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Nerve Blood Flow Control in Health & Disease: The Effects of Type 1 Diabetes and Exercise Training

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Graduate Program in Kinesiology
A thesis submitted in partial fulfillment of the requirements for the degree in Doctor of Philosophy
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Nerve Blood Flow Control in Health & Disease:
The Effects of Type 1 Diabetes and Exercise Training

(Thesis format: Integrated Article)

by

T. Dylan Olver

Graduate Program in Kinesiology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctorate of Philosophy

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Peripheral nerve blood flow (NBF) is critical to nerve health. Impaired NBF control may contribute to the progression of diabetes peripheral neuropathy. The purposes of this dissertation were: i) to investigate the acute and chronic effects of hyperglycemia on basal sciatic NBF (measured via Doppler ultrasound), ii) to examine the potential vasodilatory effects of insulin (euglycemic hyperinsulinemic clamp; $10 \text{ m}\mu\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) on NBF control in healthy rats and rats with insulin-treated type 1 diabetes (DS) and iii) to determine if exercise training ($\sim 75\% \text{ VO}_2\text{max}$, 60 min/day, 5 days/wk for 10 wk) improves vasa nervorum reactivity to insulin and motor nerve conduction velocity (MNCV) in rats with DS. The studies tested the overall hypotheses that i) basal NBF, the vasodilatory response to insulin, and MNCV would be attenuated in rats with DS, and ii) DS-induced deficits would be offset by concurrent exercise training. Rats with DS were hypertensive and featured reduced MNCV ($P < 0.01$). However, DS did not alter: basal NBF; the relationship between NBF and blood pressure; or the vascularization of the sciatic nerve compared to healthy controls ($P \geq 0.50$). Based on these data, hypertensive rats with DS may exhibit elevated NBF in the conscious, basal state ($P = 0.02$). Despite similar or possibly elevated basal NBF values compared with control rats, rats with DS displayed impaired insulin-mediated vasodilation ($P \leq 0.01$). Deficits in insulin-mediated vasa nervorum dilation and MNCV were prevented by concurrent endurance exercise training in a separate group of rats with DS. Enhanced insulin-mediated vasa nervorum dilation in aerobically exercise trained rats with DS may be explained partially by the observed increase in sciatic nerve endothelial NO synthase expression ($P = 0.04$). The degree of hyperglycemia was similar in both groups with DS, but exogenous insulin requirements were reduced in rats with DS that exercise trained ($P = 0.02$). Perhaps increased insulin sensitivity, and not reduced hyperglycemia, is the mechanism responsible for preserving vasa nervorum and nerve function in exercise-trained rats with DS. Improved insulin signaling in the vasa nervorum may potentiate insulin signaling in the sciatic nerve and protect against diabetic peripheral neuropathy.

Keywords: nerve blood flow, diabetes, hypertension, exercise training, insulin, NO

Co-Authorship Statement

The following is a list of co-authors that contributed to this thesis dissertation (see the appendix for details pertaining to published material):

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“Keep the tension high” – J. Kevin Shoemaker, PhD

Kevin Shoemaker – a wonderful mentor, a great scientist and a mediocre (but motivated) hockey player, is to blame for this thesis dissertation. After a few bus rides and numerous tangential discussions about science, life, and faith, we agreed that a doctoral degree was an appropriate atonement for my impractical philosophies. Kevin gave me every opportunity to be successful. For the errors hidden within the text, in plain view, I claim sole responsibility.

“Why sacrifice the present to the future, fancying that you will be happier when your fields teem with wealth and your cities with people? [...] You dream of your posterity; but your posterity will look back to yours as the golden age, and envy those who first burst into this silent, splendid nature, who first lifted up their axes upon these tall trees, and lined these waters with busy wharves. [...] Why, in your hurry to subdue and utilize nature, squander her splendid gifts? [...] You have opportunities such as mankind has never had before, and may never have again. Your work is great and noble; it is done for a future longer and vaster than our conceptions can embrace. Why not make its outlines and beginnings worthy of these destinies; the thought of which gilds your hopes and elevates your purposes?”

–The Viscount (James) Bryce

Contributors and my committee – In the beginning, I did not appreciate my lot sufficiently. I have always loved the process, but too often I glanced ahead and fantasized about “when...” and “what if...” To be clear, I love this profession and thoroughly enjoyed designing these experiments as well as collecting and analyzing these data with all of you. My projects were strengthened substantially by your contributions. Going forward, if I am able to benefit from those around me, as I have from you, every day of my career will be a success. I no longer look onward to dream, I look inward. Your efforts will not be forgotten.

“It is amazing what you can accomplish if you do not care who gets the credit”
– **Harry Truman, 33rd President of the United States**

My doctoral degree is not my own; it belongs to them, the people who came together to support my endeavours. I have demonstrated competence and hard-work, both of which I have been trained in extensively. To negotiate this passage, I placed one foot in front of the other; but it has been my loved ones and colleagues who laid themselves out to ensure my feet stayed dry.

“If destruction be our lot, we must ourselves be its author and finisher”
– **Abraham Lincoln, 16th President of the United States**

My critics – never again will I pursue my craft with such little courage. Valuable hypotheses and theories were left unexplored because I lacked conviction and fortitude. My inability to translate, formalize and address many of my experimental inquiries has left me unsatisfied. As a result, my doctoral contribution shall be considered incomplete, this I regret. Rest assured, I have learned more from this failure than from any of my successes.

“If our father had had his say, nobody who did not know how to catch a fish would be allowed to disgrace a fish by catching him” – **Norman Maclean, Author**

Pete and Kevin: My work will be a testament to your instruction.

“I am on until I am dead” – **Henry Rollins, Artist**

“Never never never give up” – **Sir Winston Churchill, 53rd Prime Minister of Britain**

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Matthew 16: 24-28

T. Dylan Olver, PhD

Table of Contents

Abstract.....	ii
Co-Authorship Statement.....	iii
Acknowledgments.....	iv
Table of Contents.....	vi
List of Tables.....	ix
List of Figures.....	x
List of Appendices.....	xiii
Chapter 1.....	1
1 « Introduction ».....	1
1.1 General perspective.....	1
1.2 Nervous system.....	1
1.3 Cardiovascular system.....	2
1.4 Type 1 diabetes and peripheral neuropathy.....	3
1.5 The blood supply of nerves.....	3
1.6 Glucose, insulin and blood flow control.....	6
1.7 Glucose, insulin and the vasa nervorum.....	7
1.8 References.....	11
Chapter 2.....	22
2 « Review of literature ».....	22
2.1 Sciatic nerve vasculature.....	22
2.2 Sciatic nerve blood flow (NBF) measuring techniques.....	23
2.3 Autoregulation and basal NBF control.....	32
2.4 Acute effects of glucose and insulin on NBF control.....	34
2.5 Effects of untreated experimental diabetes on NBF.....	36

2.6 Insulin treatment and NBF.....	39
2.7 Exercise and NBF	41
2.8 Perspectives.....	42
2.9 Purpose and hypotheses	44
2.10References.....	46
Chapter 3.....	61
3 « The relationship between blood pressure and sciatic nerve blood flow velocity in rats with insulin-treated experimental diabetes».....	61
3.1 Introduction.....	61
3.2 Methods.....	62
3.3 Results.....	68
3.4 Discussion	75
3.5 References.....	81
Chapter 4.....	87
4 « Glucose-stimulated insulin secretion causes a nitric oxide mediated vasodilation in the blood supply of the rat sciatic nerve ».....	87
4.1 Introduction.....	87
4.2 Methods.....	88
4.3 Results.....	93
4.4 Discussion	100
4.5 References.....	107
Chapter 5.....	111
5 « Exercise training enhances insulin-stimulated nerve arterial vasodilation in rats with insulin-treated experimental diabetes».....	111
5.1 Introduction.....	111
5.2 Methods.....	114
5.3 Results.....	120

5.4 Discussion.....	130
5.5 References.....	138
Chapter 6.....	147
6 « General discussion»	147
6.1 Key findings.....	147
6.2 Conclusion	152
6.3 References.....	154
Appendices.....	160
Curriculum Vitae	168

List of Tables

Table 3-1 Animal Characteristics	70
Table 3-2 Raw Data: MAP and NBV	71
Table 3-3 Blood Vessel Count.....	71
Table 4-1 Glucose and Insulin Concentrations.....	96
Table 4-2 NBV and MAP.....	97
Table 5-1 General Characteristics.....	124

List of Figures

- Figure 2-1 Colour Doppler screen shot taken in duplex imaging mode of the vasa nervorum 26
- Figure 3-1 Δ Mean arterial pressure (Δ MAP; mmHg) from baseline in the control (\square) and insulin-treated experimental diabetes (\blacksquare) group during sodium nitroprusside (SNP – NO donor) and phenylephrine (PE – α 1 adrenergic agonist) infusion. Early and late responses reflect ~50 and 100% of the peak effect of the drug..... 72
- Figure 3-2 Δ Nerve blood velocity (Δ NBV; mm/s) from baseline in the control (\square) and insulin-treated experimental diabetes (\blacksquare) group during sodium nitroprusside (SNP – NO donor) and phenylephrine (PE – α 1 adrenergic agonist) infusion. Early and late responses reflect ~50 and 100% of the peak effect of the drug..... 72
- Figure 3-3 Nerve blood velocity (NBV; mm/s) at a given mean arterial pressure (MAP; mmHg) in the control (\circ , —) and insulin-treated experimental diabetes (\blacksquare , ... \dagger) group. The regression equations were calculated for each rodent and averaged separately to create a two group means. 73
- Figure 3-4 Estimated nerve blood velocity (NBV; mm/s) based on the appropriate individual linear regression equations in the control (\circ ; larger circle denotes the mean) and insulin-treated experimental diabetes (\blacksquare ; larger box denotes the mean) group. *, Significantly greater than control (P=0.02). 73
- Figure 3-5 Representative cross-sectional images of the sciatic nerve in the control (A) and insulin-treated experimental diabetes (B) group. The arrows denote the location of blood vessels. In each image there are two small and one large blood vessel. Sections were stained with hematoxylin and eosin. 74
- Figure 4-1 Femoral artery index of vascular conductance (VCi; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) pre- and post- infusion of Low Glucose (1 g/kg). *, Significantly greater than baseline (P=0.007). ... 98

Figure 4-2 Sciatic nerve arterial index of vascular conductance (VC_i ; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) pre- and post- Low (1g/kg) and High (3 g/kg) Glucose infusions. *, Significantly greater than baseline ($P<0.001$). 98

Figure 4-3 Sciatic nerve arterial index of vascular conductance (VC_i ; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) pre- and post- hyperglycemia+euinsulinemia (NIS) and euglycemia+hyperinsulinemia (EHC). *, Significantly greater than corresponding baseline and peak NIS ($P\leq 0.004$). 99

Figure 4-4 Sciatic nerve arterial index of vascular conductance (VC_i ; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) pre- and post- glucose and L-NAME infusions. *, Significantly greater than corresponding baseline, High Glucose (3g/kg) co-infused with L-NAME (15 mg/kg), L-NAME alone and High Glucose infused 20 min following L-NAME ($P\leq 0.011$). 99

Figure 5-1 a) Nerve blood flow velocity (mm/s) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp in control sedentary (CS; dark bar) rats, diabetes sedentary (DS; grey bar) rats and diabetes exercise (DX; open bar) rats. b) Mean arterial pressure (mmHg) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp. c) Baseline and peak sciatic nerve arterial index of vascular conductance (VC_i ; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) measured during the euglycemic hyperinsulinemic clamp. d) Area under the curve (AUC $_i$) for the VC_i ($\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}\cdot\text{min}^{-1}$) measured throughout the euglycemic hyperinsulinemic clamp. †, Significantly greater than baseline ($P\leq 0.01$). *, Significantly less than CS and DX rats ($P\leq 0.01$). 125

Figure 5-2 a) Motor nerve conduction velocity (MNCV; m/s) measured following the euglycemic hyperinsulinemic clamp in control sedentary (CS; dark bar) rats, diabetes sedentary (DS; grey bar) rats and diabetes exercise (DX; open bar) rats. b) Endothelial nitric-oxide synthase (eNOS) expression (normalized to β -actin) in the sciatic nerve. *, Significantly less than CS and DX ($P\leq 0.01$). ‡, Significantly greater than DS ($P=0.04$). 126

Figure 5-3 a) Nerve blood flow velocity (mm/s) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp in control exercise (CX; hatched bar) rats and diabetes exercise (DX; open bar) rats. b) Mean arterial pressure (mmHg) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp. c) Baseline and peak sciatic nerve arterial index of vascular conductance (VC_i ; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) measured during

the euglycemic hyperinsulinemic clamp. d) Area under the curve (AUCi) for the VCI ($\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}\cdot\text{min}^{-1}$) measured throughout the euglycemic hyperinsulinemic clamp. *, Significantly less than CX ($P=0.03$). 127

Figure 5-4 a) Motor nerve conduction velocity (MNCV; m/s) measured following the euglycemic hyperinsulinemic clamp in control exercise (CX; hatched bar) rats and diabetes exercise (DX; open bar) rats. b) Endothelial nitric-oxide synthase (eNOS) expression (normalized to β -actin) in the sciatic nerve. *, Significantly less than CX ($P\leq 0.02$). 128

Figure 5-5 Linear regression between endothelial nitric-oxide synthase (eNOS) expression and area under the curve (AUCi) for the index of vascular conductance (VCI; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}\cdot 80\text{ min}^{-1}$) measured throughout the euglycemic hyperinsulinemic clamp in control sedentary (CS; ●) rats, control exercise (CX; ○) rats, diabetes sedentary (DS; ▼) rats and diabetes exercise (DX; Δ) rats. Adjusted $r^2=0.54$; $P<0.01$ 129

List of Appendices

Appendix A: Streptozotocin induction protocol (rat)	160
Appendix B: Insulin pellet insertion protocol (rat).....	162
Appendix C: Ethics approval	164
Appendix C: Permission to reproduce published materials.....	167

Chapter 1

1 « Introduction »

1.1 General perspective

To combine in a systematic method, the phenomena that together constitute the existence of living beings, and to organize, compare and contrast such phenomena to form generalizations or principles regarding physiological functions and their regulation is the domain of the *Integrative Physiologist* (1). As such, the objectives of this dissertation were fourfold: 1) to consolidate the available information pertaining to blood flow control in arteries that supply peripheral nerves, 2) to provide the context necessary to understand and justify the research projects described herein and 3) to participate in the process of discovery and 4) to produce novel and meaningful research that extends upon current understanding. Prior to a more thorough dissection of the literature, an elementary outline of the nervous and cardiovascular systems, as well as diabetes and the vascular etiology of diabetic peripheral neuropathy, is requisite.

1.2 Nervous system

The nervous system is composed of the central and peripheral components. The central nervous system consists of the brain and the spinal cord. The brain is responsible for receiving, integrating and coordinating activity in all parts of the body and the spinal cord serves primarily as the conduit of information between the brain and peripheral nervous system. The peripheral nervous system includes afferent sensory neurons that relay information from peripheral receptors to the central nervous system, as well as efferent neurons that transmit information from the central nervous system to peripheral

tissues. The somatic nervous system is a branch of the peripheral nervous system and conducts impulses from the central nervous system to skeletal muscles. The autonomic nervous system is the second branch of the peripheral nervous system and conducts impulses from the central nervous system to organs of the body as well as cardiac and smooth muscles (i.e. blood vessels). Nervous tissue is highly active and requires a continuous stream of blood flow to supply the nutrients necessary to function properly (2).

1.3 Cardiovascular system

The cardiovascular system consists of the heart, arteries, capillaries and veins. The heart is the primary source of impetus for blood flow. Oxygenated blood from the left ventricle of the heart is distributed throughout the body by arteries and smaller arterial branches called arterioles. The arterioles branch into smaller blood vessels called capillaries and this is where the bulk of nutrient and gas exchange between the blood and surrounding tissues takes place. The capillaries feed into venules, the venules converge to form veins and these veins transport deoxygenated blood to the right atrium of the heart. Once returned to the heart, deoxygenated blood is pumped into the pulmonary circulation to become re-oxygenated. Thereafter, oxygenated blood returns to the heart and is re-distributed throughout the body. The cardiovascular system provides tissues with a continuous stream of nutrients including oxygen and removes metabolic byproducts away from the site of energy use. Blood flow distribution is influenced by tissue specific control features which rely on a multitude of inputs including mechanical factors as well as neurogenic and metabolic stimuli (2). A detailed description of the blood supply and blood flow in the sciatic nerve will be provided later (Chapter 2).

1.4 Type 1 diabetes and peripheral neuropathy

Type 1 diabetes is an autoimmune disease characterized by the destruction of the insulin-producing beta cells of the pancreas. The net result is a decreased ability to produce insulin which leads to elevated blood glucose concentrations (hyperglycemia) (3). Other classical symptoms include increased thirst (polydipsia), frequent urination (polyuria), the appearance of glucose in the urine (glycosuria), increased hunger (polyphagia) and weight-loss. Although many options are available, the primary treatment for type 1 diabetes is supplemental insulin (3). Insulin treatment helps attenuate hyperglycemia, but tight blood glucose control is difficult to maintain chronically (3,4). As a result of poorly controlled blood glucose (or potentially the lack of insulin), individuals with type 1 diabetes are at an increased risk for developing both vascular disease and peripheral nerve dysfunction (5). Accordingly, researchers have proposed a vascular etiology of diabetes peripheral neuropathy. Specifically, the theory states that episodic or sustained hyperglycemia impairs microcirculation in peripheral nerves and over time this culminates in nerve dysfunction (6,7). Hyperglycemia may be caused by excess glucose production or ingestion as well as insufficient insulin to stimulate tissue glucose uptake or impaired insulin signaling. Individuals with poor glycemic control are at a greater risk of developing vascular disease and diabetic peripheral neuropathy; therefore, treatments that enhance insulin signaling or sensitivity in the microcirculation of nerves may help prevent such diabetes-related complications.

1.5 The blood supply of nerves

By the early 1900s the gross anatomical and morphological aspects of peripheral nerve blood supply (vasa nervorum) had been described. These observations generated

great interest regarding the functional significance of the vasa nervorum. Detailed historical perspectives that also serve as research summaries are available (8–10); therefore, only a brief account of the historically relevant background information will be provided.

Whereas the early literature (1627-1756) noted the existence of the vasa nervorum (11–14), it was not until 1768 that Isenflamm and Doerffler (15) devoted an entire body of work regarding its potential significance to neural health. Of note, they were able to provide an anatomical description of the epineurial vascular network by filling the arteries with wax. Approximately one-hundred years later (1859 & 1864), Hyrtl provided the first series of critical discoveries in two papers (16,17) and among other findings established that:

- i) each nerve contains feed arteries that do not branch into surrounding muscle beds,
- ii) the nerve supply arteries contain ascending and descending branches that form a continuous longitudinal anastomose between adjacent nerve supply arteries.

These observations raised the possibility of collateral circulation.

By the late 1800s similar anatomical observations were made by others (18–20), and with a more clear understanding of the nerve vasculature scientists were able to generate more specific hypotheses regarding the significance of the vasa nervorum and, as a result, employ more appropriate experimental models. At the time, understanding was that impaired circulation or nerve vascularity would compromise nerve function and

recovery from insult (18,21–23). Generally speaking, two experimental models were used to test these hypotheses, namely, nerve compression and arterial ligation. Although the results between studies vary (24–28), perhaps owing to differences in experimental design, in his 1957 review, Blunt suggested that occlusion of regional neuronal arteries may result in profound histological changes in the nerve and impair nerve regeneration following injury (9). Later he confirmed this position and demonstrated experimentally that ischemia results in neuronal degeneration (29).

Around the same time the relationship between ischemia and nerve degeneration was being unraveled (~35 years after the discovery of insulin), in the late 1950s Fagerberg was among the first to hypothesize a vascular etiology of diabetic peripheral neuropathy formally (6,30). He proposed that in patients with type 1 diabetes mellitus, sclerosis of nerve supply arteries may result in ischemia and subsequently nerve damage. Shortly afterwards, the observation of diabetes-induced platelet abnormalities in the blood (31) and vasa nervorum fibrin accumulation (32), strengthened Fagerberg's position. However, at this point a direct link between nerve blood flow (NBF) and diabetes had not been documented. Then in 1984, a hallmark study published by Tuck and co-workers (7) demonstrated for the first time that sciatic NBF was reduced in rats with untreated experimental diabetes (rodent model of type 1 diabetes). In rats with experimental diabetes (i.e. limited ability to produce insulin, no insulin treatment and chronic severe hyperglycemia), reductions in sciatic NBF occurred alongside decrements in oxygen content of the nerve and nerve dysfunction. These data confirmed over 200 years of hypotheses and speculation by linking reduced NBF with ischemia and nerve

dysfunction (7). This paper continues to serve as the foundation for studies investigating the effects of glucose, insulin and diabetes on NBF control.

1.6 Glucose, insulin and blood flow control

Early after the discovery of insulin (33), it became known that insulin administration and the subsequent hypoglycemia increased heart rate and reduced blood pressure (34,35). The possibility that insulin-hypoglycemia might alter peripheral blood flow had not received much attention until Abramson and colleagues (36) published a study in 1939, describing insulin-stimulated vasodilation in the human forearm and calf. In this study, they also noticed greater vasodilation in the lower vs upper limb and that the magnitude of vasodilation was attenuated if there was a reduction in insulin concentration alongside an increase in blood glucose. Whereas the interaction between glucose, insulin and vascular control remains to be elucidated fully, in the years following the study conducted by Abramson and co-workers (36), it was established that insulin-hypoglycemia is associated with an increase in skeletal muscle blood flow (37,38).

In 1979 a technique was developed by DeFronzo and colleagues (39) known as the euglycemic hyperinsulinemic clamp. This method involves the co-infusion of glucose and insulin, such that, normal blood glucose concentration can be maintained in the presence of hyperinsulinemia. The primary advantage of this model is that during insulin administration, hypoglycemia as well as the negative side effects of hypoglycemia could be avoided. From a vascular perspective, this enabled researchers to tease out the differences between insulin-hypoglycemia, insulin-euglycemia, insulin-hyperglycemia and glucose-stimulated insulin secretion on skeletal muscle blood flow control (40–49).

Since the seminal work of Abramson and co-workers (36), the vasodilatory effects of insulin have been studied extensively. Although many pathways may be involved, it appears that insulin-stimulated vasodilation operates primarily through an endothelium-dependent nitric oxide (NO) mechanism (50–52). This theory is based on observations that insulin-stimulated increases in muscle blood flow are attenuated dramatically when insulin and NO synthase inhibitors, L-NG-nitroarginine methyl ester (L-NAME) or L-NG-monomethylarginine (L-NMMA), are co-infused (50,52). Likewise, insulin-induced increases in skin blood flow are reduced when insulin and a NO synthase inhibitor L-N-nitroarginine (L-NNA) or phosphatidylinositol 3-kinase (PI3-K) inhibitor wortmannin are co-infused (53). In endothelial cells, NO synthase is activated by intracellular enzymes PI3-K and protein kinase B (Akt) (54). Therefore, it has been proposed that upon binding to the insulin-receptor, insulin activates intracellular enzymes, PI3-K and Akt, which phosphorylate and activate endothelial NO synthase. Endothelial NO synthase catalyzes the conversion of the substrate L-arginine to the products L-citrulline and NO, and NO can diffuse into surrounding smooth muscle where it stimulates vasorelaxation (51,54).

1.7 Glucose, insulin and the vasa nervorum

Based on the earlier results outlined above, the vascular etiology of diabetes peripheral neuropathy emphasizes that hyperglycemia impairs microvascular function in the nerve. This precedes and causes or exacerbates nerve dysfunction. Therefore, it is essential to understand how acute and chronic hyperglycemia as well as insulinemia, impact NBF control and nerve function. Unlike skeletal muscle (43,48), sciatic NBF is reduced (55,56) following glucose infusion. This reduction in NBF may relate to gluco-

inhibitory effects on adenosine-mediated vasodilation (56), NO synthase (57) or increased NO scavenging as a result of the overt hyperglycemia (58,59). In contrast to glucose, acute exposure to insulin stimulates vasa nervorum dilation (60). Why hyperglycemia and the accompanying insulin secretion results in vasa nervorum constriction, but insulin alone induces vasa nervorum dilation, is unknown. Regardless, potentially, in the presence of acute hyperglycemia, insulin-mediated dilation is impaired.

Similar to the effects of acute hyperglycemia, untreated experimental diabetes or chronic severe hyperglycemia is often associated with reduced NBF (7,55,61–63). However, these findings are inconsistent, as others have documented no change (64,65) or even increased (66–68) NBF following diabetes induction.

The physiological relevance of the untreated experimental diabetes model is questionable, as that model results in excessive and sustained high blood glucose concentrations (~25-40 mmol/L) (7,55,61). Compared with the degree of hyperglycemia observed in humans with poorly controlled type 1 diabetes (~13.5 mmol/L) (4), the experimental diabetes rodent model results in $\sim \geq 2$ -fold greater blood glucose concentrations (7,55,61). Therefore, it is important to place an emphasis on the studies that incorporate insulin treatment following diabetes induction. It has been established that in rats with experimental diabetes, insulin treatment is beneficial to vasa nervorum health (61,69–71). For example, insulin treatment administered from the onset of hyperglycemia may prevent reductions in NBF, preserve vasa nervorum reactivity and protect nerve function (69–71). Also, in rats with experimental diabetes, insulin treatment may restore, at least partially, decrements in NBF and nerve function 1-month following diabetes induction (61).

Despite the advantages of insulin treatment, individuals with type 1 diabetes continue to experience vascular and peripheral nerve dysfunction in the form of sensory and motor nerve impairments (4,5,72). This may be the result of poor glycemic control or impaired insulin signaling in the vasa nervorum of the peripheral nerve. To date the effects of insulin-treated experimental diabetes on basal NBF and insulin responsiveness in the vasa nervorum have yet to be elucidated. Such knowledge is critical for the development of treatments to preserve vasa nervorum and nerve health in humans with diabetes.

In addition to insulin treatment, researchers have investigated many pharmacological strategies to improve vasa nervorum and nerve function in a rodent model of diabetes (55,73–90). However, so far few studies have focused on the potential benefits of exercise training. In humans (91) and in rats (92) with type 1 diabetes, exercise training prevents or attenuates the progression of neuropathy. Whether exercise training improves vasa nervorum function is unknown. As early as 1914, the diabetes specialist E.P. Joslin prescribed repeated plantar flexion to improve circulation in the lower limb (93). Later, he advocated regular physical activity in order to manage blood glucose concentration and exogenous insulin requirement (94). Potentially, regular exercise increases circulation and potentiates insulin signaling in the vasa nervorum which would enhance overall nerve health.

In humans with type 1 diabetes, exercise may increase the risk of an adverse event (i.e. hyperglycemia, hypoglycemia, musculoskeletal injury, cardiovascular incident, deterioration of retinopathy and nephropathy); however, the benefits of exercise (reduced glycosylated hemoglobin, increased life span, increased fitness, increased insulin

sensitivity, increased sense of well-being and a reduced risk of cardiovascular disease, colon cancer, hypertension, obesity and osteoporosis) outweigh the potential risks (95). Therefore, it is essential to evaluate the role of exercise training in conjunction with insulin treatment in the preservation of insulin signaling, vasa nervorum and nerve health.

The purpose of the projects described herein (Chapters 3, 4 & 5) was to examine the effects of hyperglycemia on basal NBF as well as the potential vasodilatory effects of insulin on NBF control in healthy rats and rats with insulin-treated experimental diabetes. In addition, one project (Chapter 5) aimed to determine if exercise training enhances insulin-mediated dilation in the vasa nervorum of rats with diabetes.

1.8 References

1. Carpenter WB. Principles of human physiology, with their chief applications to pathology, hygiene, and forensic medicine. Second. London: Mr. Churchill & The testimony of the press; 1845. p. 1–2.
2. McArdle W. Exercise physiology: energy, nutrition, and human performance. Sixth. New York: Lippincott Williams & Wilkins; 2007.
3. Van Belle TL, Coppieters KT, Von Herrath MG. Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol. Rev.* 2011;91:79–118.
4. Riveline J-P, Schapelynck P, Chaillous L, Renard E, Sola-Gazagnes A, Penfornis A, et al. Assessment of patient-led or physician-driven continuous glucose monitoring in patients with poorly controlled type 1 diabetes using basal-bolus insulin regimens. *Diabetes Care.* 2012;35(5):965–71.
5. Tesfaye S, Chaturvedi N, Eaton SE, Ward JD, Manes C, Lonescu-Tirgoviste C, et al. Vascular risk factors and diabetic neuropathy. *N Engl J Med.* 2005;352(4):341–50.
6. Fagerberg SE. Studies on the pathogenesis of diabetic neuropathy. *Acta Med. Scand.* 1956;156(4):295–302.
7. Tuck RR, Schmelzer JD, Low PA. Endoneurial blood flow and oxygen tension in the sciatic nerves of rats with experimental diabetic neuropathy. *Brain.* 1984;107:935–50.
8. Adams WE. The blood supply of nerves I. Historical review. *Anatomy.* 1942;76:323–41.
9. Blunt MJ. Functional and clinical implications of the vascular anatomy of nerves. *Postgrad. Med. J.* 1957;33(376):68–72.

10. Paille W. The blood supply to nerves. In: Schwartz C, Werthessen N, Wolf S, editors. *Struct. Funct. Circ.* Volume 1. New York and London: Plenum Press; 1980. p. 769–803.
11. Spiegel V. *De humani corporis fabrica*. Venice; 1627. cited by Isenflamm J, Doerffler J. *De vasis nervorum*. Erlangen. Erlangen; 1768, cited by Blunt MJ. Functional and clinical implications of the vascular anatomy of nerves. *Postgrad. Med. J.* 1957;33(376):68–72.
12. Ruysch F. *Thesaurus anatomicus primus*. Amsterdam; 1701. cited by Blunt MJ. Functional and clinical implications of the vascular anatomy of nerves. *Postgrad. Med. J.* 1957;33(376):68–72.
13. Haller V. *Icones anatomicae*. Gottingen; 1756. cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy.* 1942;76:323–41.
14. Schmidel C. No Title. 1755; cited by Isenflamm, cited by Blunt MJ. Functional and clinical implications of the vascular anatomy of nerves. *Postgrad. Med. J.* 1957;33(376):68–72.
15. Isenflamm J, Doerffler J. *De vasis nervorum*. Erlangen. Erlangen; 1768.
16. Hyrtl J. *Oesterr. Z. prakt. Heilk.* 1859; cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy.* 1942;76:323–41, cited by Tonakoff V. Die arterien der intervertbral-ganglien und der cerebrospinal nerven des menschen. *Int. Mschr. Anat.* 1898;15:300–53.
17. Hyrtl J. *Denkschr. Akael. Wiss., Wein.,* 1864;23. cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy.* 1942;76:323–41, cited by Tonakoff V. Die arterien der intervertbral-ganglien und der cerebrospinal nerven des menschen. *Int. Mschr. Anat.* 1898;15:300–53.
18. Tonakoff V. Die arterien der intervertbral-ganglien und der cerebrospinal nerven des menschen. *Int. Mschr. Anat.* 1898;15:300–53. cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy.* 1942;76:323–41.

19. Quènu J, Lejars F. Les arteres et les veines des nerfs. Seanc. Acad. Sci. 1890;3(608). cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy*. 1942;76:323–41.
20. Quènu J, Lejars F. Etude anatomique sur les vaisseaux sanguins des nerfs. *Arch Neurol*. 1892;23(1).cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy*. 1942;76:323–41.
21. Tonakoff V. Die nervenbegleitenden gefasnetze beim embryo und die arteriae nutriciae nervorum biem nervi isolati. *Anat. Anz*. 1907;30:471. cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy*. 1942;76:323–41.
22. Lapinsky M. Ueber veränderungen nerven der bei der störung acuter blutzufuhr. *Dtsch. Z. Nervenheilkd*. 1899;15(5-6):364–94.cited by Adams WE. The blood supply of nerves I. Historical review. *Anatomy*. 1942;76:323–41.
23. Lapinsky M. Ueber veränderungen der nerven bei acuter störung der blutzufuhr. *Dtsch. Z. Nervenheilkd*. 1878;25:364-393.
24. Denny-Brown D, Brenner C. The effect of percussion on nerve. *J. Neurol. Neurosurg. Psychiatry*. 1944;7(3-4):76–95.
25. Causey G, Stratmann CJ. The spread of failure of conduction in degenerating mammalian nerve. *J Physiol*. 1953;121:215–23.
26. Bentley FH, Schlapp W. The effects of pressure on conduction in peripheral nerve. *J. Physiol*. 1943;102:72–82.
27. Reid C. Experimental ischemia: sensory phenomena, fibrillary twitchings, and effects on pulse, respiration and blood-pressure. *Quart. J. Exper. Physiol*. 1931;21:243.
28. Lewis T, Pickering G, Rothschild P. Observations upon muscular pain in intermittent claudication. *Heart*. 1931;15:359–83.

29. Blunt M. Ischemic degeneration of nerve fibers. *AMA. Arch. Neurol.* 1960;2(5):528–36.
30. Fagerberg SE. Diabetic neuropathy: a clinical and histological study on the significance of vascular affections. *Acta Med. Scand.* 1959;94(164 suppl. 345):1–97.
31. O'Malley BC, Ward JD, Timperley WR, Porter NR, Preston FE. Platelet abnormalities in diabetic peripheral neuropathy. *Lancet.* 1975;306(7948):1274–6.
32. Timperley WR, Ward JD, Preston FE, Duckworth T, Malley BCO. Clinical and histological studies in diabetic neuropathy. A reassessment of vascular factors in relation to intravascular coagulation. *Diabetologia.* 1976;243:237–43.
33. Banting FG, Best CH. The internal secretions of the pancreas. *J Lab Clin Med.* 1922;7(5):465–80.
34. Fletcher AA, Campbell WR. Blood sugar following insulin administration and symptom complex-hypoglycemia. *J Metab. Res.* 1922;2:637–49.
35. Edwards DJ, Page IH. Observations on the circulation during hypoglycemia from large doses of insulin. *Am J Physiol.* 1924;177–89.
36. Abramson DI, Schkloven N, Margolis MN, Mirsky IA. Influences of massive doses of insulin on peripheral blood flow in man. *Am J Physiol.* 1939;128:124–32.
37. Allwood MJ, Ginsburg J, Paton A. The effect of insulin hypoglycemia on blood flow in intact and sympathectomized extremities in man. *J Physiol.* 1957;(1955):97–107.
38. Allwood MJ, Hensel H, Papenberg J. Muscle and skin blood flow in the human forearm during insulin hypoglycemia. *J Physiol.* 1959;147:269–73.

39. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol.* 1979;237(3):E214–E23.
40. Liang C, Doherty JU, Faillace R, Maekawa K, Arnold S, Gavras H, et al. Insulin infusion in conscious dogs. *J. Clin. Investig.* 1982;69:1321–36.
41. Creager MA, Liang CS, Coffman JD. Beta adrenergic-mediated vasodilator response to insulin in the human forearm. *J. Pharmacol. Exp. Ther.* 1985;235(3):709–14.
42. Yagi S, Takata S, Kiyokawa H, Yamamoto M, Noto Y, Ikeda T, et al. Effects of insulin on vasoconstrictive responses to norepinephrine and angiotensin II in rabbit femoral artery and vein. *Diabetes.* 1988;37:1064–7.
43. Baron AD, Laaksof M, Brechtel G, Hoit B, Watt C, Edelman SV. Reduced postprandial skeletal muscle blood flow contributes to glucose intolerance in human obesity. *J. Clin. Endocrinol. Metab.* 1990;70(6):1525–33.
44. Cleland SJ, Petrie JR, Ueda S, Elliott HL, Connell JMC. Insulin-mediated vasodilation and glucose uptake are functionally linked in humans. *Hypertension.* 1999;33:554–8.
45. Hickner RC, Ungerstedt U, Henriksson J. Regulation of skeletal muscle blood flow during acute insulin-induced hypoglycemia in the rat. *Diabetes.* 1994;43:1340–4.
46. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy M-A, Simonson DC, et al. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo. *Circulation.* 1998;97:1695–1701.
47. Vincent MA, Dawson D, Clark ADH, Lindner JR, Rattigan S, Clark MG, et al. Skeletal muscle microvascular recruitment by physiological hyperinsulinemia precedes increases in total blood flow. *Diabetes.* 2002;51:42–8.

48. Olver TD, Hazell TJ, Hamilton CD, Shoemaker JK, Lemon PWR. Impaired superficial femoral artery vasodilation and leg blood flow in young obese women following an oral glucose tolerance test. *Appl. Physiol. Nutr. Metab.* 2012;37:176–83.
49. Oomen PHN, Kant GD, Dullaart RPF, Reitsma WD, Smit AJ. Acute hyperglycemia and hyperinsulinemia enhance vasodilatation in Type 1 diabetes mellitus without increasing capillary permeability and inducing endothelial dysfunction. *Microvasc. Res.* 2002;63(1):1–9.
50. Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am J Physiol.* 2003;285:E123–E129.
51. Muniyappa R, Montagnani M, Koh KK, Quon MJ. Cardiovascular actions of insulin. *Endocr. Rev.* 2007;28(5):463–91.
52. Steinberg H, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Investig.* 1994;94:1172–9.
53. Heidarianpour A, Hajizadeh S, Khoshbaten A, Niaki AG, Bigdili MR, Pourkhalili K. Effects of chronic exercise on endothelial dysfunction and insulin signaling of cutaneous microvascular in streptozotocin-induced diabetic rats. *Eur. J. Cardiovasc. Prev. Rehabil.* 2007:746–52.
54. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature Letters.* 1999;399:601–5.
55. Cameron NE, Cotter MA, Low PA. Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am J Physiol.* 1991;261(1):E1–E8.

56. Saini AK, Arun KHS, Kaul CL, Sharma SS. Acute hyperglycemia attenuates nerve conduction velocity and nerve blood flow in male Sprague-Dawley rats: reversal by adenosine. *Pharmacol. Res.* 2004;50(6):593–9.
57. Tesfamariam B, Brown ML, Cohen RA. Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J. Clin. Investig.* 1991;87:1643–8.
58. Brodsky S V, Morrishow AM, Dharia N, Gross SS, Goligorsky MS. Glucose scavenging of nitric oxide. *Am. J. Physiol. Renal Physiol.* 2001;280(3):F480–F6.
59. Beckman JA, Goldfine AB, Gordon MB, Creager MA. Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation.* 2001;103(12):1618–23.
60. Davidson EP, Coppey LJ, Calcutt NA, Oltman CL, Yorek MA. Diet-induced obesity in Sprague – Dawley rats causes microvascular and neural dysfunction. *Diabetes. Metab. Res. Rev.* 2010;26:306–18.
61. Biessels GJ, Stevens EJ, Mahmood SJ, Gispen WH, Tomlinson DR. Insulin partially reverses deficits in peripheral nerve blood flow and conduction in experimental diabetes. *J. Neurol. Sci.* 1996;140:12–20.
62. Gregory JA, Jolivald CG, Goor J, Mizisin AP, Calcutt NA. Hypertension-induced peripheral neuropathy and the combined effects of hypertension and diabetes on nerve structure and function in rats. *Acta Neuropathol.* 2012;124:561–73.
63. Sasaki H, Schmelzer JD, Zollman PJ, Low PA. Neuropathology and blood flow of nerve, spinal roots and dorsal root ganglia in longstanding diabetic rats. *Acta Neuropathol.* 1997;93(2):118–28.
64. Zochodne DW, Ho LT. Normal blood flow but lower oxygen tension in diabetes of young rats: microenvironment and the influence of sympathectomy. *Can J Physiol Pharmacol.* 1992;70:651–9.

65. Zochodne DW, Cheng C, Sun H. Diabetes increases sciatic nerve susceptibility to endothelin-induced ischemia. *Diabetes*. 1996;45:627–32.
66. Chang K, Ido Y, LeJeune W, Williamson JR, Tilton RG. Increased sciatic nerve blood flow in diabetic rats: assessment by “molecular” vs particulate microspheres. *Am J Physiol*. 1997;273(36):E164–E173.
67. Pugliese G, Tilton RG, Speedy A, Chang K, Santarelli E, MA P, et al. Effects of very mild versus overt diabetes on vascular haemodynamics and barrier function in rats. *Diabetologia*. 1989;32(12):845–57.
68. Tilton RG, Chang K, Nyengaard JR, Van den Enden M, Ido Y, Williamson JR. Inhibition of sorbitol dehydrogenase. Effects on vascular and neural dysfunction in streptozocin-induced diabetic rats. *Diabetes*. 1995;44:234–42.
69. Singhal A, Cheng C, Sun H, Zochodne DW. Near nerve local insulin prevents conduction slowing in experimental diabetes. *Brain Res*. 1997;763:209–14.
70. Stevens EJ, Carrington AL, Tomlinson DR, Mary Q. Nerve ischaemia in diabetic rats: time-course of development, effect of insulin treatment plus comparison of streptozotocin and BB models. *Diabetologia*. 1994;37:43–8.
71. Kihara M, Low PA. Impaired vasoreactivity to nitric oxide in experimental diabetic neuropathy. *Exp. Neurol*. 1995;132:180–5.
72. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet*. 2001;358:221–9.
73. Cameron NE, Cotter MA, Ferguson K, Robertson S, Radcliffe MA. Effects of chronic α -adrenergic receptor blockade on peripheral nerve conduction, hypoxic resistance, polyols, Na^+ - K^+ -ATPase activity, and vascular supply in STZ-D rats. *Diabetes*. 1991;40:1652–8.
74. Obrosova IG, Van Huysen C, Fathallah L, Cao X, Stevens MJ, Greene DA. Evaluation of $\alpha(1)$ -adrenoceptor antagonist on diabetes- induced changes in

- peripheral nerve function , metabolism , and antioxidative defense. *FASEB J.* 2000;14:1548–58.
75. Cotter MA, Cameron NE. Correction of neurovascular deficits in diabetic rats by beta2-adrenoceptor agonist and alpha1-adrenoceptor antagonist treatment: interactions with the nitric oxide system. *Eur. J. Pharmacol.* 1998;343(2-3):217–23.
76. Robertson S, Cameron NE, Cotter MA. The effect of the calcium antagonist nifedipine on peripheral nerve function in streptozotocin-diabetic rats. *Diabetologia.* 1992;35(12):1113–7.
77. Kappelle AC, Biessels GJ, Van Buren T, Erkelens DW, De Wildt DJ, Gispen WH. Effects of nimodipine on sciatic nerve blood flow and vasa nervorum responsiveness in the diabetic rat. *Eur. J. Pharmacology.* 1993;250:43–9.
78. Ueno Y, Koike H, Nakamura Y, Ochi Y, Annoh S, Nishio S. Effects on diabetic of beraprost sodium, a prostacyclin analogue, on diabetic neuropathy in streptozotocin-induced diabetic rats. *Jpn J Pharmacol.* 1996;70:177–82.
79. Hotta N, Koh N, Sakakibara F, Nakamura J, Hamada Y, Wakao T, et al. Prevention of abnormalities in motor nerve conduction and nerve blood-flow by a prostacyclin analog, beraprost sodium, in streptozotocin-induced diabetic rats. *Prostaglandins.* 1995 ;49:339–49.
80. Stevens EJ, Tomlinson DR. Effects of endothelin receptor antagonism with bosentan peripheral nerve function in experimental diabetes. *Br. J Pharmacol.* 1995;115:373–9.
81. Cameron NE, Cotter MA. Effects of a nonpeptide antagonist neurovascular function in diabetic rats: interaction with the renin-angiotensin system. *J. Pharmacol. Exp. Ther.* 1996;278(3):1262–8.

82. Yamamoto T, Takakura S, Kawamura I, Seki J, Goto T. The effects of zenarestat, an aldose reductase inhibitor, on minimal F-wave latency and nerve blood flow in streptozotocin-induced diabetic rats. *Life Sci.* 2001;68:1439–48.
83. Yoshida M, Sugiyama Y, Akaike N, Ashizawa N, Aotsuka T, Ohbayashi S, et al. Amelioration of neurovascular deficits in diabetic rats by a novel aldose reductase inhibitor, GP-1447: minor contribution of nitric oxide. *Diabetes Res. Clin. Pract.* 1998;40:101–12.
84. Nakamura J, Koh N, Sakakibara F, Hamada Y, Wakao T, Sasaki H, et al. Diabetic neuropathy in sucrose-fed Otsuka Long-Evans Tokushima fatty rats: effect of an aldose reductase inhibitor, TAT. *Life Sci.* 1997;60(21):1847–57.
85. Nakamura J, Kato K, Hamada Y, Nakayama M, Chaya S, Nakashima E, et al. A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. *Diabetes.* 1999;48(10):2090–5.
86. Kihara M, Schmelzer JD, Poduslo JF, Curran GL, Nickander KK, Low PA. Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. *Proc Natl Acad Sci USA.* 1991;88:6107–11.
87. Cameron NE, Cotter MA. Effects of chronic treatment with a nitric oxide donor on nerve conduction abnormalities and endoneurial blood flow in streptozotocin-diabetic rats. *Eur J Clin Invest.* 1995;25(1):19–24.
88. Omawari N, Dewhurst M, Vo P, Mahmood S, Stevens E, Tomlinson DR. Deficient nitric oxide responsible for reduced nerve blood flow in diabetic rats: effects of L-NAME, L-arginine, sodium nitroprusside and evening primrose oil. *Br. J Pharmacol.* 1996;118(1):186–90.
89. Coppey LJ, Gallett JS, Davidson EP, Yorek MA. Preventing superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats restores endothelium-dependent vasodilation. *Free Radic Res.* 2003;37(1):33–40.

90. Cameron NE, Cotter MA. Effects of evening primrose oil treatment on sciatic nerve blood flow and endoneurial oxygen tension in streptozotocin-diabetic rats. *Acta Diabetol.* 1994;31:220–5.
91. Balducci S, Iacobellis G, Parisi L, Di Biase N, Calandriello E, Leonetti F, et al. Exercise training can modify the natural history of diabetic peripheral neuropathy. *J. Diabetes Complications.* 2006;20(4):216–23.
92. Severo Do Nascimento P, Lovatel GA, Ilha J, Schaan BD, Achaval M. Diabetes increases mechanical sensitivity and causes morphological abnormalities in the sural nerve that are prevented by treadmill training. *Muscle Nerve.* 2012;47:46–52.
93. Joslin EP. The treatment of diabetes mellitus. *Can. Med. Assoc. J.* 1914;6(8):808–11.
94. Joslin EP, Root EF, White P. The treatment of diabetes mellitus. Philadelphia: Lea & Febiger; 1959.
95. Riddell MC, Perkins BA. Type 1 diabetes and vigorous exercise: applications of exercise physiology to patient management. *Can. J. Diabetes.* Elsevier Masson SAS; 2006;30(1):63–71.

Chapter 2

2 « Review of literature »

2.1 Sciatic nerve vasculature

The sciatic nerve is the largest peripheral nerve in the human body and contains the largest arteria nervorum. Chief among the sciatic nerve feed arteries is the arteria nervi ischiadi. It stems from the inferior gluteal artery and gives rise to 1-6 arterial branches. The sciatic nerve also contains other smaller feed arteries that arise from the femoral and popliteal arteries (1,2). The intraneurial circulation originates from the aforementioned feed arteries at intervals along the sciatic nerve. Upon reaching the sciatic nerve, the vessels bifurcate into ascending and descending branches that form an intricate epineurial network of longitudinal arterioles. These arterioles can form arterio-venous anastomoses or branch into the nerve. The arterioles that branch into the nerve traverse obliquely through the perineurium and form an endoneurial network within nerve fascicles. The endoneurial network may contain arterio-venous anastomoses or form a capillary plexus that drains into an emerging vein. Nerve vascular channels may travel for a great distance along or within the nerve. Also, because capillary beds are sometimes absent, some of these vessels are considered to be a continuous anastomotic network (2). Various morphological approaches have been used to study the vasa nervorum and include counting and sizing microvessels using light or electron microscopy and a number of different perfusates (3–6). Radiographic and X-ray microtomographic images of the vasa nervorum also exist and describe the sciatic nerve vascular anatomy as well as the prospect of bidirectional flow (i.e. adjacent arteries

traveling in opposite directions along the same segment of the nerve) in animal models (2,7).

2.2 Sciatic nerve blood flow (NBF) measuring techniques

The sciatic nerve vasa nervorum consists of an epi-, peri- and endoneurial vasculature. Therefore, when assessing NBF it is important to consider what technique is being used and whether the data represent gross flow or flow to specific portions of the nerve. Numerous techniques have been developed and the methods used most commonly are hydrogen clearance, microsphere distribution, laser Doppler flowometry and Doppler ultrasound. The measurement of NBF is often invasive and requires sciatic nerve exposure. The following discussion pertains to measuring sciatic NBF in an animal model.

To assess NBF using the hydrogen clearance technique, hydrogen gas is added to an inspired gas mixture. As the animal inspires the hydrogen gas, the hydrogen concentration of the nerve is measured using a platinum electrode inserted into the sciatic nerve endoneurium. When the hydrogen concentration of the nerve is stable, this indicates equilibrium has been achieved between arterial and nerve hydrogen concentration. Once equilibrium is reached, the hydrogen gas is removed and the hydrogen clearance curve is measured until the concentration reaches pre-hydrogen inspiration values (8,9). Using the rate of hydrogen clearance, blood flow values can be calculated (8) and depending on the electrode placement, they are assumed to represent endoneurial NBF (9,10).

In addition to the hydrogen clearance technique, researchers often use the microsphere tracer technique to assess NBF (11–16). Briefly, radio or fluorescent labelled microspheres are infused into systemic circulation. Before and after the microsphere infusion, reference blood samples are collected to determine the microsphere concentration in arterial blood. At a designated time following the infusion, the sciatic nerve is harvested and the labelled microsphere concentration from the reference blood samples and the sciatic nerve (or specific portions of the nerve) can be used to calculate NBF (12,15,17,18).

At approximately the same time as the microsphere distribution technique was being developed, another method known as laser Doppler flowometry was being applied to measure NBF (19–22). Unlike the aforementioned methods, laser Doppler flowometry is an imaging technique that measures moving particles directly. To measure NBF, a probe is placed superficially over the sciatic nerve and a light source emits a monochromatic laser beam. Portions of the emitted light are absorbed by moving particles, such as red blood cells, and the remaining backscattered light is detected by a photo-detector. The frequency shift (i.e. the difference in the frequency of the emitted beam vs the frequency distribution of the backscattered beam) is used to calculate net flux (red blood cell number X velocity) and serves as a surrogate for NBF (19,23). Often, researchers remove the epineurium so the net flux reflects peri- and endoneurial flow exclusively (19,21,24).

Another advanced imaging technique being applied to measure NBF is Doppler ultrasound (25). This method was employed to examine NBF in the present dissertation; therefore, it is explained in detail here. The examination of NBF using Doppler

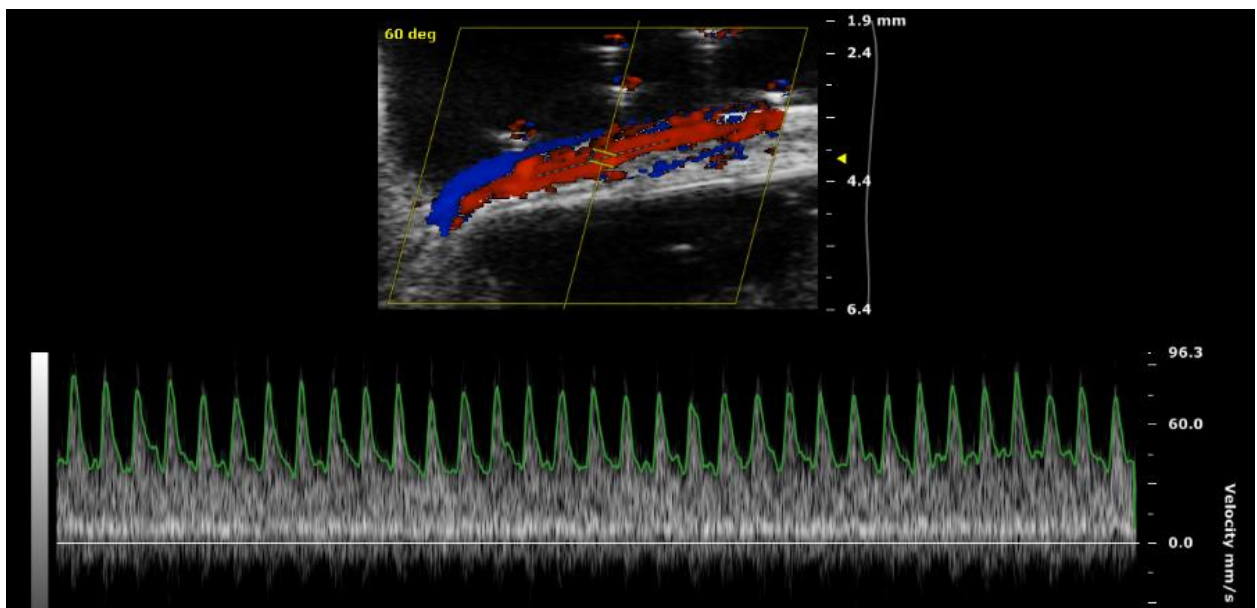
ultrasound occurs in three stages; identification of the nerve using B-mode imaging, location of the artery using power or colour Doppler and the measurement of NBF velocity through a delimited segment of the selected artery using pulsed-wave Doppler (25). Except where noted, the following theoretical descriptions of Doppler ultrasound come from the book *Medical Imaging Physics* (26) and the methodological explanations pertaining to the measurement of NBF were drawn from personal experience as well as a previous study by Twynstra and colleagues (25).

To image the sciatic nerve, a transducer containing piezoelectric crystals is placed superiorly to the dorsal aspect of the exposed sciatic nerve (coated with ultrasound gel). An electric current is applied to the transducer and the piezoelectric crystals vibrate resulting in the emission of mechanically produced high-frequency ultrasonic waves. These sound waves penetrate the surrounding tissues and are reflected by tissues in proportion to their density. The reflected sound waves are processed in terms of their distance from the probe and combined to form a B-mode image. This two-dimensional image is composed of bright dots representing the ultrasound echoes. The brightness of each dot is determined by the amplitude of the reflected echo/sound wave. This imaging technique is ideal for visualization and quantification of anatomical structures.

After the nerve has been identified the sciatic nerve vasculature is visualized using colour Doppler. This approach requires the calculation of the Doppler shift (see below) and provides a visual overview of flow within the nerve. Red blood cells moving towards to the transducer will reflect sound waves at greater frequencies than the original emitted frequency and be displayed in red. Conversely, red blood cells moving away from the transducer will reflect sound waves at lower frequencies than the original

emitted frequency and be displayed in blue. The intensity of the red and blue display reflects the velocity of the moving particles.

The flow velocity waveform of the moving particles can be examined using pulsed-wave Doppler. To achieve an optimal signal, the Doppler gate is positioned over the site of the high-power colour Doppler signal. The Doppler shift will be calculated from the sample volume demarcated by the gate. Briefly, following the identification of the nerve, visualization of an artery and the selection of an arterial segment, the Doppler shift and NBV velocity (NBV) data can be collected from a specific sciatic nerve arterial site (Figure 2-1).



The following section will briefly discuss potential limitations regarding the use of Doppler ultrasound (26). Red blood cell velocity is calculated as follows:

$$V = C (F_r - F_e) / (2F_e \times \cos\theta)$$

Figure 2-1 Colour Doppler screen shot taken in duplex imaging mode of the vasa nervorum

Where,

V = red blood cell velocity

C = the speed of sound in tissues (1540 m/s)

F_r = frequency of reflected sound waves

F_e = frequency of emitted sound wave

$\cos\theta$ = cosine of the angle of insonation

Consider that the speed of sound may vary in different tissues. However, such differences are not taken into account in the calculation of blood flow velocity. Rather, because the speed of sound in an average biological tissue is ~1540 m/s, this value is assumed to be generally representative. Both the sciatic nerve and the vasa nervorum may undergo structural changes as a result of ageing and prolonged diabetes, but whether this or other experimental conditions impact the speed of an emitted or reflected ultrasonic sound wave remains unknown.

In addition to its speed, the sound wave itself may also contribute to artifacts or measurement error. For example, sound waves may be attenuated. This occurs when sound waves are absorbed by surrounding tissues, the amplitude of the sound wave is decreased and the reflected signal is dampened. Attenuation also occurs when sound waves scatter. This is the diffusion or redirection of sound waves and occurs more often when the wavelength of a sound wave is larger than the tissue being sampled. The high-frequency transducer used to measure NBF in the studies reported in this dissertation was

operated at 32 MHz, which corresponds to a wavelength of 48 μm . In this case, because the sound wave is ~ 5 times larger than a red blood cell (6-12 μm), scattering is likely to occur. Attenuation (i.e. absorption and scattering) is an inherent limitation to Doppler ultrasound imaging and little can be done to avoid it. However, because the sciatic nerve vasa nervorum is relatively shallow (1-2 cm depth) and has a relatively low blood flow, absorption and scattering are unlikely to be limiting factors in the measurement of NBV.

There are other components that contribute to artifacts in the blood flow velocity signal, such as the angle of insonation, aliasing and motion artifacts, all of which can be accounted for partially by adjusting the Doppler ultrasound parameters. Generally, the ultrasound beam approaches the moving red blood cells at an angle (Doppler angle). This reduces the frequency shift in proportion to the cosine of this angle. To minimize error the preferred Doppler angle is $\leq 60^\circ$. At angles $>60^\circ$ the values for cosine θ change rapidly and minor errors in angle accuracy can result in large errors in the measurement of blood flow velocity (i.e. overestimating the angle of insonation produces a greater magnitude of error than underestimating) (27). Also, if the ultrasound beam is perpendicular to the direction of flow, the angle would be 90° and the cosine of 90° is equal to zero (i.e. a Doppler shift would not be detected). Thus, the appropriate Doppler angle is essential to obtain accurate measurements. To measure sciatic NBF in the studies described herein (Chapters 3, 4 & 5), the transducer was secured in a bracket positioned superiorly to the dorsal aspect of the sciatic nerve; the orientation of the transducer and the angle of insonation could be adjusted accordingly to achieve an optimal signal (Figure 2-1).

After positioning the Doppler gate over the site of the high power colour Doppler signal and achieving an appropriate Doppler angle, there are still other factors to consider. Excessive Doppler shift frequencies may also contribute to errors in flow velocity estimates. This phenomenon is known as aliasing and it occurs when the pulse repetition frequency (i.e. number of emitted sound waves per second) is too low to assess NBV accurately. The maximum Doppler shift or blood flow velocity that can be detected is half of the pulse repetition frequency (Nyquist limit). Therefore, using pulsed-wave Doppler, the pulse repetition frequency or sample rate may limit the estimation of flow velocity. This occurs more frequently when examining deeper tissues, as the sample rate will be limited by the time it takes for the sound to travel to and from the sample volume. However, because the sciatic nerve and the vasa nervorum are exposed, tissue depth is not an issue and an appropriate pulse repetition frequency can be selected.

The Doppler shift is calculated from the volume delimited by the Doppler gate or sample volume. However, because the sciatic nerve artery is too small to resolve its diameter quantitatively using B-mode or colour Doppler, it is possible that the sample volume will contain both red blood cells and the arterial wall. The pulsatile movement of the arterial wall may contribute to the spectrum of detected frequency shifts. To avoid this, an adjustable wall filter is applied that discounts selectively low frequency oscillations that occur at 40-50 Hz. Although this strategy will reduce spectral broadening on account of eliminating smaller frequency shifts, because the outer envelope of the mean blood flow velocity wave form is used when measuring NBV, the overall impact of wall motion on estimates of NBV is likely minimal.

It is important to note, that because the sciatic nerve arteries are too small to resolve their diameters quantitatively using B-mode or colour Doppler, only velocity (not flow) values can be obtained. Using 40 MHz technology, it has been demonstrated that the smallest vessels that can be resolved are $\sim 130 \mu\text{m}$ (28). Using the width of the power Doppler signal, the basal diameter of a superficial sciatic nerve artery has been estimated at $\sim 70 \mu\text{m}$. But because the resolution is limited to $\sim 15 \mu\text{m}/\text{pixel}$, changes in diameter that do not exceed 20% of the total diameter cannot be detected (25). Also, at this resolution changes in diameter can only be reported in $15 \mu\text{m}$ intervals. Limitations in the ability to assess diameter and changes in diameter accurately using Doppler ultrasound have resulted in values being reported in terms of velocity and not flow. This limits general vascular interpretations and instead of using vascular conductance (flow/pressure) researchers have been forced to use an index of vascular conductance (flow velocity/pressure) to form inferences with regards to what is occurring in downstream vascular beds (25).

Each of the aforementioned methodologies; hydrogen clearance, microsphere distribution technique, laser Doppler and Doppler ultrasound, have advantages and disadvantages. For example, to measure NBF using hydrogen clearance a paralytic agent known as d-tubocurarine is administered to reduce limb movement and a platinum electrode is inserted into the nerve which may potentially cause nerve as well as vasa nervorum damage (9,10,29). Neuromuscular blocking agents and nerve exposure have been investigated using the microsphere distribution technique and both conditions alter NBF. However, NBF is altered differentially in control rats vs rats with diabetes. The paralytic agent d-tubocurarine has no impact on NBF in control rats, but reduces NBF in

rats with diabetes. Likewise, there is a hyperemic response to surgical trauma in control rats, but this response is blunted in rats with diabetes (30). Conversely, limitations to the microsphere technique are that insufficient microspheres may be captured to assess flow accurately, dynamic changes in flow cannot be assessed and structural changes in the vasa nervorum in rats with diabetes may enhance microsphere capture resulting in increased NBF values artifactually (14,31,32). With regards to laser Doppler, it too requires nerve exposure and sometimes removal of the epineurium which may alter NBF values (19,21,33). In addition, because the sciatic nerve contains feed arteries arising from different directions and arteries as well as veins with flow traveling in opposite directions (2), laser Doppler measures of net flux are limited as they do not exclude venous contributions to total flow. To examine NBF using Doppler ultrasound, the sciatic nerve must also be exposed and only a segment of an artery can be investigated. Also, as discussed because arterial diameter cannot be determined, Doppler ultrasound relies on blood flow velocity and an index of vascular conductance ($VC_i = NBV / \text{blood pressure}$) to form inferences regarding downstream vascular control (25).

Divergent results between studies may be explained partially by differences in measurement technique. However, some studies have incorporated two measurement techniques, with each method arriving at the same conclusion (19). Conversely, other studies have used the same technique and documented conflicting results (10,34). To date no study has employed each method and validated or invalidated one or more techniques. Also, the contribution of any single measurement technique or the error associated with that technique to the overall outcome of a study remains unknown.

2.3 Autoregulation and basal NBF control

Under resting conditions organ blood flow tends to remain constant despite subtle changes in perfusion pressure. This phenomenon known as autoregulation (35) was documented in the early 1900s (36) and later described in detail by Rein in 1930 (37). Maintaining relatively constant blood flow to organs such as the kidney, liver, heart, brain facilitates nutrient exchange and the maintenance of capillary pressure which is important for fluid balance. In the 1960s five mechanisms/components were considered to contribute to autoregulation: 1) metabolic, 2) tissue pressure, 3) myogenic, 4) tubuloglomerular feedback from the kidney and 5) neural. Since then it is generally accepted that these mechanisms may operate together or independently in a tissue-specific manner to maintain tissue perfusion (35).

Unlike other tissues in the body sciatic NBF is not autoregulated. Instead, blood flow in the vasa nervorum responds passively to changes in arterial blood pressure (9,20,21,38,39). Using the hydrogen clearance method to measure NBF, Smith and co-workers (39) documented simultaneous reductions in blood pressure and NBF during exsanguination. In this study NBF values also increased at blood pressures between 80-110 mmHg. However, angiotensin-induced increases in blood pressure beyond 110 mmHg did not augment NBF further. It was speculated that NBF did not increase at blood pressures greater than 110 mmHg because of the vasoconstrictor effect of angiotensin. Thus, to avoid confounding effects of a vasoconstrictor, Low & Tuck (9) used the same measurement technique, but instead of a drug infusion they connected an arterial cannula to a reservoir (filled with heparinized saline or citrated rat whole blood) and adjusted the height of the reservoir to obtain hydrostatic pressures equivalent to 60-

160 mmHg (above the heart level). They documented a curvilinear relationship (convexity towards the abscissa) between NBF (y-axis) and blood pressure (x-axis) up to pressures of ~160 mmHg. Similar observations have been made using laser Doppler flowometry (20,21); however, these studies report a linear relationship between NBF and blood pressure between pressures of ~25-150 mmHg.

It is unclear why peripheral nerves do not have autoregulated NBF. Potentially, nerves do not rely on acute fluctuations in blood flow to meet changing metabolic requirements (40). Peripheral nerves are particularly resistant to ischemia (41,42) and NBF does not increase during hypoxia (9). Although sciatic NBF does increase modestly in response to nerve stimulation (16,43), the energy expenditure of nerves and the increase in NBF following stimulation is relatively low (16,41). Further, the peripheral nerve and vasa nervorum receive cholinergic input and express muscarinic receptors (44-47). Therefore, it is possible that nerve stimulation and neural transmission elicit an acetylcholine-induced, muscarinic receptor-dependent, NO-mediated vasa nervorum dilation (29,48).

The absence of autoregulation in the vasa nervorum is a hallmark feature of NBF control, but its significance remains unclear. Kihara and co-workers (40) documented that the relationship between blood pressure and NBF changes with age, such that during periods of growth NBF is greater for a given blood pressure. This may be explained by a relatively elevated metabolic demand or increased 'vascular volume:nerve tissue' ratio during such developmental stages (41). Despite these observations, the lack of autoregulatory control persists throughout the life span (40). Since these initial observations few studies have extended upon the general understanding of (non-)

autoregulatory sciatic NBF control (38,49). Instead, during this time period much of the vasa nervorum research has focused on the impact of diabetes and insulin treatment on basal NBF and vasa nervorum reactivity. How diabetes impacts the pressure-flow relationship in the vasa nervorum has not been studied extensively, but understanding this phenomena and how it is impacted by disease may help explain conflicting results between studies (i.e. it has been documented that experimental diabetes may decrease, increase or have no effect on basal NBF) (10,12–14,32,34,49–51), as well as clarify the link between hypertension and diabetes peripheral neuropathy (52).

2.4 Acute effects of glucose and insulin on NBF control

To test the effects of acute hyperglycemia on NBF, Cameron and colleagues (32) infused glucose intravenously until blood glucose concentrations reached values observed in untreated experimental diabetes (>30 mmol/L). Using the hydrogen clearance technique they measured NBF at baseline, peak glucose concentrations (>30 mmol/L) and in recovery. They documented ~50% reduction in NBF that corresponded to an increase in vascular resistance by ~180%. They also noted that, as blood glucose concentrations returned closer to baseline values, NBF and vascular resistance were gradually restored (32). In a similar study, using laser Doppler, Saini and co-workers (53) documented reductions in NBF following glucose infusion performed intravenously or through an intra-peritoneal injection. The peak glucose concentration obtained was ~10 mmol/L. Based on these findings, it appears only a modest level of hyperglycemia must be achieved to reduce NBF. In this study (53) the effect of glucose to reduce NBF was abolished by co-infusion with adenosine. Further, the restoration of NBF by

adenosine was abolished by co-infusion with theophylline (a non-selective adenosine receptor antagonist). Therefore, Saini and colleagues (53) concluded that acute hyperglycemia reduces NBF through an inhibitory effect on vasa nervorum adenosine receptors. Whereas the effects of adenosine on NBF have not been studied extensively, in the coronary vasculature it is generally accepted that adenosine receptors mediate NO release from the endothelium as well as smooth muscle hyperpolarization (54). In line with the aforementioned observations regarding NBF, acute hyperglycemia is also associated with impaired vascular function as a result of reduced NO functioning (55,56). In vitro, endothelial cell exposure to incremental hyperglycemia is associated with a progressive decline in NO availability (57) and NO-mediated vascular relaxation (58). Further, the impact of impaired NO function may extend beyond the immediate reduction in vasodilatory capacity and expose the vasa nervorum to the vasoconstrictor effect of other compounds, such as endothelin or norepinephrine (59–61). Therefore, reductions in NBF during acute hyperglycemia may be explained by a direct effect on adenosine receptor blockade, enhanced NO scavenging, impaired NO function and decreased smooth muscle cell hyperpolarization, as well as indirect effects of impaired dilatory capacity rendering the vasa nervorum more susceptible to vasoconstrictor influences.

The effects of glucose infusion on blood flow control may be different between the vasa nervorum and skeletal muscle vasculature. Specifically, acute rises in blood glucose appear to mediate a vasa nervorum constriction, but stimulate skeletal muscle vasodilation (62,63). Despite dissimilar responses to acute glucose administration, insulin stimulates vasodilation in both vascular beds (64–69). In skeletal muscle insulin-

mediated dilation is NO-dependent (65,68). Whether the same is true for the vasa nervorum remains unclear (69).

Acute and chronic hyperglycemia and potentially hypoinsulinemia impair NBF and nerve function (50,53,70,71). The vascular responses to glucose and insulin are fundamental to the etiology of diabetes peripheral neuropathy (10,13,14,50,72).

Exploring how glucose and insulin interact to modulate NBF and nerve function is necessary to gain an understanding of the pathology and potential treatment of diabetes peripheral neuropathy.

2.5 Effects of untreated experimental diabetes on NBF

Untreated experimental diabetes has been associated with greater (12–14), normal (22,34,73) as well as reduced resting NBF (10,32,49–51, 73). It is uncertain why such discrepancies exist, as conflicting results have been obtained using the same measurement technique (10,34) as well as similar models of untreated experimental diabetes (i.e. male Sprague-Dawley rats, STZ dose=45-60 mg/kg, diabetes duration= 4-6 weeks) (13,32,34). Following the induction of experimental diabetes, it is possible the presence (49) or absence (4) of sciatic nerve microangiopathy may account for dissimilar NBF values. However, from a metabolic stand-point it can be argued that the metabolic-milieu associated with experimental diabetes may increase, decrease or have no effect on resting NBF. A review of this topic can be found elsewhere (74).

Evidence to suggest experimental diabetes is associated with increased NBF comes largely from Williamson and colleagues (12–14) and relies on the microsphere distribution technique (discussed earlier). They speculated that diabetes-induced

elevations in NBF are caused by increased cytosolic reductive stress (14). Briefly, hyperglycemia results in increased sciatic nerve glucose uptake. Excess glucose in the nerve is reduced to sorbitol coupled with the oxidation of cofactor NADPH-to-NADP⁺ by the enzyme aldose reductase. Thereafter, sorbitol is oxidized to fructose coupled with the reduction of NAD⁺ to NADH by the enzyme sorbitol dehydrogenase. Thus, increased glucose metabolism through the sorbitol pathway results in increased formation of NADH (12,14,75). It has been postulated that the accumulation of NADH causes cytosolic reductive stress and may result in vasodilation through two primary mechanisms. Firstly, because this metabolic state (elevated NADH-to-NAD ratio) resembles the biochemical changes that result from hypoxia it may result in increased blood flow (75,76). However, Low and Tuck (9) documented previously that NBF does not autoregulate or respond to hypoxia; thus, the pseudo-hypoxic state caused by the cytosolic reductive stress is unlikely to modulate NBF control. The second proposed mechanism of vasodilation argues that reductive stress may result in an increase intracellular calcium concentration which could activate NO synthase. In addition, that elevated protein kinase C activation (which occurs as a result of hyperglycemia) (58) may contribute to the phosphorylation of endothelial NO synthase (75,77). However, this proposed mechanism is also controversial because both NO synthase and sorbitol synthesis rely on the oxidation of the cofactor NADPH and excess glucose metabolism may deplete NADPH stores resulting in decreased NO production (78).

In contrast to reports of elevated NBF, the evidence supporting the link between diabetes and reduced NBF implicate limited NO availability, but also many other factors. In endothelial cells hyperglycemia or elevated intracellular glucose metabolism may

increase reductive stress/pseudo-hypoxia (as described above), stimulate *de novo* diacylglycerol synthesis and subsequent protein kinase C activation, and result in the formation of advanced glycosylation end-products. These pathways are interrelated and the cumulative effect of chronic hyperglycemia may be reduced NO synthase activity, increased endothelin-1 activity, increased oxidative stress and microangiopathy (79).

To elucidate how exactly chronic hyperglycemia reduces NBF, many studies have focused on placebo-controlled pharmacological interventions. For example, the following is a list of pharmacological targets used to prevent diabetes-induced impairments in NBF: α 1-receptor antagonists (prazosin, doxazosin) (3,80), β 2-agonist (salbutamol) (81), catecholamine release inhibitor (guanethidine) (32), Ca^{+} channel antagonist (nifedipine) (82,83), prostacyclin analogue (beraprost) (84,85), endothelin receptor antagonist (bosentan) (86), endothelin-1a receptor antagonist, angiotensin II receptor antagonist (87), aldose reductase inhibitor (88–90), protein kinase C- β selective inhibitor (91), advanced glycosylated end-product inhibitor (aminoguanidine) (92), angiotensin converting enzyme inhibitor (cilazapril), NO donor treatment (isosorbide dinitrite) (93) and various compounds involved with lipid metabolism (94–96). Despite the wide variety of targets, many of the aforementioned treatments interact with and may enhance NO production or availability (81,92–95,97,98). Diabetes-induced oxidative stress may attenuate NBF through impaired NO synthesis or availability and because oxidative stress is multifactorial, any treatment that attenuates oxidative stress may have downstream effects on NO signaling in the vasa nervorum.

Regarding the effects of experimental diabetes on NBF control, recall that acute hyperglycemia may also impair NBF (32,53). If blood glucose was not normalized before

the NBF measurements were performed it would be impossible to discern between the acute and chronic effects of hyperglycemia. Therefore it may have been concluded inappropriately that the effects of diabetes were caused exclusively by chronic hyperglycemia, because the effects of acute hyperglycemia were not considered.

An attenuated or dysfunctional vasodilatory influence (i.e. impaired NO signaling) is the primary explanation for diabetes-related impairments in NBF. However, there is also the possibility of an enhanced vasoconstrictor influence. Topical application of adrenergic agonists, norepinephrine (non-selective α adrenergic agonist) and phenylephrine (α -1 adrenergic agonist), constrict the vasa nervorum in a dose-dependent manner (60,99). Whereas diabetes does not appear to enhance the constrictor response to phenylephrine (83), it does augment vasa nervorum responsiveness to exogenous (topical application) norepinephrine (61). However, in the aforementioned study enhanced responsiveness to norepinephrine was explained partially by defective endothelial NO signaling. Therefore, reductions in NBF following diabetes induction may reflect an interaction and imbalance between multiple vasodilator and vasoconstrictor influences. The potency and wide-spread damage of uncontrolled hyperglycemia may help explain the diversity of causes as well as successful treatments associated with diabetes-related decrements in NBF.

2.6 Insulin treatment and NBF

Whereas untreated experimental diabetes highlights the pronounced effects of chronic hyperglycemia, the physiological relevance of this model is questionable because the blood glucose values are $\sim \geq 2$ -fold greater (10,32,50) than values observed commonly in humans with poorly controlled diabetes (100). Therefore, it is essential to consider

those studies that include insulin treatment when evaluating how type 1 diabetes might impact NBF control. The sciatic nerve vasa nervorum contains insulin receptors (101), supporting the notion that insulin treatment may alter the pathophysiology of diabetes-induced vasa nervorum and nerve dysfunction. Insulin treatment initiated at the onset (71,102) or one month following the induction of experimental diabetes (50) attenuates diabetes-induced decrements in NBF. Insulin treatment also reduces blood glucose and by extension reduces glucose flux in the nerve (102). Reduced hyperglycemia and sciatic nerve glucose metabolism may result in less oxidative stress and therefore, attenuate NO scavenging. Accordingly, insulin treatment preserves vasa nervorum reactivity likely through a NO synthase mechanism (29) and possibly through increased sciatic nerve cyclic AMP content (102).

Acute and chronic exposure to insulin increases NBF (50,102,103), potentially through a NO mechanism (29,65,69,104). Though insulin does act as a vasodilator in the vasa nervorum (69), the dilatory properties of insulin may be enhanced by the simultaneous reduction in hyperglycemia associated with increasing insulin concentrations. To date, the independent contributions of glucose stimulated insulin secretion, hyperglycemia and hyperinsulinemia on NBF control are unknown.

Insulin remains the most successful and widely used treatment for type 1 diabetes. However, insulin treatment alone may be insufficient to protect individuals with type 1 diabetes from an elevated risk of developing vascular disease and nerve dysfunction (52,105). Combining insulin with drugs such as angiotensin converting enzyme inhibitors, statins and protein kinase C inhibitors has been used to treat complications associated with diabetes. These treatments are highly specific, may have negative side

effects and do not work in all individuals (105). This highlights a need to develop more effective treatment strategies for individuals with diabetes and in particular those with poorly controlled blood glucose, who are at a greater risk of developing diabetes-related complications.

2.7 Exercise and NBF

Prior to the discovery of insulin, Dr. E.P. Joslin prescribed repeated plantar flexion exercise to increase lower limb circulation in patients with type 1 diabetes (106). Following the discovery of insulin, he advocated regular exercise to help moderate blood glucose concentration and exogenous insulin requirements (107). More recently, it has been established that regular exercise may help avoid or attenuate the progression of nerve dysfunction in patients with type 1 diabetes (108) by preserving nerve fiber density, integrity and myelination (109). However, how exercise training impacts vasa nervorum function is not clear. Acute exercise improves nerve function in non-neuropathic patients with type 1 diabetes and it is believed that such improvements may be due to acute exercise-induced rises in NBF. Indeed, acute electrical nerve stimulation and skeletal muscle contraction increase NBF (16,43) and the vasodilation response to electrical stimulation remains intact in rats with type 1 diabetes. Whether repeated nerve stimulation and skeletal muscle contraction in the form of exercise training has the same effect (vasa nervorum dilation), or whether repeated increases in NBF improves vasa nervorum reactivity has not been studied. Exercise training improves brachial artery flow-mediated vasodilation (an indicator of endothelial function) in patients with type 1 diabetes (110). Likewise, regular exercise enhances insulin-mediated vasodilation in the skeletal muscle (111,112) and cutaneous tissue microvasculature (113) in control rats and

rats with type 2 and type 1 diabetes, respectively. Relative to type 1 diabetes, exercise training-induced increases in microvascular reactivity to insulin are attenuated with simultaneous infusion of PI3-K inhibitor wormannin or NO synthase inhibitor L-NNA (113). Based on these data, it appears exercise training potentiates insulin-mediated vasodilation through the activation of intracellular enzymes PI3-K and Akt, which phosphorylate and activate eNOS. The aforementioned experimental observations support Dr. E.P. Joslin's thesis that acute exercise improves lower limb circulation (106) and regular exercise potentiates the effects of insulin (indicated by a reduced need for exogenous insulin in individuals with type 1 diabetes who exercise regularly) (107). Theoretically, exercise training enhances microvascular responses to insulin in the vasa nervorum and improvements in microvascular function mediate improvements in nerve health.

2.8 Perspectives

Since the mid-1700s the potential significance of the vasa nervorum as it pertains to nerve function or dysfunction has been under examination (114). Whereas many of the early studies focused on the effects of nerve compression and vascular occlusion/ligation (115–119), the focus shifted over the next 50 years with the discovery of insulin treatment in the 1920s. As the life expectancy for humans with diabetes increased; on account of their vulnerability to peripheral nerve complications, questions regarding the impact of diabetes on vasa nervorum and nerve health emerged. Beginning in the 1950s and continuing today, an emphasis has been placed on the pathophysiology of diabetes-induced vascular disease in the vasa nervorum, as well as how such factors may contribute to overall nerve health (10,49,72,120–123). Despite the general success of

insulin treatment, individuals with type 1 diabetes are still exposed to a greater risk of developing vascular disease and diabetic peripheral neuropathy (52,105). This may occur as individuals with type 1 diabetes often experience moderate hyperglycemia (100), which impairs vascular function directly (53,58,79,124) as well as affecting insulin signaling adversely in both the vasa nervorum and the nerve (125–127). Thus, the benefits of insulin treatment on vascular health may be offset partially by poor glycemic control.

Whether poor glycemic control alters nerve function through a NBF mechanism is difficult to discern. Using a rodent model for type 1 diabetes nerve dysfunction has occurred alongside no change (34) increased (13,14) or decreased (10,32) NBF. Based on these observations, baseline NBF may not be a critical factor in the development of diabetes peripheral neuropathy, or the vascular etiology of diabetes peripheral neuropathy may change depending on the NBF status. Insulin treatment may attenuate or abolish diabetes-related alterations in NBF and nerve function (29,50,71). But insulin treatment alone is often insufficient and other treatments are often combined with insulin to help treat diabetes-related complications (50,105). That insulin treatment alone is often insufficient suggests insulin signaling in the vasa nervorum or the peripheral nerve is impaired by poor glycemic control. Also, that interventions focused on improving insulin signaling instead of inhibiting downstream targets of hyperglycemia may be more suitable to improve overall vascular and neuronal health outcomes (70,128,129). Unlike many pharmacological interventions, exercise training serves as an attractive intervention as it improves many aspects human health (130) including insulin sensitivity (131–133), vascular (110,134) and neuronal health in populations with type 1 diabetes (108,109). To

date, the effect of exercise training on insulin signaling in the vasa nervorum and vasa nervorum reactivity remains unknown. Potentially, the benefits of exercise training on nerve health are mediated in part by improvements in vasa nervorum insulin responsiveness.

2.9 Purpose and hypotheses

The purpose of the studies described herein (Chapters 3, 4 & 5) were to investigate the effects of acute and chronic hyperglycemia on basal NBF, as well as the vasoactive effects of insulin on NBF control in healthy rats and rats with insulin-treated experimental diabetes. Further, one study (Chapter 5) sought to determine if exercise training could prevent diabetes-induced declines in vasa nervorum function. These objectives were divided into three separate projects, with the following purposes and hypotheses:

1. Purpose: To determine the effects of insulin-treated experimental diabetes on basal NBF and assess NBF across a range of arterial blood pressures (i.e. examine the impact of diabetes in the absence of autoregulation).
 - a. It was hypothesized that insulin-treated experimental diabetes would reduce NBF modestly and attenuate the NBF response to dynamic changes in blood pressure.
2. Purpose: To assess to vasoactive effects of glucose and insulin on NBF control in a healthy state.
 - a. It was hypothesized that glucose infusion and the subsequent hyperglycemia would reduce NBF. However, insulin infusion would increase NBF.

3. Purpose: To examine the effects of insulin-treated experimental diabetes with and without endurance exercise training on vasa nervorum responsiveness to insulin.
 - a. It was hypothesized that insulin-treated experimental diabetes would reduce vasa nervorum reactivity to insulin, but deficits in vasoreactivity would be prevented by concurrent endurance exercise training.

2.10 References

1. Paille W. The blood supply to nerves. In: Schwartz C, Werthessen N, Wolf S, editors. *Struct. Funct. Circ.* Volume 1. New York and London: Plenum Press; 1980. p. 769–803.
2. Bell MA, Weddell AG. A descriptive study of the blood vessels of the sciatic nerve in the rat, man and other mammals. *Brain.* 1984;107:871–98.
3. Cameron NE, Cotter MA, Ferguson K, Robertson S, Radcliffe MA. Effects of chronic α -adrenergic receptor blockade on peripheral nerve conduction, hypoxic resistance, polyols, Na^+ - K^+ -ATPase activity, and vascular supply in STZ-D rats. *Diabetes.* 1991;40:1652–8.
4. Zochodne DW, Nguyen C. Increased peripheral nerve microvessels in early experimental diabetic neuropathy: quantitative studies of nerve and dorsal root ganglia. *J. Neurol. Sci.* 1999;166:40–6.
5. Zochodne DW, Nguyen C. Angiogenesis at the site of neuroma formation in transected peripheral nerve. *J Anat.* 1997 191(1):23–30.
6. Sugimoto K, Yagihashi S. Effects of aminoguanidine on structural alterations of microvessels in peripheral nerve of streptozotocin diabetic rats. *Microvasc. Res.* 1997;53:105–12.
7. Zamir M, Twynstra J, Vercnocke AJ, Welch I, Jorgensen SM, Ritman EL, et al. Intrinsic microvasculature of the sciatic nerve in the rat. *J Peripher Nerv Syst.* 2012;17:377–84.
8. Aukland K, Bower BF, Berliner RW. Measurement of local blood flow with hydrogen gas. *Circ. Res.* 1964;14(2):164–87.
9. Low PA, Tuck RR. Effects of changes of blood pressure, respiratory acidosis and hypoxia on blood flow in the sciatic nerve of the rat. *J Physiol.* 1984;347:513–24.

10. Tuck RR, Schmelzer JD, Low PA. Endoneurial blood flow and oxygen tension in the sciatic nerves of rats with experimental diabetic neuropathy. *Brain*. 1984;107:935–50.
11. Suter SP, Chang K, Marvel J, Williamson JR. Concurrent in diabetic increases in regional hematocrit rats: prevention by sorbinil and blood flow. *Am J Physiol*. 1992;263:H945–H950.
12. Pugliese G, Tilton RG, Speedy A, Chang K, Santarelli E, MA Province, et al. Effects of very mild versus overt diabetes on vascular haemodynamics and barrier function in rats. *Diabetologia*. 1989;32(12):845–57.
13. Tilton RG, Chang K, Nyengaard JR, Van den Enden M, Ido Y, Williamson JR. Inhibition of sorbitol dehydrogenase. Effects on vascular and neural dysfunction in streptozocin-induced diabetic rats. *Diabetes*. 1995;44:234–42.
14. Chang K, Ido Y, LeJeune W, Williamson JR, Tilton RG. Increased sciatic nerve blood flow in diabetic rats: assessment by “molecular” vs particulate microspheres. *Am J Physiol*. 1997;273(36):E164–E173.
15. Sladky JT, Greenberg JH, Brown MJ. Regional perfusion in normal and ischemic rat sciatic nerves. *Ann. Neurol*. 1985;17:191–5.
16. Sugimoto H, Monafo WW, Shimazaki S. Regional sciatic nerve blood flow response to limb movement. *Am J Physiol*. 1987;252:H439–H441.
17. Rudolph AM, Heymann MA. The circulation of the fetus in utero: methods for studying distribution of blood flow, cardiac output and organ blood flow. *Circ. Res*. 1967;21(2):163–84.
18. McDevitt DG, Nies AS. Simultaneous measurement of cardiac output and its distribution with microspheres in the rat. *Cardiovasc. Res*. 1976;10(4):494–8.

19. Rundquist I, Smith QR, Michel ME, Ask P, Oberg PA, Rapoport SI. Sciatic nerve blood flow measured by laser Doppler flowmetry and [14C]iodoantipyrine. *Am J Physiol.* 1985;248(3(2)):H311–H317.
20. Takeuchi M, Low PA. Dynamic peripheral nerve metabolic and vascular responses to exsanguination. *Am J Physiol.* 1987;16:E349–E353.
21. Sundqvist T, Oberg PA, Rapoport SI. Blood flow in rat sciatic nerve during hypotension. *Exp. Neurol.* 1985;90:139–48.
22. Zochodne DW, Cheng C, Sun H. Diabetes increases sciatic nerve susceptibility to endothelin-induced ischemia. *Diabetes.* 1996;45:627–32.
23. Drain LE. *The laser Doppler techniques.* Chichester, New York: Wiley; 1980.
24. Stolarczyk, A. Papierski, K. Adamczyk, G. Sinski, M. Sawionek, L. Przybylski J. Functional studies on sciatic nerve blood flow in respect to its vascular supply and tonic neural activity. *J. Physiol. Pharmacol.* 2000;51(3):483–9.
25. Twynstra J, Medeiros PJ, Lacefield JC, Jackson DN, Shoemaker JK. Y1R control of sciatic nerve blood flow in the Wistar Kyoto rat. *Microvasc. Res.* 2012;84(2):133–9.
26. Hendee W, Ritenour E. *Medical Imaging Physics.* Fourth Edi. New York: Wiley-Liss; 2002. p. 303–53.
27. Shoemaker JK, Zlatoko P, Highson RL. Forearm blood flow by Doppler ultrasound during rest and exercise: tests of day-to-day repeatability. 1996;28(9):1144–9.
28. Moran CM, Pye SD, Ellis W, Janeczko A, Morris KD, McNeilly AS, et al. A comparison of the imaging performance of high resolution ultrasound scanners for preclinical imaging. *Ultrasound Med. Biol.* 2011;37(3):493–501.

29. Kihara M, Low PA. Impaired vasoreactivity to nitric oxide in experimental diabetic neuropathy. *Exp. Neurol.* 1995;132:180–5.
30. Ido Y, Chang K, Lejeune W, Tilton RG, Monafo WW, Williamson JR. Diabetes impairs surgical trauma: sciatic nerve hyperemia implications for diabetic induced by neuropathy. *Am J Physiol.* 1997;36:E174–E184.
31. Cameron NE, Cotter MA. The relationship of vascular changes to metabolic factors in diabetes mellitus and their role in the development of peripheral nerve complications. *Diabetes. Metab. Rev.* 1994;10(3):189–224.
32. Cameron NE, Cotter MA, Low PA. Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am J Physiol.* 1991;261(1):E1–E8.
33. Stolarczyk A, Papierski K, Adamczyk G, Sinski M, Sawionek L, Przybylski J. Functional studies on sciatic nerve blood flow in respect to its vascular supply and tonic neural activity. *J. Physiol. Pharmacol.* 2000;51(3):483–9.
34. Zochodne DW, Ho LT. Normal blood flow but lower oxygen tension in diabetes of young rats: microenvironment and the influence of sympathectomy. *Can J Physiol Pharmacol.* 1992;70:651–9.
35. Johnson PC. Autoregulation of blood flow. *Circ. Res.* 1986;59:483–95.
36. Bayliss WM. On the local reactions of the arterial wall to changes of internal pressure. *J Physiol.* 1901;28(3):225–31.
37. Rein H. Vasomotorische Regulationen. *Ergebnisse der Physiol.* 1931;32(1):28–72.
38. McManis PG, Schmelzer JD, Zollman PJ, Low PA. Blood flow and autoregulation in somatic and autonomic ganglia Comparison with sciatic nerve. *Brain.* 1997;120:445–9.
39. Smith DR, Kobrine AI, Rizzoli HV. Absence of autoregulation in peripheral nerve blood flow. *J. Neurol. Sci.* 1977;33:347–52.

40. Kihara M, Weerasuriya A, Low PA. Endoneurial blood flow in rat sciatic nerve during development. *J Physiol.* 1991;439:351–60.
41. Low PA, Schmelzer JD, Ward KK. The effect of age on energy metabolism and resistance to ischemic conduction failure in rat peripheral nerve. *J Physiol.* 1986;374:263–71.
42. Seneviratne KN, Peiris OA. The effect of ischaemia on the excitability of human sensory nerve. *J. Neurol. Neurosurgery, Psychiatry* . 1968;31(4):338–47.
43. Monafo WW, Eliasson SG, Shimazaki S, Sugimoto H. Regional blood flow in resting and stimulated sciatic nerve of diabetic rats. *Exp. Neurol.* 1988;99(3):607–14.
44. Amenta F, Mione M, Napoleone P. The autonomic innervation of the vasa nervorum. *J. Neural Transm.* 1983;58:291–7.
45. Gulya K, Kása P. Transport of muscarinic cholinergic receptors in the sciatic nerve of rat. *Neurochem. Int.* 1984;6(1):123–6.
46. Wamsley JK, Zarbin MA, Kuhar MJ. Muscarinic cholinergic receptors flow in the sciatic nerve. *Brain Res.* 1981;217:155–61.
47. Beetson KA, Smith SF, Muneer A, Cameron NE, Cotter MA, Celtek S. Vasa nervorum in rat major pelvic ganglion are innervated by nitrenergic nerve fibers. *J. Sex. Med.* 2013;10(12):2967-2974.
48. Thomsen K, Rubin I, Lauritzen M. In vivo mechanisms of acetylcholine-induced vasodilation in rat sciatic nerve. *Am J Physiol.* 2000;279:H1044–H1054.
49. Gregory JA, Jolivald CG, Goor J, Mizisin AP, Calcutt NA. Hypertension-induced peripheral neuropathy and the combined effects of hypertension and diabetes on nerve structure and function in rats. *Acta Neuropathol.* 2012;124:561–73.

50. Biessels GJ, Stevens EJ, Mahmood SJ, Gispen WH, Tomlinson DR. Insulin partially reverses deficits in peripheral nerve blood flow and conduction in experimental diabetes. *J. Neurol. Sci.* 1996;140:12–20.
51. Sasaki H, Schmelzer JD, Zollman PJ, Low PA. Neuropathology and blood flow of nerve, spinal roots and dorsal root ganglia in longstanding diabetic rats. *Acta Neuropathol.* 1997;93(2):118–28.
52. Tesfaye S, Chaturvedi N, Eaton SE, Ward JD, Manes C, Lonescu-Tirgoviste C, et al. Vascular risk factors and diabetic neuropathy. *N Engl J Med.* 2005;352(4):341–50.
53. Saini AK, Arun KHS, Kaul CL, Sharma SS. Acute hyperglycemia attenuates nerve conduction velocity and nerve blood flow in male Sprague-Dawley rats: reversal by adenosine. *Pharmacol. Res.* 2004;50(6):593–9.
54. Wilson CN, Mustafa SJ. Adenosine receptors in health and disease. *Handb Exp Pharmacol.* 193rd ed. London, New York: Springer; 2009. p. 161–89.
55. Giugliano D, Marfella R, Coppola L, Verrazzo G, Acampora R, Giunta R, et al. Vascular effects of acute hyperglycemia in humans are reversed by L-arginine. *Circulation*. 1997;95:1783–90.
56. Beckman JA, Goldfine AB, Gordon MB, Creager MA. Ascorbate restores endothelium-dependent vasodilation impaired by acute hyperglycemia in humans. *Circulation* . 2001;103(12):1618–23.
57. Brodsky SV, Morrishow AM, Dharia N, Gross SS, Goligorsky MS. Glucose scavenging of nitric oxide. *Am. J. Physiol. Renal Physiol.* 2001;280(3):F480–F486.
58. Tesfamariam B, Brown ML, Cohen RA. Elevated glucose impairs endothelium-dependent relaxation by activating protein kinase C. *J. Clin. Investig.* 1991;87:1643–8.

59. Zochodne DW, Ho LT, Gross PM. Acute endoneurial ischemia induced endothelin in the rat sciatic nerve by epineurial. *Am J Physiol.* 1992;32:H1806–H1810.
60. Zochodne DW, Low PA. Adrenergic control of nerve blood flow. *Exp. Neurol.* 1990;109:300–7.
61. Maxfield EK, Cameron NE, Cotter MA. Effects of diabetes on reactivity of sciatic vasa nervorum in rats. *J. Diabetes Complicat.* 1997;11(1):47–55.
62. Olver TD, Hazell TJ, Hamilton CD, Shoemaker JK, Lemon PWR. Impaired superficial femoral artery vasodilation and leg blood flow in young obese women following an oral glucose tolerance test. *Appl. Physiol. Nutr. Metab.* 2012;37:176–83.
63. Baron AD, Laaksof M, Brechtel G, Hoit B, Watt C, Edelman S V. Reduced postprandial skeletal muscle blood flow contributes to glucose intolerance in human obesity. *J. Clin. Endocrinol. Metab.* 1990;70(6):1525–33.
64. Abramson DI, Schkloven N, Margolis MN, Mirsky IA. Influences of massive doses of insulin on peripheral blood flow in man. *Am J Physiol.* 1939;128:124–32.
65. Steinberg H, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Investig.* 1994;94:1172–9.
66. Creager MA, Liang CS, Coffman JD. Beta adrenergic-mediated vasodilator response to insulin in the human forearm. *J. Pharmacol. Exp. Ther.* 1985;235(3):709–14.
67. Liang C, Doherty JU, Faillace R, Maekawa K, Arnold S, Gavras H, et al. Insulin infusion in conscious dogs. *J. Clin. Investig.* 1982;69:1321–36.
68. Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am J Physiol.* 2003;285:E123–E129.

69. Davidson EP, Coppey LJ, Calcutt NA, Oltman CL, Yorek MA. Diet-induced obesity in Sprague – Dawley rats causes microvascular and neural dysfunction. *Diabetes. Metab. Res. Rev.* 2010;26:306–18.
70. Brussee V, Cunningham FA, Zochodne DW. Direct insulin signaling of neurons reverses diabetic neuropathy. *Diabetes.* 2004;53:1824–30.
71. Stevens EJ, Carrington AL, Tomlinson DR, Mary Q. Nerve ischaemia in diabetic rats: time-course of development, effect of insulin treatment plus comparison of streptozotocin and BB models. *Diabetologia.* 1994;37:43–8.
72. Fagerberg SE. Studies on the pathogenesis of diabetic neuropathy. *Acta Med. Scand.* 1956;156(4):295–302.
73. Kalichman MW, Dines KC, Bobik M, Mizisin AP. Nerve conduction velocity, laser Doppler flow, and axonal caliber in galactose and streptozotocin diabetes. *Brain Res.* 1998;810:130–7.
74. Zochodne DW. Nerve and ganglion blood flow in diabetes: an appraisal. *Int. Rev. Neurobiol.* 2002;50:161–202.
75. Williamson JR, Chang C, Frangos M, Hasa KS, Ido Y, Kawamura T, et al. Perspectives in diabetes hyperglycemic pseudohypoxia and diabetic complications. *Diabetes.* 1993;42:801–13.
76. Cameron NE, Cotter MA. The relationship of vascular changes to metabolic factors in diabetes mellitus and their role in the development of peripheral nerve complications. *Diabetes. Metab. Rev.* 1994;10(3):189–224.
77. Nakane M, Mitchell J, Forstermann U, Murad F, Laboratories A, Park A. Phosphorylation by calcium calmodulin-dependent protein kinase II and protein kinase C modulates the activity of nitric oxide synthase. *Biochem. Biophys. Res. Commun.* 1991;180(3):1396–402.

78. Stevens MJ, Dananberg J, Feldman EL, Lattimer SA, Kamijo M, Thomas TP, et al. The linked roles of nitric oxide, aldose reductase and (Na⁺, K⁺)-ATPase in the slowing of nerve conduction in the streptozotocin diabetic rat. *J. Clin. Investig.* 1994;94:853–9.
79. Schalkwijk CG, Stehouwer CDA. Vascular complications in diabetes mellitus: the role of endothelial dysfunction. *Clin. Sci.* 2005;109:143–59.
80. Obrosova IG, Van Huysen C, Fathallah L, Cao X, Stevens MJ, Greene DA. Evaluation of alpha(1)-adrenoceptor antagonist on diabetes- induced changes in peripheral nerve function , metabolism , and antioxidative defense. *FASEB J.* 2000;14:1548–58.
81. Cotter MA, Cameron NE. Correction of neurovascular deficits in diabetic rats by beta2-adrenoceptor agonist and alpha1-adrenoceptor antagonist treatment: interactions with the nitric oxide system. *Eur. J. Pharmacol.* 1998;343(2-3):217–23.
82. Robertson S, Cameron NE, Cotter MA. The effect of the calcium antagonist nifedipine on peripheral nerve function in streptozotocin-diabetic rats. *Diabetologia.* 1992;35(12):1113–7.
83. Kappelle AC, Biessels GJ, Van Buren T, Erkelens DW, De Wildt DJ, Gispen WH. Effects of nimodipine on sciatic nerve blood flow and vasa nervorum responsiveness in the diabetic rat. *Eur. J. Pharmacology* 1993;250:43–9.
84. Ueno Y, Koike H, Nakamura Y, Ochi Y, Annoh S, Nishio S. Effects on diabetic of beraprost sodium, a prostacyclin analogue, on diabetic neuropathy in streptozotocin-induced diabetic rats. *Jpn J Pharmacol.* 1996;70:177–82.
85. Hotta N, Koh N, Sakakibara F, Nakamura J, Hamada Y, Wakao T, et al. Prevention of abnormalities in motor nerve conduction and nerve blood-flow by a prostacyclin analog, beraprost sodium, in streptozotocin-induced diabetic rats. *Prostaglandins.* 1995;49:339–49.

86. Stevens EJ, Tomlinson DR. Effects of endothelin receptor antagonism with bosentan peripheral nerve function in experimental diabetes. *Br. J Pharmacol.* 1995;115:373–9.
87. Cameron NE, Cotter MA. Effects of a nonpeptide antagonist neurovascular function in diabetic rats: interaction with the renin-angiotensin system. *J. Pharmacol. Exp. Ther.* 1996;278(3):1262–8.
88. Yamamoto T, Takakura S, Kawamura I, Seki J, Goto T. The effects of zenarestat, an aldose reductase inhibitor, on minimal F-wave latency and nerve blood flow in streptozotocin-induced diabetic rats. *Life Sci.* 2001;68:1439–48.
89. Yoshida M, Sugiyama Y, Akaike N, Ashizawa N, Aotsuka T, Ohbayashi S, et al. Amelioration of neurovascular deficits in diabetic rats by a novel aldose reductase inhibitor, GP-1447: minor contribution of nitric oxide. *Diabetes Res. Clin. Pract.* 1998;40:101–12.
90. Nakamura J, Koh N, Sakakibara F, Hamada Y, Wakao T, Sasaki H, et al. Diabetic neuropathy in sucrose-fed Otsuka Long-Evans Tokushima fatty rats: effect of an aldose reductase inhibitor, TAT. *Life Sci.* 1997;60(21):1847–57.
91. Nakamura J, Kato K, Hamada Y, Nakayama M, Chaya S, Nakashima E, et al. A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. *Diabetes.* 1999;48(10):2090–5.
92. Kihara M, Schmelzer JD, Poduslo JF, Curran GL, Nickander KK, Low PA. Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. *Proc Natl Acad Sci USA.* 1991;88:6107–11.
93. Cameron NE, Cotter MA. Effects of chronic treatment with a nitric oxide donor on nerve conduction abnormalities and endoneurial blood flow in streptozotocin-diabetic rats. *Eur J Clin Invest.* 1995;25(1):19–24.

94. Omawari N, Dewhurst M, Vo P, Mahmood S, Stevens E, Tomlinson DR. Deficient nitric oxide responsible for reduced nerve blood flow in diabetic rats: effects of L-NAME, L-arginine, sodium nitroprusside and evening primrose oil. *Br. J Pharmacol.* 1996;118(1):186–90.
95. Coppey LJ, Gellett JS, Davidson EP, Yorek MA. Preventing superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats restores endothelium-dependent vasodilation. *Free Radic Res.* 2003;37(1):33–40.
96. Cameron NE, Cotter MA. Effects of evening primrose oil treatment on sciatic nerve blood flow and endoneurial oxygen tension in streptozotocin-diabetic rats. *Acta Diabetol.* 1994;31:220–5.
97. Kihara M, Mitsui MK, Mitsui Y, Okuda K, Nakasaka Y, Takahashi M, et al. Altered vasoreactivity to angiotensin II in experimental diabetic neuropathy: role of nitric oxide. *Muscle Nerve.* 1999;22:920–5.
98. Cameron NE, Cotter MA. Rapid reversal by aminoguanidine of the neurovascular effects of diabetes in rats: modulation by nitric oxide synthase inhibition. *Metabolism.* 1996;45(9):1147–52.
99. Kihara M, Low PA. Regulation of rat nerve blood flow: role of epineurial α -receptors. *J Physiol.* 1990;422:145–52.
100. Riveline J-P, Schapelynck P, Chaillous L, Renard E, Sola-Gazagnes A, Penfornis A, et al. Assessment of patient-led or physician-driven continuous glucose monitoring in patients with poorly controlled type 1 diabetes using basal-bolus insulin regimens. *Diabetes Care.* 2012;35(5):965–71.
101. Sugimoto K, Murakawa Y, Zhang W, Xu G, Sima AAF. Insulin receptor in rat peripheral nerve: its localization and alternatively spliced isoforms. *Diabetes. Metab. Res. Rev.* 2000;16:354–63.
102. Hotta N, Koh N, Sakakibara F, Nakamura J, Hamada Y, Hara T, et al. Effects of beraprost sodium and insulin on the electroretinogram, nerve conduction, and

- nerve blood flow in rats with streptozotocin-induced diabetes. *Diabetes*. 1996;45(3):361–6.
103. Kihara M, Zollman PJ, Smithson IL, Langerlund TD, Low PA. Hypoxic effect of exogenous insulin and diabetic peripheral nerve on normal. *Am J Physiol*. 1994;29:E980–E985.
 104. Ii M, Nishimura H, Kusano KF, Qin G, Yoon Y, Wecker A, et al. Neuronal nitric oxide synthase mediates statin-induced restoration of vasa nervorum and reversal of diabetic neuropathy. *Circulation* . 2005;112:93–102.
 105. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* . 2001;358:221–9.
 106. Joslin EP. The treatment of diabetes mellitus. *Can. Med. Assoc. J.* 1914;6(8):808–11.
 107. Joslin EP, Root EF, White P. The treatment of diabetes mellitus. Philadelphia: Lea & Febiger; 1959.
 108. Balducci S, Iacobellis G, Parisi L, Di Biase N, Calandriello E, Leonetti F, et al. Exercise training can modify the natural history of diabetic peripheral neuropathy. *J. Diabetes Complications*. 2006;20(4):216–23.
 109. Severo Do Nascimento P, Lovatel GA, Ilha J, Schaan BD, Achaval M. Diabetes increases mechanical sensitivity and causes morphological abnormalities in the sural nerve that are prevented by treadmill training. *Muscle Nerve* . 2012;47:46–52.
 110. Fuchsjager-Mayrl G, Pleiner J, Wiesinger GF, Sieder AE, Quittan M, Nuhr MJ, et al. Exercise training improves endothelial function in patients with type 1 diabetes. *Diabetes Care*. 2002;25(10):1795–801.
 111. Mikus CR, Fairfax ST, Libla JL, Boyle LJ, Vianna LC, Oberlin DJ, et al. Seven days of aerobic exercise training improves conduit artery blood flow following

- glucose ingestion in patients with type 2 diabetes. *J Appl Physiol.* 2011;111:657–64.
112. Rattigan S, Wallis MG, Youd JM, Clark MG. Exercise training improves insulin-mediated capillary recruitment in association with glucose uptake in rat hindlimb. *Diabetes.* 2001;50(12):2659–65.
 113. Heidarianpour A, Hajizadeh S, Khoshbaten A, Niaki AG, Bigdili MR, Pourkhalili K. Effects of chronic exercise on endothelial dysfunction and insulin signaling of cutaneous microvascular in streptozotocin-induced diabetic rats. *Eur. J. Cardiovasc. Prev. Rehabil.* 2007;14:746–52.
 114. Isenflamm J, Doerffler J. *De vasis nervorum.* Erlangen. Erlangen; 1768.
 115. Lewis T, Pickering G, Rothschild P. Observations upon muscular pain in intermittent claudication. *Heart.* 1931;15:359–83.
 116. Reid C. Experimental ischemia: sensory phenomena, fibrillary twitchings, and effects on pulse, respiration and blood-pressure. *Quart. J. Exper. Physiol.* 1931;21:243.
 117. Bentley FH, Schlapp W. Experiments on the blood supply of nerves. *J Physiol.* 1942;102:62–71.
 118. Denny-Brown D, Brenner C. The effect of percussion on nerve. *J. Neurol. Neurosurg. Psychiatry.* 1944;7(3-4):76–95.
 119. Blunt MJ. Functional and clinical implications of the vascular anatomy of nerves. *Postgrad. Med. J.* 1957;33(376):68–72.
 120. Fagerburg SE. Studies on the pathogenesis of diabetic neuropathy. *Acta Med. Scand.* 1957;159(1):59–62.

121. Fagerberg SE. Diabetic neuropathy: a clinical and histological study on the significance of vascular affections. *Acta Med. Scand.* 1959;94(164 suppl. 345):1–97.
122. Gregersen G. A study of the peripheral nerves in diabetic subjects during ischaemia. *J Neurol Neurosurg Psychiat.* 1968;31:175–81.
123. Greene DA, Lattimer SA. Sodium- and energy-dependent uptake of myo-inositol by rabbit peripheral nerve. Competitive inhibition by glucose and lack of an insulin effect. *J. Clin. Invest.* 1982;70(5):1009–18.
124. Williams SB, Goldfine AB, Timimi FK, Ting HH, Roddy M-A, Simonson DC, et al. Acute hyperglycemia attenuates endothelium-dependent vasodilation in humans in vivo. *Circulation.* 1998;97:1695–701.
125. Federici M. Insulin-dependent activation of endothelial nitric oxide synthase is impaired by O-linked glycosylation modification of signaling proteins in human coronary endothelial cells. *Circulation.* 2002;106(4):466–72.
126. Bakker W, Eringa EC, Sipkema P, van Hinsbergh VWM. Endothelial dysfunction and diabetes: roles of hyperglycemia, impaired insulin signaling and obesity. *Cell Tissue Res.* 2009;335(1):165–89.
127. Nawano M, Ueta K, Oku A, Arakawa K, Saito A, Funaki M, et al. Hyperglycemia impairs the insulin signaling step between PI 3-kinase and Akt/PKB activations in ZDF rat liver. *Biochem. Biophys. Res. Commun.* 1999;266:252–6.
128. Sugimoto K, Baba M, Suzuki S, Yagihashi S. The impact of low-dose insulin on peripheral nerve insulin receptor signaling in streptozotocin-induced diabetic rats. *PLoS One.* 2013;8(8):1–12.
129. Pierson CR, Zhang W, Murakawa Y, Sima AAF. Insulin deficiency rather than hyperglycemia accounts for impaired neurotrophic responses and nerve fiber regeneration in type 1 diabetic neuropathy. *J. Neuropathol. Exp. Neurol.* 2003;62(3):260–71.

130. Booth FW, Gordon SE, Carlson CJ, Hamilton MT. Waging war on modern chronic diseases: primary prevention through exercise biology. *J Appl Physiol.* 2000;88(2):774–87.
131. Wasserman D, Davis S, Zinman B. Fuel metabolism during exercise in health and diabetes. In: Ruderman NB, Devlin JT, Schneider S, et al., editors. *Handb Exer Diabetes*. Alexandria, VA: American Diabetes Association; 2002. p. 63–100.
132. MacDonald MJ. Postexercise late-onset hypoglycemia in insulin-dependent diabetic patients. *Diabetes Care.* 1987;10(5):584–8.
133. Landt KW, Campaigne BN, James FW, Sperling MA. Effects of exercise training on insulin sensitivity in adolescents with type I diabetes. *Diabetes Care.* 1985;8:461–5.
134. Fuchsjager-Mayri G, Pleiner J, Wiesinger GF, Sieder AE, Quittan M, Nuhr MJ, et al. Exercise training improves vascular endothelial function in patients with type 1 diabetes. *Diabetes Care.* 2002;25:1795–801.

Chapter 3

3 « The relationship between blood pressure and sciatic nerve blood flow velocity in rats with insulin-treated experimental diabetes »

3.1 Introduction

The etiology of diabetic peripheral neuropathy (DPN) may be explained partially by declining peripheral vascular function (1). In rats with experimental diabetes, deficits in motor nerve conduction velocity (MNCV) are often linked with reduced basal nerve blood flow (NBF) (2–6). The mechanism responsible for reducing NBF is multifactorial and may include abnormalities in autonomic function, calcium handling, endothelial function, lipid/glucose metabolism or the renin-angiotensin system [for review see (7)]. Reduced NBF is believed to contribute to neural ischemia resulting in nerve dysfunction and DPN (1–3). However, decrements in NBF do not always precede DPN (8–10). Indeed, some authors have documented no change (8) or increased (9,11,12) NBF following diabetes induction. The relationship between basal NBF and nerve function remains to be elucidated fully. However, it appears diabetes-induced nerve dysfunction may occur independently of reduced NBF.

Hypertension is an independent risk factor for DPN (13). Theoretically, because NBF does not autoregulate, but instead responds passively to changes in blood pressure (14–19), elevated blood pressure should result in augmented NBF. However, Gregory and co-workers (6) documented that rats with diabetes had reduced NBF in the presence of normal and elevated blood pressure. Likewise, rats with diabetes presented with

peripheral nerve microangiopathy. Based on these data, it appears the non-autoregulatory relationship between blood pressure and NBF is altered in a diabetic state.

In a recent study, patients with poorly controlled type 1 diabetes presented with ~9% glycosylated hemoglobin values (20). This corresponds to a blood glucose concentration of ~13.5 mmol/L (21) and contrasts with values obtained during experimental diabetes (blood glucose=27-43 mmol/L) (2,3,4) emphasizing the problem that chronic, severe hyperglycemia may not reflect a moderately hyperglycemic insulin-treated, type 1 diabetes state. In rats with experimental diabetes, insulin treatment partially restores resting NBF (4,22), improves vasa nervorum reactivity (23) and enhances nerve function (4,24–26). However, whether insulin treatment impacts microangiopathy remains unclear (26–28) and if it alters the relationship between blood pressure and NBF is unknown.

The purpose of this study was to examine the effect of chronic, moderate hyperglycemia (i.e. more clinically relevant blood glucose concentrations) on the relationship between blood pressure and NBF. We tested the hypothesis that moderately hyperglycemic insulin-treated experimental diabetes (DS) would reduce basal sciatic NBF and also alter the non-autoregulatory relationship such that NBF would be reduced across a range of mean arterial pressures (MAP).

3.2 Methods

Moderately hyperglycemic rat model (29)

Eight-week-old male Sprague-Dawley rats (Charles River Laboratories; Saint-Constant, Quebec, CA) were injected with 20 mg/kg streptozotocin (STZ; Sigma-

Aldrich, St. Louis, MO, USA; citrate buffer pH 4.5) over five consecutive days. Post-confirmation of two blood glucose readings ≥ 18 mmol/L, rats received a surgically implanted subcutaneous insulin pellet (Linplant, Linshin, Toronto, ON, CAN; release rate 1 IU/12 h; one pellet per 150 g mass and one pellet for each additional 100 g). To maintain moderate hyperglycemia and replicate blood glucose values observed in humans with poorly controlled type 1 diabetes (20), blood glucose was monitored daily during the first week of insulin therapy and weekly thereafter, and pellet size was adjusted accordingly (see table 3-1 in the results section). Experimental testing occurred at 10 weeks post-implantation of the first insulin pellet.

Experimental design

All procedures complied with the Animal Care guidelines and ethics approval board from The University of Western Ontario. Rats were housed in pairs at a constant temperature of $20 \pm 1^\circ\text{C}$ with a 12-hour light/dark cycle and had *ad libitum* access to commercial chow (PROLAB RMH 3000, Brentwood, MO, USA) and water. Rats were divided randomly into two groups; control (n=8, mass= 560 ± 31 g) and DS (n=9, mass= 456 ± 33 g). Saphenous vein blood samples were collected weekly during the fed-state, and blood glucose was measured (data presented as average blood glucose throughout the study). At the end of the 10th week, blood pressure in the conscious animal was measured. Subsequently, under anesthesia, nerve arterial vasomotor control and MNCV were assessed. Thereafter, sciatic nerves were harvested for histological analyses.

Conscious blood pressure testing (control, n=7; DS, n=9)

Mean, systolic and diastolic blood pressure (SBP; DBP) as well as heart rate were measured using the tail-cuff method (CODA; Kent Scientific CO. Torrington, CT. USA) in conscious rats. Prior to data collection, all rats were familiarized with the CODA system on multiple occasions.

Surgical procedures

Rats inhaled isoflurane gas (4%) and received an intra-peritoneal injection of urethane (25 mg/kg) and α -chloralose (4 mg/kg) prior to the onset of surgery. After ~20 min, the gas was removed and the urethane α -chloralose mixture alone maintained surgical depth. A warming blanket was used to maintain rat body temperature at 37°C (rectal probe). A catheter inserted into the right jugular vein facilitated all anaesthetic and drug infusions. A catheter inserted into the right carotid artery was connected to a pressure transducer (PX272, Edwards Lifesciences, Irvine, CA, USA), and enabled continuous blood pressure recordings. Thereafter, the left sciatic nerve was exposed via blunt dissection (30).

Nerve blood flow velocity protocol (control, n=6; DS, n=6)

After surgery, following 1 h of stabilization, MAP and nerve blood flow velocity (NBV; Vevo 2100 ultrasound system VisualSonics, Toronto, ON, CA) were measured at baseline 1 (pre-sodium nitroprusside; SNP; NO donor), during SNP (60 μ g/kg, 110 μ g/mL) infusion (early and late), baseline 2 (pre-phenylephrine; PE; α 1 adrenergic agonist) and during PE (12 μ g/kg, 10 μ g/mL) infusion (early and late).

Vascular conductance and estimated conscious nerve blood flow velocity

Sciatic NBV and MAP values were used to calculate the index of vascular conductance ($VC_i = BV/MAP$). Sciatic NBV was plotted as a function of MAP for the time points: baseline 1, baseline 2, SNPearly, SNPlate, PEEarly and PELate; and regression equations for each animal were created, averaged and plotted separately for each group. Conscious MAP values were substituted into the appropriate individual regression equations to estimate conscious NBV values for each rodent, in both groups.

Motor nerve conduction velocity protocol (control, n=7; DS, n=6)

Following the pharmacological intervention, MNCV was determined in the sciatic-tibial nerve. The left sciatic nerve was stimulated first at the sciatic notch, and then at the popliteal fossa. Using a constant-voltage clinical electrical stimulator (NeuroscanComperio, Compumedics Medical Systems, El Paso, TX, USA), nerve stimulation consisted of a series of single 100 μ s, supramaximal pulses delivered through a bipolar stimulating electrode that was held over the exposed nerve. Supramaximal stimulation was achieved at ~20 mA. The evoked compound muscle action potentials (CMAP) were recorded from the tibialis anterior muscle using intramuscular, tungsten recording electrodes inserted into the muscle.

Sciatic nerve harvesting (control, n=6; DS, n=6)

After the MNCV experiment, rats were euthanized and their sciatic nerves were harvested, fixed in 4% paraformaldehyde for 72 h and embedded in paraffin blocks for histological analysis. Tissue blocks were sectioned transversely 8 μ m thick, mounted on

positively charged slides (VWR Suprafrost Plus; Batavia, IL, USA) and stained with a routine hematoxylin (H) & eosin (E) protocol. The H&E stain was used to expose blood vessels and quantify total sciatic nerve fascicular blood vessel number (31).

Data acquisition and analysis

The analog blood pressure signal sampling occurred at 1000 Hz (Powerlab; ADInstruments, Colorado Springs, CO, USA). Pulsatile blood pressure over 5 beats (corresponding to the same 5 NBV waveforms) was averaged to calculate MAP.

NBV was measured using Doppler ultrasound and a 40 MHz linear array probe (MS550D) transducer placed over an artery lying superficially along the sciatic nerve. Power Doppler was used to locate the superficial nerve artery. This arterial segment was studied using the duplex imaging mode (frequency of 32 MHz with 100% power, PRF between 4-5kHz and wall filter of 40-50Hz) with the pulsed wave Doppler gate (~0.12 mm width) positioned over the site of the high power Doppler signal (insonation angle of 60°). This ultrasound system did not have sufficient resolution to measure changes in the sciatic nerve supply artery diameter in B-mode images. Thus, peak NBV (outer envelope of the flow-velocity waveform) represents a surrogate for NBF rate.

Compound muscle action potentials were displayed and stored on a digital electromyography (EMG) acquisition system (NeuroscanComperio, Compumedics Medical Systems, El Paso, TX, USA). Electromyography signals were sampled at 10 kHz and band-pass filtered at 10 Hz to 10 kHz. Motor nerve conduction velocity was calculated by subtracting the distal from the proximal latency (measured in milliseconds) from the stimulus artifact of the initial inflection of the evoked CMAP waveforms, and

the difference was divided into the distance between the two stimulation sites (measured in mm). The MNCV was reported in m/s.

The number of nerve blood vessels in the largest fascicle was counted independently by two observers blinded to the experimental condition. Images were taken on an Axiovert S100 brightfield microscope at 40X magnification, using Northern Eclipse imaging software (EMPIX Imaging Inc.; Mississauga, ON, CAN). To determine the total vessel count, the nerve fascicle of interest was sectioned into quadrants visually. The aggregate of each quadrant represented the total vessel count for the individual nerve fascicle. The measurements from the two observers were averaged for each nerve and the average value was used for analysis. The nerve fascicle area and blood vessel diameters were measured using image J software (<http://imagej.nih.gov/ij/>).

Statistics

Independent two-tailed t-tests were used to test the effect of group (control vs DS) for the animal characteristics (Table 3-1). A mixed model ANOVA assessed the effect of group and time on MAP, NBV (Table 3-2) and VCI during the study phases which included in sequence baseline 1, SNPEarly, SNPLate, baseline 2, PEearly and PELate time periods (Sigma Stat for Windows, version 8.0). The same analysis was completed on the Δ MAP and Δ NBV response to SNP and PE infusions (Figures 3-1 & 3-2). For each animal, NBV vs MAP was plotted and the slope and y-intercept values were averaged for each group. Independent two-tailed t-tests were used to test the effect of group (control vs DS) on the average slope and y-intercept values (Figure 3-4), as well as for MNCV (Table 3-1) and the number of sciatic nerve fascicle blood vessels (Table 3-3). Lastly, an

independent two-tailed t-test was used to test the effect of group (control vs DS) on estimated NBV in the conscious rat (Figure 3-4). The significance level was set at $P \leq 0.05$ and data are presented as mean \pm SD.

3.3 Results

General characteristics

Relative to the control group, body mass was reduced (Table 3-1; $P < 0.01$), and fasting and fed state blood glucose concentrations were greater (Table 3-1; $P < 0.01$) in the DS group. There were no differences in conscious heart rate between groups (Table 3-1; $P = 0.85$). Compared with control rats, systolic, diastolic and MAP in the conscious state were greater (Table 3-1; $P < 0.01$), while MNCV was reduced (Table 3-1; $P < 0.01$) in the DS group.

Mean arterial pressure and nerve blood flow velocity

In the anesthetized state, no group or interaction effects were observed for MAP or NBV ($P \geq 0.16$). A main effect of time was observed for MAP and NBV ($P < 0.01$). Subsequent pairwise comparisons indicated that baseline 1 and 2 were similar ($P \geq 0.60$), relative to baseline 1, SNP reduced MAP and NBV ($P < 0.01$) and relative to baseline 2, PE increased MAP and NBV ($P < 0.01$). Group data for MAP and NBV can be located in Table 3-2.

No group or interaction effects were observed for VCI ($P \geq 0.47$). A main effect of time was observed for VCI ($P < 0.01$), where all time points were significantly greater than SNPlate ($P < 0.01$; data not shown).

No group or interaction effects were observed for Δ MAP and Δ NBV ($P \geq 0.20$). A main effect of time was observed for Δ MAP and Δ NBV ($P < 0.01$). Subsequent pairwise comparisons indicated that Δ MAP and Δ NBV measured during SNPlate were greater than SNPearly ($P < 0.01$) and those measured during PElate were greater than PEEarly ($P < 0.01$). Group data for Δ MAP and Δ NBV can be located in Figures 3-1 & 3-2 respectively.

Regression equation and estimated conscious nerve blood flow velocity

The average slope of the regression between MAP and NBV (control = 1.03 ± 0.16 vs DS = 0.99 ± 0.27 ; $P = 0.77$), and the corresponding y-intercept (control = -19.28 ± 9.15 vs DS = -19.45 ± 19.83 ; $P = 0.99$), were similar between groups (Figure 3-3). Estimated NBV for a conscious rat was significantly ($P = 0.02$) greater in the DS group (Figure 3-4).

Blood vessel counting

The total number of vessels as well as the total area and number of vessels/ mm^2 were similar ($P \geq 0.73$; Table 3-3) between groups (Figure 3-5). There appeared to be two sizes of blood vessels with one or three layers of smooth muscle. The proportion of smaller (~70%) and larger (~30%) blood vessels were similar ($P \geq 0.68$) between groups. The estimated radius of the larger blood vessels was similar between groups ($P = 0.50$).

Table 3-1 Animal Characteristics

Measures	Control (n=7-8)	DS (n=6-9)
Mass (g)	560±31	456±33*
Fed state blood glucose (mmol/L)	4.4±0.2	14.1±1.2*
Fasting blood glucose (mmol/L)	3.6±0.2	7.0±2.7*
Insulin Dose (IU/kg/day)	NA	6.0±2.4
Conscious heart rate (bpm)	417±33	421±26
Conscious SBP (mmHg)	149±13	171±8*
Conscious DBP (mmHg)	99±19	124±10*
Conscious MAP (mmHg)	115±17	139±8*
MNCV (m/s)	56.9±5.6	43.1±5.3*

*, Significantly different than control (P<0.05)

DS: insulin-treated experimental diabetes

SBP: systolic blood pressure

DBP: diastolic blood pressure

MAP: diastolic blood pressure

MNCV: motor nerve conduction velocity

Table 3-2 Raw Data: MAP and NBV

Time Point	MAP (mmHg)		NBV (mm/s)	
	Control (n=6)	DS (n=6)	Control (n=6)	DS (n=6)
Baseline 1	105±12	94±18	87±15	70±13
SNPearly	81±11	74±13	67±12	50±19
SNPlate	61±7	54±10	38±8	33±14
Baseline 2	101±13	95±17	86±16	75±14
PEearly	125±15	118±20	107±25	100±14
PElate	153±18	141±23	139±37	114±22

DS: insulin-treated experimental diabetes
MAP: mean arterial pressure
NBV: nerve blood flow velocity
SNP: sodium nitroprusside
PE: phenylephrine

Table 3-3 Blood Vessel Count

Nerve Fascicular Arteries	Control (n=6)	DS (n=6)
Total (#)	7±2	7±2
Area (mm ²)	0.4±0.1	0.4±0.1
Total Vessels/mm ²	20±4	20±10
Radius of Large Vessels (µm)	21±4	20±2

DS: insulin-treated experimental diabetes

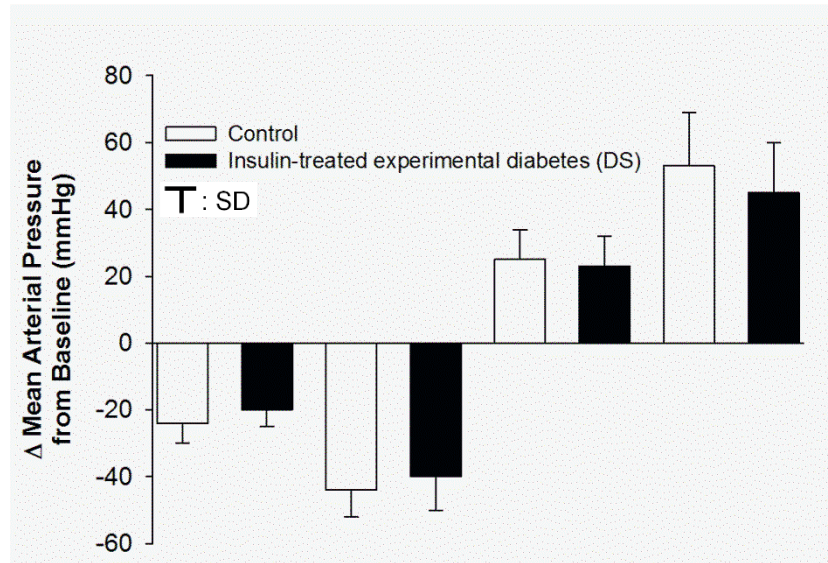


Figure 3-1 Δ Mean arterial pressure (Δ MAP; mmHg) from baseline in the control (□) and insulin-treated experimental diabetes (■) group during sodium nitroprusside (SNP – NO donor) and phenylephrine (PE – α 1 adrenergic agonist) infusion. Early and late responses reflect ~50 and 100% of the peak effect of the drug.

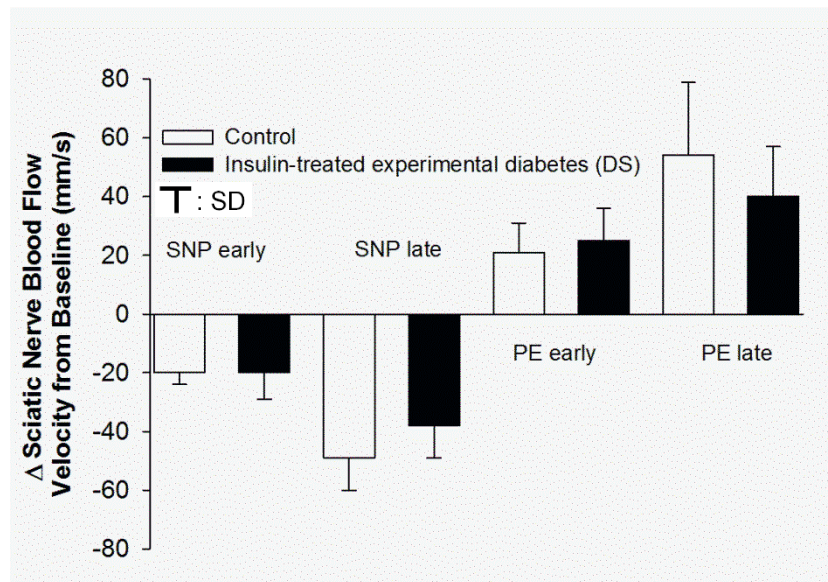


Figure 3-2 Δ Nerve blood velocity (Δ NBV; mm/s) from baseline in the control (□) and insulin-treated experimental diabetes (■) group during sodium nitroprusside (SNP – NO donor) and phenylephrine (PE – α 1 adrenergic agonist) infusion. Early and late responses reflect ~50 and 100% of the peak effect of the drug.

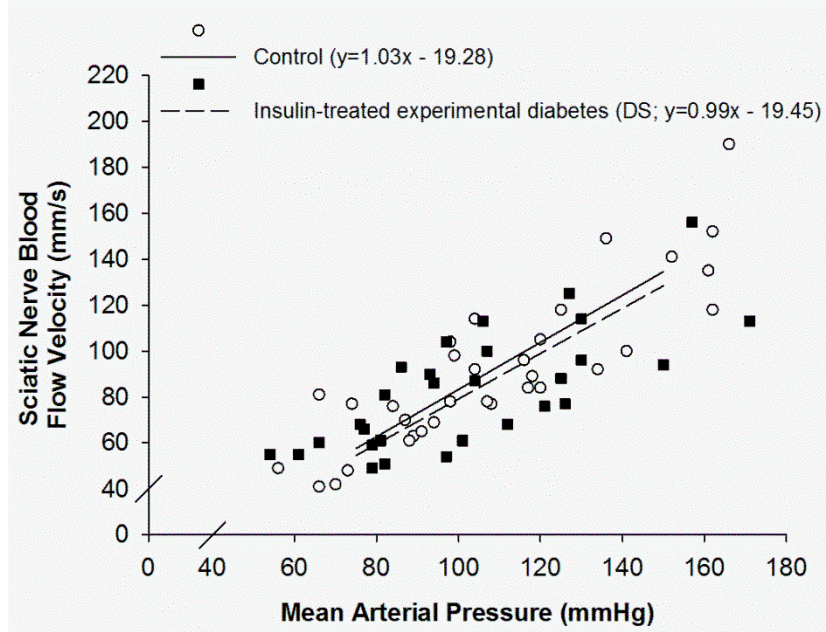


Figure 3-3 Nerve blood velocity (NBV; mm/s) at a given mean arterial pressure (MAP; mmHg) in the control (○, —) and insulin-treated experimental diabetes (■, ... -) group. The regression equations were calculated for each rodent and averaged separately to create a two group means.

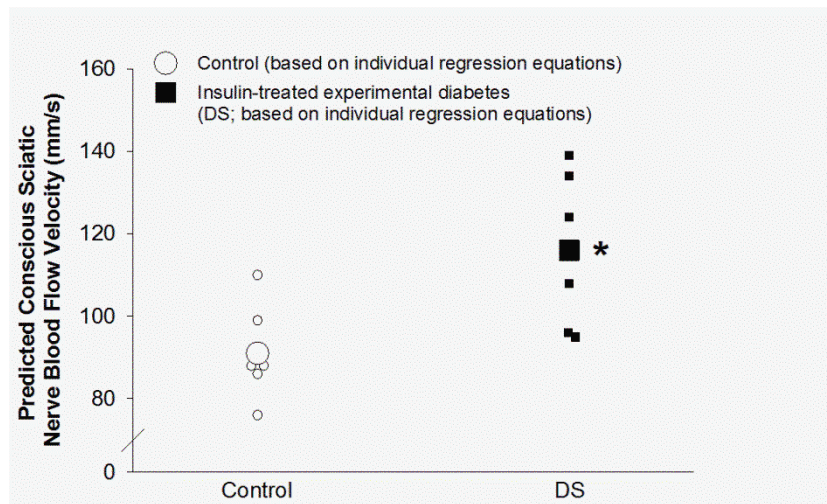


Figure 3-4 Estimated nerve blood velocity (NBV; mm/s) based on the appropriate individual linear regression equations in the control (○; larger circle denotes the mean) and insulin-treated experimental diabetes (■; larger box denotes the mean) group. *, Significantly greater than control (P=0.02).

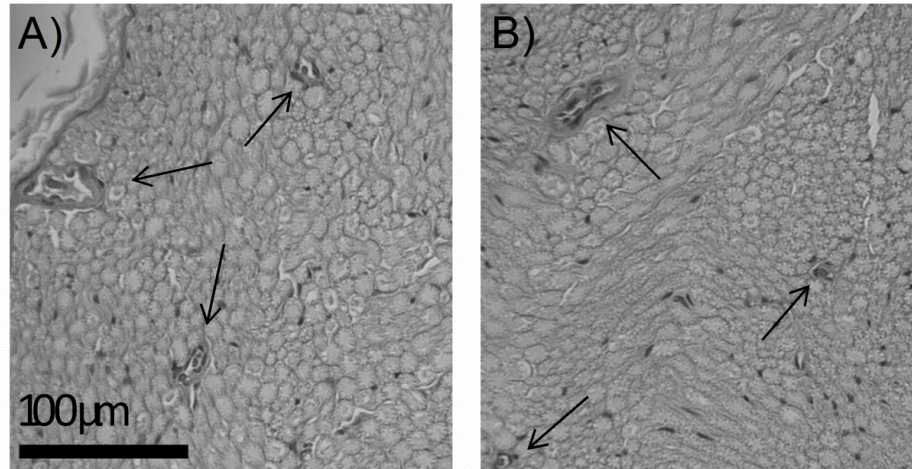


Figure 3-5 Representative cross-sectional images of the sciatic nerve in the control (A) and insulin-treated experimental diabetes (B) group. The arrows denote the location of blood vessels. In each image there are two small and one large blood vessel. Sections were stained with hematoxylin and eosin.

3.4 Discussion

The present study employed a rodent model of chronic, moderate hyperglycemia that resulted in similar blood glucose values as humans with poorly controlled type 1 diabetes. The novel finding of this study was that sciatic nerve vascularization and NBV responses to changes in MAP remained unchanged in the DS group. Therefore, NBV would have been similar or possibly elevated in the DS group because conscious blood pressure was elevated. In spite of these findings, MNCV was reduced in the DS group. In contrast to the stated hypothesis, NBV was not reduced across a range of MAP. These data suggest decrements in basal NBF do not precede nerve dysfunction in rats with chronic, moderate hyperglycemia.

Moderately hyperglycemic rat model

Unlike previous studies (2–6), experimental diabetes did not reduce NBF in the present study, confirming earlier observations (8,9,11,12). Notably, in the present study rats were chronically exposed to insulin. Insulin exposure dilates healthy nerve arterioles (32,33) and insulin treatment attenuates decrements in NBF following diabetes induction (4,22). Also, unlike previous studies that documented no change (2,3,12) or reduced (8,34) MAP in rats with chronic severe hyperglycemia, the rats in the DS group in this study had similar anesthetized and greater conscious blood pressures. The level of hyperglycemia and insulinemia appear to be important modulators of the blood pressure response to STZ, as it has been documented that insulin treatment increases blood pressure in rats with experimental diabetes (35,36). In the present study, insulin treatment prevented severe hyperglycemia and likely altered the severity and pathophysiology of disease.

Perspectives

The present study was the first to our knowledge to examine NBV across a range of MAP in rats with diabetes. There was no evidence of frank microangiopathy and because the relationship between MAP and NBV was the same between groups, NBV was similar and potentially elevated in conscious rats in the DS group. Further, because MNCV was reduced in the DS group, these data support previous observations that diabetes-related nerve dysfunction can occur independently of reductions in NBF (8–12). Further, these data provide evidence that in the absence of microangiopathy, basal NBF may be elevated in humans with combined poorly controlled type 1 diabetes and hypertension that are at risk of DPN.

Poor glycemic control increases blood pressure in rats with insulin-treated experimental diabetes (36) and given the lack of autoregulatory control in the vasa nervorum, the ultimate impact of diabetes on NBF would be determined by the balance of microvascular damage, should it occur, and perfusion pressure. While the current study did not address neuronal-glucotoxicity or ischemia-induced DPN, it does raise the possibility that heightened blood pressure and NBF may exacerbate metabolic complications associated with DPN (11,12,37). Specifically, in rats with diabetes, increased NBF is associated with elevated nerve fructose and sorbitol concentrations, and results in deficits in MNCV (9). In humans with insulin-treated diabetes nerve sorbitol content is inversely related to the number of myelinated fibers (38). Therefore, increased NBF and glucose metabolism in the nerve may contribute to nerve fiber degeneration and compromise nerve function.

The current data conflict with those from Gregory and colleagues (6), who observed low NBF values in the presence of normal and high blood pressure, in rats with diabetes. However, they did not examine NBF across a range of MAP in the same rodents. Also, their rodent model of diabetes did not include insulin treatment and their rats had ~ two-fold greater blood glucose concentrations, and expressed sciatic nerve microangiopathy. The presence of microangiopathy in the sciatic nerve may alter the relationship between MAP and NBF and help explain the divergent findings between studies (6).

Doppler ultrasound

This study employed high frequency Doppler ultrasound to assess NBV. Recent developments in high frequency ultrasound technology have enabled the measurement of blood flow velocity less than 5 mm/s, in superficial arteries as small as 20 μm (39,40). In previous tests using tissue-mimicking phantoms, arteries as small as 140 μm could be resolved accurately using B-mode images acquired with a 40 MHz transducer (41). However, in the present study, the nerve artery was too small to measure its diameter using B-mode images with 40 MHz ultrasound technology. Thus, epi- or endoneurial flow could only be assessed indirectly using NBV and VCi. In humans with poorly controlled diabetes, epineurial blood vessels may be tortuous and subject to arterio-venous shunting (27,42) resulting in a redistribution of NBF towards the epineurial vasculature. Although endoneurial flow relies on epineurial flow (43), Doppler ultrasound may not capture all reductions in endoneurial flow. However, the sciatic nerve vasculature contains feed arteries arising from multiple locations, does not express a typical capillary plexus, and has been described an anastomotic vascular network (43–

46). This pattern of tissue perfusion limits the interpretation of gross flow measurements and underlines a benefit of quantifying exclusive arterial inflow responses.

In the current experiment, failure to detect changes in arterial diameter may result in underestimated NBF during SNP infusion and overestimated NBF during PE infusion. This may occur as topical application of SNP causes vasa nervorum dilation (47) and local delivery of PE causes vasa nervorum constriction (48). However, in the current study drugs were infused systemically and MAP, rather than SNP or PE, appeared to modulate NBF. Further, because VCI was similar at all time-points (except SNPlate), any potential effects of SNP or PE on the vasa nervorum were minimal. In the case of SNPlate, it is unlikely that SNP constricted the vasa nervorum. A more plausible explanation would be that the reduction in VCI during SNPlate may have been caused by a sympathetic reflex pressor response to defend against the ~40 mmHg drop in MAP.

The current data are the first of our knowledge to use Doppler ultrasound to assess sciatic NBF/autoregulation in a rodent model of diabetes, rendering it difficult to form direct comparisons. In accordance with previous work (14–19), using a variety of techniques, we did confirm the absence of autoregulation, suggesting the results obtained using Doppler ultrasound are comparable with other methods. It is important to note conflicting results amongst techniques have been documented with laser Doppler flowometry (4,10), hydrogen clearance (2,8) as well as other methods (9,11,12,37), regarding the impact of diabetes on NBF control. Nevertheless, the current data support previous observations that diabetes is associated with either no change (8,10) or potentially increased (9,11,12) NBF.

Limitations

Sciatic NBF may be influenced by temperature. The relationship between limb temperature and NBF may be positive (49) or negative (50) and may have less of an effect on rats with diabetes (43). In the present study, although rat body temperature was maintained at 37°C, rat sciatic nerve temperature was not monitored. However, room and rat core temperature were constant, and NBV at baseline 1 and 2 were similar across conditions in each experiment, suggesting little impact of non-intervention factors, such as changes in nerve temperature.

In the present study blood pressure in the conscious state was elevated and by extension so was estimated NBV in the DS group. However, experimental diabetes often reduces blood pressure (8,34) and the current blood pressure values collected using the tail-cuff method were not validated using an arterial catheter in the same conscious rodents. This issue has been discussed with speculation that severe hyperglycemia may cause emaciation of the tail and result in artifactually high tail-cuff blood pressure recordings (51,52). However, using direct methods, it has been documented that insulin treatment increases blood pressure in rats with experimental diabetes (35,36). More importantly, in the present study the rats were not severely hyperglycemic and their tails did not appear emaciated. Also, such issues do not alter the overall conclusion that the relationship between MAP and NBV remains intact in rats with chronic, moderate hyperglycemia and reductions in NBF do not always precede diabetes-induced nerve dysfunction (8–12).

Conclusion

The novel findings of the current study demonstrated that the absence of autoregulation persisted in a rat model where moderate hyperglycemia (blood glucose values similar to those observed in humans with poorly controlled type 1 diabetes) (20) and elevated blood pressure co-existed. Therefore, elevated blood pressure in conscious rats with diabetes may result in increased NBF values. Nonetheless, rats in the DS group exhibited reduced MNCV values. Therefore, reduced NBF cannot be the sole determinant of DPN. Potentially, elevated NBF may increase glucose delivery to the nerve, exacerbating neuronal glucotoxicity and impairing nerve function. However, to date the relationship between NBF and glucose uptake in the nerve remains to be elucidated fully. Overall, the present study supports the link between poorly controlled insulin-treated type 1 diabetes, hypertension and nerve dysfunction. However, these data suggest that reduced NBF does not necessarily precede decrements in MNCV. Therefore, in humans with insulin-treated type 1 diabetes, maintaining normal blood pressure may be important not only for cardiovascular health, but peripheral nerve health as well.

3.5 References

1. Fazan VPS, Carvalho de Vasconcelos CA, Valenca MM, Nessler R, Moore KC. Diabetic peripheral neuropathies: a morphometric overview. *Int. J. Morphol.* 2010;28:51–64.
2. Tuck RR, Schmelzer JD, Low PA. Endoneurial blood flow and oxygen tension in the sciatic nerves of rats with experimental diabetic neuropathy. *Brain.* 1984;107:935–50.
3. Cameron NE, Cotter MA, Low PA. Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am J Physiol.* 1991;261(1):E1–E8.
4. Biessels GJ, Stevens EJ, Mahmood SJ, Gispen WH, Tomlinson DR. Insulin partially reverses deficits in peripheral nerve blood flow and conduction in experimental diabetes. *J. Neurol. Sci.* 1996;140:12–20.
5. Sasaki H, Schmelzer JD, Zollman PJ, Low PA. Neuropathology and blood flow of nerve, spinal roots and dorsal root ganglia in longstanding diabetic rats. *Acta Neuropathol.* 1997;93(2):118–28.
6. Gregory JA, Jolivald CG, Goor J, Mizisin AP, Calcutt NA. Hypertension-induced peripheral neuropathy and the combined effects of hypertension and diabetes on nerve structure and function in rats. *Acta Neuropathol.* 2012;124:561–73.
7. Zochodne DW. Nerve and ganglion blood flow in diabetes: an appraisal. *Int. Rev. Neurobiol.* 2002;50:161–202.
8. Zochodne DW, Ho LT. Normal blood flow but lower oxygen tension in diabetes of young rats: microenvironment and the influence of sympathectomy. *Can J Physiol Pharmacol.* 1992;70:651–9.
9. Tilton RG, Chang K, Nyengaard JR, Van den Enden M, Ido Y, Williamson JR. Inhibition of sorbitol dehydrogenase. Effects on vascular and neural dysfunction in streptozocin-induced diabetic rats. *Diabetes.* 1995;44:234–42.

10. Kalichman MW, Dines KC, Bobik M, Mizisin AP. Nerve conduction velocity, laser Doppler flow, and axonal caliber in galactose and streptozotocin diabetes. *Brain Res.* 1998;810(1-2):130–7.
11. Pugliese G, Tilton RG, Speedy A, Chang K, Santarelli E, MA Province, et al. Effects of very mild versus overt diabetes on vascular haemodynamics and barrier function in rats. *Diabetologia.* 1989;32(12):845–57.
12. Chang K, Ido Y, LeJeune W, Williamson JR, Tilton RG. Increased sciatic nerve blood flow in diabetic rats: assessment by “molecular” vs particulate microspheres. *Am J Physiol.* 1997;273(36):E164–E173.
13. Tesfaye S, Chaturvedi N, Eaton SE, Ward JD, Manes C, Lonescu-Tirgoviste C, et al. Vascular risk factors and diabetic neuropathy. *N Engl J Med.* 2005;352(4):341–50.
14. Smith DR, Kobrine AI, Rizzoli HV. Absence of autoregulation in peripheral nerve blood flow. *J. Neurol. Sci.* 1977;33(3):347–52.
15. Low PA, Tuck RR. Effects of changes of blood pressure, respiratory acidosis and hypoxia on blood flow in the sciatic nerve of the rat. *J Physiol.* 1984;347:513–24.
16. Rundquist I, Smith Q, Michel M, Ask P, Oberg PA, Rapoport SI. Sciatic nerve blood flow measured by laser Doppler flowmetry and [¹⁴C]iodoantipyrine. *Am. J. Physiol. Hear. Circ. Physiol.* 1985;248(17):311–7.
17. Sundqvist T, Oberg PA, Rapoport SI. Blood flow in rat sciatic nerve during hypotension. *Exp. Neurol.* 1985;90(1):139–48.
18. Takeuchi M, Low PA. Dynamic peripheral nerve metabolic and vascular responses to exsanguination. *Am J Physiol.* 1987;16:E349–E353.
19. McManis PG, Schmelzer JD, Zollman PJ, Low PA. Blood flow and autoregulation in somatic and autonomic ganglia Comparison with sciatic nerve. *Brain.* 1997;120:445–9.

20. Riveline J-P, Schapelynck P, Chaillous L, Renard E, Sola-Gazagnes A, Penfornis A, et al. Assessment of patient-led or physician-driven continuous glucose monitoring in patients with poorly controlled type 1 diabetes using basal-bolus insulin regimens. *Diabetes Care*. 2012;35(5):965–71.
21. Rohlfing CL, Weidmeyer H-M, Little RR, England JD, Tennill A, Goldstein DE. Defining the relationship between plasma glucose and HbA1c. *Diabetes Care*. 2002;25:275–8.
22. Stevens EJ, Carrington AL, Tomlinson DR, Mary Q. Nerve ischaemia in diabetic rats: time-course of development, effect of insulin treatment plus comparison of streptozotocin and BB models. *Diabetologia*. 1994;37:43–8.
23. Kihara M, Low PA. Impaired vasoreactivity to nitric oxide in experimental diabetic neuropathy. *Exp. Neurol*. 1995;132:180–5.
24. Singhal A, Cheng C, Sun H, Zochodne DW. Near nerve local insulin prevents conduction slowing in experimental diabetes. *Brain Res*. 1997;763(2):209–14.
25. Brussee V, Cunningham FA, Zochodne DW. Direct insulin signaling of neurons reverses diabetic neuropathy. *Diabetes*. 2004;53:1824–30.
26. Ozaki K, Yamano S, Matsuura T, Narama I. Insulin-ameliorated peripheral motor neuropathy in spontaneously diabetic WBN/Kob rats. *J. Vet. Med. Sci*. 2013; 75:1323-1328
27. Tesfaye S, Malik R, Harris N, Jakubowski JJ, Mody C, Rennie IG, et al. Arterio-venous shunting and proliferating new vessels in acute painful neuropathy of rapid glycaemic control (insulin neuritis). *Diabetologia*. 1996;39:329–35.
28. Sugimoto K, Baba M, Suda T, Yasujima M, Yagihashi S. Peripheral neuropathy and microangiopathy in rats with insulinoma: association with chronic hyperinsulinemia. *Diabetes. Metab. Res. Rev*. 2003;19(5):392–400.

29. Melling CWJ, Gris  KN, Hasilo CP, Fier B, Milne KJ, Karmazyn M, et al. A model of poorly controlled type 1 diabetes mellitus and its treatment with aerobic exercise training. *Diabetes Metab.* 2013; 39:226-235.
30. Uysal CA, Mizuno H, Hyakusoku H. Sciatic nerve anatomy in rat re-visited: a more proximal intervention. *J Plast Reconstr Aesthet Surg.* 2009;62(6):847–9.
31. Kim B, Yoo JJ, Atala A. Peripheral nerve regeneration using acellular nerve grafts. *J. Biomed. Mater. Res.* 2004;68A:201–9.
32. Davidson EP, Coppey LJ, Calcutt NA, Oltman CL, Yorek MA. Diet-induced obesity in Sprague – Dawley rats causes microvascular and neural dysfunction. *Diabetes. Metab. Res. Rev.* 2010;26:306–18.
33. Olver TD, Mattar L, Gris  KN, Twynstra J, Noble EG, Lacefield JC, et al. Glucose-stimulated insulin secretion causes an insulin-dependent nitric oxide-mediated vasodilation in the blood supply of the rat sciatic nerve. *Am J Physiol.* 2013;305(2):R157–R163.
34. Cotter MA, Ekberg K, Wahren J, Cameron NE. Effects of proinsulin c-peptide in experimental diabetic neuropathy. *Diabetes.* 2003;52:1812-7.
35. Katovich M, Hanley K, Strubbe G, Wright BE. Effects of streptozotocin-induced diabetes and insulin treatment on blood pressure in the male rat. *Proc Soc Exp Biol Med.* 1995;208(3):300–6.
36. Brands MW, Hopkins TE. Poor glycemic control induces hypertension in diabetes mellitus. *Hypertension.* 1996. p. 735–9.
37. Suter SP, Chang K, Marvel J, Williamson JR. Concurrent increases in regional hematocrit and blood flow in diabetic rats: prevention by sorbinil. *Am J Physiol.* 1992;263:H945–H950.

38. Dyck PJ, Zimmerman BR, Vilen TH, Minnerath SR, Karnes JL, Yao JK, et al. Nerve glucose, fructose, sorbitol, myo-inositol, and fiber degeneration and regeneration in diabetic neuropathy. *N. Engl. J. Med.* 1988;319:542–8.
39. Christopher DA, Burns PN, Foster FS. A high-frequency pulsed-wave Doppler ultrasound system for the detection and imaging of blood flow in the microcirculation. *Ultrasound Med. Biol.* 1997;23(7):997–1015.
40. Kruse DE, Silverman RH, Fornaris RJ, Coleman DJ, Ferrara KW. A swept-scanning mode for estimation of blood velocity in the microvasculature. *IEEE Trans. Ultrason. Ferroelectr. Freq. Control.* 1998;45(6):1437–40.
41. Moran CM, Pye SD, Ellis W, Janeczko A, Morris KD, McNeilly AS, et al. A comparison of the imaging performance of high resolution ultrasound scanners for preclinical imaging. *Ultrasound Med. Biol.* 2011;37(3):493–501.
42. Ward JD. Abnormal Microvasculature in Diabetic Neuropathy. *Eye.* 1993;7:223–6.
43. Sladky JT, Greenberg JH, Brown MJ. Regional perfusion in normal and ischemic rat sciatic nerves. *Ann. Neurol.* 1985;17:191–5.
44. Adams WE. The blood supply of nerves: II. The effects of exclusion of its regional sources of supply on the sciatic nerve of the rabbit. *J. Anat.* 1943;77(Pt 3):243–50.
45. Bell MA, Weddell AG. A descriptive study of the blood vessels of the sciatic nerve in the rat, man and other mammals. *Brain.* 1984;107:871–98.
46. Zamir M, Twynstra J, Vercnocke AJ, Welch I, Jorgensen SM, Ritman EL, et al. Intrinsic microvasculature of the sciatic nerve in the rat. *J Peripher Nerv Syst.* 2012;17:377–84.
47. Omawari N, Dewhurst M, Vo P, Mahmood S, Stevens E, Tomlinson DR. Deficient nitric oxide responsible for reduced nerve blood flow in diabetic rats: effects of L-

- NAME, L-arginine, sodium nitroprusside and evening primrose oil. *Br. J Pharmacol.* 1996;118(1):186–90.
48. Kappelle AC, Biessels GJ, Van Buren T, Erkelens DW, De Wildt DJ, Gispen WH. Effects of nimodipine on sciatic nerve blood flow and vasa nervorum responsiveness in the diabetic rat. *Eur. J. Pharmacology.* 1993;250:43–9.
49. Kihara M, Schmelzer JD, Kihara Y, Smithson IL, Low PA. Efficacy of limb cooling on the salvage of peripheral nerve from ischemic fiber degeneration. *Muscle Nerve.* 1996;19(2):203–9.
50. Dines KC, Calcutt NA, Nunag KD, Mizisin AP, Kalichman MW. Effects of hindlimb temperature on sciatic nerve laser Doppler vascular conductance in control and streptozotocin-diabetic rats. *J. Neurol. Sci.* 1999;163(1):17–24.
51. Tomlinson K, Gardiner S, Hebden R, Bennett T. Functional consequences of streptozotocin-induced diabetes mellitus, with particular reference to the cardiovascular system. *Pharmacol. Rev.* 1991;44(1):104–41.
52. Kusaka M, Kishi K, Sokabe H. Does so-called streptozocin hypertension exist in rats? *Hypertension.* 1987;10(5):517–21.

Chapter 4

4 « Glucose-stimulated insulin secretion causes a nitric oxide mediated vasodilation in the blood supply of the rat sciatic nerve »

4.1 Introduction

Nerve metabolism and function relies heavily on glucose as a substrate and, therefore, proper glucose and insulin regulation (1–7). Experimental diabetes (ie. chronic overt hyperglycemia) appears to decrease nerve blood flow (NBF) in rats (1,7,8) and insulin treatment may partially restore such decrements (1). Unlike skeletal muscle blood flow, the acute hemodynamic actions of glucose or insulin on NBF control remain unclear (1,2,6). In skeletal muscle, acute insulin infusion (9,10) or glucose-stimulated insulin secretion (GSIS) (11,12) stimulates a nitric oxide (NO)-mediated (13,14) vasodilation. This vasodilation enhances insulin binding and glucose uptake in skeletal muscle (15). Whether a similar hemodynamic pathway exists in the vasa nervorum requires further study.

Using video-microscopy, Davidson and colleagues (2) documented insulin-stimulated epineurial vasodilation. Applying the laser Doppler method, Biessels and co-workers (1) likewise observed an increase in nerve red blood cell flux following insulin infusion in streptozotocin induced diabetic rats. These data suggest that glucose may lead (via insulin stimulation) to neural artery vasodilation.

In contrast, Saini and colleagues (6) observed reductions in NBF following glucose infusion (3 g/kg). In the aforementioned experiments, insulin increased (2) or

restored (1) NBF, but GSIS did not (6). Potentially, hyperglycemia attenuates NBF and insulin-stimulated vasodilation (6). This vasoconstrictor effect of glucose on the nerve vasculature may relate to adverse effects of hyperglycemia on NO production and availability (8,16,17) or potentially to a glucose inhibitory effect on adenosine-mediated dilation (6). The discrepancies between these studies and outcomes also may result from variations in methods used to measure NBF. Nonetheless, a detailed examination of the relationship between acute glucose concentration, insulin secretion and nerve arterial vascular conductance warrants further study.

Therefore, the purpose of this study was to assess the independent vasoactive effects of acute hyperglycemia and hyperinsulinemia on an index of vascular conductance in the sciatic nerve vasculature of rats. Based on data by Saini and colleagues (6), this study tested the hypothesis that glucose infusion will reduce NBF and vascular conductance through a NO-independent vasoconstrictor mechanism. In contrast to the hypothesis, the results indicated that glucose stimulated an insulin-mediated, NO-dependent dilation in sciatic nerve blood supply.

4.2 Methods

Procedures and Animals

All procedures complied with the Animal Care guidelines and ethics approval board from The University of Western Ontario. We studied a total of 37 male Sprague Dawley rats (238-400 g) within four separate and sequential experiments whose objectives were as follows: 1) determine the effect of glucose on whole leg blood flow (femoral artery) which includes and emphasizes the skeletal muscle vasculature; 2)

determine the effect of glucose infusion at two doses (Low; 1 and High; 3 g/kg) on sciatic nerve vasomotor control; 3) determine if the glucose-stimulated change in nerve vascular control in #2 above was stimulated by concurrent insulin release; and 4) determine if the glucose-stimulated and insulin-mediated nerve vasomotor response in objectives #2 and #3 operated through an NO-dependent mechanism.

To assess the effect of hyperglycemia on nerve vasomotor control independent from the accompanying insulin response, we implemented a non-insulin secreting rat model (NIS). In this model rats received a 60 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA; citrate buffer pH 4.5) injection to impair pancreatic-beta cell function and attenuate GSIS. Forty eight hours after STZ injection (blood glucose ≥ 16 mmol/L), these rats also received a surgically implanted subcutaneous insulin pellet (Linplant, Linshin, Toronto, ON, CAN; release rate 1 IU/12 h) to reverse hyperglycemia and maintain normoglycemia. Experimental testing took place ~ 72 h post implantation of the insulin pellet.

Surgical Procedures

Surgeries were performed on the animals after a 12-h overnight fast. To achieve an appropriate surgical depth, rats inhaled isoflurane gas (5%). Thereafter, rats underwent an intra-peritoneal injection of urethane (50 mg/kg) and α -chloralose (8.0 mg/kg) at the onset of surgery. After ~ 30 min, the urethane α -chloralose mixture alone maintained surgical depth for the remainder of the experiment. A warming blanket placed beneath the animal maintained rat body temperature at 37°C (rectal probe). Catheter insertion into the right jugular vein facilitated all anaesthetic, glucose (EMD Millipore, Darmstadt,

HE, Germany.), insulin (Eli Lilly, Toronto, ON, CAN) and L-NG-nitroarginine methyl ester (L-NAME; Sigma-Aldrich, St. Louis, MO, USA) infusions. A second catheter was inserted into the right carotid artery and connected to a pressure transducer (PX272, Edwards Lifesciences, Irvine, CA, USA) to enable continuous blood pressure measurement. Subsequently, the left femoral artery or sciatic nerve blood supply was exposed. Sciatic nerve arterial exposure required sciatic nerve separation from surrounding muscle beds (gluteus maximus and biceps femoris) via blunt dissection (18). Following separation, parafilm weaved beneath the sciatic nerve and the underlying tissue provided a landmark to ensure visual consistency throughout the imaging protocol.

Experimental Protocol

Animals were allowed to stabilize for one hour after surgery. Thereafter, anaesthetized rats underwent one of seven experimental intravenous infusion protocols over the four experiments outlined above. To establish the effect of GSIS on skeletal muscle blood flow (i: n=6; 297±37 g) we measured femoral blood velocity (FBV) following the Low Glucose (50% solution) infusion via the jugular cannula (experiment 1). To examine the effect of GSIS on nerve vasomotor control, we measured nerve blood velocity (NBV) and mean arterial pressure (MAP) during infusion of Low Glucose (ii: n=5; 284±30 g), or High Glucose (iii: n=6; 291±57 g) (experiment 2). To establish whether the endogenous glucose-induced increase in insulin caused the observed effect (see Results), we assessed NBV and MAP during infusion of High Glucose alone in NIS rats (iv: n=5; 350±41 g) or during administration of a euglycemic hyperinsulinemic clamp (EHC; v: insulin: 10 mμ•kg⁻¹•min⁻¹; 0.4UI/mL; n=6; 260±9 g) (experiment 3). To determine if the GSIS affected nerve vasomotor control through a NO pathway, we co-

infused High Glucose with L-NAME (non-specific NOS blocker, 15 mg/kg) (vi: n=5; 357±48 g) (experiment 4). In a separate group (vii: n=5; 277±8 g), to assess whether the effects of L-NAME were exclusively on baseline values or if they inhibited the response to glucose infusion, L-NAME (15 mg/kg) infusion occurred alone at baseline and preceded High Glucose infusion by 20 min (experiment 4). In each of the above protocols (excluding the EHC), isovolumetric saline infusions were also made. In each protocol, measures of MAP, FBV or sciatic NBV were made at baseline, 5, 10, 20 and 30 min (and 80 min for EHC) following the infusion. The index of vascular conductance (VCi) was calculated as $VCi = \text{peak NBV}/\text{MAP}$.

Details of the Euglycemic Hyperinsulinemic Clamp

For the original description see DeFronzo et al. (19). Briefly, an infusion pump maintained insulin infusion $10 \text{ m}\mu\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (0.4 IU/mL) and induced a hyperinsulinemic state. Using a separate pump, glucose infusion commenced at $20 \text{ mg}\cdot\text{kg}\cdot\text{min}^{-1}$ (20% solution) to defend euglycemia. Maintenance of euglycemia was made through adjustments to the glucose infusion rate that, in turn, were based on blood glucose measurements made every five minutes during the first 20 min, and every 10 min thereafter.

Data Acquisition and Analysis

The analog blood pressure signal was sampled at 1000 Hz and stored online (Powerlab; ADInstruments, Colorado Springs, CO, USA). Pulsatile arterial pressure over ~ 10 heart beats was averaged to calculate MAP.

Blood flow velocity was measured using Doppler ultrasound (Vevo 2100 ultrasound system VisualSonics, Toronto, CA) and a 40 MHz linear array probe (MS550D) transducer placed superficially along the femoral artery or an artery lying superficially along the sciatic nerve. The arteries were located using power or colour Doppler. Thereafter, this segment was studied using duplex imaging mode (frequency of 32 MHz with 100% power, PRF between 4-5 kHz and wall filter of 40-50 Hz) with the pulsed wave Doppler gate (~0.12 mm width) positioned over the site of high power Doppler signal (insonation angle of 60°). The smallest vessels that could be detected in B-mode images acquired using the 40 MHz linear array probe were ~140 µm diameter (20). Based on the width of the power Doppler image, it has been approximated that the size of a sciatic supply artery vessel is ~70 - 80 µm. Moreover, the B-mode caliper resolution was ~15 µm/pixel. Therefore, insufficient resolution existed for confident measurements of either femoral or nerve arterial diameter changes that might have occurred in this study. Nonetheless, the greatest vasomotor changes occur downstream in the microvascular bed and these changes will form the dominant contribution to total changes in flow velocity through the insonated vessel segment. Therefore, blood flow velocity represents our surrogate for blood flow volume.

Blood samples obtained from the arterial catheter line enabled measurements of glucose (FreeStyle Lite, Abbot Diabetes Care, Alameda, CA, USA) and serum insulin (enzyme linked immunosorbent assay kit for human and rat insulin; ALPCO Diagnostics, Salem, NH, USA) concentrations.

Statistics

Statistical analyses were performed using Sigma Stat for Windows (version 8.0). Paired two-tailed t-tests were used to test the effect of infused agent on NBV and MAP (baseline versus peak response) for within-group comparisons. The interaction effect of group and time on the glucose, insulin and VCI changes were assessed using a mixed model (one-factor repetition) ANOVA. Where necessary, post-hoc Tukey tests determined the location of significance. The significance level was set at $P \leq 0.05$. Data are presented as mean \pm SD.

4.3 Results

General observations

Blood glucose and insulin concentrations: Glucose infusion produced a dose-dependent rise in blood glucose concentration (3 > 1 g/kg; $P < 0.001$; Table 4-1). Glucose concentration in the NIS group following High Glucose surpassed the measuring range of the glucometer (> 32 mmol/L). Insulin concentration in the High Glucose (3 g/kg) and hyperinsulinemic clamp groups exceeded those observed in the Low Glucose (1 g/kg) and NIS groups ($P < 0.001$; Table 4-1).

Nerve blood velocity and mean arterial pressure

Compared to their respective baseline levels, sciatic nerve NBV was greater in i) High Glucose, ii) High Glucose + L-NAME, and iii) insulin, ($P \leq 0.05$; Table 4-2). In all cases L-NAME significantly elevated MAP ($P \leq 0.05$; Table 4-2).

Experiment 1: Impact of glucose on femoral blood velocity, mean arterial pressure and femoral vascular conductance

In the femoral artery, infusion of Low Glucose increased FBV from 223 ± 60 mm/s at baseline to 432 ± 94 mm/s at peak ($P=0.001$) with no change in MAP (baseline= 87 ± 17 vs peak= 94 ± 19 mmHg; $P=0.153$). Consequently, femoral VCI increased from 2.7 ± 0.8 $\text{mm} \cdot \text{s}^{-1} \cdot \text{mmHg}^{-1}$ at baseline to 4.8 ± 1.5 $\text{mm} \cdot \text{s}^{-1} \cdot \text{mmHg}^{-1}$ at peak ($P=0.007$; Figure 4-1).

Experiment 2: Effect of glucose infusion at Low and High doses on sciatic nerve vasomotor control

Compared to baseline, Low Glucose did not alter VCI. However, High Glucose infusion increased VCI above baseline ($P<0.001$; Figure 4-2).

Experiment 3: Determine whether glucose stimulates an insulin-dependent or independent dilation

First, the nerve arterial vascular response to hyperglycemia was measured in the NIS group. In this model, glucose does not stimulate insulin secretion and insulin concentrations remain stable (pellet release rat1 IU/12). Compared with baseline, we observed no change in VCI following High Glucose infusion ($P=0.66$; Figure 4-3). Second, the nerve arterial vascular response was assessed during the euglycemic hyperinsulinemia clamp (EHC). A group x time interaction ($P=0.003$) was observed where the EHC caused an increase in VCI compared to both baseline EHC ($P<0.001$) and the peak NIS value ($P=0.004$; Figure 4-3).

Experiment 4: Determine if the glucose-mediated and insulin-dependent nerve vasomotor response was dependent upon NO production

This experiment examined whether NO mediated the glucose-stimulated insulin-dependent dilation. The addition of L-NAME 20 min pre-, or co-infused with, High Glucose abolished any increase in VCI induced by High Glucose alone ($P > 0.2$; Figure 4-4). A group x time interaction ($P < 0.001$) was observed where, compared with other conditions, High glucose alone stimulated increases in VCI (all comparisons $P \leq 0.011$; Figure 4-4).

Table 4-1 Glucose and Insulin Concentrations

Group	Glucose (mmol/L)			Insulin (uIU/mL)	
	Baseline	10 min	30 min	Baseline	10 min
Low Glucose (1 g/kg; n=5)	5.0±0.9	11.9±2.2 ^{^§}	6.4±0.7	8±2	43±19 [^]
High Glucose (3 g/kg; n=6)	5.0±0.9	29.1±2.5 ^{^§*}	16.2±2.9 ^{^§*}	10±7	182±42 ^{^*†}
High Glucose NIS (n=5)	4.6±1.5	>32 ^{^§*‡a}	>32 ^{^§*‡a}	38±17	43±29
EHC (n=6)	5.3±0.8	5.6±1.4	5.2±1.0	24±15	215±9 ^{^*†b}

Values are Mean ± SD.

NIS: non-insulin secreting

EHC: euglycemic hyperinsulinemic clamp

[^]Significantly greater than baseline (within group comparison; P≤0.01)

[§]Significantly greater than the EHC group (P<0.001) at the same time point

^{*}Significantly greater than the Low Glucose dose (P<0.001) at the same time point

[†]Significantly greater than the NIS group (P<0.001) at the same time point

[‡]Significantly greater than the High Glucose dose (P<0.001) at the same time point

^a Too high for the glucometer to measure (>32 mmol/L; input 32 mmol/L for stats)

^b Insulin sample acquired at 80 min

Table 4-2 NBV and MAP

Group	NBV (mm/s)		MAP (mmHg)	
	Baseline	At Peak VCI	Baseline	At Peak VCI
Low Glucose (1 g/kg; n=5)	83±30	103±24*	93±27	99±38
High Glucose (3 g/kg; n=6)	82±48	138±64*	89±24	76±24
High Glucose NIS (n=5)	62±19	73±21	82±8	86±6
EHC clamp (n=6)	75±30	167±62*	78±14	77±12
High Glucose + L-NAME (15 mg/kg; n=5)	81±23	130±36*	81±16	132±24*
L-NAME alone (n=5)	59±5	69±20	110±33	146±24*
High Glucose added to L-NAME (n=5)	83±42	110±45	99±28	110±29
Isovolumetric saline infusion (n=6)	86±36	86±38	84±16	87±18

Values are Mean ± SD. *, Significantly greater than baseline of the same protocol (P≤0.05).

NBV: nerve blood flow velocity

MAP: mean arterial pressure

VCI: index of vascular conductance

NIS: non-insulin secreting

EHC: euglycemic hyperinsulinemic clamp

L-NAME: L-NG-nitroarginine methyl ester

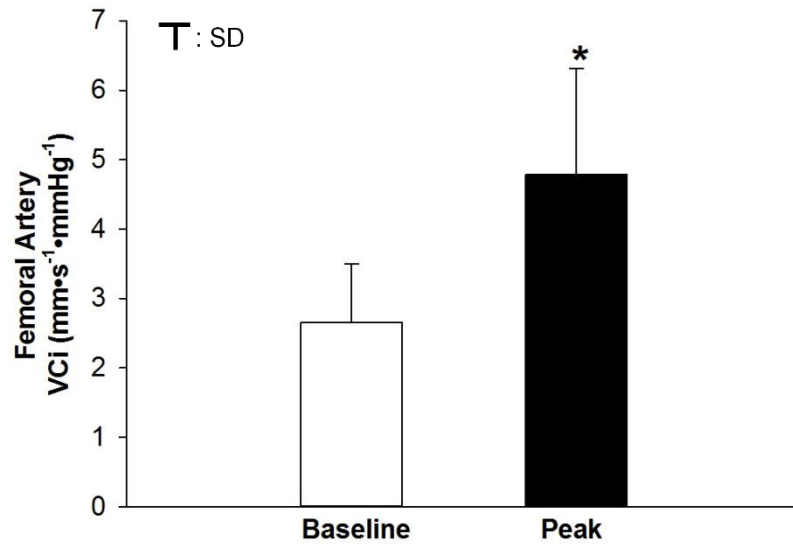


Figure 4-1 Femoral artery index of vascular conductance (VCI; mm·s⁻¹·mmHg⁻¹) pre- and post- infusion of Low Glucose (1 g/kg). *, Significantly greater than baseline (P=0.007).

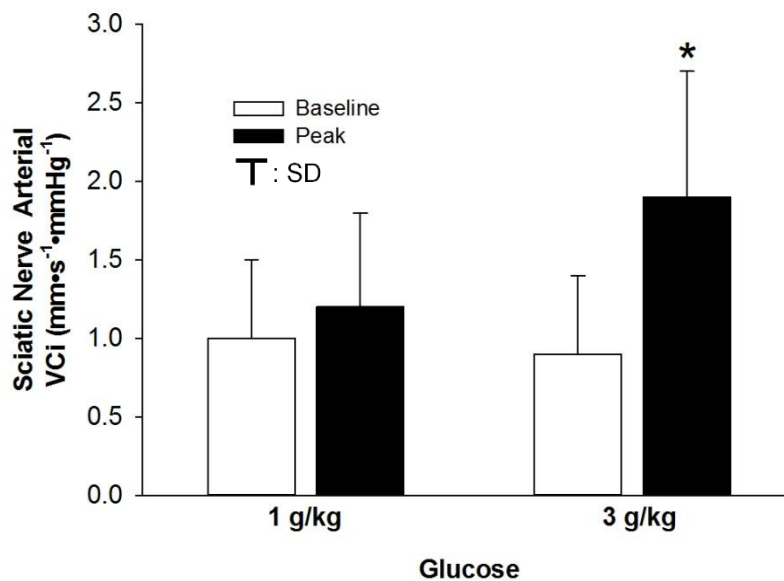


Figure 4-2 Sciatic nerve arterial index of vascular conductance (VCI; mm·s⁻¹·mmHg⁻¹) pre- and post- Low (1g/kg) and High (3 g/kg) Glucose infusions. *, Significantly greater than baseline (P<0.001).

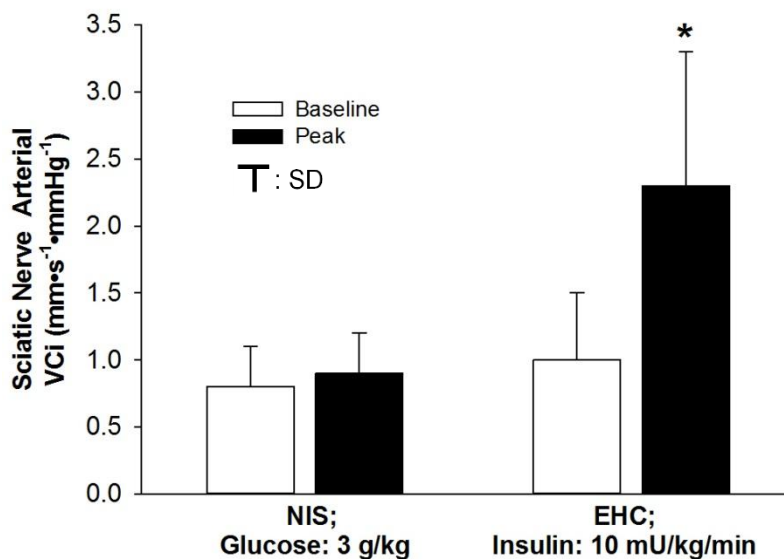


Figure 4-3 Sciatic nerve arterial index of vascular conductance (VCI; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) pre- and post- hyperglycemia+euinsulinemia (NIS) and euglycemia+hyperinsulinemia (EHC). *, Significantly greater than corresponding baseline and peak NIS ($P\leq 0.004$).

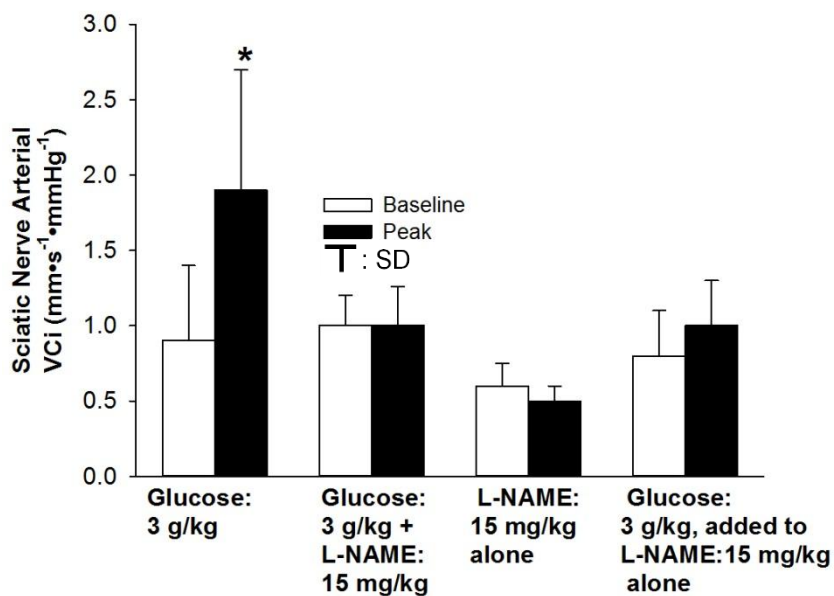


Figure 4-4 Sciatic nerve arterial index of vascular conductance (VCI; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) pre- and post- glucose and L-NAME infusions. *, Significantly greater than corresponding baseline, High Glucose (3g/kg) co-infused with L-NAME (15 mg/kg), L-NAME alone and High Glucose infused 20 min following L-NAME ($P\leq 0.011$).

4.4 Discussion

The major new findings of the current study are: i) glucose infusion did not cause a decrease in VCI, ii) High, but not Low, Glucose resulted in increases in VCI (~120% from baseline), iii) GSIS mediated the nerve arterial dilation in response to glucose infusion, and vi) GSIS-mediated dilation was caused by a NO mechanism. These findings do not support the hypothesis that acute hyperglycemia produces vasoconstriction in the rat sciatic nerve arteries, as per earlier results (6). Rather, the findings indicate that the sciatic nerve vasculature reacts similarly to skeletal muscle by vasodilating with glucose infusion through a NO mechanism in response to the acute insulinemic response.

Experiment 2: Glucose infusion increased VCI

The current data conflict with those of Saini and co-workers (6) who observed a reduction in NBF following the same intravenous glucose infusion protocol (3 g/kg). Both studies used Sprague-Dawley rats and MAP responses appear to be similar. However, we performed measurements at 0, 5, 10, 20 and 30 min following glucose infusion, whereas Saini and colleagues (6) took measurements at 0, 15, 30, 45, 60, 90 and 120 min following glucose infusion. The peak glucose values obtained following High Glucose (3 g/kg) infusion were ~three fold greater in our experiment (~ 30 mmol/L) than in the earlier study (~10 mmol/L) (6). The reason for this difference remains unclear. Conceivably, insulin secretion would be greater in the current study due to the greater glucogenic stress. Insulin values are not known from this earlier study and independent manipulation of blood glucose and insulin concentrations were not performed. In addition, in the current study, the use of fixed probe Doppler ultrasound system limited NBF velocity measures to a single segment of a superficial nerve artery. Alternatively,

Saini et al. (6) used laser Doppler to detect net sciatic nerve vascular flux. This measure of flux represents the net signal from all red blood cells flowing in any direction. The sciatic nerve contains feed arteries arising from multiple positions (for example via gluteal and popliteal arteries) located primarily at the nerve junctions (21–23). Such an arrangement led to the proposition of bidirectional flow in the nerve (i.e. arteries traveling in opposite directions along the same segment of the nerve) , as well as the presence of arterial-venous anastomoses (21–23). If this scenario exists, measures of blood flow flux in a large region of the nerve may not capture arterial inflow responses. The combined methodological differences make it difficult to compare results between the current and previous studies.

Experiment 3: insulin not hyperglycemia stimulated the rise in VCI

The novel findings from this experiment on the blood supply of the sciatic nerve compliment previous data (1,2) that suggest acute insulin administration increases NBF. Biessels and colleagues (1) speculated that the effect of insulin to increase NBF may relate to either a reduced glucose-stimulated vasoconstriction or perhaps insulin itself exhibits vasodilatory properties. Here we demonstrated that GSIS produced a dominant dilatory response in the sciatic nerve vasculature.

Using hydrogen clearance, reductions in basal NBF have been observed following chronic uncontrolled hyperglycemia (7). Employing a similar model of hyperglycemia, Biessels and colleagues (1) reversed decrements in basal NBF partially with insulin treatment. Specifically, acute insulin treatment restored basal NBF in chronically hyperglycemic rats to ~85% of healthy control values. In the present experiment the

insulin infusion rate was less than that used by Biessels and colleagues (1) but, nonetheless, a ~50% greater vasodilation was observed. Importantly, Sprague-Dawley rats were used in the current study, rather than Wistar rats, as used previously. Finally, the rats used here were not subjected to prior chronic hyperglycemia, a procedure that may impair endothelial function and NO production (8,17,24).

Experiment 4: L-NAME infusion attenuates GSIS-mediated rises in VCI

Previous studies report a role for insulin, acetylcholine (2), eNOS (8) and nNOS (17) in sciatic nerve arterial vascular control. Therefore, this study included an examination of the potential contributions of NO to the GSIS dilatory effect. The evidence that L-NAME, given before or with glucose, abolished the glucose-stimulated, insulin-mediated dilation points to an important role for NO in this response. Taken together, it appears NO influences NBF control and the vasodilatory response to combined glycemic and insulinemic stress. Whether insulin and glucose act synergistically to stimulate a NO-mediated dilation remains unclear. A study conducted by Oomen et al. (25) documented a trend towards enhanced skin blood flow following hyperglycemia + hyperinsulinemia compared with euglycemia + hyperinsulinemia in Type I Diabetic participants suggesting an interaction may occur. Nonetheless, despite differences in circulating concentrations of glucose between the High Glucose infusion (~30 mmol/L) and the EHC (~5 mmol/L) condition in the present study, GSIS and insulin infusion resulted in comparable sciatic nerve arterial vasodilation. Potentially the combination of elevated glucose and insulin enhances any vasodilatory actions; but this question requires further study.

Perspectives and Significance

The current study highlighted the independent contributions of insulin and glucose to NBF control under healthy conditions and clarifies earlier observations (1,2) regarding the potential vasodilatory actions of insulin in the rat sciatic nerve vasculature. In particular, insulin appears to vasodilate the blood supply of the sciatic nerve through an NO-mediated mechanism.

These results may have particular relevance to altered nerve function in diabetes. In the context on type I and II diabetes, the absence of chronic circulating insulin (1,7,8) or alternatively, vascular insulin resistance in the vasa nervorum (2) may contribute to impaired NO signaling and help explain previously observed decrements in sciatic NBF and nerve function. Experimental diabetes or uncontrolled hyperglycemia appears to reduce NBF and impair nerve function in rats (1,7,8). Whether this occurs as a result of hyperglycemia or hypoinsulinemia remains uncertain. Insulin treatment may partially restore NBF and nerve function (1), but these adaptations occur alongside reductions in blood glucose concentration rendering it difficult to isolate the exclusive effects of insulin. Whether stable basal circulating insulin concentrations (similar to the NIS group), or the fluctuating insulin concentrations resulting from pulsatile insulin secretion, cause differential effects on nerve health requires further study.

In the present study, NO inhibition alone did not alter baseline vascular conductance. Instead, NO inhibition impaired GSIS-mediated vasodilation. If basal insulin concentrations contribute to the maintenance of basal NBF, the mechanism may be NO-independent. Alternatively, numerous mechanisms may operate in conjunction

with some degree of redundancy to maintain basal NBF during NO inhibition.

Attenuated vasodilation during NO inhibition suggests that the sciatic nerve vasculature may operate similarly to skeletal muscle vasculature (15), in that the hemodynamic actions of insulin may serve to enhance insulin binding and perpetuate any downstream effects.

Likely, both chronic insulin concentrations and insulin-mediated vasodilation serve metabolic and vascular functions to preserve neural health. Elucidating their independent contributions to nerve health is of particular importance when evaluating the efficacy of various insulin delivery therapies (for insulin-dependent diabetes). For example, use of a continuous insulin delivery system, such as the insulin pump, may preserve vasa nervorum health to a greater extent than conventional insulin injections. Considering the current data and reports that indicate a role for insulin in the maintenance of basal (1) and vasomotor control of NBF (2), the hemodynamic actions of insulin must be considered when studying the vascular etiology of peripheral neuropathy in diabetes.

Considerations

Although core temperature was maintained at 37°C, sciatic nerve/limb temperature was not recorded. Previously, Kihara and co-workers (26) demonstrated a positive relationship between limb temperature and sciatic NBF. Presumably, in the current experiment exposure of the sciatic nerve may have cooled the surrounding tissue and reduced NBF velocity. However, any effects of cooling likely took place during the stabilization period as baseline conductance values were similar between groups and blood flow velocity did not decrease during the baseline period. Further, during all

protocols, the index of conductance either remained unchanged or increased. While the possibility remains that limb cooling may have attenuated the dilatory responses, this occurrence would not affect the overall conclusions of the current study.

The exact isoform of nitric oxide synthase was not determined in the current study as L-NAME acts as a non-selective NOS inhibitor. Although insulin stimulates the endothelial NOS pathway in skeletal muscle arterioles (13,14), evidence suggests both endothelial (2,3,8) and neuronal (17) NO may serve a role in NBF control. The current approach may affect both of these sources of NO but separate roles for endothelial, neuronal or inducible forms of NO in the insulin-dependent dilation remain uncertain.

Assuming no change in arterial diameter, the Doppler ultrasound method reflects an effective technique to detect sciatic nerve arterial flow waveforms and changes in blood flow (27). The advantage of this method includes quantifiable changes in flow and waveform patterns through arterial segments, excluding venous contributions to overall flow flux or hydrogen clearance. However, we can only measure the Doppler signal on a single artery at a time. Moreover, the inability to obtain arterial diameters forced us to rely on blood velocity measurements alone as the analog of changes in total flow. This approach, often used in studies of cerebral blood flow velocity patterns (28–30), assumes the diameter of the interrogated vessel does not change such that any changes in velocity reflect events happening to the downstream vascular bed. Certainly, the validity of this assumption requires confirmation in the sciatic nerve arterial network. Nonetheless, failure to capture any local dilation (ie. increases in arterial diameter) in interrogated supply vessel would underestimate the overall dilator responses under investigation in the current study, a limitation that does not alter the overall conclusions.

Conclusion

This experiment demonstrated that glucose infusion stimulates an insulin-dependent NO-mediated dilation along the superficial artery of the sciatic nerve. In addition, increasing the glucose infusion from 1 to 3 g/kg enhances the vasodilatory response. Lastly, glucose-stimulated insulin secretion and insulin infusion elicit a similar relative vasodilation.

4.5 References

1. Biessels GJ, Stevens EJ, Mahmood SJ, Gispen WH, Tomlinson DR. Insulin partially reverses deficits in peripheral nerve blood flow and conduction in experimental diabetes. *J. Neurol. Sci.* 1996;140:12–20.
2. Davidson EP, Coppey LJ, Calcutt NA, Oltman CL, Yorek MA. Diet-induced obesity in Sprague – Dawley rats causes microvascular and neural dysfunction. *Diabetes. Metab. Res. Rev.* 2010;26:306–18.
3. Coppey LJ, Gellett JS, Davidson EP, Yorek MA. Preventing superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats restores endothelium-dependent vasodilation. *Free Radic Res.* 2003;37(1):33–40.
4. Field RA, Adams LC. Insulin response of peripheral nerve. Effects on glucose metabolism and permeability. *Medicine (Baltimore).* 1964;43(2):275–9.
5. Kihara M, Zollman PJ, Smithson IL, Langerlund TD, Low PA. Hypoxic effect of exogenous insulin and diabetic peripheral nerve on normal. *Am J Physiol Endocrinol Metab.* 1994;29:980–5.
6. Saini AK, Arun KHS, Kaul CL, Sharma SS. Acute hyperglycemia attenuates nerve conduction velocity and nerve blood flow in male Sprague-Dawley rats: reversal by adenosine. *Pharmacol. Res.* 2004;50(6):593–9.
7. Tuck RR, Schmelzer JD, Low PA. Endoneurial blood flow and oxygen tension in the sciatic nerves of rats with experimental diabetic neuropathy. *Brain.* 1984;107:935–50.
8. Maxfield EK, Cameron NE, Cotter MA. Effects of diabetes on reactivity of sciatic vasa nervorum in rats. *J. Diabetes Complicat.* 1997;11(1):47–55.
9. Abramson DI, Schkloven N, Margolis MN, Mirsky IA. Influences of massive doses of insulin on peripheral blood flow in man. *Am J Physiol.* 1939;128:124–32.

10. Rattigan S, Clark MG, Barrett EJ. Hemodynamic actions of insulin in rat skeletal muscle. Evidence for capillary recruitment. *Diabetes*. 1997;46(9):1381–8.
11. Baron AD, Laaksof M, Brechtel G, Hoit B, Watt C, Edelman SV. Reduced postprandial skeletal muscle blood flow contributes to glucose intolerance in human obesity. *J. Clin. Endocrinol. Metab.* 1990;70(6):1525–33.
12. Vincent MA, Clerk LH, Lindner JR, Price WJ, Jahn LA, Leong-poi H, et al. Mixed meal and light exercise each recruit muscle capillaries in healthy humans. *Am J Physiol.* 2006;290:E1191–E1197.
13. Steinberg H, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Investig.* 1994;94:1172–9.
14. Scherrer U, Randin D, Vollenweider P, Vollenweider L, Nicod P. Nitric oxide release accounts for insulin's vascular effects in humans. *J. Clin. Investig.* 1994;94:2511–5.
15. Baron AD, Steinberg H, Brechtel G, Johnson A. Skeletal muscle blood flow independently modulates insulin-mediated glucose uptake. *Am J Physiol.* 1994;29:E248–E253.
16. Brodsky SV, Morrishow AM, Dharia N, Gross SS, Goligorsky MS. Glucose scavenging of nitric oxide. *Am. J. Physiol. Renal Physiol.* 2001;280(3):F480–F486.
17. Ii M, Nishimura H, Kusano KF, Qin G, Yoon Y, Wecker A, et al. Neuronal nitric oxide synthase mediates statin-induced restoration of vasa nervorum and reversal of diabetic neuropathy. *Circulation.* 2005;112:93–102.
18. Uysal CA, Mizuno H, Hyakusoku H. Sciatic nerve anatomy in rat re-visited: a more proximal intervention. *J Plast Reconstr Aesthet Surg.* 2009;62(6):847–9.

19. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol.* 1979;237(3):E214–E223.
20. Moran CM, Pye SD, Ellis W, Janeczko A, Morris KD, McNeilly AS, et al. A comparison of the imaging performance of high resolution ultrasound scanners for preclinical imaging. *Ultrasound Med. Biol.* 2011;37(3):493–501.
21. Adams WE. The blood supply of nerves I. Historical review. *Anatomy.* 1942;76:323–41.
22. Bell MA, Weddell AG. A descriptive study of the blood vessels of the sciatic nerve in the rat, man and other mammals. *Brain.* 1984;107:871–98.
23. Zamir M, Twynstra J, Vercnocke AJ, Welch I, Jorgensen SM, Ritman EL, et al. Intrinsic microvasculature of the sciatic nerve in the rat. *J Peripher Nerv Syst.* 2012;17:377–84.
24. Makimattila S, Virkamäki A, Groop P-H, Cockcroft J, Utriainen T, Fagerudd J, et al. Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. *Circulation.* 1996;94(6):1276–82.
25. Oomen PHN, Kant GD, Dullaart RPF, Reitsma WD, Smit AJ. Acute hyperglycemia and hyperinsulinemia enhance vasodilatation in Type 1 diabetes mellitus without increasing capillary permeability and inducing endothelial dysfunction. *Microvasc. Res.* 2002;63(1):1–9.
26. Kihara M, Schmelzer JD, Kihara Y, Smithson IL, Low PA. Efficacy of limb cooling on the salvage of peripheral nerve from ischemic fiber degeneration. *Muscle Nerve.* 1996;19(2):203–9.
27. Twynstra J, Medeiros PJ, Lacefield JC, Jackson DN, Shoemaker JK. Y1R control of sciatic nerve blood flow in the Wistar Kyoto rat. *Microvasc. Res.* 2012;84(2):133–9.

28. Serrador JM, Picot PA, Rutt BK, Shoemaker JK, Bondar RL. MRI Measures of Middle Cerebral Artery Diameter in Conscious Humans During Simulated Orthostasis. *Stroke*. 2000;31(7):1672–8.
29. Valdueza JM, Balzer JO, Villringer A, Vogl TJ, Kutter R, Einhüpl KM. Changes in blood flow velocity and diameter of the middle cerebral artery during hyperventilation: assessment with MR and transcranial Doppler sonography. *Am. J. Neuroradiol*. 1997;18(10):1929–34.
30. Schreiber SJ, Gottschalk S, Weih M, Villringer A, Valdueza JM. Assessment of blood flow velocity and diameter of the middle cerebral artery during the acetazolamide provocation test by use of transcranial Doppler sonography and MR imaging. *Am. J. Neuroradiol*. 2000;21(7):1207–11.

Chapter 5

5 « Exercise training enhances insulin-stimulated nerve arterial vasodilation in rats with insulin-treated experimental diabetes »

5.1 Introduction

Diabetes leads to several clinical comorbidities including vascular disease and peripheral neuropathy (1,2). The pathology of diabetes peripheral neuropathy is multifactorial and often studied in the context of a metabolic or vascular etiology (3). From a metabolic perspective, experimental diabetes in rats (model for type 1 diabetes that causes chronic severe hyperglycemia ~20-40 mmol/L) results in increased neuronal glucose, fructose, polyols, aldose reductase activity, protein kinase C activity and mono-enzymatic protein glycosylation as well as a reduction in neuronal myoinositol and sodium-potassium ATPase activity (3–8). From a vascular perspective experimental diabetes is linked with reduced endothelial function (9,10) and nerve blood flow (NBF) (10–16). Together, these factors contribute to nerve dysfunction (11–13,15). Although, reduced NBF does not always precede diabetes-induced deficits in nerve function (5,17,18), impaired vasa nervorum reactivity is implicated in peripheral nerve dysfunction and may be related to altered nitric oxide (NO) signaling or bioavailability (9,10,16,19–23) as well as enhanced adrenergic-mediated vasa nervorum constriction (23, 36, 63).

Insulin therapy is a common and effective treatment for type 1 diabetes. In rats with experimental diabetes, concurrent insulin treatment prevents the decline in NO-related vasa nervorum reactivity (9). Further, insulin treatment administered one-month

following diabetes induction partially restores basal NBF and motor nerve conduction velocity (MNCV) (15). However, in the aforementioned study, insulin treatment alone did not completely reverse the effects of chronic hyperglycemia. Conceivably, in rats with experimental diabetes, insulin treatment may restore NBF and vasoreactivity partially through a NO-related mechanism. In addition to the effects of insulin treatment on basal NBF, acute insulin administration stimulates epineurial arterial vasorelaxation and increases sciatic NBF velocity (NBV) in rats (24,25). It is uncertain what compounds mediate the insulin-induced dilation (21), but glucose-stimulated insulin-mediated dilation can be prevented by NO synthase (NOS) inhibition (25). The vasodilatory actions of insulin were documented over 70 years ago (26), and since then it has been established that insulin-mediated vasodilation occurs through the activation of the intracellular enzymes phosphatidylinositol 3-kinase (PI3-K) and protein kinase B (Akt), which phosphorylate and activate endothelial NOS (eNOS) (27,28). How the relationship between insulin sensitivity and eNOS expression influences peripheral NBF control remains to be elucidated fully.

Aside from insulin therapy, other therapeutic agents directly or indirectly targeted towards NO have been used to prevent experimental diabetes-induced reductions in NBF or vasoreactivity (10,13,19–22,29). Non-pharmacological interventions, such as concurrent exercise training, have also been used to prevent diabetes-related peripheral neuropathy (30,31). However, the effects of exercise training on the preservation of NBF and vasa nervorum reactivity have yet to be established. In skeletal muscle, insulin-stimulated vasodilation is impaired by insulin resistance (32), improved by exercise training (which is known to increase insulin sensitivity) (33) and appears to be mediated

in part by eNOS (28,34,35) as well as upstream targets PI3-K and Akt (36). Thus, it is plausible that chronic hyperglycemia-induced impairments in insulin signaling and NO-mediated vasodilation (9,10,15,16,37) may be avoided by concurrent exercise training (38,39).

Previous observations suggest impaired vasa nervorum reactivity is related to altered NO signaling and peripheral nerve dysfunction (9,10,16,19–23). Therefore, we tested the hypothesis that insulin-treated experimental diabetes reduces vasa nervorum reactivity to insulin, as well as decreases sciatic nerve eNOS expression, and that these alterations would be prevented by concurrent endurance exercise training. To address this hypothesis we compared control sedentary rats (CS) to rats with insulin-treated experimental diabetes that were either sedentary (DS) or exercise-trained (DX).

The above question relates to the ability of endurance exercise training to ameliorate the effects of diabetes on NBF control. However, it does not consider the impact of diabetes on exercise trained rats alone. For example, in a previous study (33) where insulin-stimulated NO-mediated vasodilation in the cutaneous vasculature was studied in sedentary and endurance trained rats with and without experimental diabetes, relative to their sedentary counterparts, insulin administration (10^{-4} mol/L) induced vasodilation in trained rats with diabetes but not trained control rats. These data suggest insulin-mediated vasodilation may be different in endurance trained rats with and without diabetes. However, in the aforementioned study the analyses focused on the effects of trained vs untrained and not the effects of diabetes on exercise training. Therefore, to assess the effects of insulin-treated experimental diabetes on endurance exercise trained rats alone, we conducted a secondary study whereby the responses of DX rats were

contrasted with a control exercise group (CX). This study tested the hypothesis that insulin-mediated vasa nervorum dilation would be attenuated in DX rats vs CX rats.

5.2 Methods

Study 1

Procedures

All procedures complied with the Animal Care guidelines and ethics approval board from The University of Western Ontario. Eight-week old Sprague-Dawley male rats were obtained from Charles River Laboratories (Saint-Constant, Quebec, CA) and were housed in pairs at a constant temperature of $20\pm 1^{\circ}\text{C}$ with a 12-hour light/dark cycle. Rats had *ad libitum* access to commercial chow (protein=26%, carbohydrate=60%, fat=14%; enriched with vitamins and minerals; PROLAB RMH 3000, Brentwood, MO, USA) and water. Upon arrival, rats were divided into three groups; control sedentary (CS; n=7), insulin-treated experimental diabetes sedentary (DS; n=9) and insulin-treated experimental diabetes exercised (DX; n=9). Each week all rats were weighed and their fed state blood glucose was measured (saphenous vein sample). At 10 weeks following control conditions, insulin therapy or insulin therapy and exercise training, rats were anaesthetised and underwent a euglycemic hyperinsulinemic clamp and NBV was measured at baseline and every 10 min for 80 min. Following the insulin clamp, final blood samples were collected, MNCV was measured and the right sciatic nerves were harvested, flash frozen in liquid nitrogen and (along with the plasma samples) stored at -70°C for later analyses.

Animals and diabetes induction

To achieve insulin-treated experimental diabetes (i.e. moderately hyperglycemic state), eight-week-old rats received daily intraperitoneal injections of 20 mg/kg streptozotocin (STZ; Sigma-Aldrich, St. Louis, MO, USA; citrate buffer pH 4.5) for five consecutive days. Following the confirmation of two consecutive blood glucose measures of ≥ 18 mmol/L, rats received a surgically implanted subcutaneous insulin pellet (Linplant, Linshin, Toronto, ON, CAN; release rate 1 IU/12 h). Each week, pellet size was adjusted (increased or decreased) to maintain moderate hyperglycemia (fed-state blood glucose obtained during dark cycle ~ 14.5 mmol/L) (42).

Endurance exercise training

Prior to beginning the exercise training protocol, rats were familiarized with treadmill running. Familiarization consisted of two 15 min incremental treadmill running exercise sessions. The exercise training protocol consisted of running on a motorized treadmill at 27 m/min on a 6° incline, for 60 min, 5 days/week for 10 weeks. In healthy rats, this represented an exercise intensity of ~ 75 -85% VO_2max (43).

Surgical procedures

Prior to surgery, rats were fasted for 12 h. They were anesthetized with inhaled isoflurane gas (4%) and an intra-peritoneal injection of urethane (25 mg/kg) and α -chloralose (4 mg/kg). At doses 50 times greater, urethane causes an increase of blood glucose of ~ 2 -3 mmol/L; however, in the present study, the dose of urethane was small and exogenous insulin during the euglycemic hyperinsulinemic clamp was used to

stabilize blood glucose concentrations (44). After ~20 min, the isoflurane gas was removed and the urethane α -chloralose mixture alone maintained surgical depth. To maintain body temperature at 37°C (assessed via rectal probe) a warming blanket was placed beneath the animal. To facilitate infusions of anaesthetic, insulin ($10 \text{ m}\mu\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $0.4 \text{ }\mu\text{IU/mL}$; Eli Lilly, Toronto, ON, CAN) and glucose ($20 \text{ mg}\cdot\text{kg}\cdot\text{min}^{-1}$, 0.2 g/mL ; EMD Millipore, Darmstadt, HE, Germany), a catheter was inserted into the right jugular vein. To measure continuous blood pressure, another catheter was inserted into the right carotid artery and connected to a pressure transducer (PX272, Edwards Lifesciences, Irvine, CA, USA). To measure NBV, the left sciatic nerve was exposed. This required sciatic nerve separation from surrounding muscle beds (gluteus maximus and biceps femoris) via blunt dissection (45). To identify and maintain the same nerve segment throughout the imaging protocol, a thin slice of parafilm (serving as a landmark) was weaved beneath the sciatic nerve.

Euglycemic hyperinsulinemic clamp

For the original description of the euglycemic hyperinsulinemic clamp see DeFronzo et al. (46). After surgery, all rats were stabilized for ~60 min before beginning the experiment and mean arterial pressure (MAP) as well as NBV were stable for ~15 min prior to rats undergoing the euglycemic hyperinsulinemic clamp protocol. Briefly, insulin infusion at $10 \text{ m}\mu\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ (0.4 IU/mL) was maintained using an infusion pump. Blood glucose concentrations were sustained using a separate pump. To maintain euglycemia, blood glucose was infused at a variable rate that was determined by glucose measures performed every 5 min for the first 20 min and every 10 min thereafter (FreeStyle Lite, Abbot Diabetes Care, Alameda, CA). Sciatic nerve blood velocity and

MAP values were recorded at baseline and every 10 min for 80 min. These values were used to calculate the index of vascular conductance ($VC_i = NBV/MAP$) and area under the curve with respect to increase above baseline (47) (AUC_i ; surrogate for total dilation) throughout the insulin clamp. After the euglycemic hyperinsulinemic clamp experiment, a final blood sample was collected and plasma samples were stored at -70°C for later analysis. Plasma insulin (ALPCO Diagnostics, Salem, NH, USA) and norepinephrine (Biotang, Lexington, MA, USA) concentrations were determined using enzyme-linked immunosorbent assay kits.

Data acquisition and Doppler parameters

The analog blood pressure signal was sampled at 1000 Hz (Powerlab; ADInstruments, Colorado Springs, CO, USA). Pulsatile arterial pressure was averaged over 10 beats to calculate MAP. A 40 MHz high-frequency linear array probe (MS550D, VisualSonics, Toronto, Canada) and the Vevo 2100 ultrasound system (VisualSonics, Toronto, Canada) were used to measure NBV. The probe was positioned over the sciatic nerve and an artery was located using power Doppler. In duplex imaging mode (frequency of 32 MHz with 100% power, PRF between 4–5 kHz and wall filter of 40–50 Hz) the pulsed-wave Doppler gate was positioned over the power Doppler signal (insonation angle of 60°) and this arterial segment was imaged throughout the insulin clamp. At 40 MHz, this ultrasound system did not have sufficient resolution to measure changes in the sciatic nerve supply artery diameter in B-mode images. Thus, peak blood flow velocity (outer envelope of the mean blood flow velocity waveform) represents a surrogate for blood flow rate.

Sciatic motor nerve conduction velocity

Sciatic-fibular (peroneal) MNCV was measured following the insulin clamp protocol. Single, 100 μ s square wave pulses were applied directly to the exposed nerve at the sciatic notch, and separately in the popliteal fossa via a clinical electrical stimulator (NeuroscanComperio, Compumedics Medical Systems, El Paso, TX, USA). The elicited compound muscle action potentials (CMAP) were recorded with tungsten electrodes inserted into the tibialis anterior. Subsequently, CMAP were displayed, stored and analyzed on a clinical electromyography (EMG) system (NeuroscanComperio, Compumedics Medical Systems, El Paso, TX, USA). All EMG signals were sampled at 10 kHz and band-pass filtered at 10 Hz to 10 kHz. The MNCV (m/s) was calculated as: $\text{MNCV} = \text{distance between stimulation sites} / \text{time difference in CMAP latencies}$.

Sciatic nerve eNOS expression

Following euthanasia, sciatic nerves were harvested and flash frozen in liquid nitrogen and stored at -70°C for later analysis.

Nerves were homogenized under liquid nitrogen with mortar and pestle, immersed in ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM sodium chloride, 5 mM ethylene glycol tetraacetic acid, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate and 1% Triton-X 100) containing protease inhibitors (104 mM AEBSF, 80 μ M aprotinin, 2.1 mM leupeptin, 3.6 mM bestatin, 1.5 mM pepstatin A, 1.4 mM E-64, Pierce Rockford, IL) and then sonicated. Tissue lysates were centrifuged for 15 min at 14 000 rpm at 4°C . Supernatant was collected and stored at -70°C until protein concentration was determined.

Total protein concentration of samples was determined by Bradford assay. Thirty-five micrograms of protein from each sample were loaded on a 4% to 10% gel and separated by SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes and the blocked for 4 h in 5% milk in Tris Buffer Saline +Tween 20 (0.5%) (TTBS) at 4°C. The membranes were incubated in primary antibody specific for eNOS (mouse monoclonal anti-eNOS IgG, BD 610297, 1:2000) overnight at 4°C. Membranes were subsequently incubated with horseradish peroxidase-conjugated secondary antibody specific for mouse IgG (goat anti-mouse IgG-HRP conjugate, BioRad 170–6516, 1:2000) for 1h at room temperature. Immunoreactive bands were detected using an Immun-Star WesternC© chemiluminescent kit (Bio-Rad, Hercules, CA, USA) and imaged with the ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA). To account for loading, membranes were stripped then incubated in primary antibody specific to β -actin (Anti-beta Actin, abcam ab8227, 1:5000) then secondary HRP (1:20000) and imaged. Densitometric band analysis was performed with QuantityOne 1-D Analysis Software (Bio-Rad, Hercules, CA, USA). Quantified protein expression values were normalized to β -actin.

Study 2

To examine the effects of insulin-treated experimental diabetes on endurance exercise training, we conducted a secondary study wherein the DX responses were compared with a control exercise training group (CX; n=7). The exercise intervention and experimental protocols were the same in the two groups.

Statistics

Body mass (at surgery), fed state blood glucose, insulin pellet dose and glucose infusion rate during the insulin clamp as well as norepinephrine and insulin concentrations at the end of the insulin clamp were compared using a one-way ANOVA or independent two-tailed t-tests (Sigma Stat for Windows, version 8.0). These comparisons were divided into two separate analyses; study 1 (CS rats, DS rats and DX rats; Table 5-1) and study 2 (CX rats and DX rats).

The effect of group and time on baseline and peak VCI, as well as NBV and MAP (at baseline and the peak VCI) during the insulin clamp, were studied using a mixed model ANOVA. The effects of group on area under the curve for VCI during the insulin clamp, MNCV and the eNOS:β-actin ratio were compared using a one-way ANOVA (CS rats, DS rats and DX rats) or independent two-tailed t-tests (CX rats and DX rats). Where necessary a post-hoc Tukeys tests was used to determine the location of significance. A regression analyses for eNOS:β-actin ratio vs AUC for VCI was constructed and an adjusted r^2 was calculated. All data are presented as a mean±SD and the significance level was set at $P \leq 0.05$.

5.3 Results

Study 1 (CS rats, DS rats and DX rats)

Intervention data

Body mass (at surgery), fed state blood glucose, insulin pellet dose and glucose infusion rate during the insulin clamp as well as norepinephrine and insulin

concentrations at the end of the insulin clamp are presented in Table 5-1. The 10-week average fed-state blood glucose was greater in DS rats and DX rats compared with CS rats ($P<0.001$; Table 5-1). The insulin pellet dose was greater in the DS rats compared with the DX rats ($P=0.02$; Table 5-1). Average glucose infusion rate was greater in CS rats compared with DS rats ($P=0.01$; Table 5-1). There were no statistical differences for the average glucose infusion rate between DX rats and CS rats or DS rats ($P\geq 0.09$; Table 5-1). At the end of the insulin clamp, plasma norepinephrine concentrations were elevated in the DS rats compared with DX rats ($P=0.01$; Table 5-1), but the difference in norepinephrine concentration between DS rats and CS rats did not reach significance ($P=0.09$; Table 5-1). Norepinephrine concentrations were not statistically different between CS rats and DX rats ($P=0.36$; Table 5-1). Circulating insulin concentrations at 80 min of the euglycemic hyperinsulinemic clamp were the same between CS rats, DS rats and DX rats ($P=0.94$).

Euglycemic hyperinsulinemic clamp data

Baseline NBV was similar between CS rats, DS rats and DX rats ($P>0.99$; Figure 5-1a). In DS rats, peak NBV was greater during the EHC than baseline ($P=0.01$; Figure 5-1a). However, peak NBV was attenuated in DS rats compared with CS rats and DX rats ($P<0.01$; Figure 5-1a). There were no main or interaction effects for MAP when comparing CS rats, DS rats and DX rats ($P\geq 0.15$; Figure 5-1b). Baseline VCI was similar between CS rats, DS rats and DX rats ($P\geq 0.68$; Figure 5-1c). In DS rats the peak VCI was greater than baseline ($P<0.01$; Figure 5-1c). Nonetheless, the peak VCI was attenuated in DS rats compared with CS rats and DX rats ($P\leq 0.01$; Figure 5-1c). The

AUCi for the VCI throughout the insulin clamp was reduced in DS rats compared with CS rats and DX rats ($P < 0.01$; Figure 5-1d).

Data collected following the insulin clamp

Motor nerve conduction velocity was reduced in DS rats compared with CS rats and DX rats ($P \leq 0.01$; Figure 5-2b). Expression of eNOS was greater in DX rats compared with DS rats ($P = 0.04$; Figure 5-2a). There were no differences in eNOS expression between CS rats and DS rats, or CS rats and DX rats ($P \geq 0.31$; Figure 5-2a).

Study 2 (DX rats and CX rats)

Intervention data

Body mass was greater in CX rats vs DX rats ($P < 0.01$; Table 5-1). The 10-week average fed-state blood glucose was greater in DX rats vs CX rats ($P < 0.01$; Table 5-1). Average glucose infusion rate was greater in CX rats vs DX rats ($P < 0.01$; Table 5-1). There were no differences in plasma norepinephrine between CX rats and DX rats ($P = 0.71$; Table 5-1).

Euglycemic hyperinsulinemic clamp data

There were no group or interaction effects for NBV, MAP or VCI ($P \geq 0.12$). There was a main effect of time for NBV (peak > baseline; $P < 0.01$), MAP (baseline > peak; $P < 0.01$) and VCI (peak > baseline; $P < 0.01$) in CX rats and DX rats. However, AUCi for VCI was reduced in DX rats vs CX rats ($P = 0.03$; Figure 5-3d). Group data for NBV, MAP, VCI are presented in Figure 5-3a, b and c, respectively.

Data collected following the insulin clamp

Sciatic nerve MNCV and eNOS expression were reduced in DX rats vs CX rats ($P \leq 0.02$; Figure 5-4a and b).

Group data for eNOS expression and AUCi for the VCI during the insulin clamp

The correlation coefficient between eNOS expression and AUCi for the VCI during the insulin clamp was 0.54 (adjusted r^2 ; $N=21$; $P < 0.01$; Figure 5-5)

Table 5-1 General Characteristics

Variable	CS	DS	DX
Body Mass (at surgery; g)	533±43 ^a	461±38	423±58
Fed-State Blood Glucose (mmol/L)	4.4±0.2	15.2±1.1 ^b	15.0±1.6 ^b
Insulin Pellet (IU/kg)	NA	6.6±2.7 ^c	3.8±1.5
Avg. Glucose Infusion Rate (mg·kg·min ⁻¹)	22±2 ^d	14±5	19±6
Norepinephrine at 80 min (pg/mL)	129±16	143±6	123±10 ^d
Insulin at 80 min (pmol/L)	2905±727	3038±717	2971±739

a, Significantly greater than insulin-treated experimental diabetes sedentary rats (DS) and exercise rats (DX) ($P \leq 0.02$)

b, Significantly greater than control sedentary rats (CS) ($P < 0.001$)

c, Significantly greater than insulin-treated experimental diabetes exercise rats ($P = 0.02$)

d, Significantly different from DS rats ($P = 0.01$)

Average (Avg)

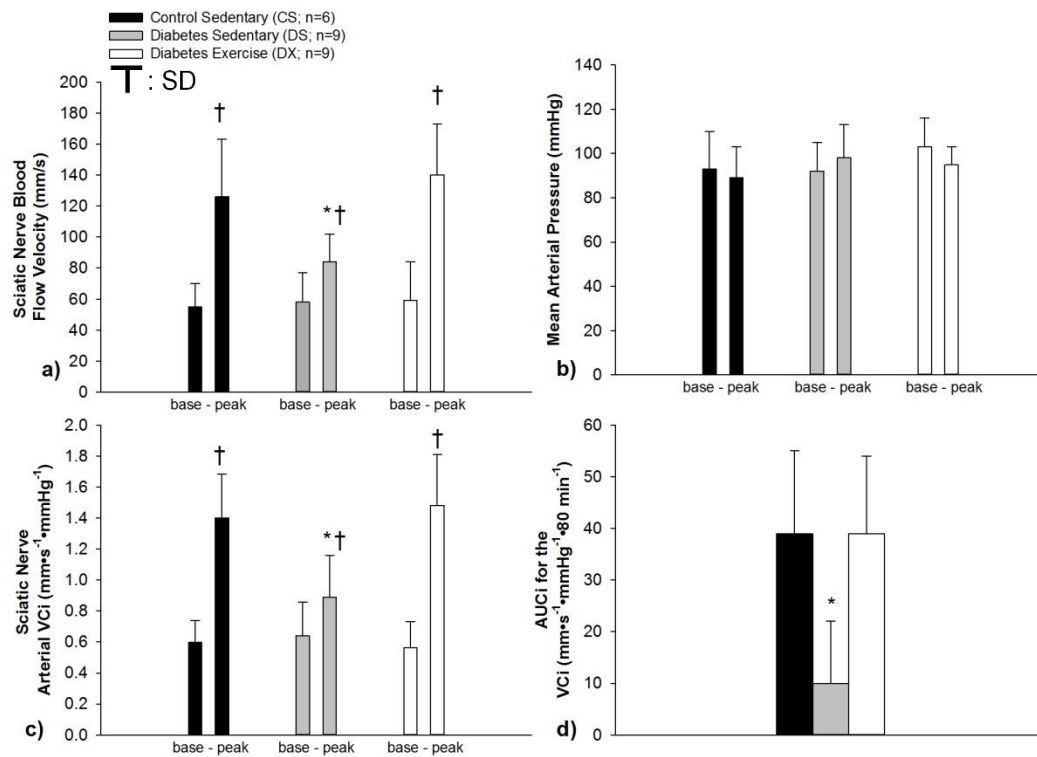


Figure 5-1 a) Nerve blood flow velocity (mm/s) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp in control sedentary (CS; dark bar) rats, diabetes sedentary (DS; grey bar) rats and diabetes exercise (DX; open bar) rats. b) Mean arterial pressure (mmHg) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp. c) Baseline and peak sciatic nerve arterial index of vascular conductance (VCI; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) measured during the euglycemic hyperinsulinemic clamp. d) Area under the curve (AUCi) for the VCI ($\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}\cdot\text{min}^{-1}$) measured throughout the euglycemic hyperinsulinemic clamp. †, Significantly greater than baseline ($P\leq 0.01$). *, Significantly less than CS and DX rats ($P\leq 0.01$).

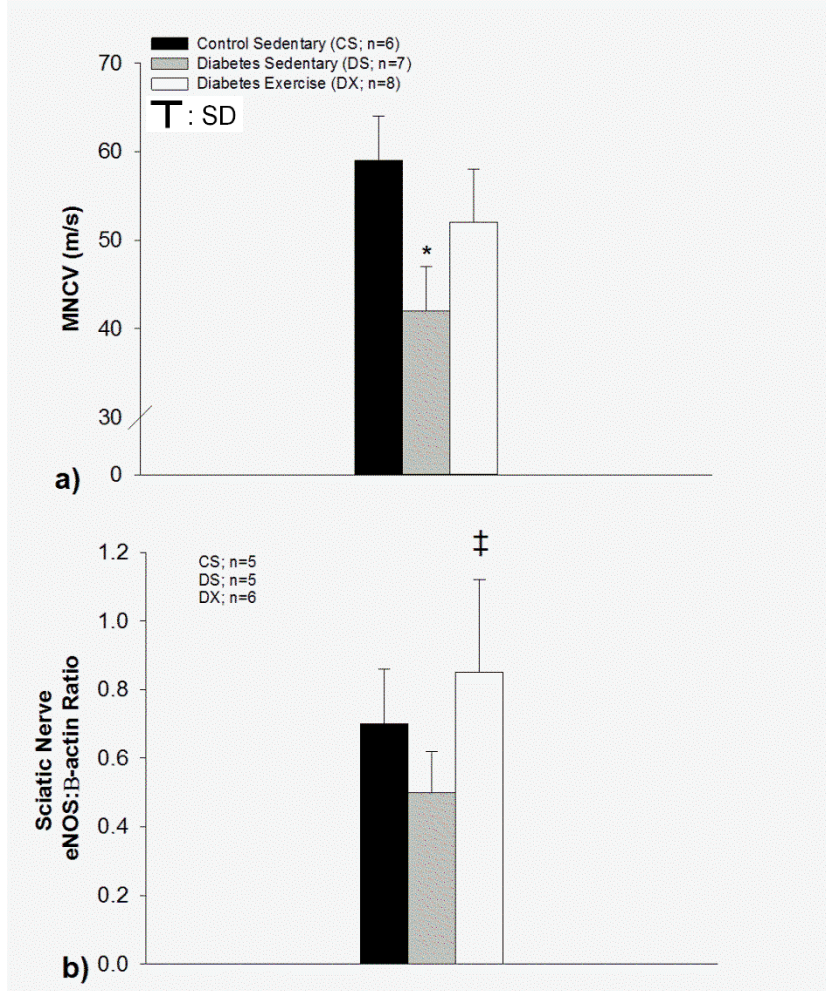


Figure 5-2 a) Motor nerve conduction velocity (MNCV; m/s) measured following the euglycemic hyperinsulinemic clamp in control sedentary (CS; dark bar) rats, diabetes sedentary (DS; grey bar) rats and diabetes exercise (DX; open bar) rats. b) Endothelial nitric-oxide synthase (eNOS) expression (normalized to β -actin) in the sciatic nerve. *, Significantly less than CS and DX ($P \leq 0.01$). †, Significantly greater than DS ($P = 0.04$).

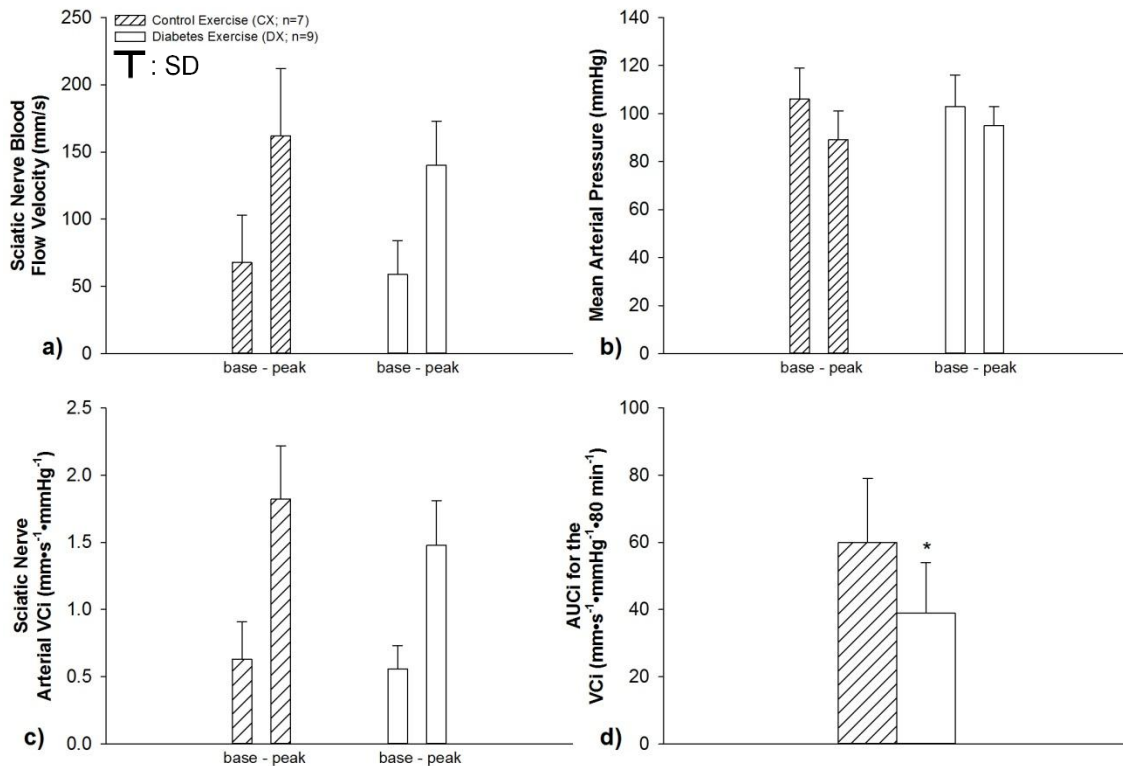


Figure 5-3 a) Nerve blood flow velocity (mm/s) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp in control exercise (CX; hatched bar) rats and diabetes exercise (DX; open bar) rats. b) Mean arterial pressure (mmHg) at baseline and peak vascular conductance during the euglycemic hyperinsulinemic clamp. c) Baseline and peak sciatic nerve arterial index of vascular conductance (VCI; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}$) measured during the euglycemic hyperinsulinemic clamp. d) Area under the curve (AUCi) for the VCI ($\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}\cdot\text{min}^{-1}$) measured throughout the euglycemic hyperinsulinemic clamp. *, Significantly less than CX ($P=0.03$).

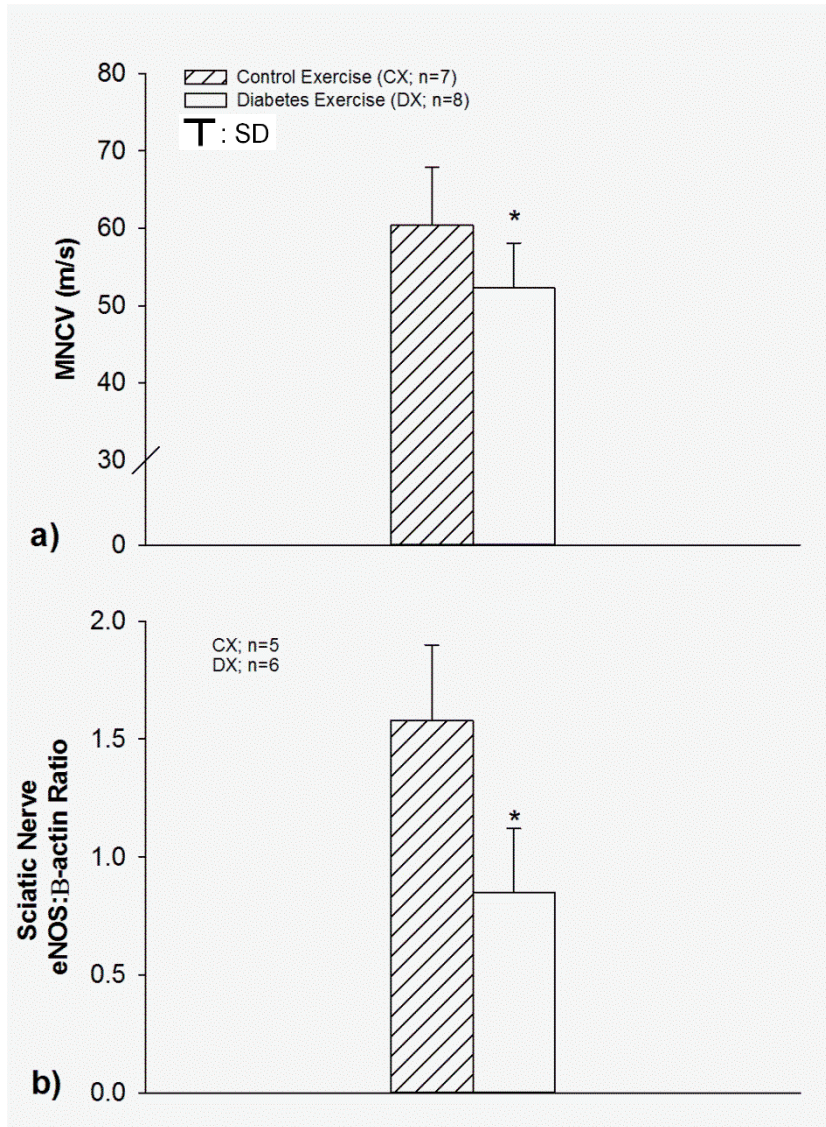


Figure 5-4 a) Motor nerve conduction velocity (MNCV; m/s) measured following the euglycemic hyperinsulinemic clamp in control exercise (CX; hatched bar) rats and diabetes exercise (DX; open bar) rats. b) Endothelial nitric-oxide synthase (eNOS) expression (normalized to β -actin) in the sciatic nerve. *, Significantly less than CX ($P \leq 0.02$).

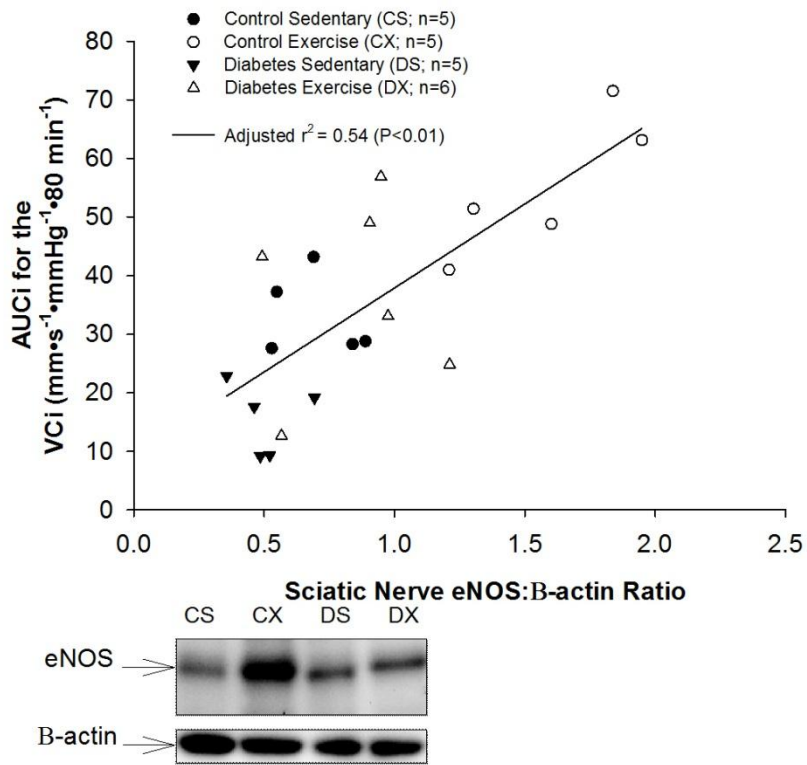


Figure 5-5 Linear regression between endothelial nitric-oxide synthase (eNOS) expression and area under the curve (AUCi) for the index of vascular conductance (VCI; $\text{mm}\cdot\text{s}^{-1}\cdot\text{mmHg}^{-1}\cdot 80 \text{ min}^{-1}$) measured throughout the euglycemic hyperinsulinemic clamp in control sedentary (CS; ●) rats, control exercise (CX; ○) rats, diabetes sedentary (DS; ▼) rats and diabetes exercise (DX; △) rats. Adjusted $r^2=0.54$; $P<0.01$.

5.4 Discussion

In study 1, insulin-treated experimental diabetes had little impact on basal VC_i, but severely diminished the VC_i response to systemic insulin infusion and decreased MNCV. In rats with insulin-treated experimental diabetes, exercise training preserved vasa nervorum NBV responses, as well as MNCV, at levels observed in the CS group. Also, in rats with insulin-treated experimental diabetes that exercise trained, eNOS expression was elevated compared with their sedentary counterparts (DS rats). In study 2 (CX rats and DX rats), diabetes was associated with reduced vasa nervorum responsiveness to insulin and nerve function. Therefore, in this model, insulin treatment combined with exercise training attenuated the effects of chronic, moderate hyperglycemia on vasa nervorum reactivity to insulin and MNCV. However, study 2 demonstrated that in exercise trained rats alone (CX vs DX), chronic, moderate hyperglycemia reduces the level of insulin-stimulated vasa nervorum dilation and reduces MNCV.

It is uncertain whether hyperglycemia or hypoinsulinemia causes the reduced NBF observed common in experimental diabetes. Chronically severe hyperglycemia often reduces basal NBF (10–16), but conflicting evidence exists (4,17,18,48,49). In the aforementioned studies linking experimental diabetes with reductions in NBF, chronic, severe hyperglycemia values in rats (21–43 mmol/L) exceeded representative ranges observed in humans with insulin-treated type 1 diabetes mellitus. The standard glyceamic status for patients with poorly controlled type 1 diabetes mellitus is ~9% glycosylated hemoglobin (40), which corresponds to ~13.5 mmol/L blood glucose concentration (41). Thus, experimental diabetes in rats that emphasizes the pronounced effects of chronic,

severe hyperglycemia may not represent the insulin-treated, type 1 diabetes mellitus observed clinically.

Based on VCI (or NBV) in the current study, baseline blood flow values were similar between insulin-treated experimental diabetes and control groups. This may have been the result of the chronic insulin treatment used in the present study or, alternatively, chronic hyperglycemia does not reduce basal NBF (4,17,18,48,49). Acute insulin administration stimulates nerve arterial vasodilation or relaxation (20, 41), and prolonged insulin treatment in rats with experimental diabetes can prevent declines in vasoreactivity (30) and restore decrements in basal NBF partially (8). In study 1, the DX rats received a reduced insulin (pellet) dose, but achieved the same degree of hyperglycemia as the DS rats. The effect of hyperglycemia on baseline VCI or NBV may have been reduced in both DS rats and DX rats, as the blood glucose concentrations observed in the current study were substantially less than those recorded during previous experimental diabetes/NBF studies (10–16). Considering blood glucose and basal VCI were similar between the two groups with diabetes, the smaller insulin dose in DX vs DS rats, suggests that insulin sensitivity and not total insulin contributes to the maintenance of the VCI under basal conditions.

In study 1, insulin-stimulated VCI responses were attenuated in DS rats. In addition, compared with CS rats, DS rats required a reduced glucose infusion rate during the clamp to maintain euglycemia. Together, these data provide evidence of depressed insulin sensitivity. Insulin-mediated vasodilation operates through the activation of intracellular enzymes PI3-K and Akt, and the subsequent phosphorylation and activation of eNOS (27,28). Therefore, reduced insulin-stimulated nerve arterial dilation in DS rats

may be explained by an impaired NO signaling mechanism. For example, glucose-stimulated insulin-mediated increases in the VCI operate through a NO mechanism (41). Also, rats with experimental diabetes express impaired NO-mediated vasa nervorum dilation (9,16), and reductions in basal NBF can be reversed with NO-donor treatment (19). Likewise, experimental diabetes impairs insulin-mediated vasodilation in the cutaneous microvasculature, likely through the PI3-K pathway (36). In the current study, sciatic nerve eNOS expression was not statistically different between CS and DS rats, but the overall impact of hyperglycemia on upstream eNOS signaling, NO bioavailability and reactivity were not examined.

Sympathetic overactivity may provide another explanation for the attenuated VCI response in DS rats. Insulin stimulates the sympathetic nervous system and elicits a robust norepinephrine response (50,51) and, in turn, norepinephrine can cause nerve arterial vasoconstriction (16,52). Furthermore, perivascular adrenergic innervation in the sciatic nerve (53), and vasa nervorum sensitivity to norepinephrine, increases in rats with experimental diabetes (16). Whether exercise training reduces perivascular adrenergic innervation in the sciatic nerve, or decreases vasa nervorum norepinephrine sensitivity, is unknown. Evidence from humans suggests insulin resistance is associated with reduced norepinephrine clearance (54), consistent with the present observations. Thus, in study 1, increased norepinephrine (potentially the result of the diabetes, the insulin infusion, or an interaction of these variables) alongside increased responsiveness to adrenergic activity in the vasa nervorum may have restrained insulin-mediated dilation.

The reduced insulin-stimulated VCI responsiveness in DS rats was abolished by concurrent endurance exercise training. The ability of insulin to stimulate a greater vasa

nervorum response in DX rats compared with DS rats may have been influenced by their enhanced insulin sensitivity (indicated by a reduced insulin dose during the study in DX rats vs DS rats). To achieve the same level of hyperglycemia in DS rats and DX rats, DX rats required less exogenous insulin. This suggests DX rats were more responsive to insulin and supports the findings that when exposed to the same level of hyperinsulinemia during the clamp, the vasodilatory response to insulin was greater in DX rats vs DS rats. Alternatively, the elevated sciatic nerve eNOS content or reduced circulating norepinephrine may help explain the preservation of the insulin-stimulated VC_i response observed in the DX rats of the current study. In rats with experimental diabetes, exercise training enhances insulin-mediated microvascular vasodilation in cutaneous tissues, likely through a PI3-K/eNOS mechanism (27,36). Although NO bioavailability was not assessed in the present study, it is important to note both DX rats and DS rats were exposed to similar levels of hyperglycemia throughout the study and were normoglycemic during the insulin clamp. Therefore, any effects of hyperglycemia on NO bioavailability should have been similar between the two groups.

Significance

By intention, blood glucose values in the current study were similar to clinical values observed in humans with poorly controlled type 1 diabetes mellitus (49). In study 1, compared with control and DX groups, sedentary rats with insulin-treated experimental diabetes had reduced insulin sensitivity and attenuated vasa nervorum responsiveness during the insulin clamp. In addition, MNCV was reduced in DS rats indicating a possible loss of large myelinated motor units and alterations in nerve physiology (31,55,56). Therefore, in spite of having similar baseline VC_i or NBV values, similar

blood glucose concentrations, and greater circulating insulin concentrations compared with the DX group, DS rats displayed impaired nerve function. These data suggest that basal blood and hemodynamic variables may be inadequate clinical markers of vasa nervorum health and that functional tests are necessary to detect the onset of decrements to vasa nervorum and neural function. Furthermore, the current data indicate that impaired insulin responsiveness and peripheral neuropathy were not caused by reduced basal NBF, hyperglycemia or hypoinsulinemia, and presumably were the result of a combination of hyperglycemia and inactivity. Insulin treatment alone was insufficient to preserve vasa nervorum and neural function in a rat model chronic, moderate hyperglycemia.

Exercise training appears to counteract the effects of insulin-treated experimental diabetes on nerve function and vasa nervorum dilation, possibly by increasing insulin responsiveness, augmenting eNOS expression and altering sympathetic function. With similar blood glucose, but reduced basal insulin concentrations in DX rats vs DS rats, it appears the effects of insulin to dilate the vasa nervorum were amplified by exercise training. Whereas exercise training has been observed to maintain nerve morphology (including large myelinated motor units) in rats with experimental diabetes (31), interventions to prevent deficits in vasa nervorum function have focused largely on pharmacological means (13,21,57–62). The current data suggest exercise training is a clinically effective treatment to avoid vasa nervorum and nerve dysfunction. Furthermore, unlike medication, exercise training offers additional benefits that prevent other chronic diseases and diabetes-related comorbidities as well as promote health and longevity in populations with and without type 1 diabetes mellitus (63,64).

Study 2

In exercise trained rats, insulin-treated experimental diabetes attenuates vasa nervorum reactivity and nerve health. For example, compared with CX rats, DX rats displayed reduced vasa nervorum responsiveness to insulin (indicated by a reduced AUC for VCI during the insulin clamp), had reduced MNCV and sciatic nerve eNOS expression. Therefore, while exercise training is beneficial, it does not abolish the effects of chronic, moderate hyperglycemia.

Considerations

A limitation in the current study may be that the artery insonated was too small to resolve accurately with the ultrasound B-mode imaging device. Therefore, it was assumed that the diameter of the artery did not change between baseline and the insulin clamp conditions. This may have resulted in an underestimation of the dilatory response.

Also, because Doppler ultrasound measures NBV from a single epineurial vessel, it may not be representative of total flow. Epineurial arteries may travel along the nerve or branch into the nerve (61) and become peri- or endoneurial arteries, but these vascular networks may possess different control features and it was not possible to quantify endoneurial-specific flow. In humans with diabetes and poor glycemic control, epineurial blood vessels are often tortuous and subject to arterio-venous shunting (65,66). This may result in a redistribution of blood flow that favours greater epineurial vs endoneurial blood flow. Thus, because Doppler ultrasound is limited to a single epineurial vessel, it may not capture reductions in endoneurial-specific flow. However, the sciatic nerve contains feed arteries originating from multiple locations that do not necessarily branch

into a capillary plexus and has been described as an anastomotic vascular network that may contain bidirectional flow (i.e. arteries traveling in opposite directions along the same segment of the nerve) (67–69). Such a pattern of tissue perfusion limits the interpretation of gross flow measurements and highlights the benefit of quantifying exclusive arterial inflow responses.

Another possible limitation is that nerve temperature may have fluctuated and influenced the dilatory response to insulin as well as MNCV (70). Kihara et al.(71) documented a positive relationship between limb temperature and NBF, while Dines et al.(72) reported an inverse relationship between limb temperature and NBF, with the effect of temperature being attenuated in rats with diabetes. In the present experiment room and body temperature were kept constant, but nerve temperature was not recorded. The exposure of the sciatic nerve may have led to a decrease in limb temperature. However, any effects of temperature likely occurred during the stabilization period and would have been consistent between groups. Also, baseline VCI values were similar between groups and, in spite of nerve exposure and possible limb cooling, all rats displayed vasodilation.

Also, during the insulin clamp, the total volume load each rat received would have been different. Insulin infusion rate was based on mass and glucose infusion rate was variable and based on insulin sensitivity. Notably, CX rats were more insulin sensitive and required a greater glucose infusion rate to maintain euglycemia. Potentially, the increased volume associated with the elevated glucose infusion rate may have contributed to their augmented VCI. However, despite a greater glucose infusion rate in CX rats, peak NBV and VCI were similar between CX rats and DX rats. Also, volume-

induced increases in flow velocity would likely occur on account of increased perfusion pressure, but during the insulin clamp there was a main effect of time (baseline>peak) for MAP in CX rats and DX rats, suggesting blood pressure decreased. The vasa nervorum does not autoregulate, but instead responds passively to changes in MAP. Therefore, the increase in the V_{Ci} (increases in NBV that are not caused by increases in MAP) during the insulin clamp, was likely not the result of the volume load, but instead the vasodilatory properties of insulin.

Although glucose-stimulated insulin-mediated dilation appears to operate through a NO mechanism (41), the exact NO-isoform mediating the insulin-dilation is unknown. The vasa nervorum dilatory response to acetylcholine and insulin may differ (20) suggesting insulin may function through an eNOS independent pathway. In the present study, eNOS expression alone was quantified, but upstream targets such as PI3-K were not. Further, post-transcriptional or translational modifications to eNOS as well as NO bioavailability were not measured. To our knowledge, no studies to date have elucidated the contribution of each NO-isoform to insulin-mediated dilation.

Conclusions

Insulin-treated experimental diabetes reduced whole-body insulin sensitivity, insulin-stimulated V_{Ci} responses as well as MNCV. These effects were offset by concurrent exercise training in a separate group of rats with insulin-treated experimental diabetes (DX rats). However, compared to CX rats, DX rats exhibited reduced whole-body insulin sensitivity, vasa nervorum responsiveness, MNCV and sciatic nerve eNOS expression.

From this perspective, exercise training is an effective treatment for insulin-treated diabetes by reducing the need for exogenous insulin and improving neural outcomes.

5.5 References

1. Tesfaye S, Chaturvedi N, Eaton SE, Ward JD, Manes C, Lonescu-Tirgoviste C, et al. Vascular risk factors and diabetic neuropathy. *N Engl J Med.* 2005;352(4):341–50.
2. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. *Diabetes.* 2005;54(6):1615–25.
3. Fazan VPS, Carvalho de Vasconcelos CA, Valenca MM, Nessler R, Moore KC. Diabetic Peripheral Neuropathies: A Morphometric Overview. *Int. J. Morphol.* 2010;28(1):51–64.
4. Pugliese G, Tilton RG, Speedy A, Chang K, Santarelli E, MA Province, et al. Effects of very mild versus overt diabetes on vascular haemodynamics and barrier function in rats. *Diabetologia.* 1989;32(12):845–57.
5. Tilton RG, Chang K, Nyengaard JR, Van den Enden M, Ido Y, Williamson JR. Inhibition of sorbitol dehydrogenase. Effects on vascular and neural dysfunction in streptozocin-induced diabetic rats. *Diabetes.* 1995;44:234–42.
6. Greene DA, Lattimer SA. Sodium- and energy-dependent uptake of myo-inositol by rabbit peripheral nerve. Competitive inhibition by glucose and lack of an insulin effect. *J. Clin. Invest.* 1982;70(5):1009–18.
7. Cameron NE, Cotter MA, Jack AM, Basso MD, Hohman TC. Protein kinase C effects on nerve function, perfusion, Na(+), K(+)-ATPase activity and glutathione content in diabetic rats. *Diabetologia.* 1999;42(9):1120–30.
8. Cameron NE, Cotter MA, Ferguson K, Robertson S, Radcliffe MA. Effects of chronic α -adrenergic receptor blockade on peripheral nerve conduction, hypoxic

- resistance, polyols, Na⁺-K⁺-ATPase activity, and vascular supply in STZ-D rats. *Diabetes*. 1991;40:1652–8.
9. Kihara M, Low PA. Impaired vasoreactivity to nitric oxide in experimental diabetic neuropathy. *Exp. Neurol.* 1995;132:180–5.
 10. Omawari N, Dewhurst M, Vo P, Mahmood S, Stevens E, Tomlinson DR. Deficient nitric oxide responsible for reduced nerve blood flow in diabetic rats: effects of L-NAME, L-arginine, sodium nitroprusside and evening primrose oil. *Br. J Pharmacol.* 1996;118(1):186–90.
 11. Tuck RR, Schmelzer JD, Low PA. Endoneurial blood flow and oxygen tension in the sciatic nerves of rats with experimental diabetic neuropathy. *Brain.* 1984;107:935–50.
 12. Cameron NE, Cotter MA, Low PA. Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am J Physiol.* 1991;261(1):E1–E8.
 13. Kihara M, Schmelzer JD, Poduslo JF, Curran GL, Nickander KK, Low PA. Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. *Proc Natl Acad Sci USA.* 1991;88:6107–11.
 14. Kappelle AC, Biessels GJ, Van Buren T, Erkelens DW, De Wildt DJ, Gispen WH. Effects of nimodipine on sciatic nerve blood flow and vasa nervorum responsiveness in the diabetic rat. *Eur. J. Pharmacology.* 1993;250:43–9.
 15. Biessels GJ, Stevens EJ, Mahmood SJ, Gispen WH, Tomlinson DR. Insulin partially reverses deficits in peripheral nerve blood flow and conduction in experimental diabetes. *J. Neurol. Sci.* 1996;140:12–20.
 16. Maxfield EK, Cameron NE, Cotter MA. Effects of diabetes on reactivity of sciatic vasa nervorum in rats. *J. Diabetes Complicat.* 1997;11(1):47–55.

17. Zochodne DW, Ho LT. Normal blood flow but lower oxygen tension in diabetes of young rats: microenvironment and the influence of sympathectomy. *Can J Physiol Pharmacol.* 1992;70:651–9.
18. Kalichman MW, Dines KC, Bobik M, Mizisin AP. Nerve conduction velocity, laser Doppler flow, and axonal caliber in galactose and streptozotocin diabetes. *Brain Res.* 1998;810(1-2):130–7.
19. Cameron NE, Cotter MA. Effects of chronic treatment with a nitric oxide donor on nerve conduction abnormalities and endoneurial blood flow in streptozotocin-diabetic rats. *Eur J Clin Invest.* 1995;25(1):19–24.
20. Cameron NE, Cotter MA. Rapid reversal by aminoguanidine of the neurovascular effects of diabetes in rats: modulation by nitric oxide synthase inhibition. *Metabolism.* 1996;45(9):1147–52.
21. Cotter MA, Cameron NE. Correction of neurovascular deficits in diabetic rats by beta2-adrenoceptor agonist and alpha1-adrenoceptor antagonist treatment: interactions with the nitric oxide system. *Eur J Pharmacol.* 1998;343(2-3):217–23.
22. Kihara M, Mitsui MK, Mitsui Y, Okuda K, Nakasaka Y, Takahashi M, et al. Altered vasoreactivity to angiotensin II in experimental diabetic neuropathy: role of nitric oxide. *Muscle Nerve.* 1999;22:920–5.
23. Coppey LJ, Gellett JS, Davidson EP, Yorek MA. Preventing superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats restores endothelium-dependent vasodilation. *Free Radic Res.* 2003;37(1):33–40.
24. Davidson EP, Coppey LJ, Kleinschmidt TL, Oltman CL, Yorek MA. Vascular and neural dysfunctions in obese Zucker rats: effect of AVE7688. *Exp Diabetes Res.* 2009;2009(ID 912327):1–8.
25. Olver TD, Mattar L, Gris e KN, Twynstra J, Noble EG, Lacefield JC, et al. Glucose-stimulated insulin secretion causes an insulin-dependent nitric oxide-

- mediated vasodilation in the blood supply of the rat sciatic nerve. *Am J Physiol.* 2013;305(2):R157–R163.
26. Abramson DI, Schkloven N, Margolis MN, Mirsky IA. Influences of massive doses of insulin on peripheral blood flow in man. *Am J Physiol.* 1939;128:124–32.
 27. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature Letters.* 1999;399:601–5.
 28. Muniyappa R, Montagnani M, Koh KK, Quon MJ. Cardiovascular actions of insulin. *Endocr. Rev.* 2007;28(5):463–91.
 29. Cameron NE, Cotter MA. Effects of a nonpeptide antagonist neurovascular function in diabetic rats: interaction with the renin-angiotensin system. *J. Pharmacol. Exp. Ther.* 1996;278(3):1262–8.
 30. Balducci S, Iacobellis G, Parisi L, Di Biase N, Calandriello E, Leonetti F, et al. Exercise training can modify the natural history of diabetic peripheral neuropathy. *J. Diabetes Complications.* 2006;20(4):216–23.
 31. Severo Do Nascimento P, Lovatel GA, Ilha J, Schaan BD, Achaval M. Diabetes increases mechanical sensitivity and causes morphological abnormalities in the sural nerve that are prevented by treadmill training. *Muscle Nerve.* 2012;47:46–52.
 32. Wallis MG, Wheatley CM, Rattigan S, Barrett EJ, Clark ADH, Clark MG. Insulin-mediated hemodynamic changes are impaired in muscle of Zucker obese rats. *Diabetes.* 2002;51(12):3492–8.
 33. Rattigan S, Wallis MG, Youd JM, Clark MG. Exercise training improves insulin-mediated capillary recruitment in association with glucose uptake in rat hindlimb. *Diabetes.* 2001;50(12):2659–65.

34. Steinberg H, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Investig.* 1994;94:1172–9.
35. Vincent MA, Barrett EJ, Lindner JR, Clark MG, Rattigan S. Inhibiting NOS blocks microvascular recruitment and blunts muscle glucose uptake in response to insulin. *Am J Physiol.* 2003;285:E123–E129.
36. Heidarianpour A, Hajizadeh S, Khoshbaten A, Niaki AG, Bigdili MR, Pourkhalili K. Effects of chronic exercise on endothelial dysfunction and insulin signaling of cutaneous microvascular in streptozotocin-induced diabetic rats. *Eur. J. Cardiovasc. Prev. Rehabil.* 2007;14:746–52.
37. Makimattila S, Virkamäki A, Groop P-H, Cockcroft J, Utriainen T, Fagerudd J, et al. Chronic hyperglycemia impairs endothelial function and insulin sensitivity via different mechanisms in insulin-dependent diabetes mellitus. *Circulation.* 1996;94(6):1276–82.
38. Fuchsjaeger-Mayrl G, Pleiner J, Wiesinger GF, Sieder AE, Quittan M, Nuhr MJ, et al. Exercise training improves endothelial function in patients with type 1 diabetes. *Diabetes Care.* 2002;25(10):1795–801.
39. Walberg-Henriksson H, Gunnarsson R, Henriksson J, DeFronzo R, Felig P, Ostman J, et al. Increased peripheral insulin sensitivity and muscle mitochondrial enzymes but unchanged blood glucose control in type I diabetics after physical training. *Diabetes.* 1982;31(12):1044–50.
40. Riveline J-P, Schapelynck P, Chaillous L, Renard E, Sola-Gazagnes A, Penfornis A, et al. Assessment of patient-led or physician-driven continuous glucose monitoring in patients with poorly controlled type 1 diabetes using basal-bolus insulin regimens. *Diabetes Care.* 2012;35(5):965–71.

41. Rohlfing CL, Weidmeyer H-M, Little RR, England JD, Tennill A, Goldstein DE. Defining the relationship between plasma glucose and HbA1c. *Diabetes Care*. 2002;25:275–8.
42. Melling CWJ, Gris  KN, Hasilo CP, Fier B, Milne KJ, Karmazyn M, et al. A model of poorly controlled type 1 diabetes mellitus and its treatment with aerobic exercise training. *Diabetes Metab*. 2013;39:226–35.
43. Bedford TG, Tipton CM, Wilson NC, Oppliger RA, Gisolfi C V, Bedford TG. Maximum oxygen consumption of rats and its changes with various experimental procedures. *J Appl Physiol*. 1979;47(6):1278–83.
44. Aisaka K, Kihara T, Koike M, Kuroki M, Ishihara T. Effect of Yohimbine on Urethane-Induced Hyperglycemia in Rats. *Jpn. J. Pharmacol*. 1989;527:523–7.
45. Uysal CA, Mizuno H, Hyakusoku H. Sciatic nerve anatomy in rat re-visited: a more proximal intervention. *J Plast Reconstr Aesthet Surg*. 2009;62(6):847–9.
46. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol*. 1979;237(3):E214–E223.
47. Fekedulegn DB, Andrew ME, Burchfiel CM, Violanti JM, Hartley T a, Charles LE, et al. Area under the curve and other summary indicators of repeated waking cortisol measurements. *Psychosom. Med*. 2007;69(7):651–9.
48. Suter SP, Chang K, Marvel J, Williamson JR. Concurrent in diabetic increases in regional hematocrit rats: prevention by sorbinil and blood flow. *Am J Physiol*. 1992;263:H945–H950.
49. Chang K, Ido Y, LeJeune W, Williamson JR, Tilton RG. Increased sciatic nerve blood flow in diabetic rats: assessment by “molecular” vs particulate microspheres. *Am J Physiol*. 1997;273(36):E164–E173.

50. Rowe JW, Young JB, Minaker KL, Stevens AL, Pallotta J, Landsberg L. Effect of insulin and glucose infusions on sympathetic nervous system activity in normal man. *Diabetes*. 1981;30(3):219–25.
51. Liang C, Doherty JU, Faillace R, Maekawa K, Arnold S, Gavras H, et al. Insulin infusion in conscious dogs. *J. Clin. Investig.* 1982;69:1321–36.
52. Zochodne DW, Low PA. Adrenergic control of nerve blood flow. *Exp. Neurol.* 1990;109:300–7.
53. Dhital K, Lincoln J, Appenzeller O, Burnstock G. Adrenergic innervation of vasa and nervi nervorum of optic, sciatic, vagus and sympathetic nerve trunks in normal and streptozotocin-diabetic rats. *Brain Res.* 1986;367(1-2):39–44.
54. Baron AD, Brechtel G, Johnson A, Fineberg N, Henry DP, Steinberg H. Interactions between insulin and norepinephrine. *J Clin Invest.* 1994;93(6):2453–62.
55. Ramji N, Toth C, Kennedy J, Zochodne DW. Does diabetes mellitus target motor neurons? *Neurobiol Dis.* 2007;26(2):301–11.
56. Allen MD, Choi IH, Kimpinski K, Doherty TJ, Rice CL. Motor unit loss and weakness in association with diabetic neuropathy in humans. *Muscle Nerve.* 2013;48(2):298–300.
57. Robertson S, Cameron NE, Cotter MA. The effect of the calcium antagonist nifedipine on peripheral nerve function in streptozotocin-diabetic rats. *Diabetologia.* 1992;35(12):1113–7.
58. Cameron NE, Cotter MA, Dines KC, Maxfield EK. Pharmacological manipulation of vascular endothelium function in non-diabetic and streptozotocin-diabetic rats: effects on nerve conduction, hypoxic resistance and endoneurial capillarization. *Diabetologia.* 1993;36(6):516–22.

59. Hotta N, Koh N, Sakakibara F, Nakamura J, Hamada Y, Hara T, et al. Effects of beraprost sodium and insulin on the electroretinogram, nerve conduction, and nerve blood flow in rats with streptozotocin-induced diabetes. *Diabetes*. 1996;45(3):361–6.
60. Nakamura J, Koh N, Sakakibara F, Hamada Y, Wakao T, Sasaki H, et al. Diabetic neuropathy in sucrose-fed Otsuka Long-Evans Tokushima fatty rats: effect of an aldose reductase inhibitor, TAT. *Life Sci*. 1997;60(21):1847–57.
61. Nakamura J, Kato K, Hamada Y, Nakayama M, Chaya S, Nakashima E, et al. A protein kinase C-beta-selective inhibitor ameliorates neural dysfunction in streptozotocin-induced diabetic rats. *Diabetes*. 1999;48(10):2090–5.
62. Obrosova IG, Van Huysen C, Fathallah L, Cao X, Stevens MJ, Greene DA. Evaluation of alpha(1)-adrenoceptor antagonist on diabetes- induced changes in peripheral nerve function , metabolism , and antioxidative defense. *FASEB J*. 2000;14:1548–58.
63. Booth FW, Gordon SE, Carlson CJ, Hamilton MT. Waging war on modern chronic diseases: primary prevention through exercise biology. *J Appl Physiol*. 2000;88(2):774–87.
64. Chimen M, Kennedy A, Nirantharakumar K, Pang TT, Andrews R, Narendran P. What are the health benefits of physical activity in type 1 diabetes mellitus? A literature review. *Diabetologia*. 2012;55(3):542–51.
65. Ward JD. Abnormal microvasculature in diabetic neuropathy. *J. Clin. Pathol*. 1993;7:223–6.
66. Tesfaye S, Malik R, Harris N, Jakubowski JJ, Mody C, Rennie IG, et al. Arterio-venous shunting and proliferating new vessels in acute painful neuropathy of rapid glycaemic control (insulin neuritis). *Diabetologia*. 1996;39:329–35.
67. Adams WE. The blood supply of nerves: II. The effects of exclusion of its regional sources of supply on the sciatic nerve of the rabbit. *J. Anat*. 1943;77(Pt 3):243–50.

68. Bell MA, Weddell AG. A descriptive study of the blood vessels of the sciatic nerve in the rat, man and other mammals. *Brain*. 1984;107:871–98.
69. Zamir M, Twynstra J, Vercnocke AJ, Welch I, Jorgensen SM, Ritman EL, et al. Intrinsic microvasculature of the sciatic nerve in the rat. *J Peripher Nerv Syst*. 2012;17:377–84.
70. Abramson DI, Chu LS, Tuck S Jr, Lee SW, Richardson G LM. Effect of tissue temperatures and blood flow on motor nerve conduction velocity. *JAMA*. 1966;198(10):1082–8.
71. Kihara M, Schmelzer JD, Kihara Y, Smithson IL, Low PA. Efficacy of limb cooling on the salvage of peripheral nerve from ischemic fiber degeneration. *Muscle Nerve*. 1996;19(2):203–9.
72. Dines KC, Calcutt NA, Nunag KD, Mizisin AP, Kalichman MW. Effects of hindlimb temperature on sciatic nerve laser Doppler vascular conductance in control and streptozotocin-diabetic rats. *J. Neurol. Sci*. 1999;163(1):17–24.

Chapter 6

6 « General discussion »

6.1 Key findings

The major contributions of this dissertation were as follows: i) in the absence of microangiopathy, the combination of insulin-treated experimental diabetes with hypertension a) may increase nerve blood flow (NBF) and b) results in motor nerve conduction velocity deficits, ii) glucose-stimulated insulin secretion causes a NO-mediated vasodilation, iii) insulin-treated experimental diabetes attenuates insulin-mediated dilation in the vasa nervorum, likely the result of an impaired nitric oxide synthase (NOS) mechanism and iv) in rats with diabetes, exercise training improves vasa nervorum reactivity to insulin and motor nerve function. Therefore, despite similar or possibly elevated basal NBF values in rats with poorly controlled type 1 diabetes, vasa nervorum reactivity to insulin and motor nerve function are impaired. These negative side effects appear to be prevented by concurrent exercise training.

These findings support work from Zochodne and Ho (1) that suggest reduced NBF does not always precede nerve dysfunction in rats with type 1 diabetes. However, that basal NBF was not decreased does not preclude the existence of microvascular damage. In the present work, the dilatory response to insulin was reduced and sciatic nerve endothelial NOS (eNOS) expression tended to be less in rats with type 1 diabetes. Impaired NO signaling has been implicated repeatedly in diabetes-induced vasa nervorum dysfunction (2–8), but the efficacy of exercise training as a preventative treatment strategy was unknown. Based on the beneficial effects of exercise training

observed in the current study and others (9–11), a greater emphasis needs to be placed on the study of exercise training and diabetes, as well as the implementation of exercise training programs to protect against adverse cardiovascular and neural outcomes in patient populations.

Prior to commencing the present dissertation, there was evidence to suggest experimental diabetes reduced (12,13), had no effect (1) or increased (14) NBF. In all cases, the aforementioned observations were supported by varying degrees of physiological evidence including simultaneous nerve dysfunction, but there was no general consensus with regards to the impact of diabetes on basal NBF. Slight differences in the degree and duration of hyperglycemia as well as other experimental factors may have altered the pathophysiology of diabetes-induced vasa nervorum dysfunction. Such experimental inconsistencies may have contributed to the conflicting results between studies. The model of diabetes used in the present dissertation attempted to replicate the blood glucose values observed in humans with poorly controlled insulin-treated type 1 diabetes (15). This model of type 1 diabetes did not induce microangiopathy or alter basal NBF (in the anesthetized state). However, it did attenuate vasa nervorum reactivity to insulin and reduce motor nerve conduction velocity. Therefore, although basal NBF values are likely important, they provide insufficient information and separate vascular function tests should be performed to assess vasa nervorum health.

The functional tests used in the current studies included i) the vasa nervorum response to changes in mean arterial pressure (MAP) as well as ii) the vasa nervorum reactivity to systemic insulin infusion. Systemic infusions of sodium nitroprusside (NO-

donor) and phenylephrine (α 1-agonist) were used to achieve changes in MAP. In line with previous work (16–20), NBF did not autoregulate and responded passively to changes in MAP. A novel finding from this study demonstrated that the relationship between MAP and NBF was unaltered by insulin-treated experimental diabetes. Given the greater MAP in the conscious rats with diabetes, NBF may be expected to increase accordingly. Conversely, in a separate study, the vasa nervorum dilatory response to systemic insulin infusion was attenuated in rats with insulin-treated experimental diabetes. Therefore, the mechanisms responsible for accommodating to changes in MAP are likely unrelated to NOS, as it is a key enzyme implicated in insulin-mediated vasodilation (9,21,22). From a clinical perspective, because vasoreactivity to insulin and nerve function were attenuated by insulin-treated experimental diabetes, but the relationship between MAP and NBF remained intact, functional tests that assess the NO pathway may be better indicators of vasa nervorum health.

Based on the current observations, the negative impact of insulin-treated experimental diabetes on vasa nervorum and nerve function were prevented by concurrent exercise training. This finding was novel, but not unexpected, as the benefits of exercise training in humans and animals with type 1 diabetes has been well-documented over the last century (10,11,23–27). Regular exercise improves endothelial (11) as well as nerve function (25), increases vascular (9) and whole body insulin sensitivity (26,28) and reduces exogenous insulin requirements. The present work supports the aforementioned observations and demonstrates that the enhanced microvascular insulin responsiveness associated with exercise training extends to the vasa nervorum. In theory, enhanced insulin sensitivity in the vasa nervorum may potentiate

insulin signaling in the nerve and protect nerve health. This may help explain why exercise trained rats with diabetes displayed better nerve function than their sedentary counterparts, in spite of a reduced insulin pellet dose.

Currently, the Canadian Diabetes Association promotes participation in a regular exercise program. They recommend that individuals with diabetes accumulate a minimum of 150 min of moderate to vigorous aerobic physical activity as well as 2-3 resistance exercise sessions per week (29). Although they acknowledge that individuals with diabetes are more susceptible to adverse events associated with exercise (hypoglycemia, hyperglycemia or other adverse events related to conditions such as, neuropathy, retinopathy and angina) (10,30,31), they reason that the benefits of regular physical activity far outweigh the risks associated with physical activity as well as inactivity (29). They highlight the beneficial effects of exercise training, citing improved cardiorespiratory fitness (23), slowed progression of neuropathy (24) and decreased rates of mortality (32). Although there is much work to be done in this area, based on the present observation, improved vasa nervorum (or sciatic nerve microvasculature) function could be added to their list.

Although it is generally accepted that regular exercise is advantageous, how exercise training imparts health benefits requires further study. Granted, this line of questioning could take many different directions. As it pertains to the current data, there are a number of follow up experiments that could be helpful in isolating the stimuli for vasa nervorum and neuronal adaptation, as well as the contribution of exercise-induced increases in eNOS to improvements in vasa nervorum function.

Isolating the stimuli responsible for the observed improvement in vasa nervorum function is critical to understanding how exercise mediates adaptation and may also help refine future exercise training prescriptions. In the present work, exercise training improved insulin signaling and eNOS expression in the vasa nervorum of rats with type 1 diabetes. The mode of exercise used, was continuous aerobic exercise (60 min per day, 5 days per week, for 10 weeks) performed at ~75% VO_2 max. This type of exercise would require repeated sciatic nerve stimulation and skeletal muscle contraction, both of which increase NBF acutely (33,34). In theory, the repetitive increases in vasa nervorum shear stress associated with each exercise session may have provided the stimulus for endothelial adaptation (35). In retrospect it would have been wise to include a measure of vasa nervorum function in the optic nerve, as it may receive a similar amount of stimulation in both the active and sedentary groups. Therefore, it would have been possible to determine if the benefits of exercise training occurred as a result of repetitive increases in shear stress and were limited to the site of direct vasa nervorum stimulation or if exercise mediates a global effect and extends to all vasa nervorum networks.

In addition, the relationship between eNOS and vasa nervorum function could be explored further using an eNOS (-/-) knockout mouse model. Comparing the effects of physical activity in C57B6 vs eNOS (-/-) knockout mice with insulin-treated experimental diabetes may help elucidate the contribution of exercise-induced increases in eNOS to vasa nervorum function. However, results from such studies should be interpreted with caution as the use of transgenic mouse models does not necessarily represent preclinical or clinical models of human disease accurately.

6.2 Conclusion

Over the last three centuries, many have upheld the charge of understanding and explaining the vasa nervorum. Theirs was the task of recording and bestowing observations to posterity. Basic and complex findings have been combined to form generalizations regarding vasa nervorum blood flow control and its association with nerve function (16,17, 36–49). Presently, I have found myself the inheritor of their great body of work and it has been my duty and pleasure to devote myself heartily to the study of the vasa nervorum and diabetes. Vasa nervorum and diabetes research emerged and evolved on different timelines. However, in the last fifty years they have become irreversibly connected. In recent history, vasa nervorum research has focused on the relationship between peripheral NBF and nerve function in type 1 diabetes (1,12,14,44). Though it is clear that glucose and insulin mediate changes in the vasa nervorum, much debate still exists regarding the overall impact of acute and chronic alterations in glycemic and insulinemic status on basal vasa nervorum blood flow and blood flow control (50). However, it is my belief that such controversy is beneficial, as it breeds scientific scrutiny as well as creativity. With that in mind, my attempts to expand upon the current understanding have focused on the exploration of the basic tenets of NBF control (i.e. the absence of autoregulation) in health and disease, as well as the discovery of new features (i.e. insulin-mediated vasodilation in the vasa nervorum). Here, we have demonstrated that i) in the absence of microangiopathy, NBF is unaltered by insulin-treated experimental diabetes and the combination of diabetes with hypertension may a) increase NBF and b) results in nerve dysfunction, ii) insulin-treated experimental diabetes attenuates insulin-mediated vasodilation in the vasa nervorum, likely the result of an

impaired NOS mechanism and iii) in rats with diabetes, endurance exercise training preserves vasa nervorum reactivity to insulin and nerve function. On account of greater insulin sensitivity and sciatic nerve eNOS expression in endurance trained rats with diabetes, the exercise training-induced restoration of vasa nervorum function is likely related to improved insulin-mediated eNOS signaling (9). Based on these observations it is clear that exercise training is critical to the maintenance of vascular and nerve health in populations with poorly controlled type 1 diabetes.

6.3 References

1. Zochodne DW, Ho LT. Normal blood flow but lower oxygen tension in diabetes of young rats: microenvironment and the influence of sympathectomy. *Can J Physiol Pharmacol.* 1992;70:651–9.
2. Omawari N, Dewhurst M, Vo P, Mahmood S, Stevens E, Tomlinson DR. Deficient nitric oxide responsible for reduced nerve blood flow in diabetic rats: effects of L-NAME, L-arginine, sodium nitroprusside and evening primrose oil. *Br. J Pharmacol.* 1996;118(1):186–90.
3. Kihara M, Schmelzer JD, Poduslo JF, Curran GL, Nickander KK, Low PA. Aminoguanidine effects on nerve blood flow, vascular permeability, electrophysiology, and oxygen free radicals. *Proc Natl Acad Sci USA.* 1991;88:6107–11.
4. Kihara M, Mitsui MK, Mitsui Y, Okuda K, Nakasaka Y, Takahashi M, et al. Altered vasoreactivity to angiotensin II in experimental diabetic neuropathy: role of nitric oxide. *Muscle Nerve.* 1999;22:920–5.
5. Cotter MA, Cameron NE. Correction of neurovascular deficits in diabetic rats by beta2-adrenoceptor agonist and alpha1-adrenoceptor antagonist treatment: interactions with the nitric oxide system. *Eur. J. Pharmacol.* 1998;343(2-3):217–23.
6. Coppey LJ, Gellett JS, Davidson EP, Yorek MA. Preventing superoxide formation in epineurial arterioles of the sciatic nerve from diabetic rats restores endothelium-dependent vasodilation. *Free Radic Res.* 2003;37(1):33–40.
7. Cameron NE, Cotter MA. Rapid reversal by aminoguanidine of the neurovascular effects of diabetes in rats: modulation by nitric oxide synthase inhibition. *Metabolism.* 1996;45(9):1147–52.

8. Cameron NE, Cotter MA. Effects of chronic treatment with a nitric oxide donor on nerve conduction abnormalities and endoneurial blood flow in streptozotocin-diabetic rats. *Eur J Clin Invest.* 1995;25(1):19–24.
9. Heidarianpour A, Hajizadeh S, Khoshbaten A, Niaki AG, Bigdili MR, Pourkhalili K. Effects of chronic exercise on endothelial dysfunction and insulin signaling of cutaneous microvascular in streptozotocin-induced diabetic rats. *Eur. J. Cardiovasc. Prev. Rehabil.* 2007;14:746–52.
10. Riddell MC, Perkins BA. Type 1 diabetes and vigorous exercise: applications of exercise physiology to patient management. *Can. J. Diabetes.* Elsevier Masson SAS; 2006;30(1):63–71.
11. Fuchsjager-Mayrl G, Pleiner J, Wiesinger GF, Sieder AE, Quittan M, Nuhr MJ, et al. Exercise training improves endothelial function in patients with type 1 diabetes. *Diabetes Care.* 2002;25(10):1795–801.
12. Tuck RR, Schmelzer JD, Low PA. Endoneurial blood flow and oxygen tension in the sciatic nerves of rats with experimental diabetic neuropathy. *Brain.* 1984;107:935–50.
13. Cameron NE, Cotter MA, Low PA. Nerve blood flow in early experimental diabetes in rats: relation to conduction deficits. *Am J Physiol.* 1991;261(1):E1–E8.
14. Chang K, Ido Y, LeJeune W, Williamson JR, Tilton RG. Increased sciatic nerve blood flow in diabetic rats: assessment by “molecular” vs particulate microspheres. *Am J Physiol.* 1997;273(36):E164–E173.
15. Riveline J-P, Schapelynck P, Chaillous L, Renard E, Sola-Gazagnes A, Penfornis A, et al. Assessment of patient-led or physician-driven continuous glucose monitoring in patients with poorly controlled type 1 diabetes using basal-bolus insulin regimens. *Diabetes Care.* 2012;35(5):965–71.
16. Smith DR, Kobrine AI, Rizzoli H V. Absence of autoregulation in peripheral nerve blood flow. *J. Neurol. Sci.* 1977;33:347–52.

17. Low PA, Tuck RR. Effects of changes of blood pressure, respiratory acidosis and hypoxia on blood flow in the sciatic nerve of the rat. *J Physiol.* 1984;347:513–24.
18. Sundqvist T, Oberg PA, Rapoport SI. Blood flow in rat sciatic nerve during hypotension. *Exp. Neurol.* 1985;90:139–48.
19. Rundquist I, Smith QR, Michel ME, Ask P, Oberg PA, Rapoport SI. Sciatic nerve blood flow measured by laser Doppler flowmetry and [14C]iodoantipyrine. *Am J Physiol.* 1985;248(3(2)):H311–H317.
20. McManis PG, Schmelzer JD, Zollman PJ, Low PA. Blood flow and autoregulation in somatic and autonomic ganglia comparison with sciatic nerve. *Brain.* 1997;120:445–9.
21. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature Letters.* 1999;399:601–5.
22. Steinberg H, Brechtel G, Johnson A, Fineberg N, Baron AD. Insulin-mediated skeletal muscle vasodilation is nitric oxide dependent. A novel action of insulin to increase nitric oxide release. *J. Clin. Investig.* 1994;94:1172–9.
23. Laaksonen DE, Atalay M, Niskanen LK, Mustonen J, Sen CK, Lakka T a, et al. Aerobic exercise and the lipid profile in type 1 diabetic men: a randomized controlled trial. *Med. Sci. Sports Exerc.* 2000;32(9):1541–158.
24. Balducci S, Iacobellis G, Parisi L, Di Biase N, Calandriello E, Leonetti F, et al. Exercise training can modify the natural history of diabetic peripheral neuropathy. *J. Diabetes Complications.* 2006;20(4):216–23.
25. Severo Do Nascimento P, Lovatel GA, Ilha J, Schaan BD, Achaval M. Diabetes increases mechanical sensitivity and causes morphological abnormalities in the sural nerve that are prevented by treadmill training. *Muscle Nerve.* 2012;47:46–52.

26. Joslin EP, Root EF, White P. The treatment of diabetes mellitus. Philadelphia: Lea & Febiger; 1959.
27. Joslin EP. The treatment of diabetes mellitus. *Can. Med. Assoc. J.* 1914;6(8):808–11.
28. Landt KW, Campaigne BN, James FW, Sperling MA. Effects of exercise training on insulin sensitivity in adolescents with type I diabetes. *Diabetes Care.* 1985;8:461–5.
29. Sigal RJ, Armstrong MJ, Colby P, Kenny GP, Plotnikoff RC, Reichert SM, et al. Physical Activity and Diabetes. *Can. J. Diabetes. Canadian Diabetes Association;* 2013;37:S40–S44.
30. MacDonald MJ. Postexercise late-onset hypoglycemia in insulin-dependent diabetic patients. *Diabetes Care.* 1987;10(5):584–8.
31. Brazeau AS, Rabasa-Lhoret R, Strychar I, Mircescu H. Barriers to physical activity among patients with type 1 diabetes. *Diabetes Care.* 2008;31:2108–9.
32. Moy CS, Songer TJ, LaPorte RE, Dorman JS, Kriska AM, Orchard TJ, et al. Insulin-dependent diabetes mellitus, physical activity, and death. *Am. J. Epidemiol.* 1993;137:74–81.
33. Sugimoto H, Monafo WW, Shimazaki S. Regional sciatic nerve blood flow response to limb movement. *Am J Physiol.* 1987;252:H439–H441.
34. Monafo WW, Eliasson SG, Shimazaki S, Sugimoto H. Regional blood flow in resting and stimulated sciatic nerve of diabetic rats. *Exp. Neurol.* 1988;99(3):607–14.
35. Tinken TM, Thijssen DHJ, Hopkins N, Dawson EA, Cable NT, Green DJ. Shear stress mediates endothelial adaptations to exercise training in humans. *Hypertension.* 2010;55:312–8.

36. Isenflamm J, Doerffler J. De vasis nervorum. Erlangen. Erlangen; 1768.
37. Hyrtl J. Oesterr. Z. prakt. Heilk. 1859;cited by Tonakoff
38. Lapinsky M. Ueber veränderungen der nerven bei acuter störung der blutzufuhr. Dtsch. Z. Nervenheilkd. 1878; 25:364-393.
39. Quènu J, Lejars F. Les arteres et les veines des nerfs. Seanc. Acad. Sci. 1890;3(608).cited by Adams
40. Tonakoff V. Die arterien der intervertebralganglien und der cerebrospinalnerven des menschen. Int. Mschr. Anat. 1898;15:300-53.cited by Adams
41. Bentley FH, Schlapp W. Experiments on the blood supply of nerves. J Physiol. 1942;102:62-71.
42. Adams WE. The blood supply of nerves I. Historical review. Anatomy. 1942;76:323-41.
43. Durward A. The blood supply of nerves. Postgrad. Med. J. 1948;24(267):11-4.
44. Fagerberg SE. Studies on the pathogenesis of diabetic neuropathy. Acta Med. Scand. 1956;156(4):295-302.
45. Blunt MJ. Functional and clinical implications of the vascular anatomy of nerves. Postgrad. Med. J. 1957;33(376):68-72.
46. Lundborg G. Ischemic nerve injury. Scand. J. Plast. Reconst. Surg. 1970;6(6):1-113.
47. Bell MA, Weddell AG. A descriptive study of the blood vessels of the sciatic nerve in the rat, man and other mammals. Brain. 1984;107:871-98.
48. Tilton RG, Chang K, Nyengaard JR, Van den Enden M, Ido Y, Williamson JR. Inhibition of sorbitol dehydrogenase. Effects on vascular and neural dysfunction in streptozocin-induced diabetic rats. Diabetes. 1995;44:234-42.

49. Zochodne DW, Low PA. Adrenergic control of nerve blood flow. *Exp. Neurol.* 1990;109:300–7.
50. Zochodne DW. Nerve and ganglion blood flow in diabetes: an appraisal. *Int. Rev. Neurobiol.* 2002;50:161–202.

Appendices

Appendix A: Streptozotocin induction protocol (rat)

PURPOSE:

To induce type I diabetes in rats

MATERIALS:

Gloves

Lab Coat

Streptozotocin (STZ)

5X Stock Citric Acid/Citrate Buffer

- Anhydrous Citric Acid
- Sodium Citrate Dihydrate
- MilliQ Deionized Water

13M HCl

3 Falcon Tube

Sterile Filter

EQUIPMENT:

Biological Safety Cabinet

Weigh Scale

pH Meter

PROCEDURE:

Preparing 5X Citric Acid/Citrate Buffer

1. For a pH 4.6 buffer at 765 mM (5X stock solution), in a beaker, Add
 - i. 13.8g Anhydrous Citric Acid (Sigma) or 15.1g Citric Acid Monohydrate
 - ii. 23.8g Sodium Citrate Dihydrate (Sigma)

Mix into iii. 175mL of MilliQ water

The pH should be at 4.6, Add HCl or NaOH to adjust (do not over-shoot pH)
2. Once the proper pH is obtained, add MilliQ water until you are close to the 200 ml mark (pH will move slightly). If satisfied with the pH, adjust volume in a 250 ml graduated cylinder and filter in a 0.2µm filter.
3. Store at room temperature. This is your 5X stock solution.

Making up Streptozotocin (STZ) for Injection

****NOTE** Animals should be pre-weighed prior to making up STZ to ensure accurate amounts of STZ to be prepared.

1. Using pre-made buffer, put 1 mL of buffer in a 50 mL Falcon Tube and add 4 mL of distilled water filtered through a 0.2µm syringe filter. Check the pH. This gives you a working concentration of 153 mM

2. The desired pH is between 4.5-4.7. Under the fume hood, add 1 drop at a time of concentrated HCl to the buffer, checking pH in between until desired pH is reached.
3. Once pH is reached, add 1 mL distilled water (sterile filtered through a 0.2µm syringe filter as before). If pH is below 4.5, restart.
4. Weigh out an appropriate amount of STZ for the number of animals (see calculations below) that will be injected in a 15 minute time frame.

Ex. Rats will be injected at 20mg/kg, so for 10 animals at an ideal weight of 200g (avg. weight of rats to be injected), you will require a minimum of 40mg.

$$20\text{mg/kg} \times 0.2\text{kg} = 4\text{mg per animal}$$

The amount of STZ weighed out should be more than the minimum as some solution will be lost in filtering. (4mg (per animal) X 12 rats = 48mg total (0.048g)

5. Dissolve the STZ into buffer (keeping in mind a comfortable injection volume). Shake to dissolve powder (approx. 1min). Sterile filter using a 0.2µm syringe filter.
 Ex. $48\text{mg STZ} \div 3 \text{ mL buffer} = 16\text{mg/mL solution}$
 $4\text{mg} \div 16\text{mg/mL solution} = 0.25\text{mL}$
6. STZ is time dependent and must be used within 15 minutes

Injecting and Follow-Up of the Animals

1. Promptly inject each rat with the solution (intraperitoneal) at a dosage rate of 20mg/mL (in this example, 0.25mL). Do not use anymore STZ solution more than 15 minutes after it has been dissolved in the sodium citrate buffer.
2. Dispose of any container having come into contact with the STZ (in either powder or dissolved form) into a biohazardous waste receptacle. Dispose of needles into a sharps container.
3. Return injected rats to their cage. Record the date of STZ injection and add a biohazard label to the cage (leave biohazard label on cage for at least 3 days following the last injection).
4. Repeat this procedure the following day.
5. Check blood glucose daily. Diabetes is achieved with two non-fasting blood glucose readings of >18 mmol. Diabetes should be achieved after 5-8 injections (i.p. 20mg/kg).

Reference:

Low dose STZ induction protocol. Animal Models of Diabetic Complications Consortium AMDCC Protocols.2003

Appendix B: Insulin pellet insertion protocol (rat)

MATERIALS:

LinShin LinPlant Insulin Pellet
Rat anesthetic - Isoflurane
Ampicillin
Sterile water
1ml syringe with 25 g needle
10% providone-iodine solution
gauze (or swab)
Tissue forceps
Scalpel handle and blades (or scissors)
Silk suture
Needle drivers

EQUIPMENT:

Isofluorane Anaesthetic Machine
Hair clippers
Heat lamp

Special Safety:

Must put on lab coat and gloves before handling rodents. Any bite or scratch that breaks the skin must be thoroughly scrubbed with soap and water (report to Occupational Health and Safety).

PROCEDURE:

Pellet implantation (for a rat):

1. Anesthetize the animal using the isofluorane machine by placing it in the induction chamber. Set isoflurane to 4-5% with an O₂ flow rate of 1L/min. Open the stopcock valve so gas reaches the chamber. Keep in chamber until the animal is unconscious.
2. Remove the animal and place its nose in the nose cone, reduce the isofluorane to 3% to maintain the plane of anesthesia.
3. Shave the area where the pellet is to be implanted.
4. Using gauze (or a swab), apply 10% providone-iodine solution to the skin, followed by 70% ethanol, to disinfect the site of insertion.
5. Hold the skin with forceps and make a subcutaneous incision.
6. Cleanse a 12g trocar with 10% providone-iodine solution and insert it through the puncture site to a depth of at least 2 cm.

7. Using forceps, briefly immerse the pellet in 10% providone-iodine solution, rinse with saline and insert into the subcutaneous region.
8. Use 1 pellet for the first 350g of body weight.
9. Pinch the skin closed after the last pellet is inserted. Place a drop of 10 % providone-iodine solution over the opening.
10. Close the incision by suturing.
11. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
12. Record on the cage card that insulin pellets have been implanted.

Pellet removal:

1. Anesthetize the animal as described above for implantation.
2. Shave and palpate the area of implantation to locate pellets. Sterilize this area by applying 10% providone-iodine solution followed by 70% ethanol.
3. Using a scalpel (or scissors), make an incision through the skin superficial to the location of the pellets.
4. Using forceps, remove the pellet. Some connective tissue may need to be cut away using scissors. Discard the pellet.
5. Close the incision by suturing.
6. Place the animal under a heat lamp and monitor until it recovers from anesthesia.
7. Record on the cage card that the pellets have been removed.

References:

<http://www.linshincanada.com>

Appendix C: Ethics approval



11.01.10

*This is the 2nd Renewal of this protocol
*A Full Protocol submission will be required in 2012



Your Animal Use Protocol form entitled:

Innovation to Reduce Cardiovascular Complications of Diabetes at the Intersection of Discovery, Prevention and Knowledge Exchange

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **11.01.10** to **11.01.11**

The protocol number for this project remains as **2008-095**

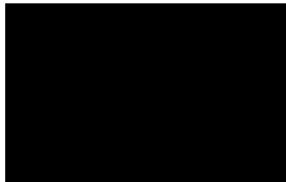
1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS

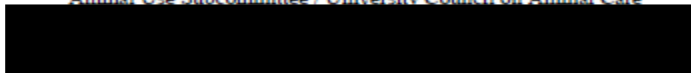
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c.



The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care



AUP Modification #1

10/27/2010 #2010-246


PI / PROTOCOL INFORMATION			
PI Name		Protocol Number: 2010-246	
Protocol Title: Regulation of Blood Flow to the Sciatic Nerve			
1. REQUESTED MODIFICATIONS TO PREVIOUSLY APPROVED ANIMAL USE PROTOCOL			
Identify all requested changes to the AUP identified above			
AUP Ref. #	Form Elements		
12, 21	Procedures Narrative, Categories of Invasiveness	D	<input checked="" type="checkbox"/>
18	Non-Hazardous Agent(s) / Drug(s) Information	G	<input checked="" type="checkbox"/>
22	Protocol Personnel & Their Training Requirements	H	<input checked="" type="checkbox"/>
2. PROVIDE JUSTIFICATION FOR PROPOSED CHANGES OUTLINED IN THIS PROTOCOL MODIFICATION			
We are requesting to change the neuromuscular blocking agent, tubocurarine, to a different neuromuscular blocking agent, rocuronium, as rocuronium has less side effects. Specifically, rocuronium does not affect histamine release and it has minimal cardiovascular effects. Also, we are adding atropine administration (subcutaneous) to minimize tracheal secretions during artificial ventilation. Lastly, we are adding one new personnel to the project to increase our productivity.			
3. PROCEDURAL CONSEQUENCES – See Section 20, AUP Reference			
1. From BOTH the Project OVERVIEW & DETAIL perspectives, identify and describe specific procedural or other elements (eg. drugs, chemicals, agents, materials and devices) associated with this revised Animal Use Protocol that may produce Pain, Distress or Impairment and identify all possible consequences (Behavioural, Physical, Biochemical, Physiological and Reproductive) for this species.		The changes being proposed are on administration of one different pharmacological agent (rocuronium) and one new (atropine) that will be administered while the animal is under general anaesthesia. We don't expect any additional pain, distress or impairment from the original protocol. Consequences as stated in original ethics are: rocuronium administration is expected to induce complete skeletal muscle paralysis of the animal. The animal will be artificially ventilated as described in section D (12). Because the animal is paralyzed, common indicators of the depth of anaesthesia such as, palpebral, pedal, ear twitch and corneal reflexes, cannot be used. Instead, heart rate will be monitored before and after paralysis and used for assessment of anaesthesia depth. Also, end tidal CO2 will be continuously monitored and maintained within 30-35mmHg range. "Lightness" of the animal is not expected as the anaesthetic	

2010-246 Shoemaker 10.27.10 Modification #1 Approved.doc

Page 1 of 4

AUP Modification #1

10/27/2010 #2010-246

	drug used has very long longevity. As all protocols will be terminal, there will not be behavioural, physical, biochemical, physiological and reproductive consequence. All other procedural elements are not expected to cause any pain, distress or impairment.
2. Detail Relief to be provided in response to the possible consequences identified within #1, or offer Scientific Justification for not providing relief for this species.	We don't expect any additional pain, distress or impairment from the original protocol. Relief procedures as stated in original ethics protocol are: Additional IV injection of the urethane, alpha-chloralose cocktail will be administered in case of increased heart rate or unstable mean arterial pressure during the experimental procedure. Urethane, alpha-chloralose mix will be continuously added until heart rate falls to baseline levels.
4. INVESTIGATOR DECLARATION	
I. All animals used in this research project will be cared for in accordance with the recommendations of the Canadian Council on Animal Care and the requirements of the provincial legislation entitled, "The Animals for Research Act," of the Province of Ontario.	
II. I confirm that this <i>Animal Use Protocol</i> accurately represents the proposed animal use.	
III. I accept responsibility for procedures performed on animals in this project.	
IV. I will ensure that any individual who will perform any animal-related procedure(s) within this protocol will complete all related mandatory training AND will be made familiar with the contents of this document.	
V. I confirm that all identified external approvals, including Institutional Safety Permits and Scientific Peer approvals, represent the stated intention of animal use within this <i>Animal Use Protocol</i> .	
1. I support the above declaration - YES <input checked="" type="checkbox"/> Today's Date <i>mm/dd/yy</i> : 10/26/10	
2. By checking 'YES' in this section, I authorize the submission of this form and its electronic delivery to auspc@uwo.ca YES <input checked="" type="checkbox"/> NO <input type="checkbox"/>	
3. I authorize Jasna Junuzovic (PI Designate) to submit this form and to receive a copy of authorization via email on my behalf. Today's Date (<i>mm/dd/yy</i>): 10/26/10	
AUS APPROVAL - AUS Office Use Only -	
Veterinary Authorization by Dr. Calder Authorization Date (<i>mm/dd/yy</i>) 10/27/10	Signature: 

2010-246 Shoemaker 10.27.10 Modification #1 Approved.doc

Page 2 of 4

D (12) CHANGES TO PROCEDURES NARRATIVE – See Section 12, AUP Reference

Provide a concise description of the procedural events experienced by the animals in each experiment. The intent is to chronologically order, name and briefly describe the procedural events that animals of each experimental cohort will experience. The species-specific events should be presented numerically in chronological order. Indicate the experimental groups experiencing each procedural event and evaluate the procedure-specific potential to cause pain.

Procedure Number	SPECIES & Sect 9 (Animal Groups Overview) Group ID#s	Procedure/ Experiment NAME	Concise Narrative Description of the Procedural Event or Experiment
1	Click Here ID# ALL	Paralysis via Rocuronium and Artificial ventilation	We have changed the neuromuscular blocking agent from tubocurarine to rocuronium and added subcutaneous administration of atropine to minimize tracheal secretions. Previous description of the procedure is as following: After the surgery, baseline nerve blood flow measurements will be taken using Vevo 2100 Doppler Ultrasound machine. The animals will be attached to a ventilator and the end tidal CO2 levels will be continuously monitored. A volume ventilator will be used and we will carefully calculate the lung volume of each animal. Atropine (0.004mls/kg) will be administered (subcutaneous) to minimize tracheal secretions. Afterward, the animals will be given rocuronium (150ug/kg) to induce paralysis. The rate of ventilation will be adjusted such that the end tidal CO2 levels are in the same range as prior to rocuronium administration (approximately between 30 and 35mmHg). As the animals will be under complete paralysis, the experimental (light) plane of anaesthesia for each animal will be maintained via continuous monitoring of heart rate (ECG) and mean arterial pressure. We do not anticipate any "lighting" of the animal as the urethane alpha-chloralose mixture has been shown to produce very stable and long lasting anaesthesia. The entire experimental procedure (approx 3hrs) is shorter than the longevity of the urethane mixture (approx 6hr). However, should heart rate increase, or blood pressure become unstable, additional top-up doses of anesthesia will be infused. Further, should muscle twitching become visible during the course of the study the rocuronium will be topped up (50-100ug/kg). SOP 351: Anaesthetic Monitoring will be followed throughout the experiment.

G (18) CHANGES TO NON-HAZARDOUS AGENT(S)/ DRUG(S) INFORMATION – See Section 18, AUP Reference

1. Agent Name & Common / Other Name (if applicable):	2. Species	3. Agent Type	4. Dose	5. Route(s) of Administration	6. Volume, if administered in liquid formulation	7. Frequency of Administration	8. Is this a Controlled Substance? (If 'Yes', please
--	------------	---------------	---------	-------------------------------	--	--------------------------------	--

1.1. Rocuronium 1.2.	Rat	Nervous System	150ug/kg	I.V.	0.2mls	x3 (as needed)	Yes <input type="checkbox"/> No <input checked="" type="checkbox"/>
1.1. Atropine 1.2.	Rat	Cardiovascular	0.004mg/kg	subcutaneous	0.1mls	x1	Yes <input type="checkbox"/> No <input checked="" type="checkbox"/>

H (22) PROTOCOL PERSONNEL - See Section 22, AUP Reference

TRAINING INFORMATION

CCAC Mandated Training Requirements – All personnel working with live animals require CCAC mandated training including the 'Basic Animal Care & Use Web-CT Course' and related hands-on 'Workshops'. Completion of the 'Basic Animal Care & Use Web-CT Course' once every 5 years is mandatory for ALL personnel, including the Principal Investigator. The Animal Use Subcommittee will be informed of all personnel with incomplete training beyond 1 month of notice.

Workshop Enrolment Detail – The 'Workshop' requirements are determined by the species and procedures associated with each individual listed below. All personnel listed below will be contacted directly via the email address listed below for auto-enrolment in all 'Workshop' requirements. Previous hands-on 'Workshops' attended at another research institution may be accepted; please submit training documentation with this form. For additional training requirement detail and associated costs, go to http://www.uwo.ca/animal/website/VS/Content/Teaching_and_Courses.htm

COMPLETE ALL COLUMNS BELOW PER PERSON					If 'YES' to HANDS-ON ANIMAL WORK, Complete This Section PER SPECIES														
FIRST NAME <small>Repeat For Each Species Used</small>	LAST NAME <small>Repeat For Each Species Used</small>	ROLE <small>(Role within this Protocol) Researcher Staff Student</small>	EMAIL Address <small>*Mandatory Field* UWO & Affiliated Institution Emails Preferred</small>	HANDS ON Animal Work? YES or NO	SPECIES One Species Per Row Use an Additional Row for Each Species	Expected START DATE <small>mm/dd/yy</small>	PROCEDURES PER SPECIES												
							1	2	3	4	5	6	7	8	9				
Terry	Olver	Student	tolver@uwo.ca	Yes	Rat	10/26/10	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>

Appendix D: Permission to reproduce published materials

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Chapter 4 (Am J Physiol Regul Integr Comp Physiol. 2014 Jun 15;306(12):R941-50. doi: 10.1152/ajpregu.00508.2013. Epub 2014 Apr 16.)

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Curriculum Vitae

Name:	T. Dylan Olver		
Post-secondary Education and Degrees:	University of Western Ontario London, Ontario, Canada 2004-2008 B.A. (Honors Specialization)		
	The University of Western Ontario London, Ontario, Canada 2008-2010 M.Sc. (Integrative Physiology, Coaching)		
	The University of Western Ontario London, Ontario, Canada 2010-2014 Ph.D. (Integrative Physiology)		
Honours and Awards:	Ontario Graduate Scholarship		2010, 2012-2014
	CIHR Vascular Fellow		2011-2013
	Kinesiology Research and Service Award		2011
Related Work Experience	Part-Time Professor	Fanshawe College	2014
	Research Consultant	Western University	2010-2014
	Research Assistant	Western University	2013
	Sessional Instructor	Western University	2012
	Graduate TA.	Western University	2008-2012
First Author Publications:	<ol style="list-style-type: none"> Olver TD, et al (Shoemaker JK). Evidence of bidirectional flow in the vasa nervorum. <i>Microvascular Res.</i> 2014; epub ahead of print (short report) Olver TD, et al (Shoemaker JK). Exercise training enhances insulin-stimulated nerve arterial vasodilation in rats with insulin-treated experimental diabetes. <i>J Physiol Regul Integr Comp Physiol.</i> 2014; epub ahead of print Olver TD, et al (Shoemaker JK). The relationship between blood pressure and sciatic nerve blood flow velocity in rats with insulin-treated experimental diabetes. <i>Diabetes Vasc Dis Res.</i> 2014; epub ahead of print Olver TD, et al (Shoemaker JK). Glucose-stimulated insulin secretion causes an insulin-dependent nitric oxide-mediated vasodilation in the blood supply of the rat sciatic nerve. <i>Am J Physiol Regul Integr Comp Physiol.</i> 2013; 305(2):R157-163 Olver TD, et al. Putting eggs and cigarettes in the same basket; are you yolking? <i>Atherosclerosis.</i> 2013; 227(1):184-5 (<i>letter</i>) Olver TD, et al (Lemon PWR). Impaired superficial femoral artery vasodilation in young obese women following an oral glucose tolerance test. <i>Appl Physiol Nutr Metab.</i> 2012; 37(1): 176-183 Olver TD et al. Carbohydrate conundrum: Why Canadians cannot fit into their skinny genes. <i>Health Sci Inquiries.</i> 2012; 3(1): 82-83 (<i>graduate student journal</i>) 		