The TNFα/S1P Signalling Axis Mediates Elevated Myogenic Tone in Diabetes Mellitus

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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ABSTRACT

Diabetes causes changes in microvascular function that can ultimately promote multi-organ failure. Microvascular resistance arteries, integral to blood pressure, tissue perfusion and capillary integrity, are targets for dysregulation in diabetes. Resistance arteries function by adapting their diameter in response to transmural pressure through an intrinsic property of vascular smooth muscle cells (VSMCs), termed the myogenic response. Altered myogenic tone compromises tissue perfusion and aggravates hypertension; permanent structural changes to the microcirculation can result.

The bioactive sphingolipid sphingosine-1-phosphate (S1P) is a central regulator of the myogenic response, with the pressure-induced activation of the S1P-generating enzyme sphingosine kinase 1 (Sphk1) and activation of the S1P₂ receptor (S1P₂R) as key elements. This signalling pathway and hence, myogenic tone are governed by the cytokine tumour necrosis factor alpha (TNFα). Given the association of diabetes with TNFα-induced inflammation, I hypothesized that TNFα recruits the S1P pathway to induce functional changes in resistance arteries.

This thesis utilized pressure myography techniques on isolated resistance arteries. To understand the mechanisms by which myogenic tone is affected, I titrated a standard diabetes mouse model (high fat diet + streptozotocin; HFD/STZ), to induce a mild increase in blood glucose levels. HFD/STZ treatment produced a progressive myogenic tone augmentation in mesenteric and olfactory cerebral arteries; HFD alone had no effect on blood glucose or resistance artery myogenic tone. Using gene deletion models that eliminate TNFα or Sphk1, I demonstrated that
VSMC TNFα drives augmented myogenic tone via enhanced S1P signalling. Inhibiting both TNFα (etanercept) and S1P (JTE013) signalling was therapeutically utilized to specifically correct this defect.

My results add diabetes to a diverse group of microvascular diseases driven by pathological TNFα/S1P signalling. My data demonstrate that microvascular reactivity is an early disease marker and advocates establishing therapies that strategically target the microcirculation in diabetes patients.
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During the course of this investigation, I personally witnessed the need for new microvascular-based therapy for the treatment of diabetes complications. My father has been living with type 1 diabetes for 42 years and was recently diagnosed with peripheral artery disease. Following a visit with one of the top vascular surgeons in Toronto, he was told that there was nothing that could be done beyond intensive blood glucose control. My goal then is that this work provides the rationale for the repurposing of existing medications and the development of new interventions to treat the vascular complications of diabetes, so that individuals like my father and many friends have something they can do to alleviate part of the challenges of life with diabetes.
DEDICATION

For Dr. Eric K. Patterson

a devoted scientist and outstanding educator

who took a chance on a first year undergraduate student;

you inspired the scientist within me and ultimately the accomplishment of this thesis

and

For Jack Prins

who, by some miraculous twist of fate,

ended up living on MAH6 to forever alter my life for the better;

you radiate and exemplify an important message that has motivated me every day of my PhD:

never, ever give up.
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<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ACh</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ACCORD</td>
<td>Action to Control Cardiovascular Risk in Diabetes trial</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ADVANCE</td>
<td>Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation trial</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end product</td>
</tr>
<tr>
<td>AICAR</td>
<td>5-Aminoimidazole-4-carboxamide ribonucleotide</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine monophosphate</td>
</tr>
<tr>
<td>AMPK</td>
<td>5’ adenosine monophosphate-activated protein kinase</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>AUC</td>
<td>Area under the curve</td>
</tr>
<tr>
<td>BBDZR</td>
<td>Bio-breeding Zucker diabetic rat</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney fibroblast cells</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>dia&lt;sub&gt;active&lt;/sub&gt;</td>
<td>Active vessel diameter in MOPS containing Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
</tr>
<tr>
<td>dia&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximal vessel diameter in Ca&lt;sup&gt;2+&lt;/sup&gt;-free MOPS</td>
</tr>
<tr>
<td>CABG</td>
<td>Coronary artery bypass grafting</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CBF</td>
<td>Cerebral blood flow</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNP</td>
<td>C-natriuretic peptide</td>
</tr>
<tr>
<td>CO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DMEM/F12</td>
<td>Dulbecco’s modified eagle medium nutrient mixture F-12</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EDHF</td>
<td>Endothelium-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>EPI</td>
<td>Echo planar imaging</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ET</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>ETN</td>
<td>Etanercept</td>
</tr>
<tr>
<td>ETT</td>
<td>Epoxideicosatrienoic acids</td>
</tr>
<tr>
<td>FAIR</td>
<td>Flow-sensitive alternating inversion recovery technique</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FDA</td>
<td>US Food and Drug Administration</td>
</tr>
<tr>
<td>FSK</td>
<td>Forskolin</td>
</tr>
<tr>
<td>G6PD</td>
<td>Glucose-6-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GK</td>
<td>Goto-Kakizaki</td>
</tr>
<tr>
<td>GPCR</td>
<td>G protein-coupled receptor</td>
</tr>
<tr>
<td>HbA1C</td>
<td>Glycosylated haemoglobin</td>
</tr>
<tr>
<td>HFD</td>
<td>High fat diet</td>
</tr>
<tr>
<td>HFD/STZ</td>
<td>High fat diet plus streptozotocin (type 2 diabetes mouse model)</td>
</tr>
<tr>
<td>HMBS</td>
<td>Hydroxymethylbilane synthase</td>
</tr>
<tr>
<td>HR</td>
<td>Heart rate</td>
</tr>
<tr>
<td>IBMX</td>
<td>Isobutylmethylxanthine</td>
</tr>
<tr>
<td>i.p.</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>LC20</td>
<td>20-kDa regulatory light chain subunits of myosin II</td>
</tr>
<tr>
<td>L-NNA</td>
<td>N(^6)-nitro-L-arginine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LV</td>
<td>Left ventricle/ventricular</td>
</tr>
<tr>
<td>LZR</td>
<td>Lean Zucker rat</td>
</tr>
<tr>
<td>memTNF(\alpha)</td>
<td>Membrane TNF(\alpha)</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>MLCP</td>
<td>Myosin light chain phosphatase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(n-morpholino)propanesulfonic acid buffer</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NAD/NADP</td>
<td>nicotinamide adenine dinucleotide/nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NC</td>
<td>Normal chow</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear factor-κB</td>
</tr>
<tr>
<td>Nrf2</td>
<td>NF-E2-related factor 2</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral glucose tolerance test</td>
</tr>
<tr>
<td>OZR</td>
<td>Obese Zucker rat</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCA</td>
<td>Posterior cerebral artery</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PGH₂</td>
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<td>Poly(ADP-ribose) polymerase</td>
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<td>SMA</td>
<td>Spiral modiolar artery</td>
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SMMHC  Smooth muscle cell-specific heavy chain promoter
SNP    Sodium nitroprusside Na$_2$[Fe(CN)$_5$NO]
SPNS2  Spinster 2 homolog-2
Sphk1  Sphingosine kinase-1
Sphk1  Sphingosine kinase-2
sTNF$\alpha$ Soluble TNF$\alpha$
STZ    Streptozotocin
TACE   TNF$\alpha$ converting enzyme
TCMM   Toronto Centre for Microvascular Medicine
TNF$\alpha$ Tumour necrosis factor alpha
TNFR1  TNF receptor type 1
TNFR2  TNF receptor type 2
TXA$_2$ Thromboxane A$_2$
UHN    University Health Network
UKPDS  United Kingdom Prospective Diabetes Study
VDAT   Veterans’ Affairs Diabetes Trial
vs.    versus
VSMC   Vascular smooth muscle cell
Wt     Wild-type

Nucleic Acid Abbreviations
A  adenosine
C  cytosine
G  guanine
T  thymine
Methodological Abbreviations

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Prefixes

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CHAPTER 1. GENERAL INTRODUCTION
1.1 INTRODUCTION

1.1.1 Diabetes Mellitus

1.1.1.1 Definition, global impact and associated complications

Diabetes mellitus (commonly referred to as diabetes) is characterized by chronic hyperglycaemia and disturbances in protein, fat and carbohydrate metabolism as a result of defects in insulin secretion, insulin action or a combination of the two. Diabetes is a present-day health crisis affecting a staggering 382 million people worldwide. The World Health Organization forecasts diabetes to be the 7th leading cause of death in 2030. In Canada, approximately 2.5 million individuals are affected.

Diabetes is classified into two broad categories, defined as ‘type 1’ and ‘type 2’ diabetes. The development of type 1 diabetes is due to the autoimmune destruction of pancreatic β-cells resulting in a loss of insulin production. Type 2 diabetes affects more individuals than type 1, and is characterized by disordered β-cell function, peripheral insulin resistance and eventual β-cell loss.

Hyperglycaemia is often present long before the diagnosis of diabetes is made, causing pathological tissue damage. Ultimately, both type 1 and type 2 diabetes are primary risk factors for several diseases that determine the patient’s fate (e.g., heart failure, stroke, renal failure) and quality of life (e.g., retinopathy, neuropathy, cognitive dysfunction and depression). Consequently, diabetes has a pronounced negative socioeconomic impact with the long term potential to overburden the public health systems.

Cardiovascular disease, specifically, accounts for more than half of the mortality occurring in the diabetes population. These cardiovascular disorders include atherosclerosis, resulting in myocardial infarction and stroke, as well as impaired cardiac diastolic function. Other complications include diabetic nephropathy, a major cause of end stage renal failure, diabetic retinopathy, a leading cause of blindness in adults aged 20-74 years and neuropathy, with a lifetime risk of one or more lower extremity amputations. Finally, diabetes is accompanied by depression, cognitive decline and dementia, and sexual dysfunction.
1.1.1.2 Known causes of diabetes complications

The wide-ranging complications in diabetes are, at least in part, due to chronic elevation of blood glucose levels damaging blood vessels. Diabetes complications are grouped under either ‘macrovascular’ or ‘microvascular’ complications, based on the size of the arteries in which the damage occurs\(^\text{18}\), but it is important to recognize that many diabetes complications contain an element of vascular dysfunction.

Glucose as the principal mediator of diabetes complications

The most effective way to reduce the risk of vascular complications in both type 1 and type 2 diabetes is to control blood glucose, aiming to reach levels as close to normoglycaemia as early as possible in the course of the disease\(^\text{19},\text{20}\). In clinical trials that employed intensive diabetes treatment to lower blood glucose in either type 1\(^\text{19}\) or type 2\(^\text{21}\) diabetes patients, it was demonstrated that elevation in the intermediate AGE haemoglobin A\(_1\text{C}\) (HbA\(_1\text{C}\)) is a useful prognostic indicators of cardiovascular risk in patients with diabetes.

However, in patients with type 2 diabetes, the clinical trials *Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation* (ADVANCE)\(^\text{21}\), *Action to Control Cardiovascular Risk in Diabetes* (ACCORD)\(^\text{22}\) and the *Veterans’ Affairs Diabetes Trial* (VADT)\(^\text{23}\) unexpectedly demonstrated that intensive glucose control does not necessarily reduce the risk of macrovascular disease. In fact, the intensive glycemic control arm of the ACCORD study was halted three years early due to an unexpected increase in overall mortality\(^\text{22}\).

Molecular mechanisms implicated in diabetes complications

The molecular mechanisms by which hyperglycaemia promotes vascular complications are numerous, thoroughly reviewed by Forbes *et al.*\(^\text{24}\). The scope of this review speaks to the substantial number of complex pathways that have been identified and those that still remain to be elucidated.
In brief, increased glucose concentrations can activate nuclear factor-κB (NFκB)\textsuperscript{25}, a key mediator in the regulation of multiple pro-inflammatory and pro-atherosclerotic genes in endothelial cells, vascular smooth muscle cells (VSMCs), and macrophages. High glucose also promotes glycation of a number of proteins, resulting in the formation of advanced glycation end product (AGE) protein cross-linking, and reactive oxygen species formation. Finally, hyperglycaemia stimulates oxidative stress, which has been strongly implicated as a cause of atherosclerosis. Other mechanisms associated with diabetic vascular disease secondary to hyperglycaemia include stimulation of the aldose reductase and hexosamine pathway, increased protein kinase activation, and oxidative stress and redox imbalances\textsuperscript{26}.

1.1.1.3 **Present treatment strategies for the management of diabetes complications**

Early intervention and avoidance or delay of the progression to type 2 diabetes by controlling modifiable risk factors in the population, such as overweight and obesity, sedentary lifestyle and dietary factors, is a primary aim for the prevention of diabetes complications. In concert with this goal, a better understanding of this multifaceted disease also holds the promise to interrupt diabetes-induced pathogenic cascades before irreversible damage occurs.

Medication for the control of blood glucose is often the first line therapy for patients with type 2 diabetes. As demonstrated in patients with type 1 diabetes by the *Diabetes Control and Complications Trial* (DCCT), there is no specific glycemic threshold for the development of either macro- or microvascular complications. This means that any increase in blood glucose, as well as the length of time that glucose is elevated, can result in diabetes complications\textsuperscript{27}. Therefore, the treatment plan for patients with diabetes should be individualized based on clinical presentation and should involve attaining blood glucose levels as close to normal as possible without increasing the risk of unacceptable side effects, such as hypoglycaemia. The medications presently available for achievement of this clinical goal of patient care include orally administered insulin secretagogues, insulin sensitizers, alpha-glucosidase inhibitors, peptide analogues and proteinase inhibitors, the new glycosurics and injectable insulin.

Second to antihyperglycemic therapy, each diabetic complication has its own treatment approaches. In diabetic retinopathy, there are benefits of early and timely laser photocoagulation
therapy to reduce the risk of loss of sight, even in mild cases. Controlling diabetic kidney disease includes management of hypertension with antihypertensive therapy\(^{28}\). For neuropathy, pain management is the only treatment available. There are agents that have shown promise in clinical trials, however none have been approved. Medications to treat cardiovascular disease associated with diabetes consist of the therapies used to treat cardiovascular disease in the absence of diabetes. These include aspirin, \(\beta\)-blockers, angiotensin-converting enzyme inhibition, anti-platelet agents, thrombolytic therapy, cholesterol-lowering therapy and treatment of hypertension.

1.1.1.4 The complications of diabetes are vascular in origin

Several studies have demonstrated that vasomotor dysfunction of the microvessels is an early manifestation of the vascular complications in diabetes\(^{29,30}\). Pathological alterations in the vasculature of patients with diabetes can alter organ perfusion, particularly affecting the functional and structural integrity of tissues like the kidneys, retina and peripheral nervous system. The most consistent structural defect observed in diabetic microvasculature is a thickening of the capillary basement membrane in the retina, muscle, and glomeruli\(^{31}\). This thickening alters vessel function, promoting hypertension, reduced wound healing, and tissue hypoxia. Furthermore, vascular compliance (i.e. the ability of a microvessel to expand and increase volume in the face of increasing transmural pressure) is potentially affected by changes in the structure of the vessel wall. Although microvascular dysfunction is clearly relevant in diabetes, the molecular mechanisms that underlie the diabetes-mediated microvascular changes are not well understood.

1.1.2 The microvasculature

1.1.2.1 Definition and function

The microvasculature is an important component of the cardiovascular system that includes arterioles, capillaries and venules. The primary function of the microvasculature in a general sense is the transport and exchange of substances between the blood and the tissue. The efficiency of these processes depends on microvascular pressure and flow\(^{32}\). Resistance arteries are the precapillary vessels that allow for the regulation of both resting resistance and active
control of blood flow depending on tissue demand. Resistance arteries have a dual function: to allow for blood flow to individual capillary beds and to regulate mean arterial blood pressure. Poiseulle’s law states that laminar flow rate of incompressible fluids along a tube is directly proportional to the fourth power of the tube’s radius. Therefore, even small changes in the radius of the artery can meaningfully impact blood flow, vascular resistance and blood pressure.

1.1.2.2 Resistance artery structure

Arterial blood vessels are structurally comprised of the tunica intima, tunica media and tunica adventitia. The tunica adventitia is made of connective tissue designed to secure the blood vessel in place and hold in place the nervous input supply. Spindle-shaped vascular smooth muscle cells of the tunica media, arranged circularly surrounded by extracellular collagen and elastin, provide the vessel with elasticity, distensibility, and mechanical and contractile functions. The tunica intima is a monolayer of endothelial cells covering the surface of the lumen of the blood vessel that mediates the interactions between circulating blood and VSMCs.

1.1.2.3 Endothelial cells

The endothelial layer plays a key role in maintaining vascular homeostasis. The endothelium regulates vascular tone and permeability to nutrients and leukocytes, the balance between coagulation and fibrinolysis, the composition of the subendothelial matrix, and vessel wall inflammatory activity. In order to perform these functions, the endothelium produces a variety of regulatory mediators. Among the important molecules synthesized by the endothelium is nitric oxide (NO), primarily produced by the constitutively active enzyme endothelial nitric oxide synthase (eNOS), through enzymatic oxidation of the guanidine group of L-arginine in the presence of several important cofactors such as FAD, NAD(P)H and tetrahydrobiopterin. Many of the physiological actions in resistance arteries of NO, a small hydrophobic molecule, are regulated through its binding to guanylyl cyclase (GC)-coupled receptors in a specialized heme group, resulting in conformational changes that trigger GC activity, catalyzing the generation of cyclic guanosine monophosphate (cGMP) from GTP. In the vasculature, one of
the ways in which vasodilation occurs is through cGMP mediated NO-dependent relaxation of VSMCs. cGMP activates myosin light chain phosphatases and the opening of ATP-dependent potassium channels. NO-mediated dilation also relies on direct nitrosylation of ATP-dependent potassium channels leading to VSMC hyperpolarization and inhibition of calcium entry. NO also possesses anti-platelet, anti-proliferative, permeability-decreasing and anti-inflammatory properties. Other vasoactive relaxing factors secreted by endothelial cells include endothelium-derived hyperpolarizing factor (EDHF), prostacyclin (PGI₂), adenosine, epoxyeicosatrienoic acids (ETTs) and C-natriuretic peptide (CNP). Conversely, the endothelial layer also releases potent vasoconstrictory factors such as endothelin (ET-1), prostaglandin H₂ (PGH₂), thromboxane A₂ (TXA₂), superoxide anion, H₂O₂ and angiotensin II.

A disruption in the balance of any of these functions results in “endothelial dysfunction”, a term coined in the 1980’s following the seminal study conducted by Furchgott and Zawadzki identifying the requirement of endothelial cells for relaxation of VSMCs in response to acetylcholine. Endothelial dysfunction has come to refer to the condition in which the endothelium loses normal physiological properties, either in the basal state or in response to a stimulus, and shifts toward a vasoconstrictor, pro-thrombotic and pro-inflammatory state that ultimately may jeopardize organ function.

The earliest and one of the hallmarks of endothelial dysfunction is a disturbance in the NO pathway that occurs through several potential mechanisms, including a) reduced eNOS expression levels, b) reduced eNOS activity due to insufficient amounts of eNOS substrates and co-factors and c) impaired eNOS activation or accelerated NO degradation, especially by reactive oxygen species (ROS). In the normal physiological state, the redox balance and production of superoxides is regulated by key enzymes such as NADPH-dependent oxidases and superoxide dismutase. If endothelial cells can no longer scavenge superoxide anions, this highly reactive intermediate reacts with NO to form peroxynitrites, which are strong oxidants that can alter both structure and function of cell components.

Endothelial dysfunction is evaluated indirectly in vivo by measuring responses to certain agonists like acetylcholine, or changes in flow, and in vitro in isolated arteries. Current evidence links endothelial dysfunction with a variety of disease states, from hypertension and atherosclerosis, to
aging, heart and renal failure, sepsis, rheumatoid arthritis, sleep apnea, and so on\(^{40}\). Important for this thesis, endothelial dysfunction has been described in diabetes\(^{45-48}\) and contributes to the development of diabetic complications. The metabolic disturbances that occur in diabetes such as hyperglycaemia, excess free fatty acid release and insulin resistance and compensatory hyperinsulinemia may all contribute to the disruption of the molecular mechanisms that regulate NO synthesis and degradation\(^{43}\). Although many mechanisms have been proposed to explain the causes of impaired endothelial function in the setting of diabetes as a result of abnormal metabolism, the definitive mechanism remains unclear.

Diabetes is often associated with many other homeostatic imbalances along with hyperglycaemia (i.e., insulin signalling abnormalities, inflammation, oxidative stress) that, in combination, all disrupt endothelial function. Furthermore, clinical studies are unavoidably confounded by the presence of other cardiovascular diseases that affect the endothelial layer, such as dyslipidemia and hypertension.

Of note, in type 1 diabetic patients, some reports show normal endothelial-dependent vasodilation\(^{49-51}\). An explanation for these findings is that these data were obtained from a patient population with normoalbuminuria and relatively good blood glucose control. In type 2 diabetes patients, endothelial function is more often found impaired\(^{30}\). However, no matter how thorough the patient selection, comorbidities could not be entirely selected out of the study population. Thus, as suggested by De Vriese et al.\(^{30}\), “it remains unclear whether diabetes type II per se affects endothelial function”.

**Mechanisms of endothelial cell dysfunction in diabetes**

Elevated blood glucose is one of the primary culprits suggest to influence the reported endothelial dysfunction in diabetes.

Hyperglycaemia increases mitochondrial superoxide anion production\(^{52}\) that leads to peroxinitrite production from NO, which reacts with the eNOS co-factor tetrahydrobiopterin\(^{53}\). If tetrahydrobiopterin levels are low, eNOS is uncoupled and transfers electrons not to L-arginine, but to molecular oxygen, producing superoxide rather than NO\(^{54}\). Diabetes patients given tetrahydrobiopterin supplements have improved endothelial-dependent vasodilation, supporting the uncoupled eNOS hypothesis\(^{55}\). NO formation by eNOS is also inhibited in diabetes through a
hyperglycaemia-induced oxidative stress mechanism: levels of asymmetric dimethylarginine, a structurally similar molecule to L-arginine and thus competitive antagonist of eNOS, are increased. Finally, ROS production as a result of oxidative stress in hyperglycaemia settings directly inhibits the production of NO; ROS enhances serine phosphorylation of IRS-1 inhibiting insulin-stimulated NO release\textsuperscript{56} and ROS scavenges NO to produce peroxynitrites that damage endothelial cells\textsuperscript{39}.

Hyperglycaemia induces \textit{de novo} synthesis of diacylglycerol (DAG) activating protein kinase C isoforms (PKCs) that in turn inhibit eNOS phosphorylation and NO production in response to insulin\textsuperscript{57}. Along with a reduction in insulin-stimulated NO production, PKC isoforms also enhance VSMC contractility through the release of endothelial ET-1\textsuperscript{58}.

Another important mechanism by which hyperglycaemia contributes to endothelial dysfunction is through the formation of AGE, a heterogeneous group of modified proteins, lipids and nucleic acids formed through nonenzymatic Maillard reactions between amino groups and glucose derivatives carbonyls\textsuperscript{59}. AGEs bind to their AGE receptors (RAGEs) to increase superoxide production, accelerate eNOS mRNA degradation\textsuperscript{60,61} and enhance ET-1 expression\textsuperscript{62}, culminating in reduced NO bioavailability and a proconstrictive state of the vasculature. Notably, all of the above mentioned mechanistic pathways overlap and intersect to promote and support the dysregulation of endothelial cell homeostasis. Most importantly, the described mechanisms of endothelial dysfunction in diabetes are context dependent; there is variation depending on the size of the vessel studied, its anatomical location and its function.

\textbf{1.1.2.4 Vascular smooth muscle cells}

In an adult organism, the VSMCs of resistance arteries are highly specialized to perform the functions necessary for contraction and regulation of blood vessel tone, thus controlling blood pressure and blood flow distribution. As such, VMSCs contain a particular collection of contractile proteins, ion channels and signalling molecules that promote contraction\textsuperscript{63}.

In a healthy blood vessel, VSMCs display a low level of proliferation and synthetic activity. However, VSMCs are plastic and can undergo changes in their phenotype in response to local
environmental cues to display a multitude of other functions beyond contraction that vary at different developmental stages or during vascular repair. Consequently, abnormal changes in the environment, such as those that occur in disease states, may lead to an unfavorable phenotypic switch that could contribute to the development/progression of vascular disease. For example, in atherosclerosis, the normally low proliferation rate and production of small amounts of extracellular matrix proteins of mature VSMCs is greatly accelerated. Further evidence of this ‘phenotype switching’ effect has been shown to occur in cancer and hypertension as well. There is the potential then in diabetes for local environmental changes such as hyperinsulinemia and hyperglycaemia to directly affect the walls of microvessels. Emerging evidence supporting this hypothesis is reviewed in Porter et al. VSMC phenotype and function is influenced by the local metabolic milieu, exacerbating diabetes complications.

1.1.3 The myogenic response

1.1.3.1 Definition and function

Sir William Bayliss, who noted that an increase in resistance artery transmural pressure resulted in arterial constriction, first made the recognition of a phenomenon now understood to be the myogenic response in 1902. The ‘Bayliss effect’ describes the intrinsic ability of the VSMC of resistance arteries to antagonize stretch with contraction. This mechanism is necessary for the maintenance of constant blood flow to tissues no matter the overall blood pressure. In human arteries, the autoregulatory pressure range within which the myogenic response acts is widely accepted as 60-150 mmHg. The myogenic response contributes to the protection of the capillary network from substantial fluctuations in blood pressure and collective resistance artery constriction is a primary determinant of systemic blood pressure and total peripheral resistance.

1.1.3.2 An overall picture of the myogenic response mechanisms

The myogenic response is the result of a summation of input mechanisms that regulate smooth muscle cell contraction, summarized in Schubert et al. and Schubert & Mulvany. Broken down to simplicity, this mechanism is a local phenomenon that begins with a change in arterial
wall tension that is converted into an intracellular biochemical signalling reaction within VSMCs. A number of potential sensors on the VSMC surface lead to an increase in intracellular calcium concentration. This increased intracellular Ca\(^{2+}\) results in the binding of calcium to calmodulin, activating myosin light chain kinase (MLCK) to phosphorylate the two 20-kDa regulatory light chain subunits (LC\(_{20}\)) of myosin II. This triggers the interaction of myosin II with actin, leading to cross-bridge formation and ultimately contraction.

### 1.1.3.3 Confirmed myogenic tone mechanisms

Myogenic mechanisms are notably complex. Our understanding of the signalling components and pathways comes from numerous investigations that depend on the vascular bed studied and on information that has been obtained mainly using in vitro isolated vessel preparations. Below is a brief summary of the mechanisms that have been established.

**Initiation of the myogenic response**

It is well accepted that the pressure-induced change in microvessel wall tension is the stimulus for the myogenic response. The vessel then constricts and artery wall tension is reduced. Thus, the wall tension stimulus is attenuated during the myogenic response by a negative feedback mechanism, limiting the amount of myogenic constriction. The element(s) that act as the sensor(s) for the detection of the wall tension stimulus are presently unknown. It is also understood that the myogenic response is contingent on the VSMC specifically (i.e., neural or endothelial input is not necessary for myogenic tone). The mechanism behind VSMC membrane depolarization in response to pressure is also unidentified.

**The myogenic response sensor**

Presently we do not have direct experimental evidence for the mechanosensory mechanism that translates a physical stimulus into electrical and biochemical signalling resulting in artery constriction. Possible candidates include cation channels that directly sense stretch and integrins, extracellular glycoproteins that link the extracellular matrix and the cytoskeleton through collagen, laminin and fibronectin to ultimately transduce a mechanical force across the cellular plasma membrane.
The role of calcium

Importantly, an increase in VSMC intracellular Ca\(^{2+}\) accompanies the increase in microvessel luminal pressure. The source of Ca\(^{2+}\) is the extracellular space (i.e., resistance arteries cannot constrict when submersed in Ca\(^{2+}\)-free buffer). However, Ca\(^{2+}\) release from intracellular stores also plays a well-documented role. Voltage-operated calcium channels have been shown to allow extracellular Ca\(^{2+}\) to enter the VSMC resulting in depolarization, required for the myogenic response to occur. The elevated intracellular Ca\(^{2+}\) activates MLCK, which phosphorylates the 20-kDa myosin LC\(_{20}\) subunits, resulting in contraction\(^{69}\).

Historically, studies into myogenic mechanisms focused on the Ca\(^{2+}\)-dependent pathway in the regulation of MLCK. A growing body of data also exists showing that there are Ca\(^{2+}\)-independent signalling mechanisms that also mediate the myogenic response through inhibition of myosin light chain phosphatase (MLCP). Thus myosin LC\(_{20}\) phosphorylation can be regulated while the activity of MLCK remains constant, supporting the observation that myogenic vasoconstriction continues although intracellular levels of Ca\(^{2+}\) do not change\(^{70}\). This effect has been designated as Ca\(^{2+}\)-sensitization, referring to the inhibition of MLCP, resulting in a blockade of the dephosphorylation of the 20-kDa myosin LC\(_{20}\) subunits. In more detail, ligand interaction with VSMC membrane G protein-coupled receptors linked to the G\(_{12/13}\) family of heterotrimeric G proteins, results in activation of guanine nucleotide exchange factors (LARG, PDZ-RhoGEF and p115RhoGEF) that in turn activate the GTPase RhoA via catalyzed GDP-GTP exchange. RhoA-GTP activates RhoA/Rho-associated kinase, which phosphorylates one of the regulatory subunits on MLCP, inhibiting its activity. Additionally, studies that have utilized protein kinase C (PKC) inhibitors suggest a role for PKC in Ca\(^{2+}\) sensitization, however the mechanisms involved are unclear\(^{63,80}\).

Reorganization of the cytoskeleton in force generation

Several lines of evidence suggest that the dynamic reorganization of the cytoskeleton influences force in smooth muscle contraction\(^{81}\). The formation of actin filaments in VSMCs potentially underlies myogenic mechanotransduction via an addition of sites for myosin interaction that may enhance force in response to a pressure stimulus. The inhibition of actin polymerization using cytochalasins (which cap the fast-growing, barbed end of actin filaments) or jasplakinolide (which enhances polymerization) results in the loss of the myogenic response\(^{82}\). Presently the
mechanism by which actin polymerization is activated by pressure is unknown, but is believed to
be through integrin-mediated stimulation of signal transduction pathways known to result in
VSMC contraction\textsuperscript{82}.

1.1.4 Myogenic tone mechanisms: the sphingosine-1-phosphate/tumour necrosis factor alpha signalling pathway

Recent work from our laboratory has highlighted the importance of the bioactive sphingolipid
sphingosine-1-phosphate (S1P) and the cytokine tumour necrosis factor alpha (TNF\textalpha) in the
regulation of myogenic tone in both health and disease. A summary of the definition, regulation
and function of these molecules follows below.

1.1.4.1 Sphingosine-1-phosphate

Sphingolipids are components of the outer leaflet of the eukaryotic cell plasma membrane lipid
bilayer, made up of a ceramide molecule plus a serine head group. Sphingolipid synthesis \textit{de novo} begins at the cytoplasmic face of the endoplasmic reticulum (ER) via the condensation of
serine and palmitoyl-CoA by serine palmitoyltransferase. From this initial reaction, through a
series of steps, ceramide is eventually formed\textsuperscript{83}. Ceramide is the precursor of sphingosine, an 18-
carbon amino alcohol with an unsaturated hydrocarbon chain (produced following \textit{N}-
deactylation by the enzyme ceramidase) that can be phosphorylated by sphingosine kinases to
form S1P\textsuperscript{83}.

In general, ceramide participates in cellular apoptosis, whereas S1P promotes cell survival and
proliferation\textsuperscript{84}. The finely regulated balance between these two molecules ultimately controls
cellular fate and thus, is labeled the ‘sphingolipid rheostat’\textsuperscript{84}. Beyond cell growth and survival,
S1P is associated with cell motility and invasion, angiogenesis, vascular function and regulation
of myogenic tone, lymphocyte trafficking, vascular integrity, immune regulation and normal
embryonic development\textsuperscript{68,85}. 
1.1.4.1.1 Regulation of S1P levels

*Please refer to Figure 1.1.* Two types of sphingosine kinases can catalyze the intracellular, adenosine triphosphate (ATP)-dependent, phosphorylation of sphingosine to form S1P: sphingosine kinase-1 and sphingosine kinase-2 (Sphk1 and Sphk2, respectively). Enzymes that degrade S1P further regulate S1P levels. These include S1P lyase and two S1P-specific phosphatases, sphingosine phosphatase-1 (SGPP1) and sphingosine phosphatase-2 (SGPP2).

*Sphingosine kinases*

The sphingosine kinases are ubiquitously distributed, with higher levels found in the brain, heart, lung and spleen. Erythrocytes\textsuperscript{86} and the endothelium\textsuperscript{87} also express sphingosine kinases in large quantities. Sphk1 is a constitutively active cytosolic enzyme of 49-kDa that regulates intra- and extracellular S1P levels. Sphk1 includes three calcium/calmodulin-binding consensus sequences and several potential protein kinase phosphorylation sites, with no transmembrane binding
Sphingosine is generated in the membrane of the endoplasmic reticulum and other membranes from the precursor ceramide. Sphingosine kinase 1 (Sphk1) is located in the cytosol and upon activation, translocates to the cell membrane, where its substrate sphingosine is located. Sphingosine kinase 2 (Sphk2) is localized to the nuclear plasma membrane Sphingosine-1-phosphate (S1P) is formed following phosphorylation of sphingosine by Sphk1, which is then transported out of the cell by various transporters. S1P binds to S1P receptor(s) (S1PR) to initiate downstream signalling pathways. S1P is transported back into the cell for degradation by the cystic fibrosis transmembrane regulator (CFTR) and potentially other unidentified transporters. At the endoplasmic reticulum, S1P is reversibly dephosphorylated by S1P phosphatases 1 and 2 (SGPP1/SGPP2) back to sphingosine, or irreversibly degraded by S1P lyase into phosphoethanolamine (PE). ABC: ATP binding cassette.
domains. Specificity of Sphk1 is for the trans isomer of D-erythro-sphingosine. Sphk1 is stimulated by a variety of different molecules including agonists of growth factor receptors, ligands for G protein-coupled receptors (GPCRs), transforming growth factor beta and TNFα.

Sphk2 has a nuclear localization signal and is involved in the inhibition of cell growth and activation of apoptosis. Sphk2 has 80% similarity and 50% identity with Sphk1, with an additional 200 amino acids resulting in a molecular weight of 68-kDa. Sphk1 and Sphk2 do not share enzymatic kinetic properties, tissue distribution, or expression patterns during development; thus, they carry out distinct cellular functions that are differentially regulated.

Generation of S1P by Sphk1

In order to phosphorylate its substrate, plasma membrane-located sphingosine, Sphk1 must be activated; activation involves Sphk1 translocation from the cytosol to the plasma membrane. Therefore, the generation of S1P is spatially limited to the inner leaflet of the plasma membrane. From this location, S1P can signal “inside-out” of the cell in an autocrine or paracrine manner, stimulating S1P receptors on the cell surface of the same from which it was released, or nearby cells. To bind to cell surface receptors, S1P must be either flipped or secreted. The transporter(s) responsible for this function remain incompletely understood. This process is believed to involve the ATP-binding cassette (ABC) transporter family members, as demonstrated in in leukocytes. A recent study also characterized the spinster 2 homolog-2 (SPNS2) as a transporter of S1P into the extracellular space.

In healthy adult humans, tissue levels of S1P are low. Blood plasma concentration of S1P (primarily bound to albumin, ~30%, or high-density lipoprotein, ~65%) is much higher, falling between a reported range of 0.2 to 0.9 µmol/L. This S1P gradient is critical for homing of immune cells to lymphoid organs and for regulation of immune cell egress into blood and lymph. The cellular sources of plasma S1P include platelets, erythrocytes and vascular endothelial cells.
**S1P degradation by S1P lyase or SGPPs**

S1P inactivation involves the reversible removal of a phosphate group by the SGPP enzymes for reversion back to sphingosine. Alternatively, S1P can be irreversibly degraded into ethanolamine phosphate and hexadecenal by S1P lyase. The *in vivo* turnover of S1P is rapid (i.e., 15 minute half-life), lending to the idea that the synthesis and degradation of S1P are continually active.

S1P lyase is an integral ER membrane protein on the cytosolic face of the ER that functions as the main enzyme degrading S1P, cleaving it at the C2-C3 bond. It is most highly expressed in the liver. Our lab has characterized the effect of degradation on endogenous S1P signalling by assessing SGPP1 involvement in myogenic tone regulation. SGPP1 belongs to a family of magnesium-dependent, N-ethylmaleimide-insensitive type 2 lipid phosphate phosphohydrolases and is also localized to the ER. SPP1 expression is highest in the placenta and kidney. We found that overexpression of SGPP1 significantly reduces myogenic responsiveness and SGPP1 inhibition (with antisense oligonucleotides) enhances myogenic tone. SGPP1 overexpression also reduces vasoconstriction in response to exogenous S1P and inhibition of SGPP1 can enhance S1P-dependent vasoconstrictory responses. Therefore, S1P dephosphorylation via intracellular S1P phosphatases is a physiologically relevant regulator of S1P bioavailability (see Figure 1.1).

1.1.4.1.2 **S1P signalling via S1P receptors**

S1P is capable of activating five high-affinity, class A, G protein-coupled S1P receptors (S1P₁-₅R). These receptors all contain seven transmembrane domains, with an extracellular N-terminus and intracellular C-terminus. Most cells express one or more S1PR subtypes and thus signalling through these GPCRs regulates diverse physiological processes. In the vascular system in particular, S1PRs link to pathways mediating vascular tone, vascular permeability, angiogenesis, vascular hyperplasia following injury, atherosclerosis and heart function.
1.1.4.1.3 Lessons from mice lacking components of the sphingosine-1-phosphate signalling pathway

Genetic deletion of S1P1R results in embryonic lethality due to severe haemorrhage associated with reduced coverage of vessels by VSMCs\textsuperscript{104}. S1P2R\textsuperscript{−/−} mice do not show significantly reduced viability or obvious phenotypic changes\textsuperscript{105}. However, genetic knockout of S1P2R demonstrates essential roles for S1P2R in vivo with respect to development of neuronal excitability\textsuperscript{106}, a functional auditory system\textsuperscript{107,108} and regulation of microvascular tone\textsuperscript{109}. S1P3R\textsuperscript{−/−} mice are also viable and show normal cage behaviour\textsuperscript{105}.

Sphk1\textsuperscript{110} or Sphk2\textsuperscript{111} knockout mice develop and reproduce normally. Tissue S1P levels in Sphk1\textsuperscript{−/−} mice are not decreased compared to their littermate controls, however S1P in the serum is half as high, thus Sphk1 is necessary for controlling plasma S1P\textsuperscript{110,112}. These mice have no histologic abnormalities of the major organs\textsuperscript{110} and no changes in kidney mRNA levels of S1P lyase, SGPPs or neutral or acid ceramidase (i.e., S1P level regulating enzymes).

Lack of both Sphk1 and Sphk2 expression in mice is embryonic lethal due to severe disruption of neurogenesis and angiogenesis, suggesting that each sphingosine kinase may compensate for the lack of the other\textsuperscript{113}. S1P levels in these embryos are undetectable. Deleting Sphk1 and Sphk2 three to five days after birth in conditional Sphk1/Sphk2 double knockout mice simultaneously results in a decrease of S1P levels to undetectable amounts in both the plasma and lymph\textsuperscript{86}. Surprisingly, these double knockout mice survive without overt phenotypic complications.

1.1.4.2 Tumour necrosis factor alpha

TNFα is a cytokine involved in systemic inflammation. TNFα is produced as a type II transmembrane protein (a homotrimer of 26-kDa)\textsuperscript{114}, from which the soluble 17-kDa homotrimeric form is released following proteolytic cleavage by the metalloproteinase TNFα converting enzyme (TACE; ADAM-17)\textsuperscript{115}. Animal models demonstrate that soluble TNFα (sTNFα) is required for the development of acute and chronic inflammation and membrane TNFα (memTNFα) underlies the development of lymphoid tissues, protects against intracellular bacterial infections, and supports chronic inflammation and autoimmunity\textsuperscript{116}. The relative amounts of membrane (memTNFα) and soluble (sTNFα) forms are determined by inducing stimuli, cell type, and amount of active TACE and TACE inhibitors.
1.1.4.2.1  TNF receptor subtypes

TNFα exerts signalling functions through interaction with its cognate membrane receptors, the TNF receptor (TNFR) family. Both TNF receptor type 1 (TNFR1, 55-kDa) and TNF receptor type 2 (TNFR2, 75-kDa) bind memTNFα and sTNFα. However, TNFR2 is fully activated only by memTNFα and sTNF preferentially binds to TNFR1. Most tissues constitutively express TNFR1, except erythrocytes; TNFR2 is strictly regulated in cells of the immune system (e.g. hematopoietic cells). The extracellular domains of both receptors can undergo proteolytic cleavage resulting in fragments of soluble receptors that may either neutralize circulating TNFα or stabilize it to prevent its degradation. Of note, the affinity for TNFα by the soluble receptor is lower than that of the membrane receptor. The development of clinically relevant TNFα neutralizing therapy was based on the function of these soluble receptors. TNFR2 is cleaved by TACE; the enzyme(s) responsible for processing TNFR1 are presently unknown.

1.1.4.2.2  TNFα production, function and signalling

TNFα is produced by macrophages and T-cells in response to inflammatory stimuli. One of the main stimulants for TNFα production is bacterial lipopolysaccharide (LPS). Within 30 minutes, TNFα mRNA is induced, however TNFα is primarily post-transcriptionally regulated. Other activators include viral, mycotic and parasitic antigens, enterotoxin and TNFα itself. Many tissues and cell types including lymphoid cells, mast cells, endothelial cells, smooth muscle cells, fibroblasts, cardiomyocytes, adipocytes and neuronal tissue are also capable of producing TNFα.

The biological functions of TNFα are extensive and widespread, as virtually every nucleated cell shows some TNFα responsiveness. Low concentrations of tissue TNFα are associated with beneficial effects, such as amplification of host defense mechanisms when confronted with infection; high concentrations of TNFα result in excess inflammation and tissue injury. Briefly, TNFα is a major proinflammatory mediator, which also has the ability to induce cellular apoptosis/cellular survival, production of other cytokines (e.g. Interleukin-1), and induction of insulin resistance. TNFα has known roles in: rheumatoid arthritis, asthma, septic shock, irritable bowel disorder and cachexia, as well as atherosclerosis, endothelial dysfunction,
stroke\textsuperscript{130}, ischemic brain injury\textsuperscript{131} and importantly, the progression of diabetes (i.e., the development and exacerbation of insulin resistance) and its associated complications\textsuperscript{132,133}.

TNF$\alpha$ signalling is elaborate; discrete signalling pathways are mediated through each TNFR, and transmembrane TNF$\alpha$, as it can act as both a ligand and a receptor. Receptor-mediated effects result in alternative activation of NF$\kappa$B or an apoptotic cascade. Due to the inherent complexity of TNF$\alpha$ regulation and its emerging involvement in numerous pathologies, TNF$\alpha$ signalling continues to be an active area of research. For the purpose of this thesis, I will review the emerging relevance of TNF$\alpha$ signalling in regulation of vascular function.

1.1.4.2.3 Etanercept
Etanercept (ETN) is a genetically engineered protein (biologic) composed of a dimer of the extracellular portions of human TNFR2 fused to the Fc portion of human IgG1. ETN is designed to bind sTNF$\alpha$ with high affinity. Each molecule of ETN binds to a single sTNF$\alpha$ trimer, resulting in small 1:1 binding complexes\textsuperscript{134}. Assessing the consequences of ETN binding to memTNF$\alpha$ is more challenging, as memTNF$\alpha$ concentrations on macrophages and T cells are kept low even following cellular activation and memTNF$\alpha$ is cleaved by TACE\textsuperscript{126}. ETN is also capable of binding members of the lymphotoxin family, however the biological consequences of this interaction remain unknown.

In the USA, ETN (trade name Enbrel\textsuperscript{®}) is F.D.A. approved to treat rheumatoid arthritis, juvenile rheumatoid arthritis, psoriatic arthritis, plaque psoriasis and ankylosing spondylitis. ETN is administered subcutaneously, either 25 mg twice a week or 50 mg once a week. The molecule has a half-life of 4 days and a clearance rate of 72±5 mL/hour\textsuperscript{126}. Combined data from randomized controlled trials and safety registries have indicated that there is an increased risk for certain infections, particularly tuberculosis and other infections caused by intracellular microbes, due to treatment with TNF$\alpha$ antagonists\textsuperscript{135-140}.

1.1.4.2.4 TNF$\alpha$ in the pathogenesis of insulin resistance and diabetes
In 1993, Hotamisligil \textit{et al.}\textsuperscript{141} identified a connection between TNF$\alpha$, obesity and insulin resistance (defined as, a state in which physiological concentrations of insulin produce a less than normal response). This seminal study demonstrated that TNF$\alpha$ is expressed in white adipose
tissue and TNFα levels are increased in the fat of obese, insulin resistant rodents. TNFα is also found in human adipose tissue, produced primarily by adipocytes and resident adipose macrophages, and adipose mRNA and circulating plasma protein levels show a positive correlation with both the degree of obesity and hyperinsulinemia\textsuperscript{142-144}. Additionally, circulating TNFα levels are elevated in the plasma of patients with either type 1 or type 2 diabetes\textsuperscript{145,146}.

Consequently, significant research has investigated the notion that adipocyte-generated TNFα contributes to obesity-associated insulin resistance, and eventually the development of type 2 diabetes. TNFα neutralization improves insulin resistance in obese Zucker fa/fa rats\textsuperscript{141}. Mice deficient of TNFα are protected against the development of insulin resistance when challenged with a HFD\textsuperscript{147}. It has been suggested that TNFα regulates insulin action through several potential mechanisms. These include: (i) downregulation of gene expression of the insulin-responsive glucose transporter GLUT4, (ii) antagonizing the synthesis or action of the nuclear peroxisome proliferator-activator receptor γ (PPARγ), a regulator of adipogenesis and adipocyte function, (iii) direct effects on insulin signalling via decreased insulin-receptor mediated activity\textsuperscript{148,149}.

Given the results of these studies, anti-TNFα therapy has been suggested to ameliorate insulin resistance in patients by those in the inflammation/diabetes field\textsuperscript{132}. Disappointingly, published studies using TNFα blockade therapy in insulin resistant patients did not show beneficial effects on insulin resistance and blood glucose control with treatment\textsuperscript{150-154}. However, a short-term investigational period and low participant number limits these studies. A retrospective analysis was conducted by Gupta-Ganguli \textit{et al.}\textsuperscript{155} on the effect of a therapeutic dosage of ETN or infliximab (a TNFα neutralizing antibody) over a ten-year period used to treat Crohn’s disease and rheumatoid arthritis. This group demonstrated that patients with rheumatoid arthritis plus type 2 diabetes or Crohn’s plus type 2 diabetes had a significant improvement in fasting blood glucose and HbA\textsubscript{1c} levels following treatment. More studies are undoubtedly needed to conclusively resolve the effectiveness of TNFα neutralization on insulin sensitivity in patients with diabetes.
1.1.4.2.5 Lessons from TNFα knockout mice

Mice that are genetically engineered to lack TNFα cytokine expression are both viable and fertile, displaying no obvious phenotypic abnormalities – indicating that TNFα is not required for mouse development\textsuperscript{156}. TNFα\textsuperscript{−/−} mice demonstrate that TNFα is critical for coordinating host reactivity against pathogenic bacteria and the response to LPS-mediated toxicity. TNFα\textsuperscript{−/−} mice, particularly males, have a modest decrease in body weight, epididymal fat weight and percent body fat\textsuperscript{157}. Furthermore, these mice have lower insulin, triglyceride and leptin levels compared to wild-type littermate controls\textsuperscript{157}. Interestingly, high fat diet (HFD)-induced (50% cal/fat) obese mice lacking TNFα are protected from insulin resistance\textsuperscript{147}.

1.1.4.3 Myogenic tone regulation by S1P

Our laboratory has discovered that under physiological conditions, the S1P signalling pathway is a key regulator of the myogenic response\textsuperscript{80,98,158-163}. Of note, the degree of vascular response to S1P differs depending on the context, the species and the vascular bed studied\textsuperscript{158,162,163}. S1P controls both the Ca\textsuperscript{2+}–dependent and –independent mechanisms directing myogenic tone, following generation by the enzyme Sphk\textsubscript{1}\textsuperscript{98}. S1P is pro-constrictive, an effect that is mediated through S1P\textsubscript{2}R coupled to G\textsubscript{12/13}, via RhoA/Rho kinase, resulting in the ultimate inhibition of MLCP and also controlled through intracellular S1P increasing the Ca\textsuperscript{2+} concentration inside the cell to regulate MLCK-mediated constriction\textsuperscript{80,98,164}. Furthermore, Sphk1 plays a role in determining resting arterial tone and is integral to myogenic vasoconstriction\textsuperscript{80} following translocation from the cytosol to the plasma membrane in response to an increase in transmural pressure\textsuperscript{161}. Resting and myogenic tone are alternatively negatively regulated by S1P degradation; the intracellular S1P-degrading enzyme SGPP1 is a functional antagonist of Sphk1, thus regulating S1P bioavailability\textsuperscript{98}. Importantly, recent work has also implicated cystic fibrosis transmembrane regulator (CFTR) as a mediator of myogenic tone, as it allows for the import of extracellular S1P for inactivation by SGPP1\textsuperscript{98,163}. An exact understanding of how transmural pressure directly activates Sphk1 (and ultimately the resultant S1P-mediated constriction) remains on ongoing area of research in our laboratory.
1.1.4.4 **Myogenic tone regulation by TNFα**

Important for the focus of this thesis, there is mounting evidence establishing a role for TNFα in the disruption of microvascular function, both in vivo and in vitro. Many investigations suggest that TNFα mediates its vascular effects via the endothelium. TNFα has been shown to alter NOS expression, impairing endothelium-dependent and NO-mediated vasodilation in vascular beds; TNFα can either diminish the production of NO or enhance its removal. In healthy humans, an intra-arterial TNFα infusion resulted in impaired endothelium-dependent vasodilation and increased vasoconstrictory prostaglandin in combination with reduced basal availability of NO, providing direct evidence of vascular dysfunction caused by TNFα.

Our laboratory has outlined the emerging relationship between TNFα and VSMCs. In diseases associated with inflammation, we were the first to report that TNFα hijacks the physiological mechanisms that govern the VSMC-controlled myogenic response, resulting in augmented microvascular tone. In vivo and in vitro experiments provide convincing evidence for the regulation of myogenic tonicity by TNFα in hearing loss, heart failure and subarachnoid haemorrhage.

1.1.4.4.1 **TNFα and inner ear microcirculation**

Scherer et al. investigated TNFα-stimulated S1P signalling in the microcirculation of the inner ear. An in vivo TNFα infusion in a guinea pig cochlear window preparation (i.e., in situ observation of cochlear microcirculation) rapidly reduces cochlear blood flow and decreases small resistance artery and capillary diameter. This effect is dependent on S1P signalling, as a superfusion of the S1PR antagonist JTE013 prior to the addition of TNFα prevents the decrease in blood flow and capillary diameter. Additionally, in an ex vivo spiral ligament capillary bed preparation, TNFα stimulates capillary constriction, an effect abolished by JTE013. The in vitro measures collected from spiral ligament capillaries shows that the TNFα-stimulated capillary diameter reduction was the result of active constriction, dependent on S1PRs and responsive to exogenous S1P.

In an ex vivo isolated gerbil spiral modiolar artery (SMA) model, TNFα augments Ca²⁺ sensitivity (i.e., promotes a pro-constrictive state). ETN both blocks and reverses the TNFα-
stimulated increase in Ca$^{2+}$ sensitivity. Moreover, JTE013 also reverses the constriction elicited by TNFα. Importantly, this effect relies on a functional Sphk1 enzyme, as TNFα does not increase Ca$^{2+}$ sensitivity in SMAs that express a dominant-negative Sphk1 mutant, or in SMAs that are treated with chemical inhibitor of Sphk1, dimethyl-sphingosine. Interestingly, TNFα stimulates Sphk1 translocation from cytosol to plasma membrane. This consequence of TNFα was observed using transfected SMA VSMCs containing a green fluorescent protein-tagged Sphk1, so the translocation of Sphk1 following TNFα treatment could be visualized. Furthermore, TNFα stimulates the phosphorylation of an ERK1/2 site on Sphk1 (serine 225) within SMA smooth muscle cells. In summary, this study supports the conclusion that regulation of cochlear blood flow involves TNFα-dependent activation of S1P signalling.

1.1.4.4.2 TNFα and microvascular function in heart failure

Our laboratory’s studies involving a mouse model of heart failure (left anterior descending coronary artery ligation) have provided significant further insight into the relationship between TNFα and the microvasculature. TNFα mRNA and protein expression is upregulated in the VSMC layer of posterior cerebral microarteries (PCAs) of mice with heart failure. Augmented myogenic responsiveness occurs in isolated PCAs from mice with heart failure; this phenomenon is normalized following in vitro TNFα blockade with ETN. Additionally, TNFα applied to isolated PCAs from healthy mice elevated myogenic tone to levels comparable to those observed in the mice with heart failure. These data suggest that the vessel walls are the source of regulatory TNFα in a heart failure setting.

Yang et al. showed that the mechanism by which TNFα mediates myogenic tone in heart failure is dependent on the S1P signalling pathway. PCAs isolated from several different mice that lack: (i) Sphk1 (Sphk1$^{-/-}$), or (ii) Sphk1 and one Sphk2 allele (Sphk1$^{-/-}$Sphk2$^{+/-}$), or (iii) Sphk2 (Sphk2$^{-/-}$), established that it is the relationship between TNFα and Sphk1 specifically that augments myogenic tone in heart failure. Exogenous TNFα is unable to increase tone in Sphk1$^{-/-}$ and Sphk1$^{-/-}$Sphk2$^{+/-}$ mice, while lack of Sphk2 does not protect against the development of TNFα-stimulated elevation in tone. Furthermore, loss of S1P signalling via a genetic knockout of S1P3R, or in vitro treatment with JTE013, results in a prevention/reversal of enhanced myogenic tone as a consequence of heart failure or exogenous TNFα treatment in isolated PCAs.
Therefore, we conclude that similar to the inner ear microcirculation, elevated myogenic tone associated with heart failure relies on a TNFα-dependent activation of S1P signalling.

1.1.4.4.3 **TNFα downregulates the cystic fibrosis transmembrane conductance regulator in heart failure**

A study from Meissner *et al.*\(^{163}\) further enhances our laboratory’s mechanistic understanding of the VSMC TNFα/S1P signalling network in disease through the introduction to a new key player, CFTR. CFTR is a transmembrane anion channel responsible for the secretion of chloride (Cl\(^-\)) into the extracellular space and is a member of the ATP-Binding Cassette class of transporters\(^{167}\). First, we show that CFTR mRNA is expressed throughout the vascular tree, including the PCAs and mesenteric arteries. Second, CFTR is essential for S1P import into VSMCs in order to be targeted to the endoplasmic reticulum for degradation. Third, PCAs and mesenteric arteries from CFTR knockout mice have stronger myogenic tone compared to littermate controls and these responses are abolished following *in vitro* S1PR blockade with JTE013.

Again, we show that myogenic tone is elevated in the PCAs and in mesenteric arteries\(^{158}\) of mice with heart failure. Interestingly, CFTR mRNA and protein expression is diminished in these arteries with elevated tone. Treating heart failure with ETN at six weeks post-myocardial infarction restores the downregulated microvascular expression of CFTR and normalizes myogenic tone\(^{163}\). Heart failure is unable to augment myogenic tone in the PCAs and mesenteric arteries of TNFα\(^-/-\) mice. Importantly, in TNFα\(^-/-\) mice with heart failure, CFTR protein expression did not change, showing that the CFTR downregulation observed in wild-type mice with heart failure depends on TNFα signalling. Finally, primary mouse VSMCs exposed to TNFα have a significant reduction in CFTR mRNA and protein, with a concomitant reduction in uptake of FITC-labeled S1P. Taken together, these data demonstrate that TNFα modifies microvascular tone in heart failure via TNFα, S1P and CFTR.

1.1.4.4.4 **TNFα and myogenic reactivity in subarachnoid haemorrhage**

Yagi *et al.*\(^{166}\) have provided evidence for an operational TNFα/S1P signalling pathway in subarachnoid haemorrhage (SAH) (manuscript in submission). In a mouse model of SAH, TNFα expression is increased in the microvascular wall (co-localized with smooth muscle α-actin).
SAH also augments myogenic tone in olfactory arteries of mice. SAH mice have decreased cerebral blood flow, which is normalized with ETN. Additionally, *in vitro* administration of TNFα increases myogenic tone in wild-type mice olfactory arteries. Knocking out of (i) TNFα, or (ii) TNFR1, or (iii) TNFα specifically in the VSMC, prevents the SAH-stimulation of myogenic tone. *In vitro* sequestration of TNFα with ETN or prevention of TNFα release via inhibition of TACE reverses the augmented myogenic tone in SAH. *In vivo* ETN also prevents the SAH-induced increase in the myogenic response. TNFα does not elevate the myogenic response in olfactory arteries from Sphk1−/− mice and *in vivo* JTE013 treatment reverses the SAH increase in tone, linking TNFα to intact S1P signalling in SAH. Cerebral artery CFTR protein is downregulated in SAH and three interventions targeting TNFα; *in vivo* ETN, knockout of TNFα, and smooth muscle cell specific knockout of TNFα, abolished this downregulation.

**1.1.4.4.5 TNFα as a constitutive contributor to myogenic signaling under physiological (non-pathological) conditions**

In a smooth muscle cell specific knockout of TNFα, total peripheral resistance in anaesthetized mice is significantly reduced by 20%, with no associated change in mean arterial pressure due to a compensatory increase in cardiac output168. This reduction in total peripheral resistance correlated with an attenuation of myogenic responsiveness in skeletal arteries isolated from these mice. Systemic sequestration of TNFα with etanercept acutely and rapidly reduced mean arterial pressure (~50 mmHg) without affecting heart rate, suggesting that the drop in mean arterial pressure is caused by an etanercept-dependent decrease in total peripheral resistance. In bath blockade of TNFα in skeletal muscle resistance arteries attenuated myogenic vasoconstriction and baseline tone; this effect was dose dependent, ineffective in skeletal muscle resistance arteries isolated from smooth muscle cell-specific TNFα knockout mice, and prevented by boing ETN. Taken together, these results suggest that constitutively expressed smooth muscle cell TNFα is an important mechanosensor regulating myogenic vasoconstriction. Further evidence from our lab implies that the membrane-bound TNFα is responsible for this mechanosensitization under physiological conditions, as inhibiting the TNFα-cleaving enzyme does not affect myogenic responsiveness and exogenously applied sTNFα does not stimulate vasoconstriction168.
In conclusion, this work elegantly demonstrates that TNFα is capable of mediating VSMC myogenic signalling mechanisms in both health and disease via regulation of S1P through Sphk1 activation and CFTR downregulation.

1.1.4 Pathophysiological alteration of myogenic tone in diabetes

Although the myogenic mechanism is a critical regulator of vascular tone in both health and disease\textsuperscript{69}, and understanding this phenomenon further holds therapeutic potential\textsuperscript{169}, a limited number of studies have examined the consequence of diabetes on the myogenic response. Reports have assessed altered vascular responsiveness in multiple preclinical models of diabetes, although the results of these investigations remain contradictory. The variety of diabetic animal models, the model of the type of diabetes studied (i.e., type 1 vs. type 2), diabetes severity/duration and vascular bed examined can all account for observed tone variability in the literature.

Elevated myogenic tone in diabetes

The vast majority of studies demonstrate elevated myogenic tone in diabetes. Elevated tone has been reported in: 1) coronary arterioles\textsuperscript{170,171}, mesenteric arteries\textsuperscript{170,172-175} and skeletal muscle arterioles\textsuperscript{176} of a genetic model of type 2 diabetes, the \textit{db/db} mouse, 2) mesenteric and middle cerebral arteries from Goto-Kakizaki (GK) rats, a lean spontaneous genetic model of type 2 diabetes\textsuperscript{177,178}, 3) posterior cerebral arteries of the type 2 inbred Bio-breeding Zucker diabetic rat (BBZDR)/Wor rat strain\textsuperscript{179}, 4) proximal middle cerebral arteries\textsuperscript{180,181} and first-order arterioles of gracilis muscle\textsuperscript{182} of type 1 diabetic STZ-treated rats, and 5) posterior cerebral arteries isolated from type 1 diabetic STZ-treated mice\textsuperscript{183}.

Myogenic tone in arteries isolated from patients with diabetes

Schofield, \textit{et al.}\textsuperscript{184} have conducted the only investigation of small vessel function in \textit{human} subjects with type 2 diabetes. Arteries dissected from a gluteal fat biopsy of hypertensive patients with type 2 diabetes (n=22 patients) display a loss of the myogenic response when compared with arteries from nondiabetic normotensive patients (n=18 patients). The norepinephrine vasoconstrictor response was normal in diabetic patients, however endothelium-dependent vasodilation was significantly reduced compared with the nondiabetic controls. It is
understood that studies of microvessel function in humans are necessarily complicated, as patients often present with other confounding syndromes known to modulate the vasculature (i.e., obesity and/or hypertension). In this study, patients with diabetes were on their respective anti-diabetic therapy at the time of the investigation, however ceased taking hypertension medication four weeks before the study took place. The biochemical mechanism responsible for reduced tone in these arteries was not investigated.

1.1.4.1 The mechanisms of myogenic tone elevation in diabetes

Endothelial-independent mechanisms

Multiple molecular mechanistic pathways have been implicated in diabetes-induced increases in myogenic tone. Ungvari et al. show that the increase in gracilis muscle arteriole tone in STZ-treated Wistar rats is independent of disrupted endothelial function. Instead, voltage-dependent Ca\(^{2+}\) channels and increased protein kinase C expression in the VSMC mediate elevated tone. Additionally, studies conducted by Erdei et al. and Velmurugan et al. demonstrate that an elevation in microvessel myogenic tone persists following the removal of the endothelium, suggesting the involvement of the VSMC specifically.

Endothelial-dependent mechanisms

In contrast, in the STZ-treated Sprague-Dawley rat, the endothelium-dependent acetylcholine (ACh) response of cerebral resistance arteries is blunted, and the authors suspected that the loss of endothelial NO results in myogenic over-constriction. Additionally, in cerebral arteries from STZ-treated Wistar rats, increased myogenic tone is attributed to ET-1 (a potent endogenous vasoconstrictor).

Elevated tone in the mesentery of db/db mice has been attributed to abnormal endothelial-dependent signalling. These mechanisms include: disruption of endothelial function that results in VSMC constriction due to AGE formation, decreased phosphorylation of eNOS, decreased mitochondrial antioxidant manganese superoxide dismutase expression and increased nicotinamide adenine dinucleotide phosphate (NAPDH) subunit gp91 expression and (nicotinamide adenine dinucleotide) NADH/NAPDH activity (i.e., increased oxidative stress).
increased PKC beta activation resulting in production of vasoconstrictory prostanoids; increased pro-inflammatory transcription factor NFkB activity via a poly(ADP-ribose) polymerase (PARP)-1/transcription factor Sp-1 and cyclooxygenase (COX)-2-dependent mechanism, augmentation of the NF-E2-related factor 2 (Nrf2) pathway; and phosphorylation of the epidermal growth factor receptor.

In skeletal muscle arterioles of db/db mice, tone is elevated as a result of enhanced production of prostaglandin, which is dependent on H2O2 activation of COX-2 expression in the arteriolar wall.

Increased phospholipase C activity is shown to increase myogenic tone in BBZDR/Wor rats, a relatively new model of type 2 diabetes, produced by crossing the Zucker fatty rat and the non-diabetic BB/Wor rat. In the GK rat, a diabetes model in which the rat is neither hyperlipidemic nor hypertensive, augmented tone in mesenteric arteries is normalized by administration of the anti-diabetic therapy metformin (which ultimately lowered blood glucose). These data suggest that resistance artery dysfunction in this model is likely the result of hyperglycaemia.

**Myogenic tone reduction and associated mechanisms in diabetes**

Far fewer reports have been published showing depressed resistance artery myogenic tone in diabetes. In the kidney afferent arteriole of an STZ-induced diabetic rat, pressure-induced vasoconstriction is significantly attenuated compared to that in the normoglycaemic rat kidney arteriole. Controlling hyperglycaemia in STZ-rats with exogenous insulin injections partially restores the myogenic response to normal, which is further restored by the administration of *in vitro* prostaglandin inhibition (ibuprofen and indomethacin). This study suggests a role for both elevated glucose and vasodilatory prostaglandins in blunting of myogenic tone.

Hill *et al.* report that the rate of development of pressure-induced vasoconstriction in cremaster muscle arterioles of STZ-induced diabetic rats is markedly reduced compared to control rats, both *in situ* and in isolated arterioles. Vasodilatory responses to ACh are also significantly impaired. Chronic treatment of diabetic rats with aminoguanidine (a diamine oxidase and NOS inhibitor that acts to reduce levels of advanced glycation end products through interaction with 3-deoxyglucosone) prevents the diabetes-induced changes in cremaster artery myogenic tone.
Ito et al.\textsuperscript{192} studied the myogenic responsiveness of the ophthalmic artery from BBZDR/Wor rats. Three months following the development of obesity and diabetes, ophthalmic arteries show decreased myogenic tone compared with lean control rats. Following the removal of the endothelial cell layer of arteries, myogenic tone is normalized in diabetic rats, suggesting that the endothelium releases an unidentified factor to decrease the reactivity of the ophthalmic artery to pressure.

Although a variety of aspects of the complex regulatory network that governs myogenic responsiveness have been reported to be affected in diabetes, no link has been proposed between diabetes and altered myogenic tone as a result of aberrant TNFα/S1P signalling.
1.2 RATIONALE

The significant disruption in resistance artery functionality within the microcirculation in diabetes can greatly compromise the regulation of tissue perfusion and systemic blood pressure in this disease setting. This may ultimately lead to the trophic and degenerative symptoms that clinically emerge as part of the diabetic disease progression. Preclinical models of diabetes display a variety of myogenic phenotypes, but the overwhelming majority suggests a pro-constrictive phenotype in the microvasculature. The present work is focused on furthering our mechanistic understanding of the consequences of diabetes on myogenically active blood vessels.

As described, VSMCs are the primary mediator of microartery myogenic tone and utilize a TNFα/S1P signalling axis to do so. For this reason, the investigations undertaken in this thesis began with the following questions:

*What is the myogenic phenotype of HFD/STZ mice, an established model of type 2 diabetes?*

The vascular phenotype of diabetic animals is determined by the model used and the vascular bed studied. The HFD/STZ mouse model I used allows for ‘titration’ of the severity of diabetes, and reflects the clinical situation of type 2 diabetes by including obesity and peripheral insulin resistance in combination with decreased β-cell function. Furthermore, we have the ability in our laboratory to investigate the microvascular function of multiple microarteries from different vascular beds in the same model.

*If hyperglycaemia is normalized in diabetes, will this prevent vascular dysfunction?*

Exposure to hyperglycaemia is indisputably a major factor in the pathogenesis of diabetic complications. First line therapy for diabetes complication prevention seeks to normalize blood glucose and prevent future hyperglycaemia. It follows then that the achievement of euglycaemia in HFD/STZ mice may prevent any negative outcomes on vascular function in diabetes.

*Do TNFα and/or S1P contribute to the induction of myogenic tone dysfunction in diabetes?*

I propose that TNFα links diabetes to altered control of microvascular function as i) systemic inflammation is strongly implicated as a potential mediator in diabetes, ii) TNFα levels are significantly increased in patients with type 1 and 2 diabetes, and iii) at the cellular level,
high glucose promotes smooth muscle TNFα synthesis\textsuperscript{195}. In addition, TNFα potently activates microvascular Sphk1 in an autocrine and paracrine manner\textsuperscript{196-201}, further supporting the potential of TNFα as a candidate for altering myogenic responsiveness. As described, our laboratory is the first to report that TNFα augments resistance artery myogenic responsiveness via an S1P-dependent mechanism in disease conditions associated with inflammation, including heart failure\textsuperscript{202}, SAH\textsuperscript{166}, and vascular-based sudden hearing loss\textsuperscript{203}. The mechanistic consistency across these disease models suggests the presence of a fundamental mechanism common to inflammatory conditions.

**Does the presence of obesity alone have an effect on myogenic regulation?**

In 1921, Elliott P. Joslin published a study showing that the risk of developing diabetes (type 2, according to present criteria) was greater in obese individuals vs. their nonobese counterparts\textsuperscript{204}. Obesity is now known to be one of the major risk factors for the development of type 2 diabetes\textsuperscript{205}. Although the exact mechanism(s) linking obesity and type 2 diabetes have yet to be uncovered, it is likely there are consequences of the biologically active adipocyte on fat, muscle and liver tissue resulting in an impairment of insulin action or insulin resistance. Specifically, the expansion of adipose tissue in the abdominal region (i.e., visceral fat) has been correlated to insulin resistance and cardiovascular disease in both animals and humans\textsuperscript{206}. Adipose also contains an increased content of macrophages that are capable of secreting numerous inflammatory cytokines, such as TNFα and interleukin-6\textsuperscript{207}. Small resistance arteries from preclinical obesity models and obese humans have undergone hypertrophic structural remodelling and show endothelial dysfunction\textsuperscript{208-224}. The associated mechanisms causing this vascular damage are largely unknown.
These questions resulted in the following three hypotheses:

1.3 HYPOTHESES

1. Myogenic tone is augmented in HFD/STZ mice and this increase is mediated by activation of Sphk1, and therefore S1P, acting upon the S1P receptor.

2. The connection between diabetes and increased myogenic tone in arteries from HFD/STZ mice is the result of increased TNFα and its activation of the S1P signalling pathway.

3. Normalization of glucose in HFD/STZ mice via treatment with anti-diabetic therapy will prevent the development of myogenic tone abnormalities.

1.4 SIGNIFICANCE
The present treatment modalities for diabetes complications are limited, demonstrating the incongruence between what is known about diabetes disease mechanisms and our ability to translate these findings into therapies for patients. Importantly, the prevention of hyperglycaemia with anti-diabetic therapy is not sufficient to entirely prevent the development of diabetes complications. This thesis aims to characterize molecular mechanisms that could serve as novel and innovative therapeutic targets to alleviate the detrimental microvascular effects of diabetes.
CHAPTER 2. MATERIALS AND METHODS
2.1 ANIMAL MODELS

This investigation adhered to the *Guide for the Care and Use of Laboratory Animals* published by the NIH (Publication No. 85-23, revised 1996). All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committees at the University of Toronto and the University Health Network (UHN), Toronto and were conducted in accordance with Canadian animal protection laws.

All mouse strains used in this thesis were either commercially available or have been previously described. These strains include: wild-type C57BL/6N mice (Charles River Laboratories; Montréal, QC, Canada), germ-line TNFα knockout mice (TNFα−/−; Taconic laboratories; Hudson, NY, USA), germ-line sphingosine kinase 1 (Sphk1) knockout mice (Sphk1−/−; a gift from Dr. Richard L. Proia; Bethesda, MD, USA) and inducible, smooth muscle cell targeted TNFα knockout mice, generated by crossing floxed TNFα mice with mice expressing a recombinant Cre recombinase under the control of a smooth muscle cell-specific myosin heavy chain promoter. All mice were housed under a 14 h:10 h light-dark cycle and had access to food and water ad libitum. All animals were euthanized by anaesthetization with isoflurane (IsoFlo, Abbott Laboratories, Saint-Laurent, QC, Canada), followed with either cervical dislocation or decapitation.

### 2.1.1 Generation of smooth muscle cell-targeted TNFα knockout mice and related controls

A mouse strain with a ‘floxed’ TNFα gene (i.e., the TNFα gene is flanked by LoxP sites; TNFα<sup>flox/flox</sup>) was crossed with a transgenic mouse strain expressing a chimeric Cre recombinase (Cre-ER<sup>T2</sup>) under the control of the smooth muscle myosin heavy chain promoter (of note, the Cre transgene is located on the Y chromosome). The resulting progeny, which were heterozygous at the TNFα allele, were crossed. Breeding pairs homozygous for the floxed (TNFα<sup>flox/flox</sup>) or wild-type TNFα allele (TNFα<sup>wt/wt</sup>) were selected from the resulting second-generation progeny (all male progeny express the Cre allele). Only male mice were used in experiments (i.e., all experimental mice express the Cre transgene). The recombinase activity of the Cre-ER<sup>T2</sup> is controlled by a tamoxifen ligand-binding domain (ER<sup>T2</sup>), which allows for temporal control of Cre recombinase activity. Activation of Cre recombinase induces the
excision of the target gene, resulting in complete targeted deletion within 5 days$^{226}$. Tamoxifen was administered to 8 week-old mice for 5 consecutive days (100 µL, 10 mg/mL suspended in ethanol and solved in corn oil, one i.p. injection per day). TNF$\alpha^{\text{wt/wt}}$ mice given tamoxifen served as controls for the tamoxifen treatment.

### 2.2 DIABETES AND OBESITY MODEL INDUCTION

Mice (8 weeks old) were randomized into three groups. One third of these mice were fed normal chow (NC; 10% calories from fat). Two thirds were fed a HFD (45% calories from fat; Teklad Custom Research Diets TD.06415, Harlan Laboratories; Madison, WI, USA). After 4 weeks, mice were fasted for 5 hours. After fasting, one group of HFD mice was injected with a single dose of streptozotocin (HFD/STZ; 100 mg/kg i.p. injection in 0.1 mmol/L sodium citrate, pH 5.5); the other two groups (NC and HFD) received a sodium citrate vehicle injection. Tail vein blood samples at 72 hours post-STZ injection confirmed beta cell toxicity (i.e., elevated blood glucose). Mice were maintained on their respective diets for 12 additional weeks (Figure 2.1).

<table>
<thead>
<tr>
<th>Time (weeks)</th>
<th>Normal chow (10%)</th>
<th>Normal chow (10%) + vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>High fat diet (45%)</td>
<td>High fat diet (45%) + vehicle</td>
</tr>
<tr>
<td>15</td>
<td>High fat diet (45%)</td>
<td>High fat diet (45%) + STZ</td>
</tr>
<tr>
<td>16</td>
<td>STZ 100 mg/kg or vehicle</td>
<td>100 mg/kg or vehicle</td>
</tr>
<tr>
<td>16</td>
<td>Endpoint HbA$_{1C}$</td>
<td>Isolated mesentery, posterior cerebral and olfactory microarteries</td>
</tr>
</tbody>
</table>

Figure 2.1 Timeline for the method of diabetes induction.

Eight week-old male C57BL/6 mice either remain on normal chow diet (NC; 10% cal/fat) or are placed on high fat diet (HFD; 45% cal/fat) for 4 weeks. At the start of week 5, high fat diet plus streptozotocin (HFD/STZ) mice are injected with streptozotocin (STZ) (100 mg/kg), and NC and HFD mice are injected with vehicle (0.1 mmol/L sodium citrate, pH 5.5). Mice remain on respective diets for an additional 12 weeks. At week 15, an oral glucose tolerance test (OGTT) is performed with mice. At week 16 (endpoint), glycated haemoglobin (HbA$_{1C}$) is measured prior to sacrifice and posterior cerebral, olfactory and mesenteric arteries are isolated from mice.
2.2.1 Ten-month duration high fat diet C57BL/6 mice

A separate group of C57BL/6 mice were either placed on HFD (45% kcal from fat) at 8 weeks of age or remained on normal chow (NC controls, 10% kcal from fat) for 10 months (Figure 3.5). These mice were kindly donated by Dr. Mansoor Husain (Toronto General Research Institute, Toronto, ON, Canada).

Random fed blood glucose levels were measured in mice throughout the study using a Glucometer (Bayer Healthcare; Mississauga, ON, Canada), using blood drawn from the tail vein. Glycated haemoglobin (HbA1C) was measured using an A1C Now+ kit (Bayer Healthcare). For oral glucose tolerance tests (OGTTs), mice were fasted for 16 hours, administered 1.5 mg/g body weight glucose by oral gavage and blood glucose levels were detected at time 0 (i.e., prior to gavage), 10, 20, 30, 60, 90 and 120 minutes.

2.3 ISOLATION AND FUNCTIONAL ASSESSMENT OF MICROVASCULAR ARTERIES

Mesenteric (207±3 µm in diameter, n=178), olfactory (113±2 µm, n=140) and posterior cerebral arteries (PCAs) (147±3 µm, n=28) were microsurgically isolated as previously described\textsuperscript{158,162,163,166} (Figure 2.2).
Figure 2.2  Photographs displaying the site of microartery isolation from a mouse brain and mesentery.

(A) Organs collected from mice are placed in a petri dish with ice-cold ‘MOPS’ buffer (kept cool by surrounding ice) for careful isolation under the dissecting microscope, using ultra fine forceps (Fine Science Tools; North Vancouver, BC, Canada) and 3 mm spring scissors (Fine Science Tools). (B) Olfactory (arrow 1) and posterior cerebral (arrow 2) microarteries are isolated from sites indicated with red arrows of an intact mouse brain. (C) Mesenteric microarteries are isolated from a section of mouse intestine following dissection of the surrounding layer of connective tissue and fat.
2.3.1 **Pressure myography**

Functional experiments utilized a Living Systems Instrumentation (St. Albans, VT, USA) pressure myography system in ‘no perfusion’ mode. Microarteries were carefully cannulated onto micropipettes, pressurized and warmed to $37^\circ\text{C}$ in MOPS-buffered saline, as previously described\textsuperscript{158,162,163,166}. Mouse olfactory and PCAs were pressurized to 45 mmHg, mouse mesenteric resistance arteries were pressurized to 60 mmHg, and all treatments were diluted in MOPS-buffered saline (Figure 2.3).

Figure 2.3  **Living Systems Inc. pressure myography system.**

*Pressure myography equipment is developed and sold by Living Systems Instrumentation Inc. (St. Albans, Vermont, USA). Microarteries are placed in a single vessel chamber (CH-1) mounted between two glass micropipettes, pressurized and heated using a temperature controller (TC-09S) and thermistor temperature.*
sensor (THRS). A pressure servo controller with peristaltic pump (PS-200) is used to precisely control the lumen pressure in the isolated cannulated microartery. A flow-through pressure transducer (PT-F) placed in-line with the blood vessel senses the pressure and a miniature peristaltic pump maintains pressure at the experimenter-specified set point. Arteries are imaged using a monochrome CCTV camera (Panasonic; Scarborough, ON, Canada) attached to an inverted light microscope (Leica Microsystems Inc.; Concord, ON, Canada). The image of the artery is displayed on a television monitor attached to data acquisition systems designed to measure changes in real time of the interior vessel lumen diameter. Detection is based on differences in contrast ratios of the microartery wall by a video dimension analyzer (VDA-10, not shown) via an analog video signal. All data is recorded on a computer with DI-720, (DATAQ Instruments; Akron, OH, USA) and MP100 (BIOPAC Systems Inc.; Montréal, QC, Canada) software. *Copyrighted photos are used by permission of Dr. Gerald M. Herrera of Living Systems Instrumentation, Inc. © 2013.*

2.3.2 Myogenic and contractile response calculations

Myogenic tone was calculated as the percent steady state constriction following step-wise (20 mmHg increments) increases in transmural pressure, in relation to the maximal diameter:

\[
\text{tone (\% of } \text{dia}_{\text{max}}) = \left[ \frac{\text{dia}_{\text{max}} - \text{dia}_{\text{active}}}{\text{dia}_{\text{max}}} \right] \times 100,\]

where \( \text{dia}_{\text{active}} \) is the vessel diameter in MOPS buffer containing \( \text{Ca}^{2+} \) and \( \text{dia}_{\text{max}} \) is the diameter in \( \text{Ca}^{2+} \)-free MOPS buffer, both at the specified transmural pressure. Vasomotor responses (e.g., to phenylephrine, PE; acetylcholine, ACh; sodium nitroprusside, SNP) used the same calculation, only in this case, \( \text{dia}_{\text{active}} \) represents the vessel diameter at steady state following application of the given agent and \( \text{dia}_{\text{max}} \) represents the maximal diameter (measured under \( \text{Ca}^{2+} \)-free conditions); both of these measures were obtained at the transmural pressure set at the beginning of the experiment (i.e., 45 mmHg for olfactory and PCAs; 60 mmHg for mesenteric arteries).

2.3.3 Evidence for acquisition of the pressure myography technique

The initial goal for investigating myogenic responses of the microvasculature from all mice involved acquisition of the specific and challenging skill set of pressure myography. In pressure myography, a small, intact segment of a microartery is mounted onto two small glass cannulas and then pressurized. This *ex vivo* experimental protocol allows for establishing of near physiological conditions for investigation of intrinsic microvessel responses. I showed using
pressure myography that resistance arteries isolated from a C57BL/6 mouse mesentery (mesenteric arteries; Figure 2.4A-B) and brain (olfactory arteries; Figure 2.4C-D) displayed myogenic tone and vasoconstriction to phenylephrine. Additionally, a pressure ramp from 20-120 mmHg performed sequentially multiple times in a cannulated artery does not affect the strength of myogenic tone in mesenteric arteries (Figure 2.5A). However, myogenic tone increases after the second pressure ramp experienced by olfactory arteries and then stabilizes by the third pressure ramp replicate (Figure 2.5B). These experiments demonstrate the effect of time on ex vivo microartery function; all experimental designs for the remainder of the study took this finding into account. For example, posterior cerebral and olfactory arteries were subjected to two pressure ramps from 20-100 mmHg performed sequentially and only the diameters recorded following the second pressure ramp were included for myogenic response calculations; mesenteric arteries were subjected to only one pressure ramp.

Figure 2.4 Isolated mesenteric and olfactory arteries display intact vasomotor responses.
Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric (top) and olfactory cerebral (bottom) arteries isolated from wild-type mice.

**Panels A-C:** Mesenteric resistance arteries (n=9) isolated from healthy, wild-type mice respond to an increasing pressure ramp by constricting (i.e., they are myogenically active); mesenteric arteries (n=5) constrict in response to increasing doses of phenylephrine (0.003 µmol/L–3 µmol/L) added to the organ bath. **Panels D-F:** Similar to mesenteric arteries, olfactory resistance arteries (n=9) isolated from healthy wild-type mice respond to an increasing pressure ramp by constricting (i.e., they are myogenically active); olfactory arteries (n=9) also constrict in response to increasing doses of phenylephrine.

**Figure 2.5** Myogenic responses in resistance arteries over time (“time controls”).

(A) Subjecting the same isolated mesenteric arteries (n=6) to transmural pressure changes from 20-120 mmHg, 30 minutes apart, has no impact on the strength of myogenic activity. (B) Subjecting the same isolated olfactory arteries (n=9) to transmural pressure changes from 20-100 mmHg, 30 minutes apart, shows that these arteries have myogenic tone that is stronger in response to the second replicate of pressure changes. However, between pressure change replicates 2 and 3, myogenic tone is nearly identical. * denotes $P<0.05$ for multiple paired comparisons.
2.4 REAGENTS

JTE013 was purchased from Tocris Bioscience (Ellisville, MO, USA), etanercept (Enbrel®) from Amgen Canada (Mississauga, ON, Canada) and metformin hydrochloride from LKT Laboratories (St. Paul, MN, USA). The low and high doses of metformin administered *in vitro* were chosen based on a related study that incubated mesenteric arteries from rats with metformin *in vitro*\(^{227}\). Metformin diets were administered to mice as indicated in the timeline shown in Figure 3.9A. Standard mouse chow diet (10% kcal/fat) plus metformin (TD.110848; 450 mg/kg drug/weight of food) and HFD (45% kcal from fat) plus metformin (TD.110847; 450 mg/kg drug/weight of food) were supplied by Harlan Laboratories (Madison, WI, USA). The dose of Metformin was chosen based on review of related studies\(^{228,229}\). Of note, C57BL/6 mice food intake is ~3.6 g/mouse/day\(^{230}\). Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich (Oakville, ON, Canada). MOPS-buffered salt solution contained [mmol/L]: NaCl 145, KCl 4.7, CaCl\(_2\) 3.0, MgSO\(_4\)•7H\(_2\)O 1.17, NaH\(_2\)PO\(_4\)•2H\(_2\)O 1.2, pyruvate 2.0, EDTA 0.02, MOPS (3-morpholinopropanesulfonic acid) 3.0, and glucose 5.0.

2.5 MOLECULAR PROCEDURES

Standard procedures were used for cerebral resistance artery mRNA isolation, conversion to cDNA and subsequent quantitative RT-PCR.

2.5.1 RNA Isolation and Reverse Transcription

Cerebral resistance artery RNA was isolated with Norgen Biotek (Thorold, ON, Canada) “Total RNA Purification Micro” spin columns, using the proteinase K digestion and DNA removal procedures, as directed by the manufacturer’s instructions. The eluted RNA was quantified with an Agilent Technologies RNA 6000 Pico Kit and Bioanalyzer; the analysis confirmed that high-quality RNA was retrieved from all samples (RNA Integrity [RIN]: NC=8.5±0.1, n=5; HFD=8.2±0.6, n=5; HFD/STZ=8.2±0.3, n=6). RNA was converted to cDNA using a “Superscript III” reverse transcription kit (Invitrogen Life Technologies; Burlington, ON, Canada), according to the manufacturer’s directions. Residual RNA was removed by incubating the resulting cDNA with RNAsase H (0.125 U/µL; New England Biolabs Canada; Whitby, ON, Canada).
2.5.2 Quantitative PCR

Quantitative PCR was performed using an Applied Biosystems Viia7 Real Time PCR system and Power SYBR® Green PCR master mix (both distributed by Invitrogen Life Technologies). Each primer set (400 nmol/L in each reaction; Appendix Table 1) was rigorously validated to ensure specificity and comparable efficiency. Gene targets were assessed in triplicate, using 1 ng of cDNA generated from the reverse transcription; negative controls received water. The PCR amplification consisted of 10 minutes denaturation at 95°C, followed by 40 cycles of amplification (15 s at 95°C + 60 s at 60°C). Following amplification, the amplicons were melted: the resulting dissociation curve confirmed the production of single product. Transcript expression levels were calculated from the ΔCt values relative to the standard housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). To confirm that GAPDH was reliable for normalization, transcript expression levels were also calculated from ΔCt values relative to glucose-6-phosphate dehydrogenase (G6PD) and hydroxymethylbilane synthase (HMBS), which returned similar results (Appendix Table 1).

2.6 SYSTEMIC HEMODYNAMIC MEASURES

Standard techniques were used to invasively measure blood/intraventricular pressures (Millar SPR-671 micro-tip mouse pressure catheter211; Inter V Medical Inc., Montréal, ON Canada).

2.7 CEREBRAL PERFUSION MEASURES

Cerebral perfusion was non-invasively measured via magnetic resonance imaging (BioSpec 70/30 USR; Bruker BioSpin, Ettlingen, Germany)162,166,232.

2.7.1 Magnetic Resonance Imaging-based measurement of cerebral perfusion

As described in our previous investigation162,166, the flow-sensitive alternating inversion recovery (FAIR) technique232 is a magnetic resonance imaging (MRI) approach that evaluates brain perfusion with a 7 Tesla micro-MRI system (BioSpec 70/30 USR, Bruker BioSpin, Ettlingen, Germany), including the B-GA12 gradient insert, 72 mm inner diameter linear volume resonator for radio frequency (RF) transmission, and anteriorly placed head coil for RF reception. FAIR isolates perfusion as an accelerated T1 signal relaxation following slice-selective compared to non-selective inversion preparation, as per the following equation: CBF (cerebral blood flow)
\( = \lambda \cdot \left( \frac{1}{T_{1, ss}} - \frac{1}{T_{1, ns}} \right) \) (mL/(100 g*min)), where ‘ss’ and ‘ns’ denote slice-selective and non-selective measurements and \( \lambda \) is the blood-brain partition coefficient, defined as the ratio between water concentration per g brain tissue and per ml blood. This coefficient is approximately 90 mL/100 g in mice\(^{233}\).

FAIR optimization was a single-shot echo planar imaging (EPI) technique with preceding slice-selective or non-selective adiabatic inversion pulse. Parameters included echo time of 12.5 ms, repetition time of 17 s, 18 inversion times ranging from 25 to 6825 ms in 400 ms increments, 3 mm slice-selective inversion slab, 18x18 mm field-of-view with 72x72 matrix for 250 \( \mu \)m in-plane resolution, 1 mm slice thickness, and 10 min 12 s data acquisition time. Acquisitions included vertical sections in the fore-, mid- and hindbrain regions, which correspond to the anterior, mixed and posterior circulations.

FAIR images were evaluated by manual prescription (MIPAV, NIH, Bethesda, MD, USA; http://mipav.cit.nih.gov) of sub-hemispheric regions-of-interest (ROIs), termed ‘global’, and local ROIs corresponding to cortical and sub-cortical parenchyma in forebrain sections. ROIs were drawn directly on \( T_1 \)-weighted signal images to enable manual correction for intra-scan motion. ROIs were registered with parametric CBF maps to verify absence of bias from high perfusion vessels and meninges. \( T_1 \) regressions and CBF calculations were performed using Matlab (Mathworks, Natick, MA, USA).

### 2.8 STATISTICAL ANALYSES

Analyses were performed using Prism 6 software (GraphPad; San Diego, CA, USA). All data are expressed as mean ± SEM, where \( n \) is the number of independent experiments. For comparison of multiple independent groups, a non-parametric one-way ANOVA with exact \( p \)-value computation was performed (Kruskal-Wallis), followed by a Dunn’s multiple comparison correction as appropriate. For the assessment of myogenic responses, dose-response relationships and passive diameters, data were analyzed with a two-way ANOVA, followed by a Bonferroni corrected multiple comparisons. Differences were considered significant at error probabilities of \( P<0.05 \).
CHAPTER 3. TUMOUR NECROSIS FACTOR α / SPHINGOSINE-1-PHOSPHATE SIGNALLING AUGMENTS RESISTANCE ARTERY MYOGENIC TONE IN A MOUSE MODEL OF DIABETES
Some of the work presented in this chapter corresponds to the following publication in preparation:


**Author contributions:**

Dr. Warren D. Foltz performed all FAIR-MRI measurements and cerebral blood flow calculations in Figure 3.7. Dr. M. Abdul Momen performed the surgical procedure for the invasive hemodynamic studies and conducted the hemodynamic measurements in Table 3.1. Dr. Mansoor Husain supervised M. Sauvé and Dr. M.A. Momen. Dr. Sergei Nedospasov generated and provided the transgenic TNF<sup>fl</sup>ox/<sup>fl</sup>ox mice, utilized to produce data for Figure 3.13. Dr. Stefan Offermanns generated and provided the transgenic mouse line expressing a fusion protein of the Cre recombinase with the modified oestrogen receptor-binding domain (Cre<sup>ER</sup><sup>T2</sup>) under the control of the smooth muscle myosin heavy chain promoter, utilized to produce data for Figure 3.13. Dr. Darcy Lidington for RNA preparation, reverse-transcription, quantitative real-time polymerase chain reaction experimental protocol optimization and mRNA technical assistance for Figure 3.11A and review of the high fat diet/streptozotocin mice resistance artery data. Dr. Steffen-Sebastian Bolz provided expert advice with experimental design and data analyses.

M. Sauvé gratefully acknowledges: Dr. M. Hossein Noyan-Ashraf for maintenance of ten month duration high fat diet C57BL/6 mice (Figures 3.5 and 3.6), Eric Aki Shikatani for computation of invasive hemodynamic data (Table 3.1), Amy Cao for assistance with mouse husbandry and Dr. Patricia L. Brubaker for critical review of the manuscript in preparation that this chapter is based on.

Meghan Sauvé designed all experiments, generated data for all remaining figures (Figures 3.1–3.6, 3.8–3.16) and tables (Table 3.1 and 3.2), performed data analyses, prepared figures and produced all text within this chapter.
3.1 ABSTRACT

Diabetes is associated with widespread end-organ damage dominating the clinical situation and patient morbidity and mortality. Injury results from microvascular dysfunction and hence, a progressive loss in tissue perfusion. This pattern in diabetes suggests a pathogenic role for resistance arteries, the key regulators of blood flow to bodily organs.

Resistance arteries, localized at the interface between the arterial tree and the capillaries, control systemic blood pressure and appropriate organ blood supply. I hypothesize that resistance artery VSMCs are direct targets of metabolic dysregulation in diabetes, through alteration of molecular mechanisms that underlie the intrinsic ability of vascular smooth muscle cells (VSMCs) to adapt resistance artery tone to transmural pressure (i.e., myogenic responsiveness).

I used a mouse model of diabetes, the high fat diet/streptozotocin (HFD/STZ) mouse, to investigate the role of the TNFα/S1P signalling network in the alteration of myogenic tone in this disease state. The high fat diet (HFD) mouse controls required for this study also provided a high fat diet-induced mouse model of obesity, allowing for investigation of the regulation myogenic responsiveness in this additional context. HFD/STZ animals developed mildly elevated blood glucose and HbA1c levels, statistically higher than HFD and normal chow (NC) controls. Myogenic tone in HFD/STZ mice was enhanced in both mesenteric and olfactory resistance arteries, an effect that was absent in HFD mice not treated with STZ. Tone in proximal posterior cerebral arteries, which exhibit low myogenic responsiveness under physiological conditions, was not changed by HFD/STZ treatment. This is consistent with unaltered cerebral blood flow in HFD/STZ mice. Elevated myogenic tone was not the result of hypertension or endothelial dysfunction, as HFD/STZ mice were normotensive and displayed unchanged endothelial dilatory responses to acetylcholine, compared to HFD and NC controls. First line anti-diabetic therapy metformin was used in an attempt to normalize blood glucose in HFD/STZ mice for prevention of the development of vascular tone dysregulation. Surprisingly, twelve weeks of metformin did not normalize the mildly elevated blood glucose and HbA1c in HFD/STZ mice to levels comparable to HFD and NC controls. However, the HFD/STZ-stimulated increase in myogenic tone was absent in mesenteric and olfactory resistance arteries of metformin treated mice. In fact, metformin treatment appeared to blunt myogenic responsiveness to levels below normal in mesenteric arteries isolated from NC and HFD
metformin-treated groups as well.

Interestingly, HFD/STZ TNFα and Sphk1 knockout mice displayed normalized resistance artery myogenic tone. A smooth muscle cell-specific knockout of TNFα also prevented the HFD/STZ-induced increase in the myogenic response. Concomitantly, *in vitro* treatment of isolated resistance arteries with etanercept (TNFα scavenger) normalized myogenic tone. Pharmacological intervention, targeting the S1P pathway utilizing *in vivo* treatment with JTE013 (S1PR antagonist) or the clinically relevant (i.e., FDA-approved) etanercept also normalized the enhanced myogenic tone in HFD/STZ resistance arteries. In conclusion, my data demonstrate that a mouse model of diabetes activates VSMC TNFα/S1P signalling to augment myogenic tone. Delineation of this underlying molecular mechanism may provide valuable microcirculatory targets to interrupt this process in patients with diabetes.
3.2 INTRODUCTION

3.2.1 Diabetes complications: the association with hyperglycaemia

Microvascular dysfunction, underlying systemic hemodynamic changes and reductions in tissue perfusion, is a key pathogenic element driving the development of diabetes-associated diseases. The involvement of circulating plasma glucose in the development of diabetic vascular complications is undeniable. In particular, the risk of developing diabetic retinopathy, nephropathy and neuropathy depends on the duration and severity of hyperglycaemia. As such, treating diabetes includes a primary aim of decreasing the likelihood that tissues become injured by high glucose. The principal therapies for treatment of diabetic complications are medications that aim to normalize glucose by preventing and controlling hyperglycaemia.

3.2.2 High glucose directly impairs vascular tissue functionality

High glucose can exacerbate damage specifically to the microvessel endothelial and VSMC layer. Elevated glucose has the ability to cause excessive growth, hyperproliferation and a change in contractile phenotype in arterial VSMCs. These changes are associated with aberrant biochemical signalling related to non-enzymatic glycation of proteins that results in free radical production, polyol and diacylglycerol accumulation and activation of protein kinase C promoting growth and proliferation. VSMCs also upregulate transcriptional activities and downregulate the glucose transporter 1 resulting in an insulin-resistant state. All of these mechanisms together result in compromised function of the vascular system.

3.2.3 Treating mild hyperglycaemia early to prevent the vascular complications of diabetes

Clinical data from the 10-year post study follow-up of the United Kingdom Prospective Diabetes Study (UKPDS) population outlined the distinct benefit of intensive glycaemic control in patients who have had diabetes for a shorter duration (i.e., received intensive glucose regulatory treatment soon after diagnosis) and those with a lower HbA1C and the absence of pre-existing
cardiovascular disease\textsuperscript{235}. These patients had a significantly reduced rate of microvascular disease. They also had lower rates of any diabetes-related endpoint, diabetes-related death and death from any cause. Therefore, it is sensible to promote aggressive hyperglycaemia management in newly diagnosed diabetes patients or in patients who exhibit satisfactory glucoregulatory control to dramatically lessen the burden of cardiovascular disease in the future.

It is important to recognize that this relationship between high glucose and vascular injury is a continuous one; as the disease progresses and hyperglycaemia is worsened for a longer period of time, the risk of permanent damage to vascular reactivity is greater\textsuperscript{237,238}. Elucidating these microvascular alterations in the early stages of diabetes when hyperglycaemia is mild is thus necessary for the development of treatments to altogether avoid permanent vascular complications.

3.2.4 Obesity: a primary risk factor for the development of diabetes and a potential contributor to vascular dysfunction

Since 1980, worldwide obesity has doubled; presently 500 million adults and 42 million children are obese. This alarming rate of growth in a moderately short time period is set to increase further, as an additional 1.4 billion adults are considered overweight\textsuperscript{239}. ‘Overweight’ and ‘obesity’ are both described by body mass index (BMI; weight in kilograms/height\textsuperscript{2} in meters). Overweight is defined by the World Health Organization as a BMI between 25 and 29.9 kg/m\textsuperscript{2} and obesity as a BMI of 30 kg/m\textsuperscript{2} or greater. The most medically relevant definition of obesity is a weight that confers morbidity\textsuperscript{239}.

Importantly, overweight and obesity are primary risk factors for wide-ranging, and often fatal, chronic diseases that include diabetes\textsuperscript{239}. This global epidemic hinders almost all aspects of health and wellness (i.e., physical, mental and emotional); obesity is associated with coronary heart disease, stroke, hypertension and cancer\textsuperscript{239}, and obesity limits mobility, reproductive capacity, respiration, sleep, and learning and memory\textsuperscript{240,241}.

Successful treatment of obesity includes the sustained attainment of normal body weight and composition without producing treatment-associated morbidity, a feat that is rarely achieved in
the clinical setting\textsuperscript{242}. Therefore, combinations that include diet, exercise, drugs, surgery and behavioural therapy are successful only with motivation and commitment from the patient, in concert with the support, understanding and knowledge of the physician and healthcare team.

3.2.5 **Vascular complications of obesity**

It is well documented that the macrocirculation is a target for functional and structural dysregulation in obesity. Large and medium-sized arteries become stiff\textsuperscript{243}, less compliant and distensible\textsuperscript{244} and display disruption in endothelial cell function (i.e., nitric oxide bioavailability is reduced)\textsuperscript{245}, contributing to the clinical establishment of obesity-associated complications. Far less information exists regarding the microvasculature in obesity, demonstrating a substantial gap in our understanding of the pathogenesis of fatness on tissue known to be a primary regulator of local and overall peripheral resistance.

The limited studies available provide two important obesity-manifested consequences on microarteries: (i) endothelial dysfunction and (ii) structural remodelling. In preclinical models of obesity (i.e., genetic and diet-induced rodent models), vasodilator dysfunction is found in the skeletal muscle\textsuperscript{208,209}, mesenteric\textsuperscript{210,211} and cerebral\textsuperscript{212,246} microvascular networks. Additionally, small resistance arteries from obese humans undergo hypertrophic structural remodelling and display marked endothelial dysfunction\textsuperscript{213,214,247}. Presently, the direct effect of obesity on the microvascular smooth muscle cell has been underappreciated.

3.2.6 **Understanding the functional and molecular basis of diabetes and obesity-associated vascular complications**

The present chapter focuses on two elements: diabetes-induced effects on resistance artery function when the blood glucose elevation is mild and obesity-induced effects on resistance artery function. The target parameter studied is myogenic responsiveness because it is a most important mechanism regulating resistance artery function\textsuperscript{248}. The myogenic response, or Bayliss effect\textsuperscript{67}, is defined as the intrinsic ability of microvascular smooth muscle cells to adapt their contractile state to prevailing transmural pressure levels. This strictly local phenomenon provides
the mechanistic basis for the autoregulation of tissue blood flow\textsuperscript{68}. As previously discussed, several animal models of diabetes demonstrate changes in myogenic tone\textsuperscript{170-183,187,189-192}. However, the molecular mechanisms that link the metabolic changes in diabetes to vascular dysfunction are currently unclear.

It stands to reason that local metabolic disruption in resistance arteries as a consequence of diabetes profoundly changes the molecular signalling in VSMCs that underlies the myogenic response. In this regard, our laboratory has identified a complex regulatory network of molecules that control the bioavailability of the sphingolipid metabolite S1P, a key mediator of myogenic responsiveness\textsuperscript{80,98,108,159,161,164,202,249}. Importantly, these signalling elements are affected by the cytokine TNFα\textsuperscript{250}.

Moreover, elevated glucose is associated with our reported TNFα and S1P mechanistic pathway, activating TNFα and Sphk1 to increase S1P levels in vascular smooth muscle cells. In isolated human aortic VSMC, hyperglycaemia causes a robust activation of Sphk1, resulting in the protein kinase C phosphorylation-mediated and ROS-dependent translocation of Sphk1 to the plasma membrane. This effect leads to increased intracellular S1P levels that protect VSMC from apoptosis\textsuperscript{251}. In STZ-treated diabetic rats with significant hyperglycaemia (i.e., >14mmol/L random fed blood glucose), Sphk1 activity is increased in the intact aorta two weeks after diabetes induction. Sphk1 activity is then normalized following the attainment of euglycaemia\textsuperscript{252}. High glucose also increases TNFα synthesis and secretion in rat and human aortic VSMCs in cultured conditions\textsuperscript{195}. The greater cleavage and release of TNFα from the aortic VMSC membrane was later shown by the same group to be the result of increased TACE phosphorylation and protease catalytic activity\textsuperscript{253}.

Additionally, the conceivable link between obesity and known molecular myogenic regulatory pathways is two-fold.

First, S1P, a principal regulator of myogenic tone, is significantly elevated in the plasma of high-fat diet induced\textsuperscript{254} and genetically obese mice\textsuperscript{254,255}. Moreover, in obese, un-medicated individuals with normal fasting glucose and HbA\textsubscript{1C} levels, circulating S1P is elevated 28% above lean counterparts\textsuperscript{254}.
Second, there is a well-documented association of obesity with the myogenic mediator TNFα\(^{141}\). Secreted TNFα in obesity mainly comes from the population of macrophages that have infiltrated the adipose tissue\(^{256}\); the adipocytes in obesity produce primarily membrane bound TNFα\(^{257}\). Furthermore, circulating levels of adipokines have been implicated in the progression of vascular dysfunction in obesity\(^{258}\) and may directly affect the endothelial and vascular smooth muscle cells of the microarteries.

3.2.7 Hypothesis and experimental approaches

The core hypothesis of this chapter is that diabetes, which is associated with a TNFα-mediated inflammatory reaction\(^{259}\), activates S1P signalling to induce functional microvascular changes that alter myogenic responsiveness in resistance arteries. In order to assess the effects of diabetes on VSMC signalling and to identify targets for therapeutic intervention, I titrated a standard high fat diet HFD/STZ mouse model to induce a mild increase in blood glucose levels. Furthermore, a necessary control group of this study, the HFD only mouse, provided the ability to investigate the possibility that obesity may affect the microartery myogenic response through influence on the underlying molecular signalling pathways. In order to represent the human pathogenesis of obesity, I used the diet-induced obese C57BL/6 mouse, which is prone to gain weight based on an excess intake of calories via fat\(^{260,261}\). Diets rich in fat result in obesity in humans and animal models, demonstrating a positive relationship between level of fat in the diet and body weight or fat gain\(^{262}\). Contributions of TNFα/S1P signalling were investigated utilizing both genetic (i.e., different gene knockout strategies) and clinically relevant (i.e., FDA-approved) pharmacological approaches. Given the importance of increased plasma glucose concentrations on the development of aberrant microvascular tone in diabetes, I also addressed the small elevation in blood glucose in HFD/STZ mice using the first-line diabetes therapy metformin to potentially ameliorate the associated microvascular dysfunction.
3.3 RESULTS

3.3.1 Blood glucose is mildly elevated in HFD/STZ mice

I developed a murine model of type 2 diabetes with a mild blood glucose elevation as observed in the early stages of the progression of the disease. STZ injection in HFD mice increased blood glucose within 6 days (Figure 3.1A); terminal blood glucose (Figure 3.1B) and HbA1C (Figure 3.1C) measures confirmed sustained blood glucose elevation at the time of sacrifice, 16 weeks following placement on HFD. In all HFD mice, STZ reliably increased blood glucose within a relatively narrow range (range: 10-15 mmol/L, n=100). An oral glucose tolerance test revealed significantly impaired glucose tolerance in HFD/STZ mice only, as determined by comparing the areas under the curve (Figure 3.1D-E).

![Figure 3.1](image)

Figure 3.1 HFD/STZ treatment compromises blood glucose control.
(A) Mice fed a high fat diet (HFD: n=4) have similar blood glucose levels as normal chow (NC; n=8) controls. Injecting HFD mice with streptozotocin (HFD/STZ; 100 mg/kg; n=5) increases blood glucose within 6 days; the elevation persists for at least 120 days. (B) Blood glucose (NC: n=25; HFD: n=22; HFD/STZ: n=28) and (C) HbA1c measures (NC: n=24; HFD: n=20; HFD/STZ: n=27) at the time of sacrifice confirm that HFD/STZ mice experience a persistent elevation in blood glucose. (D) Plotted are absolute blood glucose measures following oral glucose challenge (1.5 mg/g; NC: n=4; HFD: n=6; HFD/STZ: n=6). Following (E) baseline correction (i.e., the glucose level measured immediately prior to challenge is subtracted, so that only the kinetic of response is displayed), the (F) area under the curve measures confirm that oral glucose tolerance is altered in HFD/STZ mice, but not HFD mice (relative to NC controls). * denotes P<0.05 for multiple unpaired comparisons.

3.3.2 Myogenic tone is selectively and progressively augmented in resistance arteries isolated from HFD/STZ mice

I examined mesenteric, olfactory (cerebral) and posterior cerebral arteries, since our laboratory has previously reported that TNFα pathologically augments myogenic tone in these arteries and subsequently characterized the mechanisms involved160,162,163. Interestingly, 16 weeks of HFD alone had no effect on myogenic tone, phenylephrine responses or passive diameter in any vessel, relative to NC controls (Figure 3.2A-I). In contrast, HFD/STZ specifically augmented myogenic tone in both mesenteric and olfactory resistance arteries (measured at 12 weeks post-STZ injection); neither artery type displayed differences in phenylephrine responsiveness or passive diameter between the HFD/STZ group and the NC control group (Figure 3.2A-F). Surprisingly, HFD/STZ did not alter PCA myogenic tone (Figure 3.2G-I); phenylephrine responses were similar and a small, but significant decrease in passive diameter was observed. At 4 weeks post-STZ injection in HFD mice (i.e., an earlier time point, described visually in Figure 3.3), neither mesenteric (Figure 3.4A-C) nor olfactory arteries (Figure 3.4D-F) displayed increased myogenic tone compared to NC and HFD mice.
Figure 3.2  Myogenic tone is specifically augmented in HFD/STZ mice.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric (top), olfactory cerebral (middle) and posterior cerebral (bottom) arteries isolated from wild-type mice fed a normal chow (NC) diet, a high fat diet (HFD) or HFD plus streptozotocin injection (HFD/STZ; 100 mg/kg). **Panels A-C:** Mesenteric resistance arteries isolated from HFD/STZ mice (n=13) have augmented myogenic tone, relative to arteries isolated from NC (n=12) and HFD (n=14) mice; phenylephrine responses and passive
diameter are unchanged. **Panels D-F:** Similar to mesenteric arteries, myogenic tone is augmented in olfactory cerebral resistance arteries isolated from HFD/STZ mice, with no differences in phenylephrine responses or passive diameter (NC: n=15; HFD: n=5; HFD/STZ: n=6). **Panels G-I:** In contrast to mesenteric and olfactory arteries, posterior cerebral arteries display no differences in myogenic tone. Phenylephrine responses are unchanged; however, passive diameter is slightly reduced in the HFD/STZ group (NC: n=12; HFD: n=8; HFD/STZ: n=8). + denotes a significant difference ($P<0.05$) in passive diameter, relative to the NC control (multiple unpaired comparisons); * denotes a significant difference ($P<0.05$) in myogenic tone, relative to the NC control (multiple unpaired comparisons).

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**Figure 3.3  Timeline for the 4 weeks post-STZ diabetes induction experimental protocol.**  
A separate group of eight week-old male C57BL/6 mice are sacrificed 4 weeks post-streptozotocin (STZ) injection. Mice either remain on normal chow diet (NC; 10% cal/fat) or are placed on high fat diet (HFD; 45% cal/fat) for 4 weeks. At the start of week 5, high fat diet plus streptozotocin (HFD/STZ) mice are injected with STZ (100 mg/kg), and HFD and NC mice are injected with vehicle (0.1 mmol/L sodium citrate, pH 5.5). Mice remain on respective diets for an additional 4 weeks. Glycated haemoglobin (HbA1c) is measured prior to sacrifice and mesenteric and olfactory microarteries are isolated.
Figure 3.4  Myogenic tone is not augmented at 4 weeks post-STZ injection.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric (top) and olfactory cerebral arteries (bottom) isolated from wild-type mice fed a normal chow (NC) diet, a high fat diet (HFD) or HFD plus streptozotocin injection (HFD/STZ; 100 mg/kg). Panels A-C: At 4 weeks post-streptozotocin (STZ) or vehicle injection, mesenteric resistance arteries isolated from HFD/STZ mice (n=5) have comparable myogenic tone relative to arteries isolated from NC (n=6) and HFD (n=7) mice; phenylephrine responses and passive diameter are unchanged. Panels D-F: Similar to mesenteric arteries, myogenic tone is unchanged in olfactory cerebral resistance arteries isolated from HFD/STZ mice, with no differences in phenylephrine responses or passive diameter (NC: n=7; HFD: n=4; HFD/STZ: n=5).

At the time of sacrifice (i.e., 4 weeks post STZ), random fed blood glucose levels (NC: 8.5±0.9 mmol/L, n=5; HFD: 9.1±0.5 mmol/L, n=4; HFD/STZ: 17.64±2.7 mmol/L*; n=5; P<0.05 HFD/STZ relative to NC) and HbA$_{1C}$ levels (NC: 4.7±0.1%, n=5; HFD: 5.0±0.1%, n=4; HFD/STZ: 6.5±0.4%, n=5; P<0.05 HFD/STZ relative to NC) were elevated in HFD/STZ mice, relative to NC controls.
3.3.3 Ten months of high fat feeding does not influence myogenic tone in mouse resistance arteries

Given that four months of high fat feeding alone in mice did not appear to alter myogenic tone (Figure 3.2), I investigated the consequence of an extended period of high fat feeding. C57BL/6 mice were maintained on HFD (45% kcal/fat) for ten months (mice were placed on HFD at 8 weeks of age) (described visually in Figure 3.5). Myogenic tone, phenylephrine vasomotor responses and passive diameter of mesenteric arteries were comparable between one-year-old NC and HFD mice (Figure 3.6A-C), suggesting that an prolonged period of time on a high fat diet does not affect resistance artery vasomotor responses.

**Figure 3.5 Timeline for the 10 months of HFD experimental protocol.**

A separate group of male C57BL/6 mice are sacrificed following 10 months of high fat feeding. Mice remain either on normal chow diet (NC; 10% cal/fat) or are placed on high fat diet (HFD; 45% cal/fat) at two months of age and continue to consume their respective diets for an additional ten months. Mesenteric microarteries are isolated at the time of sacrifice (when mice are 12 months old).
Figure 3.6  Myogenic tone in resistance arteries remains unchanged following ten months of high fat feeding.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric arteries isolated from wild-type mice fed a normal chow (NC) diet or a high fat diet (HFD) for ten months. No differences in mesenteric resistance artery (A) passive diameter, (B) myogenic tone or (C) phenylephrine responses are observed (NC, 12 months old: n=5; HFD, 12 months old: n=4).

3.3.4  Systemic hemodynamic measurements and cerebral blood flow are unchanged in HFD/STZ mice

The characteristics of the cohort that included NC, HFD and HFD/STZ mice (16 week protocol outlined in Figure 2.1) are provided in Table 3.1. HFD/STZ mice had similar body weights to NC controls, whereas HFD mice were significantly heavier (Table 3.1). Systemic blood pressure and cardiac parameters were similar across all groups (Table 3.1), with the exception of heart rate, which was significantly elevated in HFD mice (Table 3.1, bottom row). Cerebral perfusion in HFD/STZ, HFD and NC mice was also similar (Figure 3.7). Taken together, neither the mild STZ-induced blood glucose elevation in HFD mice nor HFD alone resulted in an obvious hemodynamic phenotype.
Table 3.1 Wild-type normal chow, high fat diet and high fat diet/streptozotocin mouse characteristics

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Figure 3.7 Noninvasive cerebral blood flow measures.
Shown are representative FAIR-Magnetic Resonance Imaging perfusion maps of the fore- (left column), mid- (center column) and hind- (right column) brain regions from mice fed a normal chow diet (NC n=5; top row, A-C), high fat diet (HFD n=5; middle row, D-F) or HFD plus 100 mg/kg streptozotocin injection (HFD/STZ n=6; bottom row, G-I). Cerebral blood flow in the (J) fore-, (K) mid-, and (L) hindbrain regions is similar between NC, HFD and HFD/STZ mice.

3.3.5 Endothelial function in NC, HFD and HFD/STZ mice

I investigated whether endothelial dysfunction and/or impaired vasodilator responsiveness were responsible for the augmented myogenic tone in HFD/STZ mice. I found that acetylcholine (ACh) and sodium nitroprusside (SNP) responses were unchanged between NC, HFD and HFD STZ mice in mesenteric (Figure 3.8A-B) and olfactory arteries (Figure 3.8C-D).
Figure 3.8  Vasodilator responses in resistance arteries.

Mesenteric and olfactory cerebral resistance arteries are isolated from mice fed a normal chow (NC) diet, high fat diet (HFD) or high fat diet plus streptozotocin injection (HFD/STZ; 100 mg/kg). Arteries are pre-constricted with 3 µmol/L phenylephrine and co-treated with increasing concentrations of acetylcholine (1 nmol/L–30 µmol/L) or sodium nitroprusside (1 nmol/L–30 µmol/L). Mesenteric arteries dilate in response to (A) acetylcholine (NC: n=5; HFD: n=9; HFD/STZ: n=9) or (B) sodium nitroprusside (NC: n=6; HFD: n=9; HFD/STZ: n=9). In contrast, olfactory arteries do not appreciably dilate in response to (C) acetylcholine (NC: n=8; HFD: n=8; HFD/STZ: n=10), but do dilate in response to (D) sodium nitroprusside (NC: n=4; HFD: n=8; HFD/STZ: n=11). No differences between the treatment groups are found.
3.3.6 TNFα and Sphk1 mediate the augmented myogenic tone in resistance arteries from HFD/STZ mice

TNFα is emerging as a central regulator of myogenic tone and the present chapter’s core hypothesis is that it also mediates the microvascular effects in HFD/STZ mice. Indeed, TNFα mRNA expression was greater in cerebral resistance arteries isolated from HFD/STZ mice, relative to those isolated from HFD and NC mice (Figure 3.9A). Pharmacologically neutralizing TNFα in vitro (ETN; a recombinant soluble TNFα receptor fusion protein that scavenges/sequesters TNFα; 1 mg/mL for 30 minutes) significantly attenuated the augmented myogenic tone in mesenteric arteries isolated from HFD/STZ mice, with no effect on phenylephrine responses (Figure 3.9B-C).

![Figure 3.9](image_url)

**Figure 3.9 TNFα expression in resistance arteries.**

Cerebral microvascular resistance arteries are isolated from mice fed a normal chow (NC) diet, high fat diet (HFD), or HFD plus streptozotocin injection (HFD/STZ; 100 mg/kg) and processed for quantitative PCR. (A) TNFα mRNA expression is higher in resistance arteries isolated from HFD/STZ mice (n=6) relative to HFD mice (n=5) and NC controls (n=5). Pharmacologically antagonizing TNFα in HFD/STZ mesenteric arteries (1 mg/mL etanercept for 30 minutes; ETN) attenuates (B) myogenic tone (n=5), but (C) does not alter phenylephrine responsiveness (n=5). * denotes P<0.05 for multiple unpaired comparisons in panel A and paired comparisons in panels B and C.
Correspondingly, germ-line TNFα gene deletion prevented the HFD/STZ-stimulated myogenic tone augmentation in both mesenteric and olfactory arteries (Figure 3.10A,D); phenylephrine responses were unchanged (Figure 3.10B,E), as was the HFD/STZ-induced blood glucose elevation (Table 3.2). Interestingly, HFD/STZ treatment in TNFα−/− mice increased passive diameters (Figure 3.10C,F), an effect not observed in wild-type HFD/STZ mice (Figure 3.2).

**Figure 3.10**  TNFα gene deletion prevents HFD/STZ treatment from augmenting myogenic tone.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric (top) and olfactory cerebral arteries (bottom) isolated from germ-line TNFα knockout mice (TNFα−/−) fed either a normal chow (NC) diet, a high fat diet (HFD) or a high fat diet plus streptozotocin injection (HFD/STZ; 100 mg/kg). **Panels A-C:** Mesenteric artery myogenic tone and phenylephrine responses are similar in the TNFα−/− NC (n=9), TNFα−/− HFD (n=7) and TNFα−/− HFD/STZ (n=7) groups; passive diameter is significantly larger in arteries isolated from TNFα−/− HFD/STZ mice. **Panels D-F:** Similarly, olfactory cerebral artery myogenic tone and phenylephrine responses are similar in the TNFα−/− NC (n=4), TNFα−/− HFD (n=5) and TNFα−/−
HFD/STZ (n=7) groups; passive diameter is significantly larger in arteries isolated from TNFα+/− HFD/STZ mice. + denotes a significant difference (P<0.05; multiple unpaired comparisons) in passive diameter.

Table 3.2. Gene knockout and treated normal chow, high fat diet and high fat diet/streptozotocin mouse characteristics

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Using a tissue-specific conditional TNFα gene deletion model, I next determined whether vascular smooth muscle cells provide a relevant TNFα source, as our mechanistic model predicts. Notably, chronically removing smooth muscle cell TNFα (i.e., treating SMMHC-CreERT2 / TNFα^flox/flox mice with tamoxifen 17 weeks prior to functional assessment) significantly impacted myogenic responsiveness in mesenteric arteries. Specifically, a proportion of arteries lose myogenic tone, although general contractility is unchanged (i.e., phenylephrine maximal constriction and EC50 values were similar in arteries with and without myogenic responses; data not shown). This loss in myogenic responsiveness may be attributed to the long-term removal of smooth muscle cell TNFα, since arteries isolated from Cre-expressing, tamoxifen-treated wild-type mice (HFD/STZ-treated SMMHC-CreERT2 / TNFα^wt/wt mice) all had normal myogenic responses (Figure 3.11A-C). Comparing the NC and HFD/STZ-treated treatment groups, similar proportions of arteries displayed the ‘loss of myogenic tone’ phenotype (NC: 4 out of 8; HFD/STZ: 5 out of 7). Under the conditions studied (i.e., smooth muscle cell TNFα knockout arteries), the NC and HFD/STZ groups did not differ in terms of myogenic tone, phenylephrine responses or passive diameter (Figure 3.11).

In contrast to mesenteric arteries, targeted smooth muscle cell TNFα gene deletion did not cause a loss of myogenic responsiveness in olfactory arteries (Figure 3.11D). Smooth muscle cell TNFα gene deletion prevented the HFD/STZ-stimulated augmentation of myogenic tone in olfactory arteries; phenylephrine responses and passive diameter were similar to NC controls (Figure 3.11E-F).
Figure 3.11  Smooth muscle cell-targeted TNFα gene deletion prevents HFD/STZ treatment from increasing myogenic tone.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric (top) and olfactory cerebral arteries (bottom) isolated from transgenic mice fed either a normal chow (NC) diet or a high fat diet plus streptozotocin injection (HFD/STZ; 100 mg/kg). Panels A–C: In mesenteric arteries isolated from smooth muscle cell-targeted TNFα knockout mice (tamoxifen-treated SMMHC-CreERT2 / TNFα<sup>lox/lox</sup> mice; henceforth denoted as TNFα<sup>smKO</sup>), myogenic tone, phenylephrine responses and passive diameter are similar in the NC (n=7) and HFD/STZ (n=7) treatment groups. Mesenteric arteries isolated from transgenic control HFD/STZ mice (tamoxifen-treated SMMHC-CreERT2 / TNFα<sup>wt/wt</sup> mice; henceforth denoted as TNFα<sup>smWT</sup>; n=7) possess higher levels of myogenic tone compared to either TNFα<sup>smKO</sup> treatment group; these arteries also display differences in their phenylephrine dose-response relationship, primarily due to a shift in baseline tone. All 3 groups have similar passive diameters. Panels D–F: In olfactory resistance arteries isolated from smooth muscle cell-targeted TNFα knockout mice (TNFα<sup>smKO</sup>),
myogenic tone, phenylephrine responses and passive diameter are similar in the NC (n=6) and HFD/STZ (n=7) treatment groups. * denotes $P<0.05$ for multiple unpaired comparisons.

Our group has previously demonstrated that TNFα augments myogenic tone by activating the S1P-generating enzyme Sphk1 and consequently, enhancing pro-constrictive S1P signalling\textsuperscript{160,162,163}. Germ-line Sphk1 gene deletion prevented the HFD/STZ-stimulated myogenic tone elevation in both mesenteric and olfactory arteries (Figure 3.12A,D), consistent with my proposed mechanistic link. HFD/STZ did not affect phenylephrine responses or passive diameters in arteries isolated from Sphk1\textsuperscript{-/-} mice (Figure 3.12B,C,E-F).

STZ injection in HFD mice consistently increased blood glucose in all gene knockout models utilized in this investigation; mouse characteristics for each genotype are presented in Table 3.2. Myogenic tone, phenylephrine responses and passive diameters in mesenteric and olfactory arteries isolated from TNFα\textsuperscript{-/-} (Figure 3.10) and Sphk1\textsuperscript{-/-} (Figure 3.12) mice on HFD alone were not different, relative to NC controls.
Figure 3.12  Sphk1 gene deletion prevents HFD/STZ treatment from augmenting myogenic tone.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric (top) and olfactory cerebral arteries (bottom) isolated from germ-line Sphk1 knockout mice (Sphk1<sup>-/-</sup>) fed either a normal chow (NC) diet or a high fat diet plus streptozotocin injection (HFD/STZ; 100 mg/kg). Panels A-C: Mesenteric artery myogenic tone, phenylephrine responses and passive diameter are similar in the Sphk1<sup>-/-</sup> NC (n=7), Sphk1<sup>+/+</sup> HFD (n= 5) and Sphk1<sup>-/-</sup> HFD/STZ (n=12) groups. Panels D-F: Similarly, olfactory cerebral artery myogenic tone, phenylephrine responses and passive diameter are similar in the Sphk1<sup>-/-</sup> NC (n=6), Sphk1<sup>+/+</sup> HFD (n=5) and Sphk1<sup>-/-</sup> HFD/STZ (n=10) groups.
3.3.7 Therapeutically targeting TNFα or S1P signalling ameliorates the augmented myogenic tone in HFD/STZ mice

My gene knockout models identified both TNFα and S1P signalling as key elements in the HFD/STZ-induced augmentation of resistance artery myogenic tone. I therefore validated the therapeutic feasibility of targeting either signalling pathway using medications that are either clinically available (ETN; an anti-TNFα medication) or in clinical trials (S1PR antagonists)²⁶³.

Neither systemic anti-TNFα therapy (ETN; 1 injection of 1 mg/kg s.c. twice per week 14 days prior to sacrifice¹⁶²,¹⁶³) nor systemic S1PR antagonism (JTE013; 3 mg/kg i.p. daily for 14 days prior to sacrifice¹⁶⁶) affected the HFD/STZ-induced blood glucose elevation (Table 3.2). However, both pharmacological interventions successfully reversed the HFD/STZ-induced increase in tone in mice, normalizing it in both mesenteric and olfactory arteries to the level of ETN - (Figure 3.13A,D) or JTE013 - (Figure 3.14A,D) treated NC controls. Neither ETN nor JTE013 altered phenylephrine responses or passive diameters (Figure 3.13B-C and Figure 3.14B-C).
Figure 3.13 Augmented myogenic tone is normalized by antagonizing TNFα signalling.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric and olfactory cerebral arteries isolated from systemically-treated mice fed either a normal chow (NC) diet or a high fat diet plus streptozotocin injection (HFD/STZ; 100 mg/kg). Mice were injected with etanercept (ETN; 20 mg/kg intraperitoneal injection 2x weekly) for 2 weeks prior to sacrifice. **Panels A-C:** Mesenteric artery myogenic tone, phenylephrine responses and passive diameter are similar in the ETN-treated NC (n=4), ETN-treated HFD (n=5) and ETN-treated HFD/STZ (n=9) groups. **Panels D-F:** Similarly, olfactory artery myogenic tone, phenylephrine responses and passive diameter are similar in the ETN-treated NC (n=4), ETN-treated HFD (n=4) and ETN-treated HFD/STZ (n=5) groups.
Figure 3.14  Augmented myogenic tone is normalized by antagonizing S1P signalling.

Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric and olfactory cerebral arteries isolated from systemically-treated mice fed either a normal chow (NC) diet or a high fat diet plus streptozotocin injection (HFD/STZ; 100 mg/kg). Mice were injected with JTE013 (3 mg/kg intraperitoneal injection, once per day) for 2 weeks prior to sacrifice. Panels A-C: Mesenteric artery myogenic tone, phenylephrine responses and passive diameter are similar in the JTE013-treated NC (n=7), JTE013-treated HFD (n=5) and JTE013-treated HFD/STZ (n=7) groups. Panels D-F: Similarly, olfactory artery myogenic tone, phenylephrine responses and passive diameter are similar in the JTE013-treated NC (n=5), JTE013-treated HFD (n=8) and JTE013-treated HFD/STZ (n=6) groups.
3.3.8 Metformin treatment does not lower blood glucose in HFD/STZ mice

To assess the influence of increased blood glucose driving elevated myogenic responses in HFD/STZ, I treated mice with the anti-diabetic medication metformin (450 mg/kg food) for twelve weeks (Figure 3.15A).

Mice fed HFD alone + metformin had comparable blood glucose and HbA$_{1C}$ levels to NC + metformin mice (Figure 3.15B-D). HFD/STZ + metformin mice displayed increased random fed blood glucose throughout the course of the study (Figure 3.15B), meaning that metformin treatment in HFD/STZ mice unexpectedly did not normalize blood glucose, as terminal blood glucose (Figure 3.15C) and HbA$_{1C}$ (Figure 3.15D) were significantly elevated compared with NC + metformin mice. HFD + metformin mice were significantly heavier than NC + metformin mice (Figure 3.15E).
Figure 3.15  Twelve weeks of *in vivo* Metformin treatment does not lower blood glucose or HbA$_{1C}$ in HFD/STZ mice.

(A) A separate cohort of eight week-old male C57BL/6 mice either remain on normal chow diet (NC; 10% cal/fat) or are placed on high fat diet (HFD; 45% cal/fat) for 4 weeks. At the start of week 5, HFD/STZ mice are injected with STZ (100 mg/kg) and NC and HFD mice are injected with vehicle (0.1 mmol/L sodium citrate, pH 5.5). All groups of mice are then placed on new diets. HFD and HFD/STZ mice are fed a special high fat diet containing metformin (450 mg/kg drug/weight of food) and NC mice are fed a special standard diet containing metformin (450 mg/kg drug/weight of food). Mice remain on
their respective metformin diets for an additional 12 weeks. At week 16, glycated haemoglobin (HbA1C) is measured prior to sacrifice and olfactory and mesenteric microarteries are isolated. (B) Random fed blood glucose levels increase 6 days post-STZ injection in HFD/STZ mice and remain elevated, even with 120 days of metformin treatment. (C) Random fed blood glucose and (D) HbA1C taken prior to sacrifice is significantly elevated in HFD/STZ + metformin mice. (E) Body weight is significantly increased in HFD + metformin mice (NC + metformin: n=6; HFD + metformin: n=6; HFD/STZ + metformin: n=6). * denotes P<0.05 for multiple unpaired comparisons.

### 3.3.9 Metformin decreased myogenic tone in HFD/STZ mice

Twelve weeks of metformin therapy prevented the HFD/STZ-stimulated increase in myogenic tone in both mesenteric and olfactory arteries (Figure 3.16A,D). In fact, metformin reduced myogenic tone in mesenteric arteries isolated from all three treatment groups (i.e., NC, HFD and HFD/STZ mice) to levels below what is observed in normal mice (Figure 3.16A), and moderately reduces tone in olfactory resistance vessels (Figure 3.16D). Phenylephrine responses were unchanged in both microarteries (Figure 3.16B,E), as were passive diameters (Figure 3.16C,F). Given the surprising finding of reduced myogenic tone in mesenteric arteries from all metformin treated mice, a separate group of isolated mesenteric arteries from NC mice were incubated for 30 minutes in the presence of low (100 µmol/L) or high (1 mmol/L) dose metformin to test for possible acute, in vitro effects of the drug. Neither dose of metformin had any acute in bath effects on myogenic (Figure 3.17A,C) or phenylephrine (Figure 3.17B,D) responsiveness of mesenteric arteries.
Figure 3.16  Metformin reduces myogenic tone in HFD/STZ mesenteric and olfactory arteries.  Shown are measured active and passive diameters (left), the corresponding myogenic tone calculations (center) and phenylephrine dose-response relationships (right) for mesenteric (top) and olfactory cerebral (bottom) arteries isolated from wild-type mice fed a normal chow (NC) diet plus metformin (450 mg/kg drug/weight of food), a high fat diet (HFD) plus metformin (450 mg/kg drug/weight of food) or HFD plus streptozotocin injection (HFD/STZ; 100 mg/kg) plus metformin (450 mg/kg drug/weight of food).  Panels A-C: Mesenteric resistance arteries isolated from metformin-treated NC (n=11), HFD (n=13) and HFD/STZ mice (n=21) have blunted myogenic tone; phenylephrine responses and passive diameter are unchanged.  Panels D-F: In isolated olfactory resistance arteries, there are no differences in myogenic tone, phenylephrine responses or passive diameter between the groups (NC + metformin: n=7; HFD + metformin: n=9; HFD/STZ + metformin: n=6).
Figure 3.17  Acute low or high doses of metformin *in vitro* do not alter vasomotor responses in mesenteric arteries from NC mice. Myogenic tone and vasoconstriction to phenylephrine are assessed in mesenteric arteries isolated from NC mice. These arteries are then treated *in vitro* with either 100 µmol/L or 1 mmol/L of the anti-diabetic medication metformin (for 30 minutes) and re-assessed. Neither low (n=8) nor high (n=5) dose treatment with metformin had any effect on *(A and C)* myogenic tone or *(B and D)* response to phenylephrine.
3.4 DISCUSSION

This thesis demonstrates that diabetes progressively augments the myogenic responsiveness of resistance arteries, a main mechanism by which the body controls both systemic blood pressure and tissue blood flow. The present chapter uncovers altered signalling processes in microvascular smooth muscle cells and provides strategies to interfere with this undesirable process through identification and validation of two new therapeutic targets (i.e., TNFα and S1P signalling).

3.4.1 Myogenic tone in HFD/STZ mice

As this chapter set out to identify the molecular mechanisms controlling resistance artery tone in diabetes with the idea of validating curative options, I used a mouse model of diabetes that is easy to generate for the achievement of a specifically mild blood glucose level elevation. I used these mice to assess an early time point in the disease progression, when functional changes in the vasculature begin to appear. High fat fed C57BL/6 mice develop obesity, hyperglycaemia and hyperinsulinemia. Subsequent treatment with STZ induces β-cell death, resulting in a model of diabetes that reflects the metabolic disturbance in clinical type 2 diabetes: peripheral insulin resistance combined with a loss of pancreatic beta cell function. The combination of HFD and chemical β-cell destruction resulted in augmented myogenic tone in two distinct vascular beds (i.e., mesenteric and cerebral) twelve weeks following STZ treatment. A separate cohort of mice that were sacrificed four weeks post-STZ did not show this alteration in myogenic tone, although blood glucose was indeed elevated at this time point. Catching this interval of non-elevated myogenic responsiveness between STZ treatment and the onset of microvascular changes argues against a possible direct cytotoxic effect of STZ on microvessels. Importantly, these data indicate that the pathologic microvascular phenotype develops over a more prolonged period of mild hyperglycaemia, potentially leaving a window open for therapeutic intervention.
3.4.2 Myogenic tone in HFD mice

A variety of alterations occur in the vascular structure and function of obese individuals as the amount of adipose deposition accumulates. The majority of patients with hypertension are overweight\textsuperscript{267} and weight gain is a potent risk factor for the subsequent development of hypertension\textsuperscript{268}. Thus, it is important to investigate the mechanisms that link obesity to increased vascular resistance and abnormalities in blood pressure. One of the secondary aims of this chapter was to therefore ascertain if obese mice (used as a control for the HFD/STZ group) display vascular dysfunction by assessing myogenic tone, a key determinant of peripheral resistance. The obesity-prone C57BL/6 mice diet induced obesity model is one of the most commonly used mouse strain for the purpose of studies of obesity, where weight gain is based on an excess intake of calories from fat\textsuperscript{269}.

3.4.2.1 HFD mice and blood glucose regulation

In this study, diet-induced obese C57BL/6 mice had significantly increased body weight, but did not develop differences in blood glucose or HbA$_{1C}$ over the course of 16 weeks of high fat feeding.

These data are in contrast to some studies indicating that C57BL/6 mice placed on high fat diet for five months become hyperglycaemic, hyperinsulinemic, hypercholesterolemic and hypertensive. They have increased adipose tissue accumulation particularly in the mesentery and subcutaneous fat depots, due to increased numbers of fat cells\textsuperscript{260,270}. As a result of this phenotype, HFD C57BL/6 mice are often designated as a model of metabolic syndrome\textsuperscript{260}.

To speak to the discrepancy between the literature and my investigation, it is difficult to compare HFD mice across studies because the exact macro- and micronutrient content of these diets is often not reported. Any consequence of dietary fat may be confounded with the effects of other components in the diet. High fat diets consist of products such as casein, cornstarch, maltodextrine or sucrose and soybean or lard, supplemented with minerals and vitamins. Sucrose, which is an ingredient in the HFD used in my study, can influence food and water intake, anxiety related behaviour, locomotor activity, fat deposition, blood insulin and leptin.
levels. Therefore, differences in the various ingredients of the HFD used in this study may explain why blood glucose levels were not changed.

### 3.4.2.2 Obesity and altered microvascular vasomotor responsiveness

In humans, obesity is associated with disrupted endothelial function. Abnormal forearm microvascular flow responses to SNP are also found in obese patients, suggesting the function of VSMCs is also impaired. The exact mechanisms by which obesity impairs microvessel vasomotor function in humans are largely unknown. A study conducted by Grassi et al. evaluated vasodilatory responses in isolated microarteries from obese, normotensive patients. Small arteries were dissecting from biopsies of subcutaneous fat tissue from the anterior abdominal region. The obese patient group consisted of 17 subjects with severe abdominal obesity that underwent laparoscopic-adjustable gastric banding. The lean group had 16 subjects hospitalized for laparoscopic surgery for cholecystectomy. Small arteries from obese patients displayed blunted vasodilation to ACh, while the response to SNP was unchanged compared to arteries from lean patients. Georgescu et al. isolated arterioles from a periumbilical subcutaneous fat biopsy taken from patients undergoing surgery for a variety of reasons. Arterioles from obese patients relaxed very little to ACh and in the presence of ACh, produced 5.7-11.6-fold less NO compared to lean control arterioles. Together, these studies implicate impaired NO release in artery dysfunction in obesity.

Genetic and diet-induced obese animal models also suggest that reduced dilation of microarteries is primarily due to impaired endothelial function, as the majority of obese animal models display reduced responses to ACh. In obese Zucker rats (OZR), mesentery and skeletal muscle resistance arteries have impaired endothelial-dependent vasodilation. Endothelial dysfunction appears to be age dependent, as 20-week-old rats have intact ACh responses in the mesentery, whereas in 32-week-old OZR, ACh-dependent vasodilation is absent. In high-fat fed rats (60% cal/fat), mesenteric and skeletal muscle arteriolar dilation to various vasodilatory agents including ACh is also reduced. These preclinical models suggest that obesity is associated with reduced vascular NO bioavailability. Further evidence implicates increased vascular superoxide anion production as causative through inactivation of endothelial NO, as free radical scavenging using tiron restored the diminished microartery
vasodilation\textsuperscript{209,217}. Vasodilation in response to ACh and SNP in the microarteries from HFD mice used in this study was not statistically different than responses found in microvessels from NC mice, suggesting that endothelial dysfunction was not present in this model of obesity.

3.4.2.3 **Obesity and the myogenic response**

Many rodent obesity models display abnormalities in vascular function. Thus, it was of interest to investigate whether our control group of HFD mice possessed altered microvascular regulation. HFD mice, after either eight or sixteen weeks of high fat feeding, displayed no abnormalities in resistance artery myogenic tone. Furthermore, HFD mice were not hypertensive and had normal cerebral blood flow. Additionally, keeping mice on the HFD for ten months did not change myogenic tone in mesenteric resistance arteries.

These findings are in accordance with two studies from the literature that assessed myogenic tone in diet-induced obese rodent models. A cafeteria-style diet (normal chow including cakes, pies and biscuits, totalling 32.1±1% cal from fat) given to Sprague-Dawley rats did not induce functional myogenic tone changes in cremaster arterioles and middle cerebral arteries\textsuperscript{221}, although rats are significantly heavier and have increased nonfasting blood glucose and plasma insulin levels compared to NC control rats\textsuperscript{221}. Erdei \textit{et al.}\textsuperscript{209} also demonstrate no change in HFD rat coronary arteriolar myogenic responses.

Normal myogenic tone in HFD mice is also unlike studies that investigated tone in genetically obese rat models. In overweight, hyperinsulinemic, hypertensive OZR, middle cerebral artery myogenic tone is enhanced compared to lean Zucker rats (LZR)\textsuperscript{222-224}. Furthermore, middle cerebral arteries do not dilate in response to ACh, but respond appropriately to SNP. Thus, the assumed mechanism behind the increased myogenic tone in OZR is impaired dilator reactivity. OZR skeletal muscle resistance arteries also display increased myogenic tone compared with LZR\textsuperscript{219}. In this vascular bed, the activation of myogenic tone was only partially dependent on the endothelial layer, as endothelium denudation caused a small, but significant reduction in myogenic tone. Finally, Butcher \textit{et al.}\textsuperscript{223} show augmented myogenic tone in both OZR middle cerebral arteries and gracilis arterioles, compared with LZR, an effect that was abolished after the endothelium was removed.
A discernible difference between these findings and my study is the model used. Notably, data from diet induced obese rats support my observation of normal myogenic tone in HFD mice, whereas abnormal tone was seen only in genetically induced obese rodent models. The OZR has genetically disrupted leptin signalling that results in profound metabolic dysregulation that may influence myogenic tone regulatory mechanisms. It appears that I am the first to investigate the consequences of HFD on myogenic tone in mice, and this work supports the notion that changes in myogenic responsiveness in an obese setting require the addition of hyperglycaemia in combination with other metabolic disturbances, such as hyperinsulinemia and hyperlipidemia.

3.4.3 Blood pressure, endothelial function and cerebral blood flow in HFD/STZ mice

Systemic blood pressure and related hemodynamic parameters of HFD/STZ mice were unchanged compared to NC controls, resulting in a normotensive diabetic model; excluding the possibility of altered systemic hemodynamic parameters exacerbating microvascular responsiveness in this study\textsuperscript{185}. Our normotensive diabetes model is in line with other studies reporting diabetic mouse models that do not develop hypertension\textsuperscript{48,272} and a report where hypertension had no effect on myogenic responsiveness (i.e., myogenic responsiveness in vessels from human type 2 diabetes was not affected by the presence or absence of hypertension)\textsuperscript{184}.

In contrast to several other investigations\textsuperscript{45-48}, endothelial dysfunction, a concept explaining changes in vascular behaviour preferentially with an impairment of endothelial autacoid availability, was absent in the arteries investigated here. Our data suggest that the endothelium does not contribute to the elevation of the myogenic response, as endothelial-dependent vasodilation in response to ACh was unchanged between NC control and HFD/STZ-treated mice. Additionally, the prevention of a myogenic tone elevation in HFD/STZ VSMC-specific knockout of TNF\textalpha{} mice further supports a localized, VSMC-specific, endothelial-independent mechanism that promotes the proconstrictive condition of the microvasculature in diabetes. A primary role for VSMC\textsuperscript{S} alone in the dysregulation of myogenic tone in diabetes has also been suggested by two studies reporting an elevation in myogenic tone in diabetes mouse models that persisted following denudation of the endothelial layer\textsuperscript{173,187}. 
Discrepancies between my study and the literature may be related to the model used, particularly with respect to the type and severity of diabetes, the size of the vessel studied and the experimental conditions under which the study took place (i.e., *in vitro* vs. *in vivo*). The reported causes of endothelial dysfunction in diabetes include hyperglycaemia, free fatty acid production and elevated blood insulin\(^4^3\). Increased blood glucose levels may affect endothelial cells differently, as these cells exhibit differences in metabolism and structure depending on the vascular bed in which they are situated\(^2^7^3\). The mildly elevated blood glucose levels in my diabetes model may also explain why the functionality of the endothelium remained intact in HFD/STZ mice. The potential for the HFD/STZ mouse to display disrupted endothelial function in other arteries remains, however, and was not tested here. For example, endothelial dysfunction was shown in the mesenteric circulation of diabetic rats, whereas the aorta from these same animals displayed normal endothelium-dependent dilation (which was used as a parameter to assess endothelial function)\(^2^7^4,2^7^5\).

Despite the significant increases in olfactory artery myogenic responsiveness, MRI-based measurements of global cerebral blood flow (CBF) *in vivo* detected no differences between HFD/STZ and NC mice. This outcome was unexpected as diabetes-associated CBF decreases have been previously reported\(^1^7^8\) and are generally thought to be the primary reason for a 1.5-2.5 fold increased risk of developing cognitive impairment in diabetes\(^1^6,2^7^6-2^8^0\). In addition, the apparent lack of a HFD/STZ-associated effect on CBF also seems to contradict our laboratory’s results from a previous study that correlated enhanced myogenic responsiveness of PCAs in heart failure with reduced CBF\(^1^6^2\). In the heart failure scenario, proximal PCAs, which have minimal myogenic activity and conduct blood at low resistance to feed the entire cerebral circulation, are recruited to the myogenic mechanism. As a consequence of this proximal resistance to flow, CBF in the entire dependent cerebrovascular tree drops. Olfactory arteries, however, are more distally located in the cerebrovascular tree and have high basal myogenic activity to protect the neighbouring capillary bed. Therefore, the effect of enhanced myogenic vasoconstriction in olfactory arteries is likely to be very localized and would not result in a detectable change in CBF. Further studies are warranted to ascertain whether the myogenic tone-enhancing effect of HFD/STZ treatment will generalize over time (i.e., spread from distal (olfactory) to proximal (PCA) arteries) and then reduce CBF. Studies are also needed to
determine whether the effect on olfactory (i.e., distal) arteries promotes cognitive impairment in HFD/STZ mice.

### 3.4.4 Myogenic tone phenotype of knockout mice

Before discussing the vascular effects observed in my HFD/STZ knockout mouse models, it is important to briefly describe the baseline vascular function in these animals prior to further intervention.

Although it has been demonstrated that S1P is a key regulator of the myogenic response, data from our laboratory demonstrates that there is no effect of genetic ablation of Sphk1 and TNFα on baseline myogenic tone or response to phenylephrine\(^\text{158,162}\). Furthermore, Sphk1 knockout mice possess normal blood pressure\(^\text{281}\). This is in contrast to our data utilizing the S1PR antagonist JTE103, in which the myogenic response is significantly reduced in mesenteric and brain vascular beds following drug treatment in wild-type mice. These data suggest that the germ-line knockout mice have developed compensatory mechanisms to maintain normal regulation of myogenic tone throughout development. Although compensatory mechanisms are likely present in knockout mice, this is not likely to be of concern should I still be able to demonstrate that the lack of the targeted protein is relevant for the diabetes-induced increase in myogenic responsiveness.

The conditional (Tamoxifen-inducible), smooth muscle cell-specific (SMMHC promoter) TNFα knockout mouse model was employed in order to further elucidate the role of TNFα in vascular tone regulation without potential for development of compensatory mechanisms.

### 3.4.5 TNFα-governed S1P signalling regulates myogenic tone in HFD/STZ mice

Although a multitude of signalling mechanisms have been suggested to play a role in the development of diabetes-induced microvascular dysfunction in a variety of diabetic rodent models, therapeutic interventions specifically tailored to correct the increase in myogenic responsiveness are presently unavailable. Most studies implicate pathogenic mechanisms that
affect the bioavailability of endothelial autacoids, either predominantly through interference with nitric oxide synthesis, release or availability\textsuperscript{172,180} or via increasing the contractility of microvascular smooth muscle cells. I am the first to establish a direct link between diabetes-induced changes in myogenic responsiveness and TNF\(\alpha\)-governed S1P signalling.

The conclusion that TNF\(\alpha\) is a central pathological mediator that augments resistance artery myogenic tone in diabetes is based on the observations that resistance artery TNF\(\alpha\) expression is elevated in HFD/STZ mice and interfering with TNF\(\alpha\) activity (with an antagonist or genetic deletion) abolishes the augmented myogenic tone. To expand on this fundamental mechanistic insight, I utilized a conditional TNF\(\alpha\) gene deletion approach to unambiguously localize the source of this relevant therapeutic target to the VSMCs. In my model, resistance arteries autonomously respond to the challenge of HFD/STZ-induced diabetes, rather than being controlled by circulating TNF\(\alpha\) from other sources (e.g., plasma TNF\(\alpha\)\textsuperscript{145,146} or activated macrophages in adipose tissue\textsuperscript{207}).

We have previously demonstrated that TNF\(\alpha\) augments myogenic responsiveness in multiple microvascular beds through enhanced S1P signalling\textsuperscript{160,162,163}. Given that Sphk1 activity provides a key source of S1P and regulates of myogenic responsiveness under healthy conditions\textsuperscript{159}, I expected that Sphk1 gene deletion should abolish myogenic tone. Although myogenic responses are retained in arteries isolated from Sphk1\textsuperscript{-/-} mice (most likely through compensatory mechanisms involving Sphk2\textsuperscript{110}), the absence of augmented myogenic tone in HFD/STZ-treated Sphk1\textsuperscript{-/-} mice confirms the central role Sphk1 plays in the pathologic mechanism.

Based on the gene knockout models that identify TNF\(\alpha\) and S1P signalling as favourable targets for therapeutic intervention, I tested \textit{in vivo} strategies to correct the HFD/STZ-induced microvascular dysfunction. HFD/STZ-treated mice either received i.p. injections of ETN, an FDA-approved medication (Enbrel\textsuperscript{\textregistered}), or JTE013, an S1PR inhibitor belonging to a group of drugs that are currently in Phase 3 clinical trials\textsuperscript{282}. Remarkably, after 2 weeks of systemic application both ETN and JTE013 fully normalized augmented myogenic responsiveness typically associated with HFD/STZ treatment. The implication of these results has translational potential, as changes in myogenic responsiveness \textit{in vivo} are reversible by pharmacologically targeting TNF\(\alpha\) and S1P signalling.
The idea of repurposing ETN to treat diabetic complications through scavenging of TNFα is not entirely new; previous attempts aimed to correct insulin resistance based on the idea that the latter results from an inflammatory process entertained by TNFα. A handful of small clinical trials have thus targeted TNFα in diabetes. The vast majority of these investigations primarily focus on blood glucose control and insulin sensitivity as clinical endpoints; a minority have indirectly assessed vasodilator responses, via forearm perfusion. Although anti-TNFα therapy successfully lowers inflammatory markers (e.g., C reactive protein), they fail to improve any other defined clinical endpoint. In the context of this thesis, I also report that anti-TNFα therapy fails to improve glucose control (Table 2.2). The microvascular endpoints central to the present investigation, however, have not been systematically assessed in clinical trials: our data establish microvascular reactivity as an early disease marker and encourage its clinical examination.

Although the beneficial effect of an S1PR-blocking agent like JTE013 on microvascular function in diabetes is entirely new, it is not surprising given a number of previous studies documenting a central role of S1P signalling in microvascular physiology and pathophysiology. At present, only one S1PR modulator has been FDA-approved for human application (Fingolimod, trade name Gilenya®), however, several compounds from different pharmacological companies are in final stages of clinical testing. Although the primary use of these new compounds is thought to be for their immunomodulatory effects, our results encourage testing and expansion in their indication to microvascular dysfunction in diabetes.

### 3.4.6 Obesity and the TNFα/S1P signalling axis

In 1993, it was discovered TNFα expression is upregulated in adipose tissue of obese mice. Both adipocytes and adipose-tissue resident immune cells (lymphocytes and macrophages) contribute to elevated circulating TNFα in obesity. Hence, I suspected that TNFα might affect myogenic regulation in an obesity setting. However, data obtained from both global TNFα knockouts and smooth muscle cell-specific TNF knockout mice showed that this inflammatory cytokine does not influence myogenic tone regulation in HFD mice. As the persistent absence of an endogenous gene in a knockout mouse may result in functional compensation that may mask
or misrepresent a myogenic phenotype, I also utilized a pharmacological treatment strategy (ETN) to block TNFα. Myogenic tone in ETN-treated HFD mice was comparable to NC mice treated with ETN. Finally, resistance artery TNFα mRNA expression was not different in HFD mice, suggesting that VSMC TNFα is not elevated in this setting of obesity.

Two studies have investigated the role of TNFα in regulating vasomotor function. In OZR, small coronary arterioles display blunted responses to ACh. This effect appears to be caused by TNFα, as anti-TNFα antibody neutralizing therapy restored ACh-mediated dilation. In small arteries isolated from the visceral fat of obese patients, ACh-induced dilatory responses were absent. Western blot analysis showed significantly higher TNFα expression in these arteries, with immunostaining demonstrating that TNFα is expressed specifically in VSMCs. In vitro blockade of TNFα with Infliximab normalized ACh-induced microvessel dilatory responses. This study suggests that elevated VSMC TNFα regulates endothelial dysfunction in subcutaneous fat arterioles. It is interesting that VSMC TNFα is elevated in VSMCs of obese patients, a finding I anticipated in my obese mice. However this effect may be limited to the vascular bed studied (in this case, resistance arteries that regulate blood flow to adipose tissue).

Total plasma S1P levels are elevated in both obese mouse models (i.e., genetic ob/ob mice and diet-induced obese mice) and in obese (BMI ~30 kg/m²), un-medicated humans. The source of this S1P in obesity is not known. Whether elevated S1P is the result of decreased cellular/tissue S1P degradation is also unclear. I did not observe changes in myogenic tone in HFD mice that lack the S1P-generating enzyme Sphk1. Furthermore, antagonizing S1PR signalling with JTE013 also did not change myogenic tone in HFD mice, compared to JTE013-treated NC mice. S1P levels were not measured in HFD mice tested in this study. I suspect given the knockout and antagonist data that S1P levels were not profoundly altered and this is why myogenic tone is intact in HFD mice.
3.4.7 Correcting hyperglycaemia in HFD/STZ mice

Glycaemic control makes a significant difference in for people with diabetes; the importance of protecting the body from hyperglycaemia cannot be overstated. Both direct and indirect effects of elevated glucose on the vascular system are causative in diabetes-associated morbidity and mortality.

Intensive glycaemic control in patients with diabetes using anti-diabetic medication has been shown to protect against the development of severe macro- and microvascular complications. Clinical trials have reported that intensive glucose lowering in patients with less “advanced” diabetes (i.e., baseline glycated haemoglobin of 8.0% or less, or those without a previous history of cardiovascular disease events) resulted in fewer cardiovascular events. Thus, intensive glycaemic control earlier on in the disease progression may translate into a profound reduction in the future burden of macrovascular and microvascular disease in type 2 diabetes patients.

Alternatively, intensive glucose control is not beneficial for macrovascular disease reduction specifically in older patients with long-standing diabetes that are either at risk, or have, cardiovascular disease. The increase in overall mortality observed in ACCORD and VDAT suggests that aggressive blood glucose control is unsafe and should be avoided in this particular patient group. Individualized glucoregulatory targets based on patients’ entire clinical situation are therefore critical.

In my HFD/STZ model of diabetes, I chose to attempt to lower blood glucose levels using the medication metformin, as it is widely used as a first-line therapy to treat type 2 diabetes. Furthermore, metformin is presently being prescribed to at least 120 million people worldwide.

3.4.7.1 Metformin was ineffective at lowering blood glucose in HFD/STZ mice

To my surprise, metformin therapy in HFD/STZ mice did not result in a lowering of blood glucose or HbA1c levels. The reason(s) for this finding are unclear. Metformin is described as an insulin-sensitizing biguanide and is clinically shown to reduce fasting glucose concentrations by 2.78-3.9 mmol/L, correspondingly reducing HbA1c by 1.3-2%. Metformin efficacy is
independent of age, body weight, ethnicity, diabetes duration and insulin and C-peptide levels\textsuperscript{292,293}. Metformin is not protein bound and widely distributes throughout the body, with maximal accumulation in the small intestine wall\textsuperscript{294}, ultimately getting excreted unchanged by the kidney\textsuperscript{295}.

The glucose lowering effect of metformin in diabetic patients is through reduced hepatic glucose output (i.e., inhibition of gluconeogenesis) and elevated insulin-stimulated glucose uptake in adipose and skeletal muscle\textsuperscript{291}. Metformin does not stimulate production of insulin\textsuperscript{296}. The exact mechanisms by which metformin inhibits liver glucose production are presently unclear, but appear to involve the disruption of mitochondrial respiratory chain oxidation of complex I substrates in hepatocytes\textsuperscript{297,298}.

One obvious possibility for the lack of a metformin effect on blood glucose could be the dosage of metformin given to HFD/STZ mice as a reason for its ineffectiveness. The amount of metformin used in our mice was 450 mg metformin/kg of food and was chosen based on past efficacy\textsuperscript{229}. This works out to a dose of \textasciitilde 0.054 mg metformin/g of mouse body weight. Diabetes patients receive on average 2000 mg metformin/day\textsuperscript{299}, translating to dose of approximately 0.04 mg metformin/g of patient body weight. Therefore, our mice received an even higher dose than what is normally used in the clinic and I conclude that the dosage of metformin is likely not the reason that it was unsuccessful at reducing hyperglycaemia.

\textbf{3.4.7.2 Metformin as a “vasculoprotective” agent}

\textit{In vivo} metformin treatment lowered myogenic tone in both mesenteric and olfactory resistance arteries isolated from HFD/STZ mice. This result is consistent with data obtained from the hyperglycaemic and hyperinsulinemic GK rat that had a reduction in augmented myogenic tone following metformin administration\textsuperscript{177}. However, metformin also reduced hyperglycaemia in GK rats, allowing the authors to suggest that glucose lowering caused the normalization of myogenic tone. Unfortunately, as metformin did not lower blood glucose in HFD/STZ mice, my experiments could not answer the question of whether treating glucose levels in diabetes would restore augmented resistance artery tone to normal.

Nevertheless, tone was normalized in HFD/STZ olfactory arteries following metformin treatment, and noticeably blunted in all mesenteric arteries, including those isolated from NC
control mice. As this finding was rather surprising, I followed up these experiments by adding low and high dose metformin to the bath of isolated mesenteric resistance arteries from NC mice to examine the outcome on vasomotor responses. A 30-minute incubation with metformin did not change myogenic tone or constriction to phenylephrine in mesenteric arteries. How is metformin then affecting the vasculature, specifically in vivo in the absence of lowering blood glucose, to result in these unpredicted findings?

Metformin has been described as a “cardioprotective” anti-diabetic therapy. This designation is the result of data from the UKPDS clinical trial that reported a risk reduction of 32% in any diabetes-related endpoint (including macro and microvascular complications), 39% for myocardial infarction, 42% for diabetes-related death and 36% for all cause mortality in patients treatment with metformin vs. other glucose-lowering agents (chlorpropamide, glibenclamide, insulin). Interestingly, HbA1C levels in diabetic patients treated with metformin were reduced to values equivalent to patients treated with other anti-diabetic therapies, suggesting that metformin has additional cardioprotective actions beyond its glucose lowering ability.

The mechanisms for this cardioprotection continue to be elucidated in both preclinical animal models and clinical trials and it is possible that metformin is providing potential vasculoprotective effects in our HFD/STZ mice to prevent the development of elevated myogenic tone.

### 3.4.7.3 Potential vasculoprotective mechanisms of metformin

#### 3.4.7.3.1 Metformin and its relationship to the TNFα/S1P myogenic tone mediating mechanism

There are no reports currently available in the literature connecting metformin and the various elements of the vasoconstriction-promoting S1P pathway (i.e., sphingosine, Sphk1, SPP1, S1PRs). The potential molecular mechanism by which for metformin may modulate S1P to affect myogenic tone in HFD/STZ mice is entirely unknown.

Another possible explanation for the tone-lowering effect of metformin therapy may be via a relationship between metformin and TNFα. However, presently available data has yielded results
showing no connection between the drug and the cytokine. In patients with impaired glucose tolerance, 1000 mg twice a day metformin does not change plasma levels of TNFα from the recorded levels prior to treatment\(^{305}\). In recently diagnosed type 2 diabetes patients (median time from diagnosis 2 years), treatment with metformin (2000 mg/day for 14 weeks) lowers glucose but does not reduce soluble TNFR2 levels from baseline measures\(^{306}\). In order to avoid any confounding influence of metformin-induced blood glucose regulation in the setting of diabetes, Carlsen \textit{et al.} \(^{307}\) determined if treatment of non-diabetic patients with metformin was associated with changes in TNFα. Sixty men with coronary heart disease were given metformin for twelve weeks (doses starting at 500 mg/day, increasing up to 2000 mg/day, mean 1749 mg/day by week 12). As a result, levels of TNFα are either unchanged in obese subjects, or surprisingly, increased, specifically in non-obese subjects. The authors concluded that metformin does not exert its action on insulin sensitivity through a reduction in circulating TNFα levels. Hence, it is not likely that metformin lowered myogenic tone by interfering with VSMC a TNFα-dependent mechanism.

\textbf{3.4.7.3.2 Metformin and AMPK activation in vascular smooth muscle cells}

One hypothesis by which metformin may have affected myogenic tone in HFD/STZ mice is through activation of the enzyme 5’ adenosine monophosphate-activated protein kinase (AMPK). Metformin is a known activator of AMPK\(^{308}\). AMPK is a serine/threonine kinase that acts as a “fuel gage”, monitoring cellular energy status and mediating cellular energy homeostasis. AMPK activation occurs as a result of increases in the intracellular AMP/ATP ratio due to an imbalance in ATP production and consumption, switching cells from an anabolic to a catabolic state to restore the energy balance. Glucose, lipid and protein synthesis and cell growth are limited and fatty acid and glucose uptake are promoted. It is postulated that metformin does not directly interact with AMPK, but rather AMPK activation is secondary to metformin’s effect on mitochondrial respiration\(^{308}\).

AMPK is found in VSMCs but its function remains poorly understood. However, AMPK activation in these cells has been associated with vasorelaxation. 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR)-mediated AMPK activation induces mouse aortic vessel
relaxation independent of NO\textsuperscript{309} and attenuates mouse smooth muscle contraction\textsuperscript{310}. Metformin itself also induced arterial relaxation and this was seen in vessels treated with a NOS inhibitor, implying a direct effect of AMPK on VSMCs\textsuperscript{311}. The downstream consequences of VSMC AMPK activation have been identified as a direct phosphorylation of MLCK by AMPK, causing MLCK inactivation and thus attenuated contraction\textsuperscript{310}. Our results of unchanged responses to phenylephrine in both \textit{in vivo} and \textit{in vitro} metformin treatment also support the idea that NO is likely not involved in vasorelaxation in resistance arteries. Whether an AMPK mechanism is at work in our HFD/STZ mice requires further study beyond the scope of this thesis work, but provides a hypothesis as to how metformin reduced myogenic tone independent of glucose regulation.

In summary, I report here on a new vasoregulatory effect of metformin, likely involving AMPK in resistance arteries. These findings may indeed contribute to our mechanistic understanding of how cardioprotective metformin “serves to protect the heart”\textsuperscript{312} and improves cardiovascular outcomes in patients with diabetes beyond its ability to ameliorate blood glucose.
3.5 CONCLUSION

In conclusion, I demonstrate in this chapter a pronounced augmentation of resistance artery myogenic responsiveness in a mouse model of diabetes. This model reveals that changes to vascular reactivity are caused by activation of TNFα / Sphk1 / S1PR signalling (summarized in Figure 3.18), making all of the molecular elements in this biochemical pathway potential new therapeutic targets for the prevention of vascular complications associated with diabetes.

I hypothesize that diabetes stimulates the expression of vascular smooth muscle cell (VSMC) TNFα through undefined mechanisms; I propose that VSMC TNFα, through the TNF receptor (TNFR) then activates sphingosine kinase 1 (Sphk1) by an autocrine/paracrine mechanism. Sphk1 activation leads to the synthesis of sphingosine-1-phosphate (S1P), which acts by an autocrine/paracrine mechanism to ultimately bind the S1P receptor (S1PR). S1P receptor activation augments microvascular tone by well-documented mechanisms. A limitation to the above-suggested pathway is that this thesis did not provide evidence of the direct activation of Sphk1/S1P by TNFα. Thus it remains to be determined whether the activation of the TNFα/Sphk1/S1P signalling network occurs in the sequential order as I propose, or whether activation of the TNFα signalling pathway and the Sphk1/S1P signalling network occurs separately but in parallel to the other.

Figure 3.18 Proposed mechanistic model of TNFα/S1P signalling in the augmentation of myogenic tone in diabetes.
CHAPTER 4. GENERAL DISCUSSION, LIMITATIONS AND FUTURE DIRECTIONS
4.1 DISCUSSION

4.1.1 Significance

Diabetes has reached global epidemic proportions. In Canada alone, approximately 2.5 million individuals are currently living with diabetes and an estimated 6 million more are in a “pre-diabetic” state according to 2010 statistics. A rising occurrence in obesity, inactive lifestyles and an aging population are expected to increase the incidence of diabetes to 10% of the total population (~3.7 million) by the year 2020. Additionally, diabetes treatment and care is causing a financial crisis for the Canadian healthcare system. The economic cost associated with diabetes is currently reported as 12.2 billion annually, and is expected to grow to at least 16.6 billion by 2020.

Diabetes associates with numerous vascular complications that contribute to the severity of this burden around the world. This thesis directly addressed this problem by seeking to make significant, original contributions in terms of knowledge, mechanistic insight and identification of therapeutic approaches for the treatment of diabetic vascular disease.

Compared with our present understanding of diabetic vascular complications, this thesis has identified novel key mediators of vascular myogenic tone in a murine model of diabetes. My studies sought to answer a variety of important questions posed in the introduction with respect to vascular function in the setting of diabetes.

4.1.2 Findings that address introductory questions and hypotheses

A key component to cardiovascular physiology in health is the development of vascular tone, or the degree of constriction experienced by blood vessels relative to the maximally dilated state. Resistance arteries located at the terminal portion of the vascular tree primarily regulate tone. The myogenic response is an intrinsic property of resistance artery VSMCs: this mechanism rapidly adjusts vascular resistance via alterations in diameter in response to changes in input pressure, thereby maintain constant tissue perfusion over a wide range of systemic blood pressures. Recognizing the critical function of resistance artery myogenic tone for control of
blood flow during altered tissue demands speaks to the relevance of studying resistance arteries in the context of diabetes.

Thus, my initial inquiry in this thesis was, “what is the myogenic phenotype of diabetic HFD/STZ mice, an established model of type 2 diabetes?” I hypothesized that myogenic tone was augmented in my model, the HFD/STZ mouse, based on literature suggesting resistance arteries play an important role in the aetiology of disease and previous work from our laboratory that reported enhanced myogenic tone in the setting of other pathologies that associate with inflammation (e.g., heart failure and SAH).

Chapter 3 contains a response to this question. The HFD/STZ model utilized in these studies was purposefully titrated so that hyperglycaemia was mild. After four months of high fat feeding and three months post-STZ injection, resistance arteries isolated from two distinct vascular beds from HFD/STZ mice displayed augmented myogenic tone. The pro-constrictive phenotype across the microvascular system occurred in the absence of hypertension, endothelial dysfunction and markedly high HbA1C, and appeared to be progressive in nature, as myogenic tone was not elevated in HFD/STZ mice after two months of high fat diet and one-month post-STZ.

Since the myogenic response is altered early on in the diabetes disease progression, it stands to reason that with worsening blood sugar, the ability of resistance arteries to appropriately regulate vascular tone and tissue perfusion would be at risk. Thus, there is a high likelihood that myogenic tone dysregulation is involved in the progression and worsening of complications. In line with this hypothesis, although myogenic vasoconstriction was enhanced in one of the two tested cerebral arteries, this effect did not translate into changes in global cerebral blood flow. This functional heterogeneity between microarteries of the cerebral circulation provides a reasonable explanation for why global cerebral blood flow does not change at this time point: the microvascular effects are localized and likely, compensated by collateral blood supply.
The consequences of elevated constriction in response to a pressure stimulus on the regulation of total peripheral resistance are alarming, as Poiseulle’s law tells us that even minute changes in vessel radii significantly impact blood flow to organs. Therefore, understanding the molecular signalling behind this effect is of great importance, particularly so that we assist in the development of treatments that target dysfunction at this early time point creating preventative therapy.

The molecular mechanisms directing myogenic tone *in vivo* are remarkably multifarious. Mechanisms can vary according to the species, strain, vascular bed and artery branch order. Furthermore, signalling may act cooperatively or exclusively, and may operate in series or parallel. This complexity is well documented, as the lack or dysfunction of one or more elements of the myogenic response can be compensated for in genetic deletion, chemical inhibition or disease models. Our laboratory has contributed to the understanding of the myogenic response by focusing on the role that the sphingolipid metabolite S1P plays in its regulation. We have shown that S1P, along with Sphk1 (a S1P-synthesizing enzyme) and the cytokine TNFα, regulate and coordinate both Ca\textsuperscript{2+}-dependent and -independent signalling mechanisms, which all together drive the myogenic response\textsuperscript{70,80,98,158-163,166,250}.

Chapter 3 addresses the following question posed in the introduction of this thesis, “*do TNFα and/or S1P contribute to the induction of myogenic tone dysfunction in diabetes?*”

I hypothesized that the increase in myogenic tone in HFD/STZ mice is mediated by activation of Sphk1, and therefore S1P, acting upon its S1PR. This hypothesis is based on the potential link between diabetes and TNFα specifically, a known activator of the S1P signalling pathway.

It was necessary to explore multiple loss-of-function models (i.e., both genetic and pharmacological) to determine the involvement of TNFα and/or S1P in the dysregulation of myogenic tone in HFD/STZ mice. In this regard, I took advantage of the global TNFα and Sphk1 knockout mice and conditional knockout mice lacking smooth muscle cell TNFα. Remarkably, in all mouse animal knockout models rendered diabetic using the HFD/STZ protocol, myogenic tone was normalized. The VSMC-specific knockout further provided evidence of the involvement of VSMC-generated TNFα, a finding that differs from the literature.
indicating TNFα mediates its vascular effects via the endothelium\textsuperscript{45,46}. These results are supported by my data showing elevated TNFα mRNA expression in isolated resistance arteries and \textit{in vitro} blockade of TNFα in isolated HFD/STZ mouse arteries abolishes myogenic tone. Taken together, these experiments indicate a fundamental role for TNFα and S1P signalling and reversibility of the HFD/STZ-mediated enhancement of myogenic tone.

From a clinically relevant perspective, I looked to test strategies that pharmacologically blocked S1P and TNFα activity in HFD/STZ mice in an attempt to alleviate aberrant myogenic tone. To accomplish this task, I used the clinically available anti-TNFα medication etanercept (ETN; Enbrel\textsuperscript{®}) and the S1PR antagonist JTE013. JTE013 is not approved for clinical use, but has been used \textit{in vivo} without overt toxicity and belongs to a class of medications that is currently under development for clinical application (i.e., they are all patent-protected entities)\textsuperscript{282}. Here I chose not to apply a ‘prevention’ strategy, since in the clinic TNFα and S1P signalling antagonists would not likely be administered prior to the onset of diabetes or without indication.

In corroboration with my genetic knock out data, disruption of TNFα and S1P signalling in HFD/STZ mice also normalized myogenic tone, demonstrating the value of this pathway was a therapeutic target and the exciting potential for the translational capacity of this research.

There are well-recognized challenges in translating success at the bench in preclinical experiments into success at the bedside. Although the new discoveries made in this thesis may hold the potential to improve health in people with diabetes in a meaningful way, this will undoubtedly take a considerable amount of time.

Therefore, what can be done in the interim for diabetes patients dealing with vascular dysfunction using the tools we already have approved? This question leads nicely into another query asked in this thesis, \textit{“if hyperglycaemia is normalized in diabetes, will this prevent vascular dysfunction?”} We have an answer to this question from numerous landmark clinical trials indicating that hyperglycaemia appears to be the primary determinant of the vascular-based pathologies observed in diabetes (e.g., retinopathy, neuropathy and nephropathy), as intensive glucose control prevents or significantly limits the progression of microvascular complications\textsuperscript{19,20}. Therefore, I \textbf{hypothesized} that augmented myogenic tone in HFD/STZ mice
is ultimately the result of abnormal circulating levels of blood glucose, and upon achievement of euglycaemia with anti-diabetic therapy, myogenic tone will be comparable to normoglycaemic controls. The tools available to lower blood glucose are numerous, and I deliberately chose to use of metformin given its cardioprotective nature and widespread clinical use in diabetes patients. Although the very obvious failure of metformin to normalize blood glucose in HFD/STZ mice limits my ability to conclusively understand the role of glucose in the development of enhanced myogenic tone, I discovered a previously unreported mechanism of myogenic regulation due to metformin treatment. I believe metformin’s biological target AMPK mediates the lowering of tone by metformin in HFD/STZ mice. However, I presently do not have more direct data to substantiate AMPK activation in myogenic responsiveness and there is quite a bit further left to do to characterize this potentially important mechanism that is beyond the scope of this thesis.

The remaining question posed in this thesis was “does the presence of obesity alone have an effect on myogenic regulation?” The analyses to answer this inquiry were conducted using my mouse model, specifically the high fat diet mice. These mice are valuable as both a control for experiments utilizing HFD/STZ mice and in and of themselves. High fat fed rodents have been used widely in the literature to understand the variety of consequences of increased fat mass. Worldwide obesity has doubled since 1980 and we are beginning to understand the magnitude of this effect on public heath. I hypothesized that obesity, a comorbidity frequently associated with diabetes, may further exacerbate disrupted myogenic tone. I also expected a mechanistic connection between increased adipose tissue and TNFα/S1P given studies suggesting this, qualifying my consideration for the involvement of this pathway in obesity.

My HFD mice were unexpectedly normoglycaemic and appeared to have no difficulty in clearing plasma glucose following an oral glucose challenge. These mice also had no phenotypic abnormalities with respect to any of the genetic or pharmacological interventions utilized to delineate the myogenic mechanism in a model of obesity. Four months of high fat feeding in TNFα−/− and Sphk1−/− knockout mice did not change myogenic or vasomotor responses compared to normal chow controls. Isolated HFD mouse resistance arteries did not have higher mRNA expression of TNFα. Additionally, pharmacological blockade of TNFα and S1P signalling left myogenic tone in HFD mice unaltered. Furthermore, HFD mice were not hypertensive and had
statistically unchanged endothelial function in resistance arteries from two distinct vascular beds. Taken together, these data suggest that the S1P/TNFα-mediated myogenic response may not be a direct target of increased adiposity, and further allows for the conclusion that elevated glucose levels are critical in the development of vascular dysfunction.
4.2 LIMITATIONS

4.2.1 The HFD/STZ model

One clear limitation in interpretation of these studies pertains to the diabetes mouse model utilized. In addition to glucose intolerance, the mice were also challenged by the increased weight gain and fat deposition resulting in numerous other biochemical alterations. However, visceral obesity is frequently present in diabetes patients. Therefore, I felt that it would be most clinically relevant to examine molecular mechanisms in an animal model that also possesses the complexities of this multi-faceted disease.

4.2.1.1 Potential confounding factors from increased fat deposition

Excessive adipose presents a potential source of many cytokines that may influence myogenic tone beyond TNFα, such as interleukin-1β, AGE product formation and prostaglandins. These molecules, and potentially others (either not listed or yet to be discovered) are candidates for the mediation of vascular tone in the setting of diabetes. However, I chose to study TNFα, as: (i) it is known to be elevated in diabetes and is an important cause of insulin resistance in obesity and diabetes, (ii) TNFα is a well-characterized activator of Sphk1 and thereby, the S1P pathway, and (iii) TNFα can be sequestered using the FDA-approved drug etanercept (ETN), representing the potential for therapeutic translation.

The inclusion of the HFD control mice not given STZ in my study was done in order to help address any confounding findings as a result of the lipid-rich diet. Interestingly, although HFD mice are significantly heavier than normal chow controls, their myogenic and vasoconstrictory responses are not different. In my hands, HbA1C levels in the HFD-only mice are also similar to those in the NC control mice, and significantly lower as compared to HFD/STZ mice. The same trend is observed following an oral glucose tolerance test, where HFD mice are able to clear glucose as well as NC mice. These data suggest that although adiposity may result in the generation of potential mediators of myogenic tone in mice, I do not see any effect of high-fat feeding on the myogenic responses of these animals with my protocol.
4.2.1.2 The role of glucose

I attempted to ascertain glucose-induced vascular modifications in my animals by using anti-diabetic therapy. Metformin treatment ultimately did not lower blood glucose and had interesting, non-glucose-dependent effects on microvascular function in all mice tested (i.e., NC, HFD and HFD/STZ mice), particularly in the mesenteric vascular bed. I acknowledge that metformin has pleiotropic effects, including antihypertensive, anti-inflammatory, vasculoprotective, and lipid lowering properties. While the literature suggests a potential effect of AMPK activation may be at play in my experimental setting, I have not provided more direct proof. Interestingly, in a rat model of diabetes (GK rats), metformin normalizes blood glucose and normalizes diabetes-related increases in myogenic tone. This effect was independent of metformin’s potential to modify blood pressure or blood lipid levels, as these rats were neither hyperlipidemic nor hypertensive, allowing for the opportunity to determine the effects of hyperglycaemia alone. Moreover, a study from Caballero et al. demonstrated that levels of TNFα remain unchanged following metformin treatment in human subjects with impaired glucose tolerance, indicating that TNFα-driven S1P activation is likely not affected in metformin treated mice. These studies reinforce the suitability of this candidate as anti-diabetic therapy for my study to understand the role of elevated glucose in HFD/STZ mice. Nevertheless, without the normalization of blood sugar levels, the contribution of glucose to myogenic tone dysregulation in this setting remains elusive. Moreover, based on HFD-only control data, I can infer that the observed changes in myogenic tone are dependent on increased blood glucose in my mouse model of diabetes. The direct contribution of glucose to the microvascular phenotype observed in HFD/STZ mice remains unknown.

A different diabetes model, such as a C57BL/6 mouse injected with STZ, may have helped delineate a more exact contributory role of elevated plasma glucose in aberrant myogenic tone. This model would have removed the confounding HFD treatment that complicates the interpretation of my results, as hyperglycaemia is not the only variable at play. Utilizing a different anti-diabetic therapy such as insulin also would have helped achieve euglycaemia without the differential effects on other tissues seen with drugs like metformin. I am limited, however, in that an STZ-treated mouse represents an entirely different and unique model of diabetes, that of chemically induced type 1 diabetes. Including this control group complicates my interpretations of the data with respect to the type of diabetes represented by my mouse models.
and ultimate applicability to a clinical setting. Additionally, insulin therapy is not without a vascular effect (insulin has direct vasodilatory effects through nitric oxide\textsuperscript{327}) and thus may also confound the interpretation of my results.

Finally, I also recognize that to fully understand the effects of hyperglycaemia on VSMCs, I must respect the heterogeneity of the smooth muscle cell phenotype that depends on the artery and location in the body in which they are located. A more interesting aspect of diabetes complications is the widely differential impact hyperglycaemia has depending on the vascular bed. For example, in diabetic retinopathy high glucose stimulates problematic angiogenesis ultimately impairing vision\textsuperscript{328}, whereas in the peripheral circulation, impaired vascular responses may result in limb amputation\textsuperscript{24}. Therefore, suggesting that any one therapy may have a global positive impact with respect to every diabetes-associated complication is highly unrealistic. Thus the consequences of the treatment modalities suggested in this thesis on each vascular bed in diabetes would need to be carefully addressed in preclinical investigations prior to moving forward to any clinical trials.

To speak to this consideration, an exciting discovery has come out of laboratory as a result of the PhD thesis successfully defended last year by my colleague Dr. Sonya Hui. Dr. Hui demonstrated for the first time that pressure-stimulated myogenic vasoconstriction is augmented in skeletal muscle resistance arteries isolated from cardiac surgery patients with type 2 diabetes, relative to arteries isolated from cardiac surgery patients without diabetes (Appendix Figure 1B), without obvious differences in either active or passive diameters (Appendix Figure 1A). PE responses (Appendix Figure 1C) were not different in these arteries and the mean HbA\textsubscript{1C} level in the diabetes patient cohort was 7.2±0.3% (range: 5.1 to 9.7%; n=15) (see Appendix 2 Methods). Given the inherent variability in patient characteristics, comorbidities and medications, the clear separation of the myogenic tone curves points to a highly consistent pathologic effect in diabetic patients: I therefore conclude that the myogenic tone augmentation in HFD/STZ is genuine, albeit derived from small differences in the active and passive diameters. The importance of this observation lies in the key role skeletal muscle resistance arteries play as regulators of blood pressure and blood distribution (e.g., in response to exercise): augmented myogenic tone in this vascular bed should aggravate diabetes-associated hypertension and deficits in tissue perfusion. Remarkably, the microvascular phenotype materializes despite reasonable blood glucose control. The diabetes patient cohort had a mean HbA\textsubscript{1C} level of 7%: although this level is elevated
compared to the 4-6% range found in non-diabetic patients, it reasonably meets current therapeutic targets and may not prompt further intervention. Consequently, this study speaks to the clinical relevance of what was observed in HFD/STZ mice and advocates establishing complementary therapies that strategically target the microcirculation.

4.2.2 The pressure myography technique

Another limitation in this thesis is related to the restrictions of pressure myography. Pressure myography is an incredibly useful technique for the functional assessment of small arteries, pressurized to a suitable transmural pressure. The set up is near physiological and allows for characterization of the intrinsic vessel response to physiological or pharmacological stimuli. However, it is not without some unique limitations. Pressure myography systems are expensive, with commercially available setups including all of the components shown in Figure 2.3 costing over $40,000 US. Furthermore, there is a lack of scalability using this system, one can only cannulated and test one artery at a time. Isolated resistance arteries are rarely less than 60 µm in diameter, as anything smaller is nearly impossible to cannulate and tie onto glass pipettes without risk of damage to the vessel. This limits our choices in vascular beds that we are feasibly able to study and also limits our ability to study the smaller, more distal regions of the vascular tree. Mesenteric arteries have been widely utilized to understand resistance artery function due to their ease of isolation and cannulation despite their small size. However, I recognize that the mesenteric vessel bed may be of limited clinical relevance with regards to diabetic complications. This is why I chose to also isolate olfactory and posterior cerebral arteries, given the association of diabetes with cognitive dysfunction and stroke.

4.2.2.1 Understanding the consequences of myogenic response dysregulation in vivo using in vitro experimental techniques

Schubert and Mulvany highlight a very important fact with respect to investigations of myogenic tone: our understanding of myogenic mechanisms comes almost entirely from in vitro isometric or isobaric microvessel preparations. The usefulness of the pressure myography technique is not to be underestimated, but microvessel function in vivo is undoubtedly complex, limiting our ability to interpret our findings in the context of whole body physiology.
This is particularly true in my thesis; it has been difficult to understand the significance of these dramatic changes in myogenic tone, as HFD/STZ mice do not display an overt vascular phenotype. Olfactory arteries of HFD/STZ mice display a marked augmentation of tone; I would have anticipated this would dramatically affect brain blood flow. However, MRI flow measures were unable to detect differences in the cerebral blood flow of HFD/STZ mice brains. Upon speaking with my collaborator from Spatio-Temporal Targeting and Amplification of Radiation Response (STTARR) program, Dr. Warren D. Foltz, it may be that the MRI does not have the capacity to investigate flow in the anterior location of the brain where the olfactory artery is located. We discussed the potential for the 7 Tesla micro-MRI system to measure volumetric flow in the olfactory artery itself, but this would require two different techniques including FAIR and Dr. Foltz would need to invest in protocol development. A more simplistic experiment to assess the consequences of elevated myogenic tone in the HFD/STZ mouse cerebrovasculature may be the measurement of cognitive decline through behavioural testing for learning and memory, such as the Morris water maze test\textsuperscript{330}.

Nevertheless, it remains to be determined if the augmentation of myogenic tone in HFD/STZ mice results in diabetes-associated pathologies. The study of a more relevant vascular network beyond the brain, such as retinal or kidney microarteries may provide greater insight into the clinical significance of myogenic dysregulation in the development of diabetes complications. Unfortunately, available mouse models of diabetes generally lack the advanced clinical features of microvascular complications. Rodents do not progress to the advanced stages of renal disease, lack overt degenerative neuropathy and do not display the pre-retinal neovascularization seen in humans\textsuperscript{24}. It remains to be determined if the development of innovative mouse strains that closely mimic the human complications of diabetes through efforts such as the National Institute of Diabetes and Digestive and Kidney Diseases-sponsored Diabetic Complications Consortium may prove to be more suitable preclinical models for these types of studies.

4.2.3 Mouse control groups
In some of the experimental groups, certain control mice are knowingly not included. These groups consist of: a) an STZ-treatment alone as a control for the NC, HFD and HFD/STZ experimental cohort, b) wild-type littermate NC, HFD and HFD/STZ control mice for the TNF\textsubscript{α} and Sphk1 knockout experimental cohorts, c) vehicle injected NC, HFD and HFD/STZ mice to
control for the ETN and JTE013 injections, and d) vehicle injected conditional (tamoxifen-inducible), smooth muscle cell-specific (SMMHC promoter) NC, HFD and HFD/STZ TNFα knockout mice. While I understand the importance of each of these experimental control groups, the main reason why they were not included here is that the number of animals, the time and the cost associated with these groups is not justified from an experimenter standpoint and thus the lack of these groups in this thesis remains a limitation in terms of interpretation of the data.

4.2.4 **Application of the treatment modalities proposed to patients with diabetes**

While it is encouraging that each of the treatment modalities proposed in this thesis to ameliorate myogenic tone in a model of diabetes had successful outcomes, I understand the limitations to drug development. The benefits of etanercept therapy lie in its clinical approval, making it a candidate for drug repositioning due to reduced risk of toxicology, reduced time for validation of a new indication in the clinic and well-characterized pharmacokinetics. Yet, the repurposing of etanercept does not necessarily have an increased chance of its success as a therapeutic in a secondary use. Five small trials have demonstrated that anti-TNFα therapy (either with etanercept or other fusion proteins and neutralizing antibodies) does not improve insulin sensitivity in obese, insulin-resistant patients and patients with T2DM. It must be stressed, however, that the effects of anti-TNFα therapy on the endpoints of microvascular function and cerebral perfusion in diabetes patients have never been systematically addressed. Although there are indications that cautious monitoring must be undertaken once anti-TNFα therapy is initiated in T2DM patients (in rare cases, hypoglycaemia has resulted), the treatment regimen appears to be well-tolerated and thus, would potentially represent a viable clinical option for these separate endpoints.

Unfortunately, there are no data available on JTE013 from clinical studies. Developed by Central Pharmaceutical Research Institute in Osaka, Japan, JTE013 is the most utilized S1P2R antagonist. Although the beneficial effect of an S1PR-blocking agent like JTE013 on microvascular function in diabetes is entirely new, it is not surprising given a number of previous studies documenting a central role of S1P signalling in microvascular physiology and pathophysiology. At present, no S1PR inhibitors have been approved for human application, however, several compounds from different pharmacological companies are in final stages of clinical testing. Although the primary use of these new compounds is thought to be
their immunomodulatory effects, our results encourage testing and possibly expanding their indication to microvascular dysfunction in diabetes.
4.3 FUTURE DIRECTIONS

4.3.1 The complexity of myogenic mechanisms

Our present understanding of the complexities of the myogenic response consists of a mix of elements candidly defined by Schubert and Mulvany as “established facts and attractive hypotheses”69. The myogenic response can be simplistically described as the conversion of an extracellular and primarily mechanical stimulus (transmural pressure) into an intracellular biochemical signal; however, recent reviews attest to its enormous complexity68,70,250,336. The potential for diabetes to interrupt this pathway at any point is a possibility. Certainly, there are other molecules that may also directly target the molecule focused on in this thesis, S1P. Beyond its role in the myogenic response, S1P signalling is complex and redundant, complicating the assignment of specific functions to specific receptors. S1P also acts as both an intra- and extracellular molecule, and S1PRs have the ability to crosstalk with other signalling pathways. All of these complications make it difficult to ascertain the exact mechanism by which S1P mediates constriction in the vascular smooth muscle cell and this remains an active area of research in our laboratory.

There is a number of remaining molecular-based experiments that require completion to further support my conclusion of the involvement of the VSMC TNFα/S1P signaling mechanism in myogenic tone in diabetes. It would be of interest to ascertain VSMC TNFα protein expression levels. I have attempted these experiments and had difficulty validating commercially available antibodies using a Western blot technique. However, the potential to utilize immunohistochemistry to measure TNFα in the wall of VSMCs isolated from HFD/STZ remains, as our laboratory has utilized collaborators in the past to complete these experiments162. The exact nature of the interaction between TNFα and S1P in myogenic tone regulation remains unidentified. Based on submitted data from Dr. Kenji Yagi using a mouse model of subarachnoid haemorrhage, our laboratory has identified a key role for the TNFR1 in the mediation of increased myogenic tone166. Given the mechanistic consistency in myogenic tone signalling between different pathologies associated with TNFα (i.e., heart failure, subarachnoid haemorrhage and hearing loss), I expect that TNFR1 may also be at play in HFD/STZ mice. The use of a TNFR1 knockout mouse (commercially available from Jackson Laboratory) or a TNFR1 antagonist337 would be valuable for future experimental work. Furthermore, elegant studies have
identified a motif on Sphk1 that binds the TNF receptor-associated factor 2 adaptor protein (TRAF2) and results in stimulation of Sphk1 activity\textsuperscript{338}. Additionally, S1P was found to be a missing cofactor required for the E3 ligase activity of TRAF2 and subsequently NF\kappa B activation\textsuperscript{339}. It would be interesting to see if any of these known pathways are involved in the activation of S1P/Sphk1 by TNF\alpha in vascular smooth muscle cell myogenic responses. Finally, measuring microvessel and plasma expression of S1P would add to my study. S1P can be measured in plasma using a commercially available competitive ELISA designed for plasma/serum determination of S1P levels (echelon, Salt Lake City, UT, USA)\textsuperscript{254}. Our laboratory maintains a collaboration with Eli Lilly in Indianapolis, IN, USA, as they have developed the technology to measure tissue S1P expression using liquid chromatography electrospray ionization tandem mass spectrometry (LC/ESI/MS/MS)\textsuperscript{340}. Microartery samples from a cohort of NC, HFD and HFD/STZ mice were sent to Indianapolis but were never received by Eli Lilly and thus these studies require repeating. Alternatively, measuring Sphk1 activity using a commercially available assay (also from echelon) that quantitates the remaining ATP levels in solution following the sphingosine kinase reaction\textsuperscript{341}, may be useful.

### 4.3.2 The specificity of JTE013 for S1P\textsubscript{2}R

The involvement of S1P\textsubscript{2}R in regulating myogenic tone in HFD/STZ mice remains to be investigated more conclusively.

In 2008, Salomon et al.\textsuperscript{342} reported that JTE013 does not appear to be selective. JTE013 was able to inhibit S1P-induced basilar artery constriction, an effect that remained present in the S1P\textsubscript{2}R\textsuperscript{-/-} mice. Therefore, it is in my best interest to add to my studies utilizing JTE013 by rendering S1P\textsubscript{2}R\textsuperscript{-/-} mice diabetic using the HFD/STZ protocol and measuring myogenic tone in mesenteric and olfactory arteries. A limitation I foresee with these experiments relates to the susceptibility of S1P\textsubscript{2}R\textsuperscript{-/-} mice to STZ-induced beta cell apoptosis and diabetes incidence, as S1P\textsubscript{2}R\textsuperscript{-/-} mice appear to be resistant to the development of diabetes\textsuperscript{343}. Given the consistency in blood glucose levels between my HFD/STZ mice no matter the genotype, it may be difficult then to compare HFD/STZ S1P\textsubscript{2}R\textsuperscript{-/-} mice to other mice if blood glucose levels are different or if more STZ is required to achieve hyperglycaemia. Nevertheless, experiments with S1P\textsubscript{2}R\textsuperscript{-/-} mice
remain important in order to further delineate the S1PR involved in regulating myogenic tone HFD/STZ mice. Our laboratory has developed a working protocol to measure S1P$_2$R mRNA expression in microarteries and it may be valuable to ascertain if expression levels are different in HFD/STZ mice.

4.3.3 Addressing the consequence of glucose in the vascular dysfunction of HFD/STZ mice

In order to add to the present study without developing and characterizing a second diabetic mouse model, I propose to use techniques established in our laboratory that will allow for information about the consequences of elevated glucose in isolated resistance arteries in vitro. We have well-defined techniques for incubation of arteries in the presence of elevated glucose, either acutely or for up to 48 hours in culture\. We could use either vessels from NC controls or microarteries from HFD/STZ mice to see if myogenic tone increases further. Given the relevance of microvascular complications in patients with type 1 diabetes, follow up studies could be conducted in mice injected with STZ alone. Not only would this group of animals better address the consequences of high glucose alone on the vasculature, but also may provide valuable diabetic complication treatment strategies applicable to those with type 1 diabetes as well.

4.3.4 The potential role of CFTR

I have one more avenue by which I can investigate and manipulate myogenic tone in the setting of diabetes – through the regulation of cystic fibrosis transmembrane receptor (CFTR). CFTR is essential for S1P uptake into VSMCs, a prerequisite for the degradation of S1P by the intracellular enzyme SPP1. Relevant to this thesis work, CFTR is transcriptionally and posttranscriptionally regulated by TNFα. Modulation of CFTR function by TNFα can enhance the bioavailability of S1P. Should diabetes modulate CFTR activity through TNFα-mediated mechanisms (e.g., via downregulation or inactivation), extracellular levels of S1P would remain elevated, augmenting S1P signalling and ultimately the myogenic response. It would be both
interesting and clinically relevant to see if CFTR participates in the pro-constrictive phenotype observed in HFD/STZ mice.

Our laboratory has a well-developed research program designed to address the involvement of CFTR in myogenic tone regulation in both health and disease\textsuperscript{98,163}. Our lab has discovered that CFTR is a target for dysregulation in heart failure\textsuperscript{163} and subarachnoid hemorrhage\textsuperscript{166} as a result of TNF\textgreek{a}. This opens the door for the potential exploitation of FDA-approved therapeutics designed to enhance CFTR activity and expression\textsuperscript{345} to achieve a normalization of myogenic tone in diabetes should this mechanism be at play. I completed a preliminary investigation with the assistance of my colleague Dr. Firhan Malik using baby hamster kidney (BHK) cells in culture stably transfected to express human epithelial isoform of wild-type CFTR, incubated in high glucose conditions.

Iodide efflux assays, a valid and reliable method to assess CFTR function\textsuperscript{346,347}, are based on CFTR’s ability to secret iodide ions as well as chloride, and iodide replaces endogenous chloride. Therefore, the iodide conductance measured with these assays reflects the behaviour of chloride allowing for conclusions regarding CFTR activity (see Appendix 2 Methods). Dr. Malik and I found that iodide efflux in CFTR-transfected BHK cells was significantly lower following a 48 hour incubation with high glucose media (25 mmol/L), compared to normal glucose media (17.5 mmol/L) and an osmotic control media (high mannitol, 17.5mmol/L glucose, up to 25mmol/L with mannitol) (Appendix Figure 2). Importantly, untransfected BHK cells express endogenous Cl\textsuperscript{-} channels but do not express CFTR, therefore the iodide assay is CFTR-specific. These results are encouraging and give good reason to believe that along with the TNF\textgreek{a}/S1P signalling pathway, CFTR activity may be affected as well in HFD/STZ mice. A fascinating research project could be built around the hypothesis that either elevated glucose or TNF\textgreek{a} downregulates CFTR activity to drive pathological S1P signalling in the HFD/STZ mouse model of diabetes.
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CHAPTER 5. COLLABORATIONS
5.1 COLLABORATIVE EFFORTS

Throughout my graduate degree, I was very lucky to participate in collaborative research, where individual accomplishments came second to achieving meaningful discoveries. I was selected to integrate into other projects in both my own laboratory and at other University of Toronto laboratories (the labs of Dr. Patricia L. Brubaker and Dr. Axel Günther) that prioritized the dissemination of research findings that provide the basis for advances in medicine. My efforts have contributed to three high-impact scientific articles beyond my own first-author manuscript work (presented in this thesis; in preparation) that are published, under review, or presently submitted for publication. The abstracts from these manuscripts and my contributions are listed in the next section.

Additionally, I have had the joy of teaching and training numerous other students who worked with the Bolz laboratory throughout the years, including a PhD trainee (Dr. Sonya Hui), an MSc (Sascha Pinto) and PhD trainee (Zhamak Abdi) from our collaboration with the Department of Mechanical and Industrial Engineering and the Institute of Biomaterials and Biomedical Engineering, and three undergraduate summer students (Ellen Langille, Farigol Hakemzadeh and Asrinus Subha).
5.2 ABSTRACTS FROM COLLABORATIVE MANUSCRIPTS

5.2.1 Tumour Necrosis Factor-α–Mediated Downregulation of the Cystic Fibrosis Transmembrane Conductance Regulator Drives Pathological Sphingosine-1-Phosphate Signalling in a Mouse Model of Heart Failure

Anja Meissner, PhD; Jingli Yang, MD; Jeffrey T. Kroetsch, MSc; Meghan Sauvé, MSc; Hendrik Dax, MD; Abdul Momen, MD; M. Hossein Noyan-Ashraf, PhD; Scott Heximer, PhD; Mansoor Husain, MD; Darcy Lidington, PhD; Steffen-Sebastian Bolz, MD, PhD


ABSTRACT

Background—Sphingosine-1-phosphate (S1P) signalling is a central regulator of resistance artery tone. Therefore, S1P levels need to be tightly controlled through the delicate interplay of its generating enzyme sphingosine kinase 1 and its functional antagonist S1P phosphohydrolase-1. The intracellular localization of S1P phosphohydrolase-1 necessitates the import of extracellular S1P into the intracellular compartment before its degradation. The present investigation proposes that the cystic fibrosis transmembrane conductance regulator transports extracellular S1P and hence modulates microvascular S1P signalling in health and disease.

Methods and Results—In cultured murine vascular smooth muscle cells in vitro and isolated murine mesenteric and posterior cerebral resistance arteries ex vivo, the cystic fibrosis transmembrane conductance regulator (1) is critical for S1P uptake; (2) modulates S1P-dependent responses; and (3) is downregulated in vitro and in vivo by tumour necrosis factor-α, with significant functional consequences for S1P signalling and vascular tone. In heart failure, tumour necrosis factor-α downregulates the cystic fibrosis transmembrane conductance regulator across several organs, including the heart, lung, and brain, suggesting that it is a fundamental mechanism with implications for systemic S1P effects.
Conclusions—We identify the cystic fibrosis transmembrane conductance regulator as a critical regulatory site for S1P signalling; its tumour necrosis factor-α–dependent downregulation in heart failure underlies an enhancement in microvascular tone. This molecular mechanism potentially represents a novel and highly strategic therapeutic target for cardiovascular conditions involving inflammation.

CONTRIBUTIONS

I provided all of the pressure myography data collected from the mesenteric arteries of wild-type, TNFα−/−, etanercept and JTE013-treated heart failure mice.
5.2.2 Restoring cerebrovascular CFTR function normalizes myogenic reactivity and cerebral perfusion in heart failure

Anja Meissner, PhD*; Firhan A. Malik, PhD*; Meghan Sauvé, MSc; Jeffrey T. Kroetsch, PhD; Jessica C. Fares, BSc; Danny D. Dinh, BSc; Abdul Momen, MD; Hangjun Zhang, MD; Roozbeh Aschar-Sobbi, PhD; Warren D. Foltz, PhD; Scott P. Heximer, PhD; Peter H. Backx, PhD, DVM; Mansoor Husain, MD; Christine E. Bear, PhD; Darcy Lidington, PhD** and Steffen-Sebastian Bolz, MD, PhD**

* denotes equal first authorship contribution; ** denotes equal senior authorship contribution

Circulation 2015; under revision.

ABSTRACT

Background—The cystic fibrosis transmembrane conductance regulator (CFTR) controls the bioavailability of cerebrovascular sphingosine-1-phosphate (S1P) and hence, its pro-constrictive effects. In heart failure, reduced microvascular CFTR expression correlates with enhanced myogenic tone and reduced cerebral blood flow. The present investigation proposes that therapeutics designed to enhance CFTR activity/expression can be exploited to normalize cerebrovascular myogenic tone and brain perfusion in heart failure.

Methods and Results—Mice carrying the ΔF508 CFTR mutation, which reduces cell surface CFTR expression, have a phenotype that mimics the effects of heart failure in wild-type mice: augmented posterior cerebral artery (PCA) myogenic tone and reduced cerebral perfusion. In two distinct cell culture models, the CFTR therapeutic “C18” (6 µmol/L) increases CFTR expression and enhances both S1P uptake and stimulated channel activity. C18 attenuates the enhanced myogenic tone in isolated PCAs (600 nmol/L in vitro) from mice with heart failure, but does not affect myogenic tone in PCAs from sham-operated mice. Systemic (in vivo) C18 treatment (0.8 mg/kg BW daily for 2 days) normalizes PCA CFTR expression, myogenic tone and cerebral perfusion in heart failure, without altering the systemic parameters of total peripheral resistance or cardiac output.
Conclusion—We present the first in vivo demonstration that CFTR therapeutics improve cerebral perfusion in heart failure. As the first new microvascular drug-target to emerge in years, CFTR and therapeutics that enhance its function represent an untapped resource for managing cerebrovascular dysfunction and compromised cerebral perfusion in heart failure.

CONTRIBUTIONS

I provided all of the pressure myography data collected from the posterior cerebral arteries of CFTR+/− mice, posterior cerebral and mesenteric arteries of in vivo and in vitro C18-treated heart failure mice, and mouse handling/C18 injections of animals for MRI cerebral blood flow measurements.
5.2.3 Chronic Exposure to Tumour Necrosis Factor α Impairs Secretion of Glucagon-like Peptide-1

Jeffrey Gagnon, PhD; Meghan Sauvé, MSc; Wen Zhao, BSc; Holly M. Stacey, BSc; Stuart C. Wiber, BSc, Steffen-Sebastian Bolz, MD, PhD; Patricia L. Brubaker, PhD

*Endocrinology* 2015; Aug 13;en20151361 [Epub ahead of print].

**ABSTRACT**

Obesity is associated with systemic inflammation and elevated levels of tumour necrosis factor-α (TNFα), leading to impaired glucose tolerance. In humans, obesity is also associated with reduced nutrient-stimulated secretion of the intestinal incretin hormone, glucagon-like peptide-1 (GLP-1). We hypothesized that TNFα plays a direct role in the impairment of GLP-1 secretion from the enteroendocrine L-cell, and that blocking TNFα can restore both GLP-1 secretion and glucose homeostasis. Expression of the TNFα receptor subtype-1 was detected in the human NCI-H716 and murine GLUTag L-cell models and in mouse ileal sections. Although TNFα acutely increased GLP-1 release from NCI-H716 cells (p<0.05-0.001), pre-incubation with TNFα for 24 hr reduced proglucagon mRNA (p<0.05) and GLP-1 cellular (p<0.05) levels without affecting cell viability. Furthermore, both NCI-H716 and GLUTag cells pre-treated with TNFα for 24 hr no longer responded to known GLP-1 secretagogues, an effect that was reversed by co-incubation with the NFκB inhibitor, 5-aminosalicylic acid, in the NCI-H716 cells. Mice given a high fat diet (HFD) for 12 wk developed impaired glucose tolerance, hyperinsulinemia and increased TNFα mRNA expression in fat and ileal tissue. Hyperglycaemia and -insulinemia were reduced in HFD-mice treated with the anti-TNFα biological, etanercept, for 2 wk. In primary intestinal cultures from these animals, HFD-control mice had impaired GLP-1 secretion, and this was not observed in the HFD-etanercept derived cultures (p<0.05). In conclusion, chronic exposure to TNFα directly impairs GLP-1 secretion at the level of the intestinal L-cell, an effect that is reversed by anti-TNFα therapy in association with improved glucose tolerance.

**CONTRIBUTIONS**

I assisted with all oral glucose tolerance tests and all tissue collection from high fat diet mice and completed treatments of mice with etanercept.
APPENDICES

Appendix 1. Sequences of quantitative PCR primers

Appendix Table 1. Quantitative PCR primers

<table>
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Appendix 2. Methods

*Human skeletal muscle resistance artery isolation*

The use of human subjects in this study conforms to the principles outlined in the *Declaration of Helsinki* and was approved by the Research Ethics Board of St. Michael’s Hospital, Toronto, Canada (Approval #11-198). Study subjects who planned to have non-emergency (elective) cardiac bypass surgery were recruited from the cardiac surgery clinic at St. Michael’s Hospital. With informed patient consent, surgeons directly provided a small piece of thoracic wall skeletal muscle (3-4 cm³) to research staff inside the operating room. The specimen was immersed in room temperature 3-morpholinopropanesulfonic acid (MOPS)-buffered saline, placed on ice and transported to the laboratory; once the specimen cooled, it was washed with ice-cold MOPS buffer and placed in a Petri dish. Resistance arteries (100-200 µm in diameter) were carefully dissected from the surrounding tissue, taking care to minimize vessel tension during the isolation process.

*Cell culture*

Baby hamster kidney (BHK) cells stably expressing the human epithelial isoform of wild-type CFTR (CFTRwt) (Yu 2010) were generously provided by Dr. Christine Bear (The Hospital for Sick Children, Toronto, Canada). The CFTR construct has an exofacial triple-HA tag (YPYDVPDYASYPYDVPDYAYPYDVPDYA) in the fourth extracellular loop, originally developed and characterized by the Lukacs laboratory (Sharma 2004).

Cells were cultured using Dulbecco’s Modified Eagle Medium Nutrient Mixture F-12 media (DMEM/F12, 50/50) (Gibco, Invitrogen Life Technologies), containing 5% (v/v) fetal bovine serum (FBS), 1% Anti-Anti (100x antibiotic solution, Gibco, Invitrogen Life Technologies), supplemented with 250µmol/L methotrexate (which activates the CFTR transgene promoter) under standard culture conditions (37°C, 5% CO₂).
High glucose treatment:

Cells were treated for 48 hours with normal cell media (DMEM/F12 + 5% FBS + 250 µmol/L Methotrexate; 17.5 mmol/L normal glucose media), or 25 mmol/L mannitol in media (osmotic control - normal cell media containing 17.5 mmol/L glucose, up to 25 mmol/L mannitol), or 25 mmol/L glucose in media (high glucose condition).

Conventional iodide efflux assay

Cells were grown onto 25 mm plastic cover slips in six-well titer plates to a confluency of 95-100%. The cover slips were then rinsed in 1X phosphate buffered saline (PBS) and incubated with 2mL of sodium iodide (NaI) uptake buffer (136 mmol/L NaI, 3 mmol/L potassium nitrate-KNO₃, 2 mmol/L calcium nitrate-Ca(NO₃)₂, 11 mmol/L glucose, and 20 mmol/L HEPES, pH 7.2, 300 osm) for one hour at 37°C and 5% CO₂ in a cell culture incubator. Another set of six-well titer plates were prepared containing 2 mL of iodide-free efflux buffer (136 mmol/L sodium nitrate-NaNO₃, 3 mmol/L KNO₃, 2 mmol/L Ca(NO₃)₂, 11 mmol/L glucose, and 20 mmol/L HEPES, pH 7.2, 300 osm) in the first four wells, and a cyclic adenosine monophosphate (cAMP) stimulation cocktail in the next eight wells. The cocktail contained (i) 10µmol/L forskolin (FSK) to activate adenylate cyclase and promote protein kinase A (PKA)-mediated phosphorylation, (ii) 1000 µmol/L isobutylmethylxanthine (IBMX) to slow channel inactivation by inhibiting phosphodiesterases, and 100 µmol/L of the cell-permeable cpt-cAMP to activate PKA. These three compounds were dissolved in 1% (v/v) dimethyl sulfoxide (DMSO) and the iodide-free efflux buffer.
A calibrated iodide ion-selective probe (Lazar Research Laboratories) was used to measure the voltage (mV), corresponding to the amount of iodide ions released out of the cells into solution. For measurements, the plates were gently shaken and the probe was immersed into each well until a stable reading was established. The probe was then washed in distilled water until a stable reading was achieved (i.e., equilibration), then immersed into the next well. The post-stimulation mV value that corresponded to the greatest difference was subtracted from the pre-stimulation value (i.e., fourth wash well). This calculated value was converted to concentration values (µmol/L) using a standard curve equation. Measuring the voltage of different concentration of iodide solutions generated the standard curve, and the curve parameters were used in the equation below:

\[
[i -] = \ln \left( \frac{(mV - (-109.4))}{107.5} \right) - 0.0515
\]
Appendix 3. Myogenic tone data collected from human patients

Appendix Figure 1. Myogenic tone is augmented in human skeletal muscle resistance arteries isolated from patients with type 2 diabetes.

Shown are (A) measured active and passive diameters, (B) the corresponding myogenic tone calculations and (C) phenylephrine dose-response relationships for thoracic wall skeletal muscle resistance arteries isolated from patients undergoing elective coronary artery bypass graft surgery. Arteries isolated from patients with diabetes (n=20 arteries from N=15 patients) display higher myogenic tone than arteries isolated from patients without diabetes (n=24, N=17). Phenylephrine responses and passive diameters are similar across the two groups. * denotes \( P<0.05 \) for an unpaired comparison.

Data collected and analyzed by Dr. Sonya Hui, Bolz Laboratory. Figure from: Sauvė M., Hui S.K., Foltz W.D., Momen M.A., Nedospasov S.A., Offermanns S., Husain M., Lidington D. and Bolz S.S. Tumour necrosis factor \( \alpha / \) sphingosine-1-phosphate signalling augments resistance artery myogenic tone in diabetes. 2015. Submitted: under review at Diabetes.
## Appendix Table 2. Patient Characteristics

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Clinical data from control (N=17) and diabetic study patients (N=15). Inclusion criteria: patients undergoing coronary artery bypass graft surgery. ‘Family history’ refers to a positive family history of coronary artery disease. ‘Smoking’ indicates any history of smoking, including currently reformed smokers. ACE inhibitor; angiotensin converting enzyme inhibitor, ASA; acetylsalicylic acid.
Appendix 4. High glucose modulates CFTR activity

Appendix Figure 2. BHK cells stably transfected to express CFTR have significantly reduced iodide efflux following incubation with high glucose media. Iodide efflux, measured in BHK cells to assess CFTR function, is significantly lower following a 48 hour incubation with high glucose media (25 mmol/L), compared to normal glucose media (17.5 mmol/L) and an osmotic control (High mannitol, 17.5 mmol/L glucose, up to 25 mmol/L with mannitol). *P<0.05, n=4 wells per treatment. Iodide efflux data collected and analyzed by Dr. Firhan Malik, Bolz laboratory.
Appendix 5. References
