppler flow was measured in the mid-sciatic nerve on the left hind limb (see section 2.2.4 and 2.2.6).

4.2.6 Muscle blood flow

Laser Doppler flowmetry was also used to measure muscle blood flow in the US study. Muscle blood flow was measured in the biceps femoris muscle, which lies directly over the sciatic nerve in the rat and underlies the skin at the site of ultrasound treatment. Muscle blood flow was assessed immediately after nerve blood flow assessment (see section 2.2.7).

4.2.7 Endoneurial blood vessel density assessment

Sciatic nerves were removed from animals at the conclusion of the study and processed for histologic analysis. Endoneurial blood vessels were counted using a light microscope at 400x magnification and the endoneurial area was measured in each sciatic nerve (see section 2.3.7).

4.2.8 Soleus muscle dissection
Soleus muscle was completely dissected from the left hind leg of animals in the exercise I study. The muscle tissue was then immediately weighed (wet weight) on a Sartorius analytical scale (Model A 100 S).

4.2.9 Sciatic nerve axon caliber assessment

Sciatic nerves cut were cut into 1-µm sections, stained with paraphenylenediamine and analyzed for quantitative morphometric study by light microscopy. Using the NIH Image program 1.55, axonal areas and perimeters were measured for each nerve section (see section 2.3.5).

4.2.10 Sciatic nerve VEGF protein content

Each sciatic nerve sample was placed in 200 µl of lysis buffer [20 mmol/L imidazole HCl, 10 mmol/L KCl, 1 mmol/L MgCl2, 10 mmol/L EGTA, 1% Triton, 10 mmol/L NaF, 1 mmol/L Na molybdate, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), pH 6.8] supplemented with a protease inhibitor cocktail, followed by sonication. The lysate was cleared of debris by centrifugation at 10,000 x g for 15 minutes (4°C), and the supernatant was assayed. Total protein was determined using the BCA kit (Bio-Rad, Hercules, CA). VEGF levels in sciatic nerve supernatants were determined using sandwich ELISA according to the manufacturers instructions (R&D Systems) and normalized to total protein. The minimum detectable levels for VEGF with this assay are 5 pg/ml.
4.3 Results

4.3.1 Effects of exercise training on indices of experimental diabetic neuropathy in STZ-diabetic rats

In exercise studies I and II, STZ-diabetic rats became severely hyperglycemic (blood glucose > 300 mg/dL; Table 4.1 and Table 4.3). Diabetic animals in both studies displayed significant (p<0.01) weight loss when compared to non-diabetic animals (Table 4.1 and Table 4.3). Weight loss was monitored closely in these animals to ensure the animals did not become cachexic or suffer from severe muscle wasting. Due to the relatively short duration of these studies, it was not necessary to provide any exogenous insulin to diabetic animals. Soleus muscle weight, measured in exercise study I was significantly (p<0.001) lower in diabetic animals compared to non-diabetic animals and was not effected by exercise (Table 4.1). Muscle weight was not measured in exercise study II.

Nerve conduction velocity measurements made for exercise study I showed no significant effects of diabetes or exercise on MNCV (Table 4.2). Despite the lack of statistical significance, there was a modest trend toward slowing of MNCV in diabetic animals compared to controls in exercise study I. There was a significant reduction in SNCV in diabetic animals compared to controls in exercise study I (p<0.05; Table 4.2). Exercise did not affect NCV of control animals in exercise study I. However, there was a trend towards protection of SNCV slowing in diabetic animals exposed to exercise. In exercise study II, diabetic animals exercised in 30° water had a reduction (p<0.05) in
MNCV compared to control animals also exercised in 30° water (Table 4.4). As with exercise study I study, animals in exercise study II showed a trend toward slowing of MNCV and SNCV in diabetic animals compared to non-diabetic controls. MNCV measurements in the 37°C exercise groups were not different between control and diabetic animals, which appeared to result from a slowing in the control group. There was no apparent effect of exercise on SNCV in exercise study II.

Sciatic nerve vascular conductance, laser Doppler flow normalized to arterial blood pressure, was measured in exercise study I. Although there was no significant difference in nerve vascular conductance amongst control animals with or without exercise, diabetic no exercise animals had markedly reduced vascular conductance compared to both control groups and diabetic animals exposed to exercise (Figure 4.1A). There was a trend towards a hypotensive effect of STZ-diabetes in exercise study I, but there were no significant differences in blood pressure between any treatment groups (Table 4.2), suggesting the reduction in conductance in the diabetic no exercise group was a result of reduced flow. There were no significant differences in endoneurial blood vessel density between any groups in exercise study I (Figure 4.1B).

Sciatic nerve vascular conductance was also measured in exercise study II. Because exercise study II utilized 6 treatment groups, unpaired t-tests were used to compare control and diabetic groups within their respective exercise treatment group. There was a significant decrease in vascular conductance in the sciatic nerve in diabetic animals compared to controls in both the no exercise
and the 30° exercise groups (p<0.05; Figure 4.2). There was no difference in vascular conductance between control 37° exercise and diabetic 37° exercise groups (Figure 4.2). Arterial blood pressure was reduced in all diabetic groups compared to control groups (p<0.05; Table 4.4).

4.3.2 Effects of near infrared energy on indices of experimental diabetic neuropathy in STZ-diabetic rats

STZ-injected rats in the NIRE study were hyperglycemic (blood glucose > 300 mg/dL; Table 4.5). Hyperglycemia was accompanied by a significant loss of body weight in diabetic animals (p<0.05; Table 4.5). Due to the length of the study, rat weights were followed very closely and any animals that lost more than 20% of their initial body weight or became cachexic were supplemented with exogenous insulin to allow for a low level of glucose utilization. The insulin supplementation did not correct hyperglycemia in these animals (see section 2.1).

There were no significant differences in mean axon caliber of the sciatic nerve between any of the experimental groups in the NIRE study. There was a subtle trend toward a diminution of axon caliber in the diabetic groups compared to the control groups but this change was not affected by treatment (Table 4.5). VEGF protein content was measured and heat or NIRE treatment did not appear to alter sciatic nerve VEGF protein content in the sciatic nerve of control or diabetic rats. There was a marked decrease in the VEGF protein content of the diabetic untreated group, but this finding was based on only one measurement and thus may not be representative of the group as a whole (Table 4.5).
Nerve conduction velocity measurements in the NIRE study demonstrated a consistent trend of nerve conduction slowing in diabetic groups compared to control groups. The impairment of nerve conduction velocity was apparent in both the left and right sciatic nerve and there was no clear effect of either heat or NIRE treatment on conduction velocities in control or diabetic animals (Table 4.6). Vascular conductance in the left (treated) sciatic was significantly reduced in untreated diabetic animals compared to all control groups (p<0.05). Both heat and NIRE treatment increased nerve vascular conductance in the diabetic groups to the extent that they no longer had vascular conductance values significantly different from control animals (Figure 4.4A). There was no significant difference in endoneurial blood vessel density between control and diabetic animals or between any of the treatment groups in the NIRE study (Figure 4.4B).

4.3.3 Effects of ultrasound on indices of experimental diabetic neuropathy in STZ-diabetic rats

As in the NIRE study, rats in the US study became significantly hyperglycemic within 2-3 days following STZ injection (p<0.01; Table 4.7). Diabetic animals had significantly reduced body weight at the end of the study and ultrasound treatment did not affect body weight (p<0.001; Table 4.7). Anesthetized blood pressure was reduced in diabetic animals compared to control animals, with placebo treated diabetic animals having the lowest mean arterial blood pressure as a group (Table 4.7). There was no difference in sciatic nerve VEGF protein content between placebo and ultrasound treated control animals or between placebo-treated diabetic animals and either control group.
However, ultrasound treated diabetic animals had a markedly increased VEGF protein content in the sciatic nerve compared to all other groups (p<0.001; Table 4.7).

Motor conduction velocity in the left (treated) sciatic nerve was significantly reduced in placebo treated diabetic animals compared to both control groups (p<0.05; Table 4.8). Ultrasound treatment in diabetic animals led to a partial protection of MNCV. Sensory conduction velocity in the left sciatic nerve was significantly reduced in both diabetic groups compared to both control groups, and ultrasound treatment did not improve sensory conduction velocity in the treated sciatic nerve (p<0.05; Table 4.8). Nerve conduction velocity measurements in the right (untreated) side demonstrated a trend of slowing of both motor and sensory conduction velocity in the sciatic nerve in the diabetic groups compared to the control animals, which was not affected by treatment (Table 4.8).

There were no significant differences in muscle vascular conductance, measured in the biceps femoris, between any of the experimental groups. There was a slight trend towards a reduction in muscle vascular conductance in the untreated diabetic animals, but this result was not statistically significant. Ultrasound treatment in diabetic animals led a modest increase in muscle vascular conductance compared to untreated diabetic animals, but again this was not a statistically significant difference (Figure 4.5A).

There was a trend towards a reduction in sciatic nerve vascular conductance in diabetic animals compared to control animals in the US study. In
control animals, ultrasound treatment led to an increase in nerve vascular conductance. Ultrasound treatment also led to a slight increase in nerve vascular conductance in the diabetic animals (Figure 4.5B). Endoneurial blood vessel densities were similar in all groups in the ultrasound study.

4.4 Discussion

This chapter describes the use of three distinct non-pharmacologic approaches in an attempt to modulate peripheral nerve blood flow and improve nerve function in a model of experimental diabetic neuropathy. As nerve ischemia has been implicated as potential causative factor in the development and progression of diabetic neuropathy, improving peripheral nerve blood flow in diabetic patients is of interest. Because pharmacologic tools, such as vasodilatory agents, have systemic actions with potential harmful side effects, it is important to develop methods to increase blood flow locally in the peripheral nerve. The exercise, NIRE and ultrasound treatments applied in this work represent an attempt to increase blood flow to the peripheral nerve without eliciting deleterious side effects associated with systemic drugs.

4.4.1 Swimming exercise

The effect of swimming exercise on the development of diabetic neuropathy was assessed. Physiologic testing of rats with four weeks of diabetes exposed a number of complications consistent with experimental diabetes and experimental diabetic neuropathy. Compared to controls, diabetic animals in these studies had high blood glucose, reduced body weight, nerve conduction slowing and reduced nerve blood flow.
There were several differences between exercise studies I and II. In exercise study I, control no exercise and diabetic no exercise animals were not exposed to water during the experiment. This design did not address the possible effect of water stress on animals in the exercise groups compared to the no exercise groups. In exercise study II, this concern was addressed by placing the no exercise animals in shallow water at body temperature for the same duration as animals performing swimming exercise. There was no apparent difference observed between control no exercise animals in exercise studies I and II, suggesting that any stress from being in water did not impact nerve function. Another difference between the studies was that in exercise study I, animals were only exercised in 37°C water. This temperature was chosen based on previous work that had shown improvement in peripheral neuropathic pain in rodents that swam for 90 minutes per day for 9 days in 37°C water (Kuphal et al., 2007). However, potential harmful effects of hyperthermia induced by exercising in water at 37°C have been reported (Mottola et al., 1993). To control for the possibility of heat stress, animals in exercise study II swam in water at either 30°C or 37°C. There was no clear indication from the experiments performed in exercise study II that nerve function or the general health of the animals was affected by the difference in water temperature in which the animals exercised.

Exercise treatment resulted in modest protection in nerve function in diabetic animals. In exercise study I, there was a modest improvement in SNCV in diabetic rats exposed to exercise, and nerve blood flow was almost completely corrected by exercise in this group. In exercise study II, there was no deficit in
MNCV observed in diabetic animals exercised at 37°C, suggesting a possible benefit to nerve function of swimming exercise at this temperature. Also, a reduction in nerve vascular conductance was observed in diabetic no exercise and diabetic 30°C exercise compared to their respective control groups in exercise study II, but diabetic rats that exercised in 37°C water did not differ significantly in nerve conductance compared to controls. This finding was consistent with the trend observed in exercise I study and suggests that swimming exercise prevents a reduction in nerve blood flow in diabetic animals exercised at 37°C.

There is an alternate interpretation of the putative improvement in MNCV in diabetic animals exercised at 37°C in exercise study II. As opposed to a protection of MNCV in diabetic animals exercised at 37°C, it could be argued that there was a slowing of MNCV in controls, accounting for the lack of difference in MNCV between control and diabetic animals. Also, the finding in exercise study II that nerve vascular conductance was not different between control and diabetic animals exercised at 37°C could be interpreted as a reduction in nerve blood flow in the control animals as opposed to an improvement in nerve blood flow in diabetic animals. Although nerve blood flow does increase modestly in the diabetic 37°C exercise animals compared to the no exercise and 30°C exercise diabetic animals, there is a decrease in nerve blood flow of similar magnitude in the control 37°C exercise compared to the other two control groups.
The limited ability of the peripheral nerve to autoregulate its own endoneurial blood supply (Smith et al., 1977) makes it dependant on blood flow to the local vasculature and to the metabolic demands of surrounding tissues, such as muscle (Dines et al., 1999). If skeletal muscle diverts blood away from the nerve in response to exercise, the nerve will receive a diminished blood supply. Muscle warming, as can occur during exercise, reduces nerve blood flow in non-diabetic rats by as much as 40% and is associated with a concomitant increase in muscle blood flow of nearly 50% (Dines et al., 1999). In healthy human subjects, muscle blood flow increases in response to exercise (Marsh and Ellerby, 2006). In diabetic patients, muscle blood flow is increased after exercise, but not to the extent that it increases in non-diabetic subjects (Menon et al., 1992). In control animals, decreases in nerve blood flow associated with increased muscle blood flow are even more substantial than in diabetic animals, likely because diabetic animals have less muscle mass due to the muscle wasting that occurs (Dines et al., 1997, 1999). The decrease in nerve blood flow seen in control animals in exercise study II exercised at 37°C could therefore be explained by an increase in muscle blood flow caused by the relatively high water temperature and additional muscle warming as a result of exercise.

In exercise study I, there was no increase in endoneurial blood vessel density in either control or diabetic animals subjected to exercise. Although there was no observed increase in nerve vascularization in response to exercise, there was also no deficit in nerve blood vessel density in diabetic animals compared to controls. Therefore, decreased nerve blood flow observed in diabetic animals in
this study was not a function of decreased vascularization of the nerves. In addition, the normalization of nerve blood flow observed in diabetic exercise animals in exercise I was not due to an increase in blood vessel density in the endoneurium. A study investigating the effects of VEGF on STZ and alloxan-induced diabetes found significantly decreased nerve blood flow and nerve blood vessel density in diabetic animals compared to controls. VEGF gene transfer to the sciatic nerve corrected both nerve blood flow and capillary density in diabetic animals (Schratzberger et al., 2001). Though other reports also suggest beneficial effects of VEGF on nerve function in models of diabetes (Chattopadhyay et al., 2005; Price et al., 2006), none have suggested reduced blood vessel density as a cause for reduced blood flow. It is currently unclear why there is a discrepancy in these findings.

The partial protective effects of exercise on nerve vascular conductance seen in exercise studies I and II could have been mediated through VEGF. Short-term exercise can lead to an increase in VEGF expression in the absence of hyperglycemia (Gustafsson et al., 1999). Thus, VEGF could be produced as a direct function of exercise or nerve ischemia generated by exercise and could benefit the nerve through angiogenesis or direct neurotrophic support. The lack of effect of swimming exercise on endoneurial blood vessel density in exercise study I also implies the role of VEGF, if any, in nerve vascular conductance improvement was neurotrophic in nature.

Although some effects of exercise on preventing indices of diabetic nerve injury were observed in this study, the effects were relatively subtle. At present,
the mechanism of action of exercise-induced protection against reduced nerve blood flow in experimental diabetes is not known. Despite controlling water depth and constantly monitoring the behavior of the animals, the rats were still able to avoid constant swimming by floating at the surface or allowing themselves to sink and then pushing off the bottom of the tank in order to get a breath at the surface. Animals also made attempts to clutch at the side of the container in order to avoid exercise. It is possible that if the animals had been forced to swim more consistently the effects of exercise on the progression of diabetic neuropathy would have been more robust.

Despite the lack of a clear mechanistic understanding, recent clinical studies have reported beneficial effects of exercise on the development and progression of diabetic neuropathy in type II diabetic patients (Balducci et al., 2006; Fisher et al., 2007). Additionally, swimming may be the most desirable mode of exercise in patients, due to the decreased risk of plantar foot injury compared to running or walking (Kanade et al., 2006). These findings highlight the importance of continued research into how modulation of blood flow and vascular factors through exercise impacts the development of diabetic neuropathy.

4.4.2 NIRE treatment

The efficacy of NIRE and ultrasound as treatments for nerve injury associated with diabetes were also assessed in this chapter. In both the NIRE and US studies, diabetic rodents developed severe hyperglycemia, significant
weight loss and nerve conduction velocity slowing. Neither NIRE or ultrasound treatment reversed any of these physiologic changes induced by STZ-diabetes.

In the NIRE study, the most encouraging finding was the partial protection of nerve vascular conductance in the diabetic animals treated with either heat or NIRE. In addition to emission of near infrared light, NIRE emitting diode arrays also produce heat. For this reason, a group of control and diabetic animals both treated with diode arrays that produced an equivalent amount of heat as the NIRE pads, but without the near infrared light, were used to determine if heat was responsible for any potential effects seen by the treatment. Based on the nerve vascular conductance data, it appears heat alone is sufficient to improve nerve blood flow and the addition of near infrared energy does not further increase nerve blood flow.

The partial protection of nerve vascular conductance observed in diabetic animals treated with either heat or NIRE could plausibly be explained by changes in VEGF expression in the nerve. As described above, heating of muscle adjacent to the peripheral can lead to an increase in muscle blood flow with a concurrent decrease in nerve blood flow. Both heat and NIRE treatment in the NIRE study could lead to a temporary drop in nerve blood flow as a result of muscle warming. In normal, healthy animals, a transient drop in nerve blood flow would not be considered harmful, as peripheral nerves are capable of losing up to 50% of their blood supply before suffering from ischemia (Conn and Dyck, 1975). However, when nerve blood flow is already compromised, as is the case
with diabetic rodents, further decreases in nerve blood flow can cause nerve hypoxia and lead to nerve injury (Nukada, 1992).

Ischemic conditions in the peripheral nerve lead to induction of the transcription factor HIF-1α and subsequent upregulation of VEGF protein (Liu and Simon, 2004). Heat applied to the hind limb of diabetic animals used in the NIRE study, either with heat alone or heat and NIRE, may direct enough blood away from the nerve to induce nerve ischemia and drive HIF-1α and VEGF expression. Increased VEGF protein in the sciatic nerve could then lead to angiogenesis. In addition to vascular actions such as angiogenesis, VEGF is also known to have a direct neurotrophic action. VEGF has been shown to play a role in neurogenesis, neuroprotection and axonal outgrowth (Raab and Plate, 2007). VEGF protein levels measured in the sciatic nerve of animals in the NIRE study suggests that diabetic animals treated with either heat or NIRE have markedly increased VEGF content compared to untreated diabetic animals. However, the data for untreated diabetic animals consists of only one measurement and VEGF levels in treated diabetic animals does not exceed that of control animals. The lack of increase in endoneurial blood vessel density in the heat or NIRE treated diabetic animals, suggests that any VEGF mediated benefit to nerve vascular conductance in the NIRE study may be due to neurotrophic actions of VEGF as opposed to an angiogenic effect.

4.4.3 Ultrasound treatment

Although there was a modest trend towards improvement, treatment of diabetic animals with ultrasound therapy did not result in a significant increase in
nerve vascular conductance compared to untreated diabetic animals. However, there was a dramatic increase in VEGF protein content in the sciatic nerve of diabetic animals treated with ultrasound in the US study compared to all other groups. It is possible that chronic effects of 12 weeks of ultrasound treatment were sufficient to induce a hypoxic state in the sciatic nerve and the subsequent upregulation of VEGF. Angiogenesis associated with VEGF upregulation may have only been in the early stages at the time nerve blood flow was measured in this study and thus was not yet sufficiently developed to significantly improve nerve blood flow. This possibility is supported by the observation that there were no significant differences in endoneurial blood vessel density between control and diabetic animals or animals treated with ultrasound. VEGF protein content in the US study was markedly higher than that measured in the NIRE study. It is unclear what accounts for the dramatic difference in VEGF protein in nerves between these two studies.

4.4.4 Conclusions

The NIRE and US studies have produced some promising results. However, neither study conclusively demonstrates a benefit to hyperglycemia-induced peripheral nerve injury. A number of reports in the literature have also found NIRE therapy to be either ineffective or only marginally beneficial to peripheral nerve function (Walsh et al., 2000; Zinman et al., 2004). There is considerable discrepancy, both in study duration and in mode, intensity and wavelength of treatment, in the protocols used in both NIRE and ultrasound
studies in the literature and this could be one reason for the conflicting reports on the efficacy of this type of approach to treat nerve injury.

At this point it is not clear that exercise, NIRE or US treatment will prove to be a useful tool in the prevention and management of diabetic neuropathy. Observed increases in nerve blood flow in the exercise and NIRE study and increased VEGF protein content in the sciatic nerve of treated diabetic animals in the US study are interesting findings and suggest some potential for these therapies to benefit the diabetic peripheral nerve. Since both NIRE and ultrasound devices are currently FDA-approved and are considered safe and non-invasive, it is worth considering these devices as possible therapeutic tools.

In addition, it is difficult to argue against the positive health benefits of regular exercise. However, more study will be required to further elucidate the physiologic actions of exercise as well as NIRE and ultrasound treatment, and their effects on the peripheral nerve so that these treatments will have a better chance to prevent, halt, or reverse diabetes-induced nerve injury.

Several follow up studies could be considered to gain a better understanding of the results reported in this chapter. Swimming, as a mode of exercise, was too inconsistent between animals. Future exercise studies should be conducted using a mode of exercise the can be more standardized. Treadmill running may provide a good alternative to swimming exercise. The general hypothesis in each of the experiments described in this chapter was that by increasing muscle blood flow, nerve blood flow might become compromised, ultimately leading to the induction of new endoneurial vessel formation, as a
result of nerve ischemia. However, nerve blood flow was only measured at the conclusion of each experiment, and at least 24 hours after the last treatment. Acute muscle and nerve blood flow measurements should be acquired in future exercise, NIRE and ultrasound studies to validate that these treatments are capable of affecting muscle and nerve blood flow. VEGF protein content in sciatic nerve and muscle would also be of interest in exercise animals to determine if exercise mediates an angiogenic response in the peripheral nerve. Again, this experiment could provide support to the hypothesis that exercise leads to angiogenesis in the nerve by directing blood flow away from the sciatic nerve, and into the surrounding musculature.
Table 4.1

Blood glucose and body and soleus muscle weight measurements of rats used in exercise study I.

<table>
<thead>
<tr>
<th>Group (n= 4-8)</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
<th>Muscle weight (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control no exercise</td>
<td>116 ± 8\textsuperscript{a}</td>
<td>263 ± 5\textsuperscript{a}</td>
<td>104 ± 3\textsuperscript{a}</td>
</tr>
<tr>
<td>Control exercise</td>
<td>127 ± 9\textsuperscript{a}</td>
<td>258 ± 6\textsuperscript{a}</td>
<td>100 ± 1\textsuperscript{a}</td>
</tr>
<tr>
<td>Diabetic no exercise</td>
<td>559 ± 22</td>
<td>216 ± 13</td>
<td>74 ± 4</td>
</tr>
<tr>
<td>Diabetic exercise</td>
<td>536 ± 43</td>
<td>199 ± 8</td>
<td>67 ± 5</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. \textsuperscript{a}, p<0.01 versus both diabetic no exercise and diabetic exercise.
Table 4.2

Physiologic measurements of rats used in exercise study I.

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP (mmHg)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control no exercise</td>
<td>94 ± 4</td>
<td>57.3 ± 1.3</td>
<td>58.9 ± 2.0a</td>
</tr>
<tr>
<td>Control exercise</td>
<td>92 ± 3</td>
<td>59.1 ± 2.0</td>
<td>60.1 ± 3.2a</td>
</tr>
<tr>
<td>Diabetic no exercise</td>
<td>88 ± 4</td>
<td>55.3 ± 1.8</td>
<td>46.8 ± 0.9</td>
</tr>
<tr>
<td>Diabetic exercise</td>
<td>77 ± 10</td>
<td>55.2 ± 0.8</td>
<td>50.9 ± 1.8</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.05 versus both diabetic no exercise and diabetic exercise. MAP, mean arterial pressure; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity.
Table 4.3

Blood glucose and body weight measurements of rats used in exercise study II.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control no exercise</td>
<td>121 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>267 ± 5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic no exercise</td>
<td>573 ± 9</td>
<td>223 ± 9</td>
</tr>
<tr>
<td>Control 30° exercise</td>
<td>125 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>274 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic 30° exercise</td>
<td>553 ± 19</td>
<td>216 ± 9</td>
</tr>
<tr>
<td>Control 37° exercise</td>
<td>123 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>278 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Diabetic 37° exercise</td>
<td>577 ± 19</td>
<td>222 ± 7</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by unpaired t-test comparing control and diabetic groups within the same treatment regime. a, p<0.001; b, p<0.01.
Table 4.4

Physiologic measurements of rats used in exercise study II.

<table>
<thead>
<tr>
<th>Group</th>
<th>MAP (mmHg)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control no exercise</td>
<td>89 ± 3a</td>
<td>61.3 ± 2.4</td>
<td>56.4 ± 1.0</td>
</tr>
<tr>
<td>Diabetic no exercise</td>
<td>76 ± 5</td>
<td>57.7 ± 1.7</td>
<td>53.4 ± 1.1</td>
</tr>
<tr>
<td>Control 30° exercise</td>
<td>92 ± 3a</td>
<td>62.3 ± 1.4a</td>
<td>57.5 ± 2.8</td>
</tr>
<tr>
<td>Diabetic 30° exercise</td>
<td>79 ± 4</td>
<td>57.7 ± 1.6</td>
<td>51.3 ± 1.7</td>
</tr>
<tr>
<td>Control 37° exercise</td>
<td>92 ± 3a</td>
<td>57.4 ± 1.9</td>
<td>58.2 ± 1.4a</td>
</tr>
<tr>
<td>Diabetic 37° exercise</td>
<td>72 ± 5</td>
<td>56.5 ± 2.0</td>
<td>53.0 ± 1.8</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by unpaired t-test comparing control and diabetic groups within the same treatment regime. a, p<0.05. MAP, mean arterial pressure; MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity.
Table 4.5

Physiologic measurements of rats used to study the effects of near infrared light on nerve function.

<table>
<thead>
<tr>
<th>Group (n = 6-8)*</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
<th>Mean axon caliber (µm)</th>
<th>VEGF protein (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated</td>
<td>132 ± 24\textsuperscript{a}</td>
<td>268 ± 3\textsuperscript{b}</td>
<td>6.1 ± 0.1</td>
<td>12.8 ± 0.6 (n=2)</td>
</tr>
<tr>
<td>Control heat</td>
<td>136 ± 17\textsuperscript{a}</td>
<td>268 ± 6\textsuperscript{b}</td>
<td>6.2 ± 0.3</td>
<td>18.8 ± 4.3</td>
</tr>
<tr>
<td>Control NIRE</td>
<td>145 ± 10\textsuperscript{a}</td>
<td>256 ± 5\textsuperscript{b}</td>
<td>6.3 ± 0.1</td>
<td>15.5 ± 2.9</td>
</tr>
<tr>
<td>Diabetic untreated</td>
<td>389 ± 6</td>
<td>212 ± 18</td>
<td>5.9 ± 0.1</td>
<td>5.2 (n=1)</td>
</tr>
<tr>
<td>Diabetic heat</td>
<td>&gt; 400\textsuperscript{†}</td>
<td>209 ±8</td>
<td>5.7 ± 0.2</td>
<td>16.9 ± 3.6</td>
</tr>
<tr>
<td>Diabetic NIRE</td>
<td>382 ± 13</td>
<td>218 ± 13</td>
<td>5.8 ± 0.2</td>
<td>14.3 ± 3.7 (n=4)</td>
</tr>
</tbody>
</table>

Data are group mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. \textsuperscript{a}, p<0.001 versus all diabetic groups; \textsuperscript{b}, p<0.05 versus all diabetic groups. *, n= 6-8 unless otherwise noted. \textsuperscript{†}, blood glucose data for the diabetic heat group does not have a sem because all animals in this group exceeded the 400 mg/dL limit of the glucometer. VEGF, vascular endothelial growth factor; NIRE, near infrared energy.
Table 4.6

Sciatic nerve motor and sensory conduction velocity in the left (treated leg) and right (untreated leg) at 12 weeks of rats used to study the effects of near infrared light on nerve function.

<table>
<thead>
<tr>
<th>Group</th>
<th>MNCV (m/s) Left leg</th>
<th>SNCV (m/s) Left leg</th>
<th>MNCV (m/s) Right leg</th>
<th>SNCV (m/s) Right leg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control untreated</td>
<td>61.9 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>56.7 ± 1.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>64.2 ± 1.8</td>
<td>60.3 ± 1.9</td>
</tr>
<tr>
<td>Control heat</td>
<td>59.0 ± 2.6</td>
<td>55.0 ± 0.9</td>
<td>63.0 ± 1.9</td>
<td>63.1 ± 2.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control NIRE</td>
<td>62.6 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>57.8 ± 1.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>67.2 ± 1.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>60.4 ± 2.0</td>
</tr>
<tr>
<td>Diabetic untreated</td>
<td>53.4 ± 1.5</td>
<td>50.0 ± 1.9</td>
<td>60.4 ± 2.5</td>
<td>53.8 ± 1.4</td>
</tr>
<tr>
<td>Diabetic heat</td>
<td>52.6 ± 2.0</td>
<td>49.3 ± 2.5</td>
<td>55.5 ± 2.7</td>
<td>54.3 ± 2.5</td>
</tr>
<tr>
<td>Diabetic NIRE</td>
<td>55.5 ± 1.5</td>
<td>51.3 ± 1.6</td>
<td>60.5 ± 3.6</td>
<td>52.8 ± 2.0</td>
</tr>
</tbody>
</table>

Data are group mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.05 versus diabetic untreated and diabetic heat; b, p<0.05 versus diabetic heat; c, p<0.05 versus all 3 diabetic groups. MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity; NIRE, near infrared energy.
Table 4.7

Physiologic measurements of rats used to study the effects of ultrasound on nerve function.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
<th>MAP (mmHg)</th>
<th>VEGF protein (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control placebo</td>
<td>139 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>278 ± 7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>96 ± 2</td>
<td>104 ± 21</td>
</tr>
<tr>
<td>Control ultrasound</td>
<td>120 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>266 ± 4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86 ± 4</td>
<td>112 ± 18</td>
</tr>
<tr>
<td>Diabetic placebo</td>
<td>535 ± 19</td>
<td>226 ± 8</td>
<td>65 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>110 ± 7</td>
</tr>
<tr>
<td>Diabetic ultrasound</td>
<td>508 ± 16</td>
<td>242 ± 8</td>
<td>79 ± 3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>247 ± 32&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are group mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups.  

- a, p<0.01 versus both diabetic groups;  
- b, p<0.001 versus both diabetic groups;  
- c, p<0.001 versus both control groups;  
- d, p<0.01 versus control placebo and diabetic placebo;  
- e, p<0.001 versus all other groups.  

VEGF, vascular endothelial growth factor; MAP, mean arterial blood pressure.
Table 4.8

Sciatic nerve motor and sensory nerve conduction velocity in the left (treated leg) and right (untreated leg) at 12 weeks of rats used to study the effects of ultrasound on nerve function.

<table>
<thead>
<tr>
<th>Group</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Left leg</td>
<td>Right leg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control placebo</td>
<td>59.2 ± 3.5</td>
<td>62.9 ± 2.2</td>
<td>59.5 ± 2.9</td>
<td>63.5 ± 2.6</td>
</tr>
<tr>
<td>Control ultrasound</td>
<td>56.2 ± 2.5</td>
<td>65.4 ± 1.8</td>
<td>57.8 ± 3.0</td>
<td>63.7 ± 1.8</td>
</tr>
<tr>
<td>Diabetic placebo</td>
<td>46.9 ± 1.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55.8 ± 2.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.2 ± 2.4</td>
<td>56.4 ± 1.7</td>
</tr>
<tr>
<td>Diabetic ultrasound</td>
<td>51.4 ± 2.4</td>
<td>49.4 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>51.5 ± 2.0</td>
<td>57.8 ± 2.2</td>
</tr>
</tbody>
</table>

Data are group mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. <sup>a</sup>, p<0.05 versus both control groups; <sup>b</sup>, p<0.01 versus both control groups. MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity.
Figure 4.1 Vascular parameters measured at week 4 in the sciatic nerve of rats used in exercise study I. A. Vascular conductance in the sciatic nerve. B. Endoneurial blood vessels counted per unit fascicular area in the sciatic nerve. Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. n=4-8. AUF, arbitrary units of flow; MAP, mean arterial blood pressure.
Figure 4.2 Vascular conductance measured at week 4 in the sciatic nerve of rats used in exercise study II. Data are presented as mean ± sem and were analyzed by unpaired t-test comparing control and diabetic groups within the same treatment regime. *, p<0.05. n=7-10. AUF, arbitrary units of flow; MAP, mean arterial blood pressure.
Figure 4.3 A. Rats being treated with near infrared energy on the left hind flank and the plantar surface of the left hind paw. B. Rats being treated with low-intensity ultrasound on the left hind flank.
Figure 4.4 Vascular parameters measured at week 12 in the sciatic nerve of rats used in the NIRE study. A. Vascular conductance in the sciatic nerve. B. Endoneurial blood vessel density in the sciatic nerve. Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. *, p<0.05 versus all control groups. n=6-8. AUF, arbitrary units of flow; MAP, mean arterial blood pressure.
Figure 4.5 Vascular parameters measured at week 12 in the biceps femoris and sciatic nerve of rats used in the ultrasound study. A. Vascular conductance in the biceps femoris muscle. B. Vascular conductance in the sciatic nerve. C. Blood vessel density in the sciatic nerve. Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. *, p<0.05 versus both diabetic groups. n=6-10. AUF, arbitrary flow units; MAP, mean arterial blood pressure.
CHAPTER 5 - CONCURRENT HYPERTENSION EXAGGERATES
PERIPHERAL NEUROPATHY IN STZ-DIABETIC RATS

5.1 Introduction

5.1.1 Risk factors for the development of diabetic neuropathy

Diabetic neuropathy is one of the most common complications associated with diabetes, affecting more than 50% of patients with a 20-year history of diabetes (Pirart et al., 1978). Neuropathy leads to increased morbidity and mortality in diabetic subjects (Vinik et al., 2000). No treatments have proven safe and effective in preventing the development and progression of diabetic neuropathy (Zochodne, 2007). At the present time, the aldose reductase inhibitor (ARI) epalrestat is commercially available only in Japan. However, data from ARI clinical trials have not provided clear evidence of efficacy in treating diabetic neuropathy (Baba et al., 2006; Zochodne, 2007). The lack of treatment options to prevent, stabilize or reverse diabetic neuropathy makes it crucial to identify potential risk factors. It is well appreciated that the two most important risk factors for diabetic neuropathy are degree of glycemic control and duration of diabetes (DCCT, 1993). However, neuropathy can still develop in patients who practice extremely strict glycemic control (DCCT, 1995). A recent study sought to identify risk factors for distal symmetric neuropathy in over 1,100 patients with type I diabetes participating in the European Diabetes Prospective Complications Study (Tesfaye et al., 2005). After correcting for duration of diabetes and level of glycemic control, the incidence of neuropathy was found to be associated with several potentially modifiable risk factors, including: increased triglyceride levels,
body-mass index, smoking and hypertension. Certain potential risk factors are not directly associated with diabetes (smoking), but others, such as hypertension are. Several other clinical studies have found a positive correlation between hypertension and the incidence of diabetic neuropathy (Maser et al., 1989; Harris et al., 1993; Forrest et al., 1997; Malik, 2000). Even with a relatively short duration of diabetes (less than 10 years), hypertension exposes patients to increased risk of neuropathy (Jarmuzewska et al., 2007). When elevated, pulse pressure (pressure oscillation between systolic and diastolic blood pressure), has also been linked to an increased risk of developing neuropathy in diabetic patients (Jarmuzewska and Mangoni, 2005). Non-diabetic subjects with unmedicated hypertension also develop sensory abnormalities, highlighting the importance of hypertension in the etiology of neuropathy (Edwards et al., 2008).

5.1.2 Vascular effects of hypertension

Hypertension is found in roughly 30% of type I diabetic subjects, and in patients with type II diabetes the incidence of hypertension is as high as 80% (Landsberg and Molitch, 2004). More than 50 million people suffer from hypertension in the United States alone (Sun and Zhang, 2005). Hypertension, as defined by an elevation of systolic blood pressure above 140 mmHg and diastolic blood pressure above 90 mmHg, leads to both macro- and microangiopathy. In larger vessels, the vascular wall can become rigid and thickened as a result of hypertension, reducing the ability of the vessels to dampen the pulse pressure generated by ventricular contraction (Beevers and Robertson, 2007). In small blood vessels, hypertension leads to thickening of the
tunica media and tunica intima, resulting in narrowing of the vessel lumen. Hypertension can also impair nitric oxide and endothelium-derived hyperpolarizing-factor activity, as well as vascular permeability and inflammatory responses mediated by endothelial cells (Cines et al., 1998; Blann et al., 2000). In addition, hypertension increases oxidative stress, which further damages the vasculature by causing increased endothelial cell dysfunction, increased blood vessel contractility, and proliferation and death of vascular smooth muscle (Touyz, 2004).

5.1.3 Experimental models of hypertension

The etiology of essential hypertension is attributed to both genetic and environmental factors. However, how these factors interact and ultimately lead to the development of hypertension remains unclear (Sun and Zhang, 2005). Because hypertensions, along with associated cardiovascular diseases, are the leading causes of death in humans, it is critical to understand the biological causes and consequences of the disease. In order to study the etiology and effects of hypertension, a number of non-genetic and genetic experimental models of hypertension have been developed. Hypertension in non-genetic models can be induced by surgical intervention, or through pharmacologic, dietary or endocrine manipulation. Genetic models of hypertension are considered to be either genotype- or phenotype-driven. Genotype-driven models typically utilize mice and involve the genetic manipulation of one or a number of genes responsible for blood pressure regulation. Phenotype-driven models are generated through selective breeding of inbred strains that demonstrate a natural
physiologic variation in blood pressure (Lerman et al., 2005). Phenotype-driven models of hypertension include the Dahl salt-sensitive rat, the obese Zucker rat and the spontaneously hypertensive rat (SHR). The SHR rat, developed in Japan over 40 years ago, is the most common experimental model of hypertension (Okamoto and Aki, 1963; Sun and Zhang, 2005).

5.1.4 Hypertension in experimental diabetic neuropathy

There are relatively few reports examining the combined effects of hypertension and diabetes on the development of neuropathy. In the central nervous system of SHR rats, cortical neuron cell number and volume is decreased compared to normotensive rats and this effect was exacerbated by the combination of hypertension and STZ-diabetes (Mignini et al., 2004). Cerebral arteries in SHR rats display wall thickening and vessel narrowing compared to control rats, and the combination of STZ-diabetes and hypertension leads to more severe vascular pathology (Tomassoni et al., 2004a). In spontaneously hypertensive rats (SHR), hypertension causes increased wall thickness and decreased luminal area in sciatic nerve blood vessels (Sabbatini et al., 1996; Sabbatini et al., 2001). Diminution of axon caliber and myelin thickness and a reduction in neurofilament density have all been observed in the sciatic nerve of SHR rats (Tomassoni et al., 2004b). However, there are no known experimental studies examining the effects of concurrent hypertension and diabetes on the development and progression of diabetic peripheral neuropathy.
Both diabetes and hypertension are common diseases. Given that the coincidence of hypertension and diabetes is as high as 75%, that the occurrence of neuropathy in diabetic subjects is more than 50%, and the establishment of hypertension as a risk factor for the development of diabetic neuropathy, there is a need to understand how hypertension affects the progression of diabetic neuropathy. The etiology of diabetic neuropathy has been linked to microvascular alterations in the vasa nervorum, and it is plausible that the microangiopathy associated with hypertension could accelerate or worsen the progression of hyperglycemia-induced nerve injury. The studies presented in this chapter are designed to describe the effects of concurrent hypertension on the development of experimental diabetic neuropathy.

5.2 Methods

5.2.1 Experimental design - General

Three studies were conducted to examine the role of concurrent hypertension on the progression and development of STZ-diabetic nerve injury and were termed SHR studies I, II and III. The initial study, SHR I, was 2 months in duration. Findings from SHR study I led to the longer term, 6-month duration SHR study II. SHR study III was 1 month long and was conducted in order to develop a more complete time course of the effects of hypertension and experimental diabetes on peripheral nerve function and structure. In all three SHR studies, rats were made hyperglycemic by intraperitoneal injection of streptozotocin (55 mg/kg) and hyperglycemia was confirmed 2-3 days later using blood collected by tail-prick and a strip-operated reflectance meter (see section
2.1). The spontaneously hypertensive rat (SHR) was used as a model of hypertension in all three SHR studies. SHR rats develop hypertension spontaneously between 80-100 days of age. In an effort to synchronize the potential effects of hyperglycemia and hypertension-induced nerve injury, hyperglycemia was induced at approximately 100 days of age in all diabetic rats. Wister Kyoto (WKY) rats are the genetic background for SHR rats and therefore served as non-hypertensive controls. In order to assess the individual and combined effects of hyperglycemia and hypertension, the SHR studies were designed to include control (WKY), diabetic (WKY D), hypertensive (SHR) and hypertensive diabetic animals (SHR D).

5.2.2 Experimental design - SHR study I

Rats used in SHR study I were assigned to one of four groups: WKY, WKY D, SHR and SHR D. Animals were maintained for 2 months following the induction of hyperglycemia. Body weight and blood glucose were monitored weekly throughout the 8-week study period. Thermal paw withdrawal latency was measured at weeks 4 and 8, and motor and sensory nerve conduction velocities were measured at weeks 0, 4 and 8. At the conclusion of the study, animals were sacrificed, and sciatic, tibial and sural nerves were carefully dissected out of the animals in order to assess blood vessel density, axon caliber and frequency of thinly myelinated nerve fibers in the peripheral nerves. Plantar skin from the hind foot was also collected from the animals at the time of sacrifice in order to assess the density of intra-epidermal nerve fibers (IENFs) in foot skin tissue.
5.2.3 Experimental design - SHR II

The longer duration SHR study II was conducted to determine whether extended periods of combined hypertension and diabetes would exaggerate neuropathy detected at earlier time points. SHR study II consisted of 5 groups: WKY, WKY D, SHR, SHR D and SHR DA. SHR DA animals were hypertensive diabetic rats that were also treated daily with the angiotensin converting enzyme (ACE) inhibitor, captopril (Product # C4202, Sigma-Aldrich, St. Louis, MO) at a dose of 80 mg/kg in drinking water. As with the SHR study I, blood glucose and body weights were monitored weekly throughout the study. Thermal paw withdrawal latency was measured every four weeks after the induction of diabetes. Motor and sensory nerve conduction velocity was measured in animals in the SHR study II at week 0 and every 4 weeks thereafter. At the conclusion of the study, the femoral artery of the rats was cannulated to assess both anesthetized and conscious blood pressure, and nerve blood flow was measured. All animals were sacrificed by perfusion of 2.5% glutaraldehyde solution in 0.1 M phosphate buffer via cardiac puncture (see section 2.2.8). Sciatic, tibial and sural nerves were collected for histologic analysis of blood vessel density, the occurrence of thinly myelinated nerve fibers and morphometric parameters including mean axon caliber, myelin thickness, axon area, and myelin area. Plantar foot skin was also removed for PGP 9.5 staining to determine the density of intra-epidermal nerve fibers.
5.2.4 Experimental design - SHR study III

The purpose of this study was to determine if indices of neuropathy observed in the 2 and 6-month SHR studies could be detected as early as 1 month. Animals in SHR study III were divided in the two groups: SHR and SHR D. Blood glucose and body weights were monitored weekly, and motor and sensory nerve conduction velocities were measured at onset and at week 4. At the conclusion of the study, anesthetized and conscious blood pressures as well as nerve blood flow were measured. Following sacrifice, sciatic nerve was collected from the animals in SHR study III to determine blood vessel density as well as the frequency of thinly myelinated fibers in the sciatic nerve.

5.2.5 Nerve conduction velocity measurement

Motor and sensory nerve conduction was measured in the sciatic nerve of the left hind leg (see section 2.2.3).

5.2.6 Measurement of thermal response latency

The thermal paw withdrawal latency was measured every four weeks in the left and right hind paw of animals used in SHR studies I and II (see section 2.2.2).

5.2.7 PGP 9.5 immunostaining

Footpad skin collected from animals in SHR studies I and II was processed to paraffin, sectioned and stained with the pan-neuronal marker PGP 9.5 (see section 2.3.3).
5.2.8 Assessment of intra-epidermal nerve fiber density

PGP 9.5 stained footpad sections were analyzed for the density of intra-epidermal nerve fibers (see section 2.3.4).

5.2.9 Measurement of conscious blood pressure

The blood pressure in alert, conscious animals was measured in SHR studies II and III (see section 2.2.5).

5.2.10 Nerve blood flow

Laser Doppler flowmetry was used to assess nerve blood flow. The right femoral artery was cannulated to monitor arterial blood flow, and laser Doppler flow was measured in the mid-sciatic nerve on the left hind limb (see sections 2.2.4 and 2.2.6).

5.2.11 Endoneurial blood vessel density assessment

Sciatic nerves were removed from animals at the conclusion of each SHR study and processed for histologic analysis. Endoneurial blood vessels were counted using a light microscope at 400x magnification and the endoneurial area of each sciatic nerve measured (see section 2.3.7).

5.2.12 Peripheral nerve morphometry

One-micron sections from sciatic nerves were stained with paraphenylenediamine and analyzed for quantitative morphometric study by light microscopy. Using the NIH Image program 1.55, axonal areas and perimeters were measured for each nerve section in SHR study I. For SHR study II morphometric analyses were performed using HistoQuant, an image analysis program developed at UCSD (see section 2.3.5).
5.2.13 Pathologic assessment of myelinated nerve fibers

Sciatic, sural and tibial nerves were examined by light microscopy in SHR studies I, II and III to assess the presence of myelinated nerve fiber damage (see section 2.3.6).

5.3 Results

5.3.1 SHR study I - physiologic measurements

WKY D and SHR D rats in SHR study I became hyperglycemic within 2-3 days of STZ injection and remained so for the duration of the study (p<0.001; Table 5.1). Age-matched SHR rats had significantly lower body weight compared to WKY animals (p<0.05; Table 5.1). STZ-diabetes led to a reduction in body weight in WKY D compared to WKY rats (p<0.05). SHR D rats had significantly reduced body weight compared SHR and WKY D rats (p<0.05; Table 5.1).

STZ-diabetes led to a significant reduction in MNCV in WKY D compared to WKY rats (p<0.05). There was also a significant reduction in MNCV of SHR D versus SHR rats (p<0.05; Table 5.1). Although non-significant, there was a trend towards a slowing of SNCV in WKY D compared to WKY rats. Diabetes led to a significant reduction in SNCV in SHR D rats versus SHR rats (p<0.001) as well as WKY D animals (p<0.05; Table 5.1). Hypertension was without effect on either MNCV or SNCV (WKY versus SHR, p>0.05).

5.3.2 SHR study I - Epidermal innervation and thermal response latency

The density of IENFs in the hind paw plantar skin was assessed in SHR study I (see Figure 5.1A). There was a trend towards an increase in IENFs/mm
in WKY D compared to WKY rats (Figure 5.1B). SHR D animals had IENFs/mm values significantly lower than that of SHR and WKY D animals (p<0.05; Figure 5.1B). Thermal response latency was similar between WKY, WKY D and SHR D rats. SHR animals had significantly faster thermal paw withdrawal responses compared to WKY and SHR D rats in SHR I study (p<0.05; Figure 5.1B).

5.3.3 SHR study I - Vascular parameters

There was no significant difference in endoneurial blood vessel density between WKY and WKY D animals in SHR study I (Table 5.2). There was a trend towards a reduction in sciatic nerve blood vessel density in SHR compared to WKY rats, though this result was not statistically significant. SHR D rats had a significantly lower sciatic nerve blood vessel density compared to WKY D animals (p<0.05; Table 5.2). In the tibial nerve, blood vessel density was highest in the WKY D group. Although there were no significant differences in blood vessel density in the sural nerve in SHR study I, WKY and WKY D animals had higher sural nerve endoneurial blood vessel densities compared to SHR and SHR D animals (Table 5.2).

5.3.4 SHR study I - Morphometric measurements and assessment of peripheral nerve pathology

There were no significant differences in mean axon caliber between any groups in either the sciatic, tibial or sural nerve (Table 5.3). The density of thinly myelinated fibers was assessed in the sciatic, tibial and sural nerves in SHR study I (see Figures 5.2A,B). In the sciatic nerve, WKY and WKY D rats had few thinly myelinated nerve fibers/mm² (Table 5.4). There was a modest increase in
thinly myelinated fibers in SHR rats. SHR D animals had a significantly greater density of thinly myelinated fibers in the sciatic nerve compared to WKY D (p<0.01) and compared to SHR rats (p<0.05; Table 5.4). In the tibial nerve, there were no significant differences in the density of thinly myelinated fibers between the WKY and WKY D or between WKY and SHR rats, but SHR D animals had significantly more thinly myelinated fibers compared to both SHR and WKY D (p<0.05; Table 5.4). There were no significant differences in the presence of thinly myelinated fibers in the sural nerve; however, there was a trend towards an increase in thinly myelinated fiber density in both diabetic groups compared to the non-diabetic animals (Table 5.4).

5.3.5 SHR study II - Physiologic measurements

As in SHR study I, diabetic animals in SHR study II became severely hyperglycemic within 2-3 days following STZ injection and the hyperglycemia persisted until the conclusion of the study (p<0.05; Table 5.5). STZ-diabetes caused a reduction in body weight in all diabetic animals compared to non-diabetic animals (p<0.05; Table 5.5). SHR animals had a lower final body weight compared to WKY animals (p<0.05; Table 5.5). Both diabetic SHR groups had significantly lower body weight than non-diabetic SHR rats (p<0.05; Table 5.5). There was no effect of ACE inhibition on body weight in SHR D animals.

STZ-diabetes led to a significant slowing of both MNCV and SNCV in WKY D versus WKY and in SHR D versus SHR rats (p<0.05; Table 5.6). Hypertension also induced both MNCV and SNCV deficits in SHR compared to WKY rats (p<0.05; Table 5.6). Combined hypertension and diabetes resulted in
MNCV and SNCV slowing in SHR D compared to WKY D rats (p<0.05; Table 5.6). Treatment of SHR DA rats the ACE inhibitor captopril led to a significant improvement in MNCV, but not SNCV, compared to SHR D animals (p<0.05; Table 5.6).

SHR rats had significantly higher blood pressure both under anesthesia and when conscious compared to WKY rats (p<0.05; Table 5.6). Anesthetized and conscious SHR D animals had significantly higher blood pressure compared WKY D rats (p<0.05; Table 5.6). In conscious rats, STZ-diabetes induced hypotension in WKY D rats, but this effect was not observed in anesthetized WKY D animals (p<0.05 versus WKY; Table 5.6). Significantly lower blood pressure was measured in SHR D compared to SHR rats, either conscious or anesthetized (p<0.05; Table 5.6). Captopril treatment of SHR diabetic rats led to a reduction in blood pressure; however, this effect did not lead to blood pressures significantly lower than that measured in either conscious or anesthetized SHR D animals (Table 5.6).

5.3.6 SHR study II - Epidermal innervation and thermal response latency

IENF density in hind paw plantar foot skin was significantly increased in WKY D animals compared to WKY and SHR D animals (p<0.001; Figure 5.3A). IENF density was similar in WKY, SHR and SHR D animals. Captopril treatment of diabetic SHR DA animals resulted in a significant increase in IENF density compared to SHR D rats (p<0.001; Figure 5.3A). There were no significant differences in thermal paw withdrawal latency in SHR study II. There was a trend
towards faster paw withdrawal latencies in all 3 SHR groups compared to both WKY groups (Figure 5.3B).

5.3.7 SHR study II - Vascular parameters

Although not statistically significant, STZ-diabetes led to a reduction in laser Doppler sciatic nerve blood flow and nerve vascular conductance in the WKY D group compared to the WKY group and in the SHR D rats compared to SHR rats (Table 5.7). Hypertension produced a significant deficit in nerve vascular conductance (p<0.01, SHR versus WKY; Table 5.7). The combination of diabetes and hypertension also led to a significant reduction in nerve vascular conductance in SHR D versus WKY D animals (p<0.01; Table 5.7). There was small, but not statistically significant, increase in nerve blood flow and nerve vascular conductance in SHR DA compared to SHR D animals (Table 5.7).

Endoneurial blood vessel density in the sciatic nerve was significantly lower in WKY D compared to WKY animals (p<0.01; Table 5.8). Although there was a trend towards fewer endoneurial blood vessels in the SHR groups compared to WKY animals, there were no other significant differences in sciatic nerve blood vessel density. In the tibial nerve, blood vessel densities were comparable in all experimental groups (Table 5.8). There were also no significant differences in blood vessel densities between any study groups in the sural nerve (Table 5.8). WKY D animals did appear to have a greater density of endoneurial blood vessels in the sural nerve but this result was not statistically significant.
5.3.8 SHR study II - Morphometric measurements and assessment of peripheral nerve pathology

There were no statistical differences in mean axon caliber, myelin thickness or g-ratio between any experimental groups in any nerve measured in SHR study II (Table 5.9 and 5.10). There were also no significant differences in axonal area or myelin area in the sciatic and tibial nerves between any of the groups (Table 5.11).

There was limited occurrence of thinly myelinated nerve fibers in WKY and WKY D rats in the sciatic, tibial and sural nerves (Table 5.12). Hypertension led to a significant increase in thinly myelinated fibers in the sciatic and tibial nerve compared to WKY rats (p<0.01; Table 5.12). A trend was seen towards an increase in thinly myelinated fibers in SHR versus WKY rats in the sural nerve as well, though this result was not statistically significant. The combination of diabetes and hypertension in the SHR D rats led to an even greater density of thinly myelinated fibers in the sciatic and tibial nerves compared to either WKY D or SHR rats (p<0.001; Table 5.12). Captopril treatment resulted in a modest, non-significant reduction in the occurrence of thinly myelinated fiber in SHR DA versus SHR D rats in all three nerves studied (Table 5.12).

5.3.9 SHR study III - Physiologic measurements

SHR D rats had significantly higher blood glucose and significantly lower body weight compared to SHR rats at the conclusion of the study (p<0.001; Table 5.13). Motor nerve conduction velocity was significantly slower in SHR D rats (p<0.01; Table 5.14). SNCVs were similar between SHR and SHR D rats.
(Table 5.14). As observed in SHR study II, a hypotensive effect of STZ-diabetes was measured in SHR study III in both anesthetized rats (p<0.05) and in conscious rats (p<0.001; Table 5.14).

5.3.10 SHR study III - Vascular parameters

Laser Doppler nerve blood flow was significantly reduced in SHR D compared to SHR rats (p<0.001; Table 5.15). Although the trend towards a reduction in nerve perfusion was observed, when blood flow was normalized to blood pressure there was no statistical difference in sciatic nerve vascular conductance (Table 5.15). SHR D rats had a higher blood vessel density than SHR rats, though this difference was not statistically significant (Table 5.16).

5.3.11 SHR study III - Assessment of peripheral nerve pathology

There was no difference in the occurrence of thinly myelinated nerve fibers between SHR and SHR D rats in SHR study III (Table 5.16). Values obtained for density of thinly myelinated nerve fibers in the sciatic nerve of SHR rats in SHR study III were comparable to that measured in SHR studies I and II. SHR D animals had fewer thinly myelinated fibers at 1 month in SHR study III than observed at 2 and 6 months.

5.4 Discussion

Based on the prevalence of hypertension and its association with diabetes and risk for the development of diabetic neuropathy, it is important to understand how the combination of hypertension and diabetes affects peripheral nerves. A large body of literature exists on the effects of diabetes, both clinical and experimental, on pathologic changes in the peripheral nervous system (e.g.
Sugimura and Dyck, 1982; Llewelyn et al., 1991; Tamura and Parry, 1994; Wright and Nukada, 1994; Ochodnicka et al., 1995; Kalichman et al., 1998). Relatively few studies have been published examining the sensitivity of peripheral nerves to hypertension (Sabbatini et al., 1996, 2001; Fazan et al., 1999, 2001; Tomassoni et al., 2004b). At the present time, there are no known experimental studies in the scientific literature that have studied the combined effects of hypertension and diabetes on peripheral nerve structure and function. The work described in this chapter compares the effects of STZ-diabetes, hypertension, and the combination of diabetes and hypertension on the development of experimental neuropathy.

5.4.1 Importance of the endoneurial microvasculature

Diabetic neuropathy is caused by a combination of pathologic processes including both metabolic and microvascular changes. Although the precise role of microvascular disease in the pathogenesis of diabetic neuropathy remains unclear, the presence of vascular damage in the endoneurium of diabetic patients with little or no clinical evidence of neuropathy suggests a vascular origin of diabetic neuropathy (Giannini and Dyck, 1995; Malik et al., 2005). In human subjects, diabetic microvascular changes include capillary basement membrane thickening, endothelial cell hyperplasia and hypertrophy, and pericyte degeneration (Yasuda and Dyck, 1987; Giannini and Dyck, 1995; Malik et al., 1993, 2005). Blood vessel wall and basement membrane thickening can lead to hypoperfusion, decreased tissue oxygen and risk of endoneurial ischemia (Sabbatini et al., 2001). An important microvascular change that directly
influences nerve perfusion and delivery of oxygen to the endoneurial compartment is capillary density. In patients with frank diabetes, capillary density is unchanged, but an increase in endoneurial capillary density has been reported in patients with impaired glucose tolerance in the absence of overt diabetes, and a reduction in capillary density has been seen in nerves from patients with mild diabetes (Malik et al., 1992, 2005; Thrainsdottir et al., 2003). With the exception of the diabetic cat model, endoneurial blood vessels from diabetic animals do not show clear evidence of microvascular disease or changes in capillary density (Mizisin et al., 2008). There was no clear effect of experimental diabetes or hypertension on blood vessel density in the sciatic, tibial or sural nerves in the 2 or 6-month SHR studies. Measurements in the sciatic nerve from animals in SHR study III suggested an increase in blood vessel density in diabetic compared to non-diabetic SHR animals. This appears to parallel pre-diabetic patients with impaired insulin sensitivity. There was a modest proximal-distal increase in blood vessel density in SHR studies I and II. Blood vessel density data are difficult to interpret, given the inconsistent findings in the literature of increased or decreased endoneurial vessel density at different stages of the disease. Perhaps as important as the vessel density, is the condition of the vessels that are present within the endoneurium. A recent study examined a number of structural blood vessel parameters in diabetic cats, including capillary size, endothelial cell hypertrophy and number, pericyte hypertrophy and basement membrane thickness (Estrella et al., 2008). Future work related to the studies described in this chapter should measure these blood vessel parameters
to better understand the relationship between endoneurial vessel density and the ability of those vessels to deliver oxygen to the endoneurium.

5.4.2 Effects of streptozotocin and genetic hypertension on blood pressure

Of importance when considering the effects of hypertension on the development of experimental diabetic neuropathy is the effect of STZ-diabetes on blood pressure. Prior to 1987, it was generally thought that STZ-diabetes induced hypertension in rats (Kusaka et al., 1987). However, the early descriptions of STZ-hypertension were based on indirect measurements (i.e. tail-cuff) and did not take into consideration certain diabetes-induced changes, such as muscle wasting and collagen deposition in the tail, that could alter blood pressure measurements (Kusaka et al., 1987). Measurements from direct cannulation of internal arteries in STZ-diabetic rodents have since consistently demonstrated a hypotensive effect of STZ-diabetes (Yamamoto, 1988; Susic et al., 1990; Rebolledo et al., 2001). Although not entirely clear, it is thought the STZ-induced reduction in blood pressure is caused by an impaired contractile response in blood vessels to extracellular Ca$^{2+}$ (Rebolledo et al., 2001). Conscious blood pressure measurements in SHR study II showed a clear reduction in blood pressure in WKY D rats compared to WKY and in SHR D compared to SHR rats. Although blood pressure was not measured in SHR study I, SHR studies II and III both confirmed hypertension in the SHR rats. Treatment of diabetic SHR animals with captopril did result in a reduction in blood pressure in those animals. However, due to the hypotensive effect of STZ-diabetes, blood pressure was not significantly different between SHR D and SHR
DA animals. This likely explains the only partial protective effect of ACE inhibition on conduction velocities, nerve blood flow and frequency of thinly myelinated fibers in SHR DA rats in SHR study II. Despite the hypotensive effects of STZ in this study, blood pressure in SHR D rats was still higher than that observed in WKY or WKY D animals, suggesting a vascular component to the increased severity of nerve injury in SHR D rats.

5.4.3 Relationship between epidermal innervation and thermal response latency

Unmyelinated intra-epidermal nerve fibers (IENFs) in skin are the sensory structures responsible for transducing heat-pain sensitivity (Kennedy and Wendelschafer-Crabb, 1993). Immunohistochemical staining with the neuronal marker PGP 9.5 allows identification and quantification of IENFs in skin biopsies (Karanth et al., 1991). Clinical studies have shown a negative correlation between duration of diabetes and IENF density in skin biopsies (Pittenger et al., 2004; Shun et al., 2004) and a decrease in IENF density in skin correlates positively with an increase in heat-pain threshold in diabetic patients (Shun et al., 2004; Quattrini et al., 2007). In the work presented in this chapter, there is evidence that IENF density is associated with thermal paw withdrawal threshold in hypertensive rats. In SHR study I, SHR D rodents displayed thermal hypoalgesia compared to SHR rats and the reduction in thermal sensitivity in SHR D rats was associated with a reduction in IENFs. In WKY rats, diabetes did not alter thermal sensitivity. There was a trend towards an increase in IENFs in WKY D compared to WKY rats that was not associated with a change in thermal sensitivity. WKY D rats in SHR study II also had an increase in IENFs that was
not associated with a change in thermal response. The increase in IENFs in WKY D compared to WKY rats with no associated change in thermal response may result from altered receptor function or nerve sprouting in WKY D rats. Transduction of heat pain by IENFs is mediated by heat-sensitive TRPV1 receptors on the unmyelinated c-fibers in the epidermis (Puntambekar et al., 2005). Diabetes-induced thermal hypoalgesia in mice has been shown to associated with a decrease in TRPV1 expression in paw skin (Pabbidi et al., 2008). Free radical imbalance can also impair normal TRPV1 expression (Puntambekar et al., 2005). It would be of interest to investigate TRPV1 expression as well as GAP-43 (growth cone associated protein) in skin biopsies of rats in these studies to determine if expression of these proteins is associated with the observed changes in IENF density and thermal response.

The observed differences in IENF density and thermal withdrawal response between WKY and SHR animals could also be explained by strain differences. Though the WKY rat has widely been accepted as an appropriate control for SHR studies, there is some concern regarding the genetic differences between WKY and SHR rats (Louis and Howes, 1990; Sun and Zhang, 2005). However, vascular changes in the peripheral nerve of SHR rats compared to WKY rodents are sensitive to treatment with hydralazine (a vasodilator), suggesting the differences are blood pressure dependent and not genetic (Sabbatini et al., 2001).
5.4.4 Diabetes and hypertension lead to nerve blood flow and nerve conduction velocity deficits

Among the earliest measurable changes in experimental diabetes are reduced nerve blood flow and nerve conduction velocity (Eliason, 1964; Cameron et al., 1991). Nerve blood flow is reduced by as much as 41% following 1 week of experimental diabetes and tends to precede NCV deficits, leading to the speculation that vascular alterations are important in the development of conduction slowing in peripheral nerves (Cameron et al., 1991). Nerve vascular conductance was reduced in all 3 SHR groups compared to both WKY groups in SHR study II. Trends of reduced nerve blood flow and nerve vascular conductance were also observed in WKY D compared to WKY and in SHR D compared to SHR rats. In SHR study III, nerve blood flow was significantly lower in SHR D rats compared to SHR rats at the one-month time point. These data are in agreement with nerve blood previously reported in diabetic rats, and also suggest that the combination of STZ-diabetes and hypertension leads to a more severe vascular insult in this model.

Diabetes-induced NCV deficits were consistently observed in SHR studies I, II and III. In the 8-week SHR study I, STZ-diabetes led to a reduction in NCV, especially when in conjunction with hypertension. Both STZ-diabetes and hypertension resulted in NCV slowing in the 24-week SHR study II. The combination of hyperglycemia and hypertension led to even further NCV slowing in SHR study II. Animals with short-term diabetes and hypertension in SHR study III had significantly slower MNCV compared to animals with hypertension
alone. Taken together, these data confirm the often reported diabetes-induced reduced NCV slowing and the NCV slowing in SHR rats (Tomassoni et al., 2004b). In addition, it is apparent that concurrent hypertension exacerbates NCV slowing in diabetic rodents.

The mechanism of diabetes and hypertension-induced NCV slowing is not entirely clear. Axon caliber, fiber density, myelin thickness and internodal distance are structural components of the peripheral nerve that can affect NCV. There was no difference in mean axon caliber or myelin thickness between any experimental groups in the SHR studies. Although fiber density was not quantified in these studies, there was no indication of a reduction in fiber density. In the absence of overt nerve pathology, functional changes must be considered. Na\+-K\+ ATPase, Na\+ and K\+ channel dysregulation have previously been suggested as a possible mechanism for NCV deficits observed in diabetic rodents (Cherian and Sima, 1993; Greene et al., 1988). The physiologic effects of Na\+-K\+ ATPase changes on NCV deficits are likely unfounded. NCV deficits occur prior to Na\+-K\+ ATPase alterations. Also, Na\+-K\+ ATPase changes are not observed in mice, a species that exhibits diabetes-induced NCV slowing (Bianchi et al., 1987; Lambourne et al., 1988). However, endoneurial hypoxia could limit ATP supply to Na\+-K\+ ATPase in vivo, thus impacting normal physiologic nerve function and NCV. Indeed, the patterns of NCV deficits closely follow nerve blood flow deficits measured in SHR studies II and III. Neurofilaments represent another component of myelinated nerve fibers and are key in axonal function, including NCV (Hoffman and Lasek, 1975). Neurofilament
damage occurs in the sciatic nerve of SHR rats (Tomassoni et al., 2004b). It would be of interest to examine tissue samples from the SHR studies for neurofilament damage to assess the potential role of neurofilaments in the functional nerve changes, such as NCV slowing observed in this study. NFP-H is the primary neurofilament component of myelinated nerve fibers in the peripheral nervous system and is crucial in regulation of nerve function (Tomassoni et al., 2004b). Immunohistochemical studies examining the levels of NFP in nerve tissue from SHR studies could determine if neurofilament damage has occurred in this model. Neurofilament damage could also be assessed ultrastructurally with the use of the electron microscope.

5.4.5 Hypertension and hypertension concurrent with STZ-diabetes leads to Schwann cell injury in rat peripheral nerves

The STZ-diabetic rat is the most widely used experimental model to study the pathogenesis and progression of diabetic neuropathy. Despite the utility of the STZ-rat as a tool to study diabetic peripheral neuropathy, there are several aspects of the model that do not accurately recapitulate pathologic changes observed in human diabetes. Perhaps the most striking difference between human diabetic neuropathy and the STZ-diabetic rat is the absence of severe pathologic changes in the peripheral nerve of diabetic rats. Pathologic changes in human diabetic neuropathy include: segmental demyelination, axonal degeneration and ultimately dramatic fiber loss. Myelinated and unmyelinated fibers are affected in human diabetic neuropathy (Richardson and De Girolami, 1995). Diabetic rodents display some subtle morphometric changes, such as
reduction in axon caliber but the dramatic structural damage observed in the peripheral nerves of diabetic patients is not recapitulated by STZ-diabetes (Ochodnicka et al., 1995; Kalichman et al., 1998; Walker et al., 1999). The short life span and shorter length of nerves in rodents may account for some of the differences in nerve pathology between humans and diabetic rats. In galactose-fed rodents, more severe structural changes occur in the peripheral nerve, primarily in Schwann cells (Mizisin and Powell, 1993; Mizisin et al., 1998). However, the galactose model does not accurately reflect other aspects of the diabetic condition. For example, galactose-fed rats have normal insulin production and generate excess polyols in nerve tissue (Dines et al., 1995b). An experimental model that has proven to be quite similar to human diabetic neuropathy is the feline diabetes model. Nerve pathology in feline diabetes is characterized by Schwann cell damage including: myelin splitting and ballooning, demyelination/remyelination, and reactive and degenerative changes (Mizisin et al., 1998, 2002, 2007). In addition, axonal pathology is observed in feline diabetes including: axonal degeneration, dystrophic accumulation of membranous debris and glycogen, and fiber loss (Mizisin et al., 2007). Although feline diabetes is an excellent model for studying the pathogenesis of diabetic neuropathy it is still of interest to develop rodent models with neuropathology that more closely resembles human diabetic neuropathy. In the studies described in this chapter, pathologic changes were observed in Schwann cells of peripheral nerves as well as in the dorsal roots. At 1, 2 and 6 months, the presence of disproportionately thinly myelinated nerve fibers was observed in the sciatic
nerves of rats with hypertension and with both STZ-diabetes and hypertension. At 2 and 6 months, but not at 1 month, the combination of STZ-diabetes and hypertension led to a significant increase in thinly myelinated fibers compared to rats with only hypertension. In the tibial nerve thinly myelinated fibers were not apparent by 2 months of hypertension or diabetes, but following at 6 months, hypertension and the combination of diabetes and hypertension induced Schwann cell injury as measured by thinly myelinated fibers. Disproportionately thin myelin is indicative of demyelination of axons, followed by an attempted reparative response of the Schwann cell to remyelinate (demyelination/remyelination; Richardson and Di Girolami, 1995). Myelin splitting and ballooning, thought to precede demyelination/remyelination was also observed in SHR and SHR D rats in this study (Figure 5.2C). The relative frequency of thinly myelinated fibers observed in this study compared to the overall population of fibers in rat sciatic nerve is relatively low (~ 20-40:5,000 fibers). However, this is the first known report indicating active demyelination in the sciatic and tibial nerve of STZ-diabetic rats. The relative frequency of thinly myelinated fibers did not increase significantly between 1, 2 or 6-month time-points suggestive of an ongoing degenerative and reparative process of Schwann cells in this model. This is supported by the presence of redundant layers of basal lamina observed in Schwann cells of thinly myelinated fibers (Figure 5.2B). The presence of redundant basal lamina as well as supernumerary Schwann cell processes are a hallmark of demyelination/remyelination in human diabetic neuropathy (Yagihashi and
Matsunaga, 1979). Teased fibers could be analyzed in order to determine if internodal distance was changed or if segmental demyelination occurred in nerves in the SHR studies. An attempt was made to analyze teased fibers from sciatic nerve in SHR study II; however, tissue-processing issues made it impossible to accurately assess the tissue.

5.4.6 Minimal evidence of changes in axon-myelin relationships

The structure of a nerve fiber in the sciatic nerve is generally considered to be that of a cylinder. However, a number of factors can lead to variations in axon and myelin thickness at different points along the length of fiber. These factors include: proximal-distal nerve fiber tapering, presence of Schwann cell nuclei, nodes of Ranvier, Schmidt-Lantermann’s clefts, random variations and pathologic changes (Sunderland and Roche, 1958). Based on these inherent variations in nerve fiber structure, microscopic morphometric analysis of any given nerve fiber may provide misleading information regarding the relative proportions of axon and myelin size. However, by sampling a relatively large number of fibers in a transverse section of an entire nerve, individual fiber variations can be accounted for and a good approximation of nerve fiber size parameters can be obtained. Previous morphometric studies have identified a number of measures that describe the overall axon-myelin relationship in a nerve. These parameters include: axon caliber (diameter), myelin thickness, g-ratio (axon diameter/fiber diameter), axon area, myelin area and axon area/myelin area (Sunderland and Roche, 1958).
G-ratio values can range between approximately 0.1 and 0.8 in nerves. It is generally accepted that g-ratio rises as nerve fiber caliber increases (Sunderland and Roche, 1958). In rats, g-ratio is reported as approximately 0.6 (da Silva et al., 2007). Rats in SHR study II all had a similar g-ratio, ranging from 0.63 - 0.65. There were no differences in g-ratio between treatment groups or between the sciatic, tibial and sural nerve. Alterations in axon caliber and myelin thickness can occur without affecting g-ratio. For example if a pathologic process led to a reduction of similar proportions in both axon caliber and myelin thickness in a nerve fiber, g-ratio would remain unchanged despite changes in nerve fiber dimensions. For this reason, myelin thickness becomes an important measure to detect changes in myelination. Previous work has reported axons with a diameter ranging between 3.3 and 11.5 µm have myelin thickness between 1.3 and 5.7 µm (Sanders, 1947). Despite the presence of thinly myelinated fibers in SHR, SHR D and SHR DA animals, there were no measurable changes in mean axon caliber or myelin thickness in SHR study II between any study groups in either the sciatic or tibial nerve (axon caliber range: 7.5-8.3 µm; myelin thickness range: 4.1-4.4 µm). This suggests that the relatively low frequency of the thinly myelinated fibers in hypertensive animals was not sufficient to alter the overall myelin thickness measurements. Also, although demyelination/remyelination was occurring in a number of nerve fibers within SHR, SHR D and SHR DA nerves, the remainder of the fiber population had myelin thicknesses comparable to WKY animals. Myelin area, axon area and myelin area/axon area ratio are other measures to assess nerve fiber
characteristics. In the rat, myelin area/axon area ratio has been reported to be 1.47 (Dunn, 1912). For rats used in SHR study II, myelin area/axon area ratio ranged between 1.4 and 1.5 and as with the other morphometric parameters, there was no difference between study groups or between sciatic or tibial nerve. Although no measurable differences were found in myelin thickness or area in the SHR studies the presence of thinly myelinated fibers does indicate Schwann cell damage is occurring in some nerve fibers. S100β is a Ca^{2+} binding protein localized in glial cells that is upregulated following nerve damage (Iwasaki et al., 1997). It would be interesting to perform immunohistochemistry on nerve sections from animals in the SHR studies to determine if S100β staining was increased in animals with increased thinly myelinated fiber density, despite the lack of overt myelin damage throughout the nerve.

Previous reports have consistently shown persistent reductions in sciatic nerve axon caliber in diabetic rats compared to non-diabetic controls in as early as 4 weeks following the induction of experimental diabetes (e.g. Jakobsen, 1976; Mattingly and Fisher, 1985; Yagihashi et al., 1990; Kalichman et al., 1998). There was no significant reduction in mean axon caliber in STZ-diabetic rats in this study in rats with either 8 or 24 works of STZ-diabetes, irrespective of hypertension. It is not entirely clear why a reduction in axon caliber did not occur. A plausible explanation is the strain of rats used in the SHR studies. Previous studies have shown axon caliber diminution in Wistar, Sprague Dawley and BB/Wor rats, but diabetes-induced axon caliber changes have not previously
been reported in WKY or SHR rats (Jakobsen, 1976; Kalichman et al., 1998; Xu et al., 2002).

5.4.7 Conclusions

The studies described in this chapter demonstrate both functional and microanatomical changes in the sciatic, tibial and sural nerves in SHR rats and especially in animals with both hypertension and STZ-diabetes. Existing models used to study the effects of experimental hyperglycemia on peripheral neuropathy have not demonstrated structural pathology that resembles the severe nerve damage observed in patients with a long history of diabetes. Also, STZ-diabetes induces hypotension in diabetic rats. This is a major physiologic distinction between the experimental model and the clinical condition, given that more than 75% of diabetic patients suffer from hypertension, a risk factor for the development of diabetic neuropathy. The discrepancy in the course of disease between clinical diabetic neuropathy and experimental diabetic neuropathy calls in to question the utility of current experimental models in accurately predicting the efficacy of potential drugs or therapeutic approaches to treat or prevent the development of neuropathy in diabetic patients. STZ-diabetes concurrent with hypertension in the SHR rats produced more severe functional deficits than either hypertension or hyperglycemia alone. Additionally, the combination of STZ-diabetes and hypertension led to the occurrence of structural pathology in peripheral nerves that has not previously been reported. Because diabetes and hypertension occur simultaneously so frequently and, since current experimental models of diabetic neuropathy do not generate structural pathology as seen in
clinical diabetic neuropathy, the SHR/STZ-diabetic rat may provide a useful tool for future studies.
Table 5.1

Physiologic measurements of rats in SHR study I after 2 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY (n= 7-8)</td>
<td>101 ± 7</td>
<td>241 ± 5</td>
<td>66.3 ± 1.8</td>
<td>63.9 ± 1.5</td>
</tr>
<tr>
<td>WKY D</td>
<td>336 ± 9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>224 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>59.7 ± 1.8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>60.4 ± 1.5</td>
</tr>
<tr>
<td>SHR</td>
<td>98 ± 5</td>
<td>213 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>64.8 ± 1.4&lt;sup&gt;d&lt;/sup&gt;</td>
<td>62.7 ± 2.3</td>
</tr>
<tr>
<td>SHR D</td>
<td>352 ± 9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>151 ± 8&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
<td>55.1 ± 1.7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>49.1 ± 1.2&lt;sup&gt;a,b,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.001 versus WKY; b, p<0.001 versus SHR; c, p<0.05 versus WKY; d, p<0.05 versus WKY D. MNCV, motor nerve conduction velocity; SNCV, sensory nerve conduction velocity.
Table 5.2

Endoneurial blood vessel density of rats in SHR study I after 2 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sciatic (blood vessels/mm²)</th>
<th>Tibial (blood vessels/mm²)</th>
<th>Sural (blood vessels/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>68 ± 5</td>
<td>78 ± 4</td>
<td>95 ± 14</td>
</tr>
<tr>
<td>WKY D</td>
<td>73 ± 3</td>
<td>96 ± 8</td>
<td>97 ± 9</td>
</tr>
<tr>
<td>SHR</td>
<td>61 ± 3</td>
<td>66 ± 9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70 ± 12</td>
</tr>
<tr>
<td>SHR D</td>
<td>55 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>78 ± 5</td>
<td>67 ± 7</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. 
<sup>a</sup> p<0.05 versus WKY D.
Table 5.3

Morphometric measurements of the sciatic, tibial and sural nerve of rats used in SHR study I after 2 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean axon caliber (µm)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sciatic</td>
<td>Tibial</td>
</tr>
<tr>
<td>WKY</td>
<td>6.3 ± 0.2</td>
<td>5.4 ± 0.3</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>WKY D</td>
<td>6.0 ± 0.1</td>
<td>5.6 ± 0.3</td>
<td>4.9 ± 0.2</td>
</tr>
<tr>
<td>SHR</td>
<td>6.2 ± 0.2</td>
<td>5.7 ± 0.2</td>
<td>5.3 ± 0.3</td>
</tr>
<tr>
<td>SHR D</td>
<td>6.3 ± 0.1</td>
<td>5.5 ± 0.1</td>
<td>4.6 ± 0.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.01 versus WKY and WKY D; b, p<0.05 versus SHR; c, p<0.05 versus all other groups.
Table 5.4

Density of thinly myelinated nerve fibers in rats used in SHR study I after 2 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Thinly myelinated fibers (#/mm²)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sciatic</td>
<td>Tibial</td>
<td>Sural</td>
</tr>
<tr>
<td>(n= 7-8)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WKY</td>
<td>4.2 ± 1.7</td>
<td>5.1 ± 3.0</td>
<td>5.1 ± 4.0</td>
</tr>
<tr>
<td>WKY D</td>
<td>5.8 ± 2.0</td>
<td>3.4 ± 1.6</td>
<td>24.0 ± 10.6</td>
</tr>
<tr>
<td>SHR</td>
<td>24.2 ± 5.4</td>
<td>2.8 ± 1.1</td>
<td>1.6 ± 1.1</td>
</tr>
<tr>
<td>SHR D</td>
<td>44.7 ± 12.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>12.6 ± 2.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>20.6 ± 17.4</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.01 versus WKY and WKY D; b, p<0.05 versus SHR; c, p<0.05 versus all other groups.
Table 5.5

Blood glucose and body weight measurements of rats in SHR study II after 6 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>121 ± 7</td>
<td>252 ± 3</td>
</tr>
<tr>
<td>WKY D</td>
<td>501 ± 14&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>210 ± 6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR</td>
<td>127 ± 5</td>
<td>223 ± 5&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR D</td>
<td>502 ± 20&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>197 ± 7&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR DA</td>
<td>497 ± 32&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>199 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are group mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups.  
<sup>a</sup>, p<0.05 versus WKY; <sup>b</sup>, p<0.05 versus SHR.
Table 5.6

Physiologic measurements of rats in SHR study II after 6 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group (n= 8-10)</th>
<th>Anesth BP (mmHg)</th>
<th>Conscious BP (mmHg)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>78 ± 10</td>
<td>121 ± 5</td>
<td>87.0 ± 2.1</td>
<td>59.1 ± 1.9</td>
</tr>
<tr>
<td>WKY D</td>
<td>76 ± 4</td>
<td>99 ± 4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>70.2 ± 2.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>51.4 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR</td>
<td>145 ± 10&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>191 ± 5&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>74.7 ± 3.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>53.0 ± 1.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR D</td>
<td>122 ± 7&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>142 ± 4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>60.6 ± 2.2&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>46.4 ± 0.9&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR DA</td>
<td>108 ± 4&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
<td>134 ± 5&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>70.9 ± 1.0&lt;sup&gt;a,d&lt;/sup&gt;</td>
<td>48.1 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are group mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups.  
<sup>a</sup>, p<0.05 versus WKY;  
<sup>b</sup>, p<0.05 versus SHR;  
<sup>c</sup>, p<0.05 WKY D;  
<sup>d</sup>, p<0.05 versus SHR D. BP, blood pressure.
Table 5.7

Nerve blood flow parameters of rats in SHR study II after 6 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Nerve blood flow (AUF)</th>
<th>Nerve vascular conductance (AUF/MAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>47 ± 7</td>
<td>0.59 ± 0.05</td>
</tr>
<tr>
<td>WKY D</td>
<td>36 ± 4</td>
<td>0.47 ± 0.05</td>
</tr>
<tr>
<td>SHR</td>
<td>32 ± 6</td>
<td>0.23 ± 0.04</td>
</tr>
<tr>
<td>SHR D</td>
<td>22 ± 3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.19 ± 0.03&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>SHR DA</td>
<td>27 ± 7</td>
<td>0.25 ± 0.06&lt;sup&gt;a,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.01 versus WKY; b, p<0.05 versus WKY; c, p<0.01 versus WKY D. AUF, arbitrary units of flow; MAP, mean arterial blood pressure.
Table 5.8

Nerve blood vessel densities of rats in SHR study II after 6 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sciatic (blood vessels/mm²)</th>
<th>Tibial (blood vessels/mm²)</th>
<th>Sural (blood vessels/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>66 ± 5</td>
<td>71 ± 5</td>
<td>66 ± 11</td>
</tr>
<tr>
<td>WKY D</td>
<td>47 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>72 ± 4</td>
<td>102 ± 9</td>
</tr>
<tr>
<td>SHR</td>
<td>53 ± 4</td>
<td>67 ± 4</td>
<td>84 ± 13</td>
</tr>
<tr>
<td>SHR D</td>
<td>53 ± 2</td>
<td>67 ± 4</td>
<td>87 ± 8</td>
</tr>
<tr>
<td>SHR DA</td>
<td>59 ± 3</td>
<td>71 ± 5</td>
<td>70 ± 10</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. a, p<0.01 versus WKY.
Table 5.9

Nerve fiber diameter measurements in the sciatic and tibial nerves of rats used in SHR study II after 6 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean axon caliber (µm)</th>
<th>Myelin thickness (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sciatic</td>
<td>Tibial</td>
</tr>
<tr>
<td>WKY</td>
<td>8.3 ± 0.4</td>
<td>7.8 ± 0.4</td>
</tr>
<tr>
<td>WKY D</td>
<td>7.7 ± 0.5</td>
<td>7.8 ± 0.2</td>
</tr>
<tr>
<td>SHR</td>
<td>7.8 ± 0.4</td>
<td>8.2 ± 0.2</td>
</tr>
<tr>
<td>SHR D</td>
<td>7.5 ± 0.5</td>
<td>7.9 ± 0.2</td>
</tr>
<tr>
<td>SHR DA</td>
<td>7.9 ± 0.3</td>
<td>7.5 ± 0.2</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups.
Table 5.10

G-ratio measurements in the sciatic, tibial and sural nerves of rats used in SHR study II after 6 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sciatic</th>
<th>Tibial</th>
<th>Sural</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>0.65 ± 0.004</td>
<td>0.64 ± 0.005</td>
<td>0.65 ± 0.01</td>
</tr>
<tr>
<td>WKY D</td>
<td>0.65 ± 0.004</td>
<td>0.64 ± 0.01</td>
<td>0.63 ± 0.01</td>
</tr>
<tr>
<td>SHR</td>
<td>0.64 ± 0.01</td>
<td>0.65 ± 0.01</td>
<td>0.64 ± 0.01</td>
</tr>
<tr>
<td>SHR D</td>
<td>0.64 ± 0.004</td>
<td>0.64 ± 0.01</td>
<td>0.63 ± 0.02</td>
</tr>
<tr>
<td>SHR DA</td>
<td>0.65 ± 0.002</td>
<td>0.64 ± 0.01</td>
<td>0.63 ± 0.01</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups.
Table 5.11

Nerve fiber area measurements in the sciatic and tibial nerves of rats used in SHR study II after 6 months of STZ-diabetes.

<p>| Group (n= 7-10) | Sciatric | | Tibial | |</p>
<table>
<thead>
<tr>
<th></th>
<th>Axon (µm²)</th>
<th>Myelin (µm²)</th>
<th>Axon (µm²)</th>
<th>Myelin (µm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>70 ± 2</td>
<td>89 ± 3</td>
<td>58 ± 4</td>
<td>78 ± 4</td>
</tr>
<tr>
<td>WKY D</td>
<td>62 ± 5</td>
<td>83 ± 6</td>
<td>55 ± 2</td>
<td>75 ± 2</td>
</tr>
<tr>
<td>SHR</td>
<td>67 ± 4</td>
<td>93 ± 5</td>
<td>61 ± 3</td>
<td>80 ± 5</td>
</tr>
<tr>
<td>SHR D</td>
<td>63 ± 4</td>
<td>87 ± 6</td>
<td>55 ± 2</td>
<td>77 ± 3</td>
</tr>
<tr>
<td>SHR DA</td>
<td>61 ± 2</td>
<td>82 ± 2</td>
<td>52 ± 2</td>
<td>72 ± 3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups.
Table 5.12

Density of thinly myelinated nerve fibers in rats used in SHR study II after 6 months of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sciatic (#/mm$^2$)</th>
<th>Tibial (#/mm$^2$)</th>
<th>Sural (#/mm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>2.9 ± 0.7</td>
<td>4.5 ± 2.4</td>
<td>1.6 ± 1.6</td>
</tr>
<tr>
<td>WKY D</td>
<td>3.2 ± 1.2</td>
<td>3.1 ± 1.0</td>
<td>4.6 ± 2.3</td>
</tr>
<tr>
<td>SHR</td>
<td>23.0 ± 4.2$^{a,b}$</td>
<td>21.1 ± 2.3$^{a,b}$</td>
<td>7.1 ± 7.1</td>
</tr>
<tr>
<td>SHR D</td>
<td>39.8 ± 2.6$^{a,b,c}$</td>
<td>24.3 ± 5.3$^{a,b}$</td>
<td>11.5 ± 7.8</td>
</tr>
<tr>
<td>SHR DA</td>
<td>31.8 ± 5.1$^{a,b}$</td>
<td>22.6 ± 3.4$^{a,b}$</td>
<td>2.3 ± 2.3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. $a$, $p<0.01$ versus WKY; $b$, $p<0.001$ versus WKY D; $c$, $p<0.001$ versus SHR.
Table 5.13

Blood glucose and body weight measurements of rats in SHR study III after 1 month of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood glucose (mg/dL)</th>
<th>Body weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>125 ± 5</td>
<td>208 ± 3</td>
</tr>
<tr>
<td>SHR D</td>
<td>526 ± 15(^a)</td>
<td>167 ± 4(^a)</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by unpaired t-test. \(a\), \(p<0.001\).
Table 5.14

Physiologic measurements of rats in SHR study III after 1 month of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group (n = 8-16)</th>
<th>Anesth BP (mmHg)</th>
<th>Consc BP (mmHg)</th>
<th>MNCV (m/s)</th>
<th>SNCV (m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>118 ± 13</td>
<td>164 ± 4</td>
<td>55.8 ± 1.2</td>
<td>49.0 ± 1.4</td>
</tr>
<tr>
<td>SHR D</td>
<td>85 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td>122 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>52.1 ± 0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>50.3 ± 0.9</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by unpaired t-test. a, p<0.001; b, p<0.01; c, p<0.05. BP, blood pressure.
Table 5.15

Nerve blood flow parameters of rats in SHR study III after 1 month of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Nerve blood flow (AUF)</th>
<th>Nerve vascular conductance (AUF/MAP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>34 ± 7</td>
<td>0.29 ± 0.05</td>
</tr>
<tr>
<td>SHR D</td>
<td>16 ± 3(^a)</td>
<td>0.19 ± 0.03</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by unpaired t-test. \(a, p<0.01\) versus SHR. AUF, arbitrary units of flow; MAP, mean arterial blood pressure.
Table 5.16

Density of blood vessels and thinly myelinated nerve fibers in the sciatic nerve of rats in SHR study III after 1 month of STZ-diabetes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Blood vessel density (#/mm²)</th>
<th>Thinly myelinated fibers (#/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR</td>
<td>51 ± 7</td>
<td>29 ± 6</td>
</tr>
<tr>
<td>SHR D</td>
<td>61± 2</td>
<td>30 ± 3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± sem and were analyzed by unpaired t-test.
Figure 5.1 A. PGP 9.5 staining in the epidermis of the rat hind paw plantar skin. Arrows point to intra-epidermal nerve fibers. B. Nerve fiber density in the epidermis of the hind paw and thermal paw withdrawal latency of rats in SHR study I. Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. +, p<0.05 versus WKY D and SHR; *, p<0.05 versus WKY and SHR D. IENF, intra-epidermal nerve fibers.
Figure 5.2 A. Light micrograph of the sciatic nerve of an SHR D rat. Arrowheads indicate nerve fibers with a disproportionately thin myelin sheath. Magnification, 600x. B. Electron micrograph of a thinly myelinated nerve fiber in the sciatic nerve of a SHR D rat. Arrow points out redundant layers of basal lamina laid down by the Schwann cell, indicating demyelination/remyelination has occurred. Magnification, 4,000x. C. Light micrograph of the sciatic nerve of an SHR D rat. Arrow points out a nerve fiber with splitting and ballooning of the myelin sheath. Magnification, 600x.
Figure 5.3 A. Nerve fiber density in the epidermis of the hind paw of rats in SHR study II. B. Thermal paw withdrawal latency for rats in SHR study II. Data are presented as mean ± sem and were analyzed by 1-way ANOVA followed by Newman-Keuls post-hoc test to assess differences between groups. *, p<0.001 versus WKY and SHR D. +, p<0.001 versus SHR D. IENF, intra-epidermal nerve fibers.
CHAPTER 6 - DISCUSSION

6.1 Discussion of results

Given the important role of oxidative stress in the etiology of diabetic neuropathy (Vincent et al., 2004), the first aim of this dissertation was to determine if antioxidant treatment in STZ-diabetic rats would be successful at preventing the development of diabetic neuropathy. α-lipoic acid, a potent antioxidant with previously reported efficacy in reversing indices of STZ-induced nerve injury (Cameron et al., 1998; Stevens et al., 2000; Coppey et al., 2001), was unsuccessful in preventing NCV slowing or nerve blood flow deficits. Taurine, another antioxidant that has been reported to convey therapeutic benefit to the peripheral nerve in experimental models of diabetes was effective at preventing the development of tactile allodynia and in preventing lipid peroxidation, a measure of oxidative stress, in the sciatic nerve. The antioxidant ellagic acid was also successful in preventing the development of tactile allodynia and lipid peroxidation in the sciatic nerve. Nerve conduction velocity deficits were also ameliorated by ellagic acid treatment in STZ-diabetic rats.

In addition to oxidative stress, reduced nerve blood flow and nerve ischemia is a process central to diabetes-induced peripheral nerve injury. Pharmacologic agents such as vasodilators have been successful at preventing indices of nerve injury in experimental models (Cameron and Cotter, 1995). However, systemic vasoactive agents may not be suitable for clinical use. Therefore, the second aim was to attempt to increase nerve blood flow using non-pharmacologic methods in an effort to prevent STZ-induced nerve
injury. There was no clear effect of exercise, NIRE or ultrasound treatment in preventing the development of STZ-induced nerve injury in these studies.

Based on the identification of hypertension as a risk factor for developing neuropathy in diabetic patients (Tesfaye et al., 2005), the third aim was to determine the effects of combined hypertension and diabetes on the development and progression of experimental neuropathy. Concurrent hypertension led to progressive nerve injury compared to diabetes or hypertension alone at the 2 and 6-month time points. Nerve blood flow and nerve conduction velocity were diminished by both diabetes and hypertension and the combination of the two diseases reduced nerve blood flow and NCVs to a greater extent. Treatment of hypertensive diabetic rats with an ACE inhibitor led to a partial protection of nerve injury. The partial protection provided by the ACE inhibitor occurred despite the fact that ACE inhibition in the SHR DA animals did not significantly reduce blood pressure compared to SHR D animals. In addition to its vasoactive actions, captopril, the ACE inhibitor used in the SHR II study, has recently been shown to possess antioxidant properties (Lopez-Real, et al., 2005). Given the importance of oxidative stress in the development of diabetic neuropathy, it is plausible that captopril led to partial protection of nerve injury in these studies by reducing oxidative stress.

Hypertension, and especially the combination of diabetes and hypertension, induced Schwann cell injury as measured by the presence of nerve fibers with a disproportionately thin myelin sheath. Given the relatively few thinly myelinated fibers counted in the sciatic nerve at the 2-month point, it was
expected that more thinly myelinated fibers would be measured at the 6-month time point. However, there was no increase in the density of thinly myelinated fibers in the sciatic nerve between 2 and 6 months, suggesting an ongoing demyelination/remyelination is occurring and that Schwann cells are actively attempting to repair nerve fibers that have been damaged. It is possible, that even at 6 month of diabetes and hypertension, not enough time has elapsed to lead to an increase in the density of thinly myelinated fibers. A longer duration study, such as 12 months, would be required to address the effect of the time component in the development of thinly myelinated fibers. Another possibility is that changes in myelination are too subtle to be detected by the eye. At the time SHR study I was conducted, the morphometry analysis software available in the laboratory (NIH Image 1.55) was only capable of measuring axon caliber and could not detect changes in myelination. The HistoQuant software, used to assess sciatic nerve morphometric parameters in the SHR II study, was developed to address the possibility that changes in myelination were occurring, but that in order to measure differences between experimental groups, a method with more resolution than that provided by NIH Image was required. Analysis of myelin thickness, myelin area, axon caliber and axon area did not reveal any differences between the experimental groups in the SHR III study. This suggests that the degenerative process, which led to demyelination and subsequent remyelination, is only occurring in a relatively few fibers. Even in SHR, SHR D and SHR DA animals, the low overall frequency of thinly myelinated fibers,
compared to the total population of nerve fibers within the sciatic nerve, was not sufficient to alter the mean morphometric parameters measured.

When the length of the nerve is considered, however, it is possible to conclude that a much larger percentage of fibers than that counted in these SHR studies are affected by demyelination/remyelination. The length of the sciatic nerves in rats used in these studies is approximately 5 cm and the internodal length of the myelinated fibers is approximately 1 mm, leading to approximately 50 internodes per nerve fiber. The frequency of thinly myelinated fibers was only about 20 thinly myelinated fibers per 5,000 fibers within the sciatic nerve, or 0.4%. However, demyelination/remyelination is often segmental in nature, with only certain internodes being affected. Given that thinly myelinated fibers were only counted in one plane of section, in the mid-sciatic region, and that each fiber examined has up to 50 internodes, if segmental demyelination is occurring up and down the sciatic nerve, as many as 20% of myelinated nerve fibers could be thinly myelinated at some point along their length. Analysis of teased fibers would provide insight into extent of segmental demyelination occurring in the SHR studies. Additionally, serial sectioning could be done perform similar thinly myelinated fiber counts as conducted in the SHR studies, but at different points along the length of the nerve.

6.2 The etiology of diabetic neuropathy remains elusive

For the past several decades, extensive research has been devoted to elucidating the underlying causes of diabetic neuropathy. Although the involvements of a number of mechanisms have been positively identified, these
discoveries have not lead to any therapeutics successful at preventing the onset or progression of the disease. Two of the most important processes implicated in the pathogenesis of diabetic neuropathy, oxidative stress and microangiopathy, were examined in this dissertation.

Perhaps the reason why the etiology of diabetic neuropathy has remained so elusive is the complex and perpetuating cycle of interactions between the various pathologic processes involved in the disease. Thus, targeting a specific pathway or process may benefit that aspect of the pathophysiology, but at the same time other mechanisms are still actively precipitating diabetes-induced nerve damage. Oxidative stress and free radical damage to peripheral nerves are good examples of this difficult problem. It has been shown that free radical production is deleterious to peripheral nerve axons and Schwann cells, as well as to the microvasculature of the vasa nervorum (Low et al., 1997, Zalba et al., 2000; Vincent et al., 2004). However, targeting oxidative stress is extremely difficult because diabetes generates free radical species in a number of ways. Hyperglycemia leads to increased polyol pathway flux and AGE/RAGE interaction, both of which lead to production of free radicals, as well as depletion of endogenous antioxidants such as glutathione, and taurine (Vincent et al., 2004). Diabetes also leads to PKC activation and mitochondrial damage, with subsequent generation of free radicals (Yamagishi and Imaizumi, 2005). Given the variety of sources of oxidative stress, it is somewhat surprising that certain selective antioxidant therapies have been successful at preventing or reversing nerve injury in experimental models. Ellagic acid was the most effective
antioxidant treatment used in these studies. In addition to antioxidant capabilities, ellagic acid also has aldose reductase inhibitor activity (Ueda et al., 2004). This may be an important finding in that successful antioxidant treatment may rely on targeting multiple pathways of oxidative stress. Existing antioxidant-based clinical trials have focused on a single antioxidant treatment and have not provided conclusive evidence for a role of antioxidants as a treatment for diabetic neuropathy. Use of a high-dose single antioxidant may disrupt normal cellular antioxidant/prooxidant regulation and the future of antioxidant therapy may lie in combinations of low-dose mixtures of a variety of antioxidant agents.

The role of microvascular damage in the etiology of diabetic neuropathy is also extremely complex and unclear. It is well established the diabetes leads to a number of endoneurial microvascular changes such as capillary basement membrane thickening, capillary closure, endothelial and smooth muscle cell proliferation and pericyte degeneration (Yasuda and Dyck, 1987; Giannini and Dyck, 1995; Malik et al., 1993). In biopsies taken from nerves of diabetic patients, axon damage tends to be patchy or multifocal, suggesting an ischemic origin to the observed pathology (Dyck et al., 1986a,b). Microvascular damage in the peripheral nerves of diabetic patients prior to the development of neuropathy is also suggestive of a vascular origin to the disease (Giannini and Dyck, 1995; Malik et al., 2005). Data reported in chapter 5 of this dissertation support a causative role of vascular damage in the etiology of diabetic neuropathy. In the SHR studies, the magnitude of nerve blood flow deficits was directly related to the extent of nerve conduction slowing and of peripheral nerve Schwann cell
injury. However, the presence of multifocal axon damage in patients with inherited neuropathy with no microvascular disease argues against a purely ischemic origin of diabetes induced axonal damage (Zochodne et al., 2007).

Hypertension leads to increased thickness of the blood vessel walls and luminal narrowing (Beevers and Robertson, 2007), and structural changes to the vasa nervorum caused by hypertension are not unlike that observed in patients with diabetic neuropathy. Clearly though, the finding that diabetes concurrent with hypertension worsens nerve injury, suggests there is a combined effect of the two diseases that results in more stress on the peripheral nerve that may involve more than just microvascular injury.

6.3 Multiple pathologic processes lead to nerve injury in the SHR/STZ-diabetic rat model

More than 80% of patients suffering from diabetic neuropathy present with the distal symmetric form of the disease (Said, 2007). In these types of cases, symptoms typically occur first in the feet. As the disease progresses, more proximal portions of the lower limbs are affected. The distal-to-proximal presentation of the disease suggests that the disease is length dependant, with the longest nerves being affected first. Pathologic changes to the nerve in patients with diabetic neuropathy include axon degeneration, demyelination (with subsequent remyelination), segmental demyelination, and axon loss, which is more pronounced distally (Llewelyn et al., 1991). The pattern of distal to proximal nerve damage has also been observed in experimental models of diabetic neuropathy. Axons from more distal nerves have been shown to exhibit
a greater degree of atrophy compared to more proximal nerves in rodents with experimental diabetes (Sima et al., 1983). The length-dependant dying-back nature of diabetic neuropathy could be at least partially explained by oxidative stress. Mitochondria in the dorsal root ganglia of peripheral nerves are a major site of reactive oxygen species production under hyperglycemic conditions. Free radicals formed in the dorsal root ganglia can damage DNA and cell membranes, impairing synthesis and transport of regulatory proteins and neurotrophic factors to distal sites in the peripheral nerve (Leinninger et al., 2006).

In the SHR studies presented in this dissertation, there was only a limited indication of a distal-to-proximal gradient of nerve injury. Axon caliber was unchanged in the sciatic nerve of diabetic animals at the 2-month time point. In the tibial nerve there was a partial reduction in axon caliber in diabetic animals compared to non-diabetic control animals, suggesting perhaps that the more distal, tibial nerve, was affected first. However, when axon caliber was examined at the 6-month time point there were no significant differences detected between any experimental groups in either the sciatic or tibial nerve. This suggests that in the WKY and SHR strains of rodents the dying-back paradigm of axon caliber changes does not apply. In fact, in SHR animals, the time-course data of thinly myelinated nerve fibers suggests a proximal-to-distal gradient of nerve injury. At the 2-month time point, thinly myelinated nerve fibers are found in SHR animals in the sciatic nerve but not in the more distal tibial nerve. By 6 months, thinly myelinated fibers are present in both the sciatic and tibial nerves.
As previously discussed, hyperglycemia-induced hypoxia and subsequent ischemia have been linked to peripheral nerve injury observed in both diabetic patients and in animal models of diabetes. Hypertension itself can also lead to reduced nerve blood flow through increased vascular resistance, blood vessel wall thickening and endothelial cell dysfunction (see Figure 6.1). In SHR rats, these effects are most pronounced in the epineurial vasculature, the site where both RAGE and aldose reductase are found (Sabbatini et al., 1996). Based on the results from the SHR studies, hypertension alone leads to a greater reduction in nerve vascular conductance compared to STZ-diabetes.

The peripheral nerve vasculature is somewhat unusual in that endoneurial blood vessels lack auto-regulatory ability (Smith et al., 1977). Thus, endoneurial blood flow is determined by epineurial arteries and arterioles of the extrinsic nerve circulation (Appenzeller et al., 1984). There is also a limited surface area for metabolic exchange in the endoneurium. Blood vessel density is reduced in the nerve vasculature, with endoneurial blood vessels being approximately 2.5 times more spaced apart than in the surrounding musculature (Bell and Weddell, 1984b). In the absence of a disease state, nerve tissue is adequately oxygenated and, in fact, peripheral nerves can sustain substantial decreases in nerve blood flow without suffering ischemia (Conn and Dyck, 1975). However, once nerve blood flow is already compromised, as occurs in both diabetes and hypertension, further decreases in nerve blood flow can induce nerve hypoxia and result in ischemic nerve injury (Nukada et al., 1992).
Given the ability of the peripheral nerve to endure a substantial decrease in nerve blood flow prior to suffering from nerve ischemia, it is possible that the metabolic and vascular changes that occur in STZ-diabetic rats are not sufficient to induce structural nerve damage. The reduction in nerve blood flow associated with STZ-diabetes is sufficient to exert a physiologic stress on the animals such that nerve conduction velocity is reduced, but not enough of a stress to lead to demyelination, with subsequent remyelination. The often reported finding of reduced axon caliber in peripheral nerves of STZ-diabetic rats suggests that the nerves may be under strain and in the early stages of neurodegeneration, but that a tipping point has not yet been reached to induce overt structural nerve injury. The relatively short length of the sciatic nerve in rodents along with the short duration of disease in most STZ-diabetic rat studies, compared to patients with diabetes, may be factors that prevent the development of more severe nerve pathology. Similarly, SHR animals appear to be close to a tipping point in terms of suffering from structural peripheral nerve damage. In SHR animals, the further reduction in nerve vascular conductance, as compared to WKY D animals, leads to nerve conduction slowing and, in addition, Schwann cell damage in the form of thinly myelinated nerve fibers.

The occurrence of thinly myelinated fibers in SHR animals occurs as early as one month and does not increase, but rather remains relatively constant over a 6-month period. There was no difference between SHR and SHR D animals in the frequency of thinly myelinated nerve fibers at the 1-month time point but by 2 months and also at the 6-month time point the co-occurrence of diabetes with
hypertension leads to a further increase in thinly myelinated nerve fibers. In addition to the decreased nerve blood flow associated with both diabetes and hypertension, other hyperglycemia-induced pathologic processes further exacerbate the nerve injury when combined with hypertension.

As discussed earlier, polyol pathway over activation is a key process involved in the pathogenesis of diabetic neuropathy. Increased polyol pathway flux can lead to neuropathy by direct tissue damage and also by inducing endothelial injury and vascular dysfunction. Increased polyol pathway flux is also intimately linked to AGE production through the generation of fructose, a glucose metabolite that acts as a potent glycating agent (Suarez et al., 1989). AGEs can cause nerve injury directly, by glycation of structural and functional nerve proteins such as neurofilaments, tubulin, actin and the myelin protein P₀ (Vlassara et al., 1994). In addition AGEs can quench nitric oxide leading to impaired vasorelaxation. AGE/RAGE interaction has also been implicated in endothelial cell dysfunction and vascular complications of diabetes through the generation of oxidative stress. In the peripheral nerve, RAGE and the critical polyol pathway enzyme aldose reductase are both localized in Schwann cells, a site where nerve injury was observed in the SHR studies. In addition, RAGE is also found in endothelial cells in the peripheral nerve and aldose reductase is also found in endothelial cells of the epineurial vasculature (Wada and Yagihashi, 2005; Jiang et al., 2006). The common localization of RAGE and aldose reductase in both Schwann cells and epineurial endothelial cells in the peripheral nerve provides the basis for a scenario in which a number of pathologic...
processes are brought together at sites that are critical to the maintenance of a healthy nerve.

AGE/RAGE interaction, increased by hyperglycemia, leads to impaired nitric oxide activity in endothelial cells, further worsening the blood flow deficit in the nerve (see Table 5.7 and Figure 6.1). In addition, increased polyol pathway flux in epineurial blood vessels and in Schwann cells in the peripheral nerve lead to further nerve injury and the observed increase in thinly myelinated fibers in SHR D animals compared to SHR animals (see Table 5.12 and Figure 6.1). The time-course of occurrence of thinly myelinated fibers indicates that the diabetes effect on myelination takes more than one month to develop. Polymorphisms in the gene AKR1B1, which codes for aldose reductase, have been shown to be associated with progression of neuropathy in diabetic patients (Thamotharampillia et al., 2006). It is possible that alternate forms of this gene play an important role in the different rates of progression of diabetic neuropathy observed in both patients with the disease and in animals models.

6.4 SHR/STZ-diabetic rat may provide a useful tool to study diabetic neuropathy

Advances in the understanding of the pathogenesis of diabetic neuropathy have resulted in large part from studies on STZ-diabetic rats. The STZ-rat model is extremely useful as a tool to study diabetic neuropathy, in that a number of functional impairments observed in diabetic patients with neuropathy are recapitulated in the STZ-diabetic rat. Reduced nerve blood flow, nerve conduction velocity slowing and progressive sensory abnormalities are examples
of indices of neuropathy seen in patients that are paralleled by the STZ-diabetic rat model. However, researchers have long been frustrated by the disconnect between the overt structural pathology observed in biopsies from diabetic patients (Richardson and De Girolami, 1995), and the relatively minor structural changes observed in diabetic rats (Jakobsen, 1976). This limits the study of experimental diabetic neuropathy primarily to the assessment of metabolic alterations of the disease. Also, an important aspect of the STZ-diabetic rat model that does not mimic the physiologic conditions found in the vast majority of patients with diabetic neuropathy is the lack of hypertension in STZ-diabetic rats. In fact, STZ-diabetes induces a mild hypotension in rats (Yamamoto, 1988; see Table 5.6).

A wide variety of therapeutic agents have proven successful at preventing or reversing the development of diabetic neuropathy in experimental models. However, there has not been success in translating these therapeutic strategies into successful treatments of human diabetic neuropathy. It is unlikely that treatment will benefit patients with severe axon loss. However, in patients with ongoing Schwann cell degeneration/regeneration, prior to axon loss, therapeutic intervention may halt or reverse the developing neuropathy. The SHR/STZ-rat studied in this thesis represents a new model to investigate the pathogenesis of diabetic neuropathy. Functional changes such as nerve blood flow reductions and nerve conduction velocity slowing are observed in the SHR/STZ-rat and in addition a structural component of nerve damage is also present. The presence of thinly myelinated nerve fibers in SHR and SHR/STZ rats indicates structural
Schwann cell injury in the peripheral nerve, a finding that has not been previously reported. In humans, demyelination/remyelination precedes outright axon loss. The stable level of Schwann cell damage seen between 2 and 6 months in the SHR studies provides an opportunity to attempt to prevent or reverse Schwann cell damage in a model that does exhibit some structural damage but does not progress to frank axon loss. Future investigations into the etiology of diabetic neuropathy can make use of the SHR/STZ-rat to better understand the pathogenesis of diabetes-induced nerve damage. Also, experimental therapies that are successful at preventing or reversing the Schwann cell damage present in the SHR/STZ-rat may have more potential for success in clinical trials.

Diabetes is the most common cause of neuropathy. Currently, the prevalence of diabetic neuropathy is remarkably high and as the incidence of diabetes increases, patients suffering from neuropathy will also increase. Despite extensive research the etiology of diabetic neuropathy and development of effective therapies has proven elusive. Clinical trials for diabetic neuropathy treatments are extremely difficult to conduct based on the long-term progression of the disease, difficulties with patient compliance and wide range of variation in the course of disease among patients. However, it is of the utmost importance that clinical trials are based on the best possible experimental data. The SHR/STZ rat offers a new model to study diabetic neuropathy and may lead to more promising future treatments for patients suffering from this disease.
6.5 Prevention is the best medicine

Unfortunately, despite the awareness that combined hypertension and diabetes leads to a more severe course of neuropathy, treatment remains a difficult challenge. As with oxidative stress, endoneurial microangiopathy is caused by several interrelated processes, including impaired NO activity, oxidative damage, AGE-induced damage and polyol pathway alterations (Bucala et al., 1991; Stevens et al., 1994; Zochodne, et al., 2007). Targeting a specific pathway may impart some therapeutic benefit to the peripheral nerve vasculature, but the remaining unaltered pathologic processes at work will likely continue to lead to microangiopathy. Thus, as suggested above with antioxidant treatment, future therapies for the microvasculature will need to target multiple pathologic mechanisms. Hypertension is treated aggressively in diabetic patients with nephropathy and retinopathy. In the absence of a treatment option to prevent or reverse diabetic neuropathy, at a minimum, diabetic patients with neuropathy should also be treated aggressively for hypertension.

Another important consideration regarding the treatment of diabetic neuropathy, and perhaps the most reasonable approach given the paucity of available treatments for the disease, is to make every attempt possible to avoid or delay developing the disease. Levels of glycemic control, along with duration of the disease, are the most important risk factors for developing neuropathy in diabetic patients. With rigor, vigilance, and few lapses of attention, it is possible for a diabetic patient to control blood sugar within reasonable limits. However, in patients suffering from diabetes for years and decades, it is not likely that
glycemic control can be monitored and tightly regulated at all times. Preventative medicine is the most effective form of treatment for any disease, and although certain risk factors for developing diabetic neuropathy, such as duration of disease and genetic predisposition, are beyond the control of a patient, other risk factors including triglyceride levels, body mass index, smoking and hypertension can be modified by patient lifestyle. Hypertension for example more than doubles the risk of developing diabetic neuropathy and this is the case even in patients with fewer than 10 years of diabetes (Tesfaye at el., 2005; Jarmuzewska et al., 2007). Because diabetic neuropathy is a progressive disease, worsening over time, if a diabetic patient can delay the onset of neuropathy through lifestyle modifications, the long-term prognosis could be dramatically improved.
Figure 6.1 Pathogenic pathway of nerve injury in the STZ and SHR/STZ-diabetic rat models
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