CARDIOVASCULAR FUNCTION IN RAT MODELS OF DIABETES – THE ROLES OF HYPERGLYCEMIA, INFLAMMATION AND OXIDATIVE STRESS

by

JOANNE YUEN TING LEUNG

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Abstract

Diabetes increases the risk of cardiovascular disease and mortality in humans. Although many diabetes-related studies have been conducted in recent years, the underlying pathogenesis still remains unclear. The aim of this thesis was to investigate if vascular and cardiac contractile dysfunction in rat models of streptozotocin-induced type 1 and fructose-streptozotocin-induced type 2 diabetes are associated with hyperglycemia, inflammation and oxidative stress, as these factors have been linked to the development of cardiovascular abnormalities in diabetes.

Cardiovascular function was assessed via measurements of blood pressure, venous tone, vascular resistance, heart rate, cardiac contractility and cardiac index in vivo in diabetic as well as control rats. Moreover, these variables were evaluated following treatment with phlorizin (a glucose lowering agent), nimesulide (a selective inhibitor of cyclooxygenase-2) or N-acetylcysteine (an antioxidant). The results showed that arterial, venous and cardiac contractile responses to noradrenaline, adrenaline and/or dobutamine were depressed, to similar extents, in both models of diabetes relative to control animals, even though the rats with fructose-streptozotocin-induced diabetes had significantly higher plasma triglyceride levels than those with streptozotocin-induced diabetes. Administration of phlorizin, nimesulide and N-acetylcysteine did not affect any of the cardiovascular variables in the control rats, but significantly improved certain vascular and/or cardiac contractile responses in the diabetic rats. Specifically, α-adrenoceptor-mediated venous constriction was augmented by phlorizin in both rat models of diabetes, whereas arterial constriction remained attenuated. Acute administration of nimesulide did not alter cardiac contractile responses, but partially restored the venous and, to a less extent, arterial constriction in the diabetic rats, while chronic treatment with N-acetylcysteine ameliorated the arterial,
venous as well as cardiac contractile functions. Collectively, these results suggest that the presence of hypertriglyceridemia does not significantly worsen *in vivo* cardiovascular function in our fructose-streptozotocin-induced rat model of type 2 diabetes, and demonstrate that hyperglycemia, inflammation and oxidative stress are, at least in part, implicated in diabetes-associated cardiovascular contractile dysfunction. Our studies have provided valuable insights into specific benefits of targeting hyperglycemia, inflammation and oxidative stress in the management of cardiovascular complications in type 1 and type 2 diabetes.
Preface

Two chapters in this thesis contain portions that have been published in peer review journals, as outlined below.

A version of Chapter 2 has been published in the following manuscript:


I designed and performed all experiments, analyzed the data, and wrote the manuscript. E.W.K and G.Y.L helped perform the experiments. C.C.P revised the manuscript.

A version of Chapter 4 has been published in the following manuscript:


I designed and performed all experiments, analyzed the data, and wrote the manuscript. C.C.P revised the manuscript.

As for the rest of the work in this thesis, I am responsible for the experimental design, data collection and data analysis.

All animal studies described in this thesis were granted ethical approval by the University of British Columbia Animal Care Committee (Animal Care Certificate A11-0146).
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<td>AGES</td>
<td>advanced glycation end-products</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>CAT</td>
<td>catalase</td>
</tr>
<tr>
<td>CI</td>
<td>cardiac index (in tables and figures only)</td>
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<tr>
<td>CO</td>
<td>cardiac output (in tables and figures only)</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>CTL</td>
<td>Control (a group in the experimental design)</td>
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<tr>
<td>CVP</td>
<td>central venous pressure</td>
</tr>
<tr>
<td>+dP/dt</td>
<td>maximal rate of increase of left ventricular pressure</td>
</tr>
<tr>
<td>–dP/dt</td>
<td>maximal rate of decrease of left ventricular pressure</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FFAs</td>
<td>free fatty acids</td>
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<tr>
<td>FRU</td>
<td>Fructose (a group in the experimental design)</td>
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<tr>
<td>FRU-STZ</td>
<td>Fructose-Streptozotocin (a group in the experimental design)</td>
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<tr>
<td>GIR</td>
<td>glucose infusion rate</td>
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<td>GLUT2</td>
<td>glucose transporter-2</td>
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<tr>
<td>GPx</td>
<td>glutathione peroxidase</td>
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<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>HbA1c</td>
<td>glycated hemoglobin</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HFCS</td>
<td>high-fructose corn syrup</td>
</tr>
<tr>
<td>HR</td>
<td>heart rate (in tables and figures only)</td>
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<tr>
<td>IDDM</td>
<td>insulin-dependent diabetes mellitus</td>
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<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
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<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
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<td>LVDP</td>
<td>left ventricular developed pressure</td>
</tr>
<tr>
<td>LVP</td>
<td>left ventricular systolic pressure</td>
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<tr>
<td>MAP</td>
<td>mean arterial pressure</td>
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<tr>
<td>MCFP</td>
<td>mean circulatory filling pressure</td>
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<td>NAC</td>
<td>N-acetylcysteine</td>
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<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NIDDM</td>
<td>non-insulin-dependent diabetes mellitus</td>
</tr>
<tr>
<td>PAI-1</td>
<td>plasminogen activator inhibitor-1</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>R_A</td>
<td>arterial resistance</td>
</tr>
<tr>
<td>RAGE</td>
<td>receptors for AGEs</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>R_V</td>
<td>venous resistance</td>
</tr>
<tr>
<td>SGLT</td>
<td>sodium-glucose cotransporter</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
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<tr>
<td>STZ</td>
<td>Streptozotocin (a group in the experimental design)</td>
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<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>UDP-GlcNAc</td>
<td>uridine diphosphate N-acetylglucosamine</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VLDL</td>
<td>very low density lipoprotein</td>
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Chapter 1: Introduction

1.1 Diabetes mellitus: a general overview

Diabetes mellitus (generally referred to as diabetes in this thesis) is a chronic metabolic disorder of multiple etiologies characterized by elevated levels of blood glucose, or hyperglycemia. Hyperglycemia can result from defects in insulin secretion, insulin action, or both. Diabetes affects many organ systems in the body, particularly the eyes, kidneys, nerves, heart and blood vessels. Long-term complications of diabetes include retinopathy, nephropathy, neuropathy, heart disease and stroke.

The prevalence of diabetes is increasing globally at an alarming rate, from 153 million in 1980 to 347 million in 2008 (Danaei et al., 2011). This number is projected to reach 439 million, or 7.7% of the adult population, by 2030 (Shaw et al., 2010). Between 2010 and 2030, the largest increases in prevalence will likely occur in developing countries. The latest report from the International Diabetes Federation (2013) has estimated that 382 million people worldwide, or 8.3% of adults aged 20-79 years, currently have diabetes, a number which will rise to 592 million by 2035. In Canada, over two million people are living with diabetes, representing approximately 6.8% of the population, or one in 15 people (Public Health Agency of Canada, 2011). From 1999 to 2009, the age-standardized prevalence of diabetes among Canadians increased by 70%. If the current incidence and mortality trends continue, there will be an estimated 3.8 million Canadians with diabetes in 2019.

The complications that arise from diabetes are a major cause of disability, reduced quality of life, and death. Diabetes is responsible for 6.8% of global (all ages) mortality, with the highest proportion (15.7% in the age group 20-79 years) being in North America (Roglic and Unwin,
In Canada, it has been estimated that 11.9% of deaths in the adult population was attributable to diabetes in 2009 (Public Health Agency of Canada, 2011). Mortality rates were at least two times higher among individuals with diabetes than those without, resulting in marked reduction in life expectancy. In fact, life expectancy may be shortened by 15 years for people with type 1 diabetes and by 5 to 10 years for people with type 2 diabetes (The Canadian Diabetes Association, 2009).

Diabetes and its complications also impose a financial burden on the healthcare system and the national economy. A study by Zhang et al. (2010b) predicted that the annual global health expenditure on diabetes in 2010 will total at least $376 billion US, or 12% of total health expenditure. By 2030, it is anticipated to reach $490 billion US. The economic burden of diabetes in Canada is estimated to be $12 billion Canadian in 2010, with direct costs (hospital care, primary care and medication) accounting for about 3.5% of public healthcare spending (The Canadian Diabetes Association, 2009). This value doubled the amount of $6 billion in 2000, and is expected to increase to $17 billion Canadian by 2020.

1.1.1 Classification of diabetes mellitus

The first generally accepted classification of diabetes mellitus was published in 1979 by the National Diabetes Data Group and endorsed by the World Health Organization one year later (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus, 2003). It categorized diabetes mellitus into two major classes: insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). These terms, however, are no longer used today because they led to confusion as patients were classified on the basis of treatment instead of pathogenesis.
The current classification adopted by the World Health Organization and the American Diabetes Association encompasses both the clinical stages and etiology of the disorder (American Diabetes Association, 2014; The World Health Organization, 1999). There are four broad categories of diabetes mellitus: type 1, type 2, “other specific types” and gestational diabetes. Type 1 diabetes (formerly called IDDM, type I or juvenile-onset diabetes) occurs in 5-10% of diabetic patients, often in comparatively younger individuals. It is due to destruction of the pancreatic β-cells, and is characterized by absolute insulin deficiency and requirement of insulin for survival. This form of diabetes includes cases ascribable to an autoimmune process, as well as those of unknown etiology. Type 2 diabetes (formerly called NIDDM, type II or adult-onset diabetes) is the most prevalent form of diabetes, accounting for at least 90% of all diabetic cases. It results from insulin resistance in peripheral tissues coupled with defective or inadequate compensatory insulin secretion. Patients with type 2 diabetes are usually overweight or obese and have a relative, rather than an absolute, deficiency of insulin. Other specific types of diabetes are associated with secondary conditions ranging from genetic defects in β-cell function or insulin action, diseases of the exocrine pancreas, endocrinopathies, drug or chemical induction, infections, and certain genetic syndromes. Gestational diabetes, which affects approximately 7% of all pregnant women, refers to glucose intolerance that begins or is first recognized during pregnancy.

1.1.2 Diagnostic criteria of diabetes mellitus

According to the current diagnostic criteria, diabetes mellitus is diagnosed when fasting plasma glucose is ≥ 7.0 mmol/L (126 mg/dL), when two-hour plasma glucose is ≥ 11.1 mmol/L (200 mg/dL) in a 75 g oral glucose tolerance test, or when glycated hemoglobin (HbA1c) is ≥
6.5% using a standardized, validated assay (American Diabetes Association, 2014; Canadian Diabetes Association Clinical Practice Guidelines Expert Committee, 2013). These diagnostic values are associated with the development of retinopathy, as demonstrated from epidemiological studies. The diagnosis of diabetes should be confirmed by repeat-testing on a different day, especially in asymptomatic individuals. In addition, patients with classic symptoms of hyperglycemia or hyperglycemic crisis can be diagnosed from a random (or casual) plasma glucose of ≥ 11.1 mmol/L (200 mg/dL).

1.2 Cardiovascular complications in diabetes

Diabetes has long been recognized as an independent risk factor for cardiovascular disease (Grundy et al., 1999). The incidence of cardiovascular disease is 2- to 4-fold higher in people with diabetes than those without after adjustment for age and other confounders (Wannamethee et al., 2011; Fox et al., 2004; Kannel and McGee, 1979). Once clinical cardiovascular disease develops, diabetic patients exhibit a poor prognosis for survival (De Groote et al., 2004; Becker et al., 2003). Cardiovascular disease is the leading cause of diabetes-related mortality and morbidity, responsible for up to 70% of all deaths in individuals with diabetes (Stratmann and Tschoepe, 2011). In a multinational study by the World Health Organization, cardiovascular disease accounted for 44% of deaths in type 1 diabetes and 52% of deaths in type 2 diabetes (Morrish et al., 2001). An 18-year follow-up study from Finland found that both type 1 and type 2 diabetes had a similar impact on cardiovascular mortality (Jutilainen et al., 2008). The risk was increased by 5.2- and 4.9-fold in type 1 and type 2 diabetic patients, respectively, with cardiovascular mortality rates of 31.2% and 43.9% compared to 7.7% in non-diabetic subjects. These findings are in agreement with another cohort study conducted in
Switzerland, which showed a substantially higher excess mortality from cardiovascular disease, by 6.6- and 5.2-fold respectively, in type 1 and type 2 diabetic patients relative to the general Swiss population (Allemann et al., 2009). Today, diabetes is not only recognized as a cardiovascular risk factor, but also a cardiovascular disease equivalent (Chiha et al., 2012). In other words, the presence of diabetes is considered equivalent to having pre-existing cardiovascular disease. With the prevalence of diabetes continuing to increase worldwide, cardiovascular disease among diabetic patients will certainly become a major global health and economic burden. More basic and clinical research is therefore needed to improve future prevention and treatment strategies.

Diabetic patients are particularly susceptible to cardiovascular complications, as described in the sub-sections below. Cardiovascular complications in diabetes consist of both cardiac and vascular abnormalities; the latter affects the function of many different organs and systems. They can be divided into three categories based on differences in pathology: macrovascular complications, microvascular complications and diabetic cardiomyopathy.

1.2.1 Macrovascular complications

Diabetic macrovascular complications involve damage to large blood vessels, such as arteries. They include coronary artery disease, cerebrovascular disease (stroke) and peripheral vascular disease, which affect respectively the heart, the central nervous system and the lower limbs (Braun and Anderson, 2007). These complications are very much associated with the development of atherosclerosis. In diabetic patients, multivessel atherosclerosis is often present, contributing to the higher occurrence of myocardial infarction and stroke (Fowler, 2008). Approximately 50% of individuals with newly diagnosed diabetes have coronary artery disease
(Frolich and Bondy, 2002), and the risk of fatal coronary events is 2- to 3.5-fold greater in those with diabetes than those without (Huxley et al., 2006). Despite a better understanding of the cardiovascular risk factors involved and the advances in disease management, macrovascular complications continue to be the most predominant cause of mortality in type 1 and type 2 diabetic patients.

1.2.2 Microvascular complications

Microvascular pathological changes affect small vessels, such as capillaries, and may lead to the development of diabetic retinopathy, nephropathy and neuropathy. The risk of developing microvascular complications is generally dependent on both the duration and severity of hyperglycemia. Retinopathy is the most common microvascular complication of diabetes and remains the leading cause of blindness among adults in the developed world (Porta et al., 2011). Nephropathy is characterized by persistent albuminuria, hypertension and a decline in glomerular filtration rate (Van Buren and Toto, 2011). It occurs in approximately 20-40% of patients with diabetes, and often progresses to end-stage renal disease (American Diabetes Association, 2004). Neuropathy, or damage of peripheral nerve fibres, typically results in one or more of the following symptoms: numbness, tingling, weakness, and sometimes pain (Callaghan et al., 2012). Many patients with severe diabetic neuropathy suffer from foot ulcers and require lower-extremity amputations. Neuropathy may also affect the autonomic nervous system, causing impairment in cardiovascular, gastrointestinal and urogenital functions (Tesfaye et al., 2010). All of these aforementioned microvascular complications are known to increase the risk of cardiovascular events in diabetic individuals (Kim et al., 2011).
1.2.3 Diabetic cardiomyopathy

Cardiomyopathy in diabetes is characterized by myocardial dysfunction in the absence of coronary artery disease, hypertension or valvular heart disease, leading to accelerated heart failure (Pappachan et al., 2013; Aneja et al., 2008). It is manifested initially by diastolic dysfunction, which can progress to systolic dysfunction (Goyal and Mehta, 2013). Diabetic patients with cardiomyopathy are presented with left ventricular hypertrophy and myocardial stiffness. Diabetic cardiomyopathy may be caused by defects in myocardial subcellular organelles (Dhalla et al., 2012), impairment of Ca\(^{2+}\) handling (Dhalla et al., 2014; Lagadic-Gossmann et al., 1996), as well as lipid accumulation within the cardiomyocytes (Yokoyama et al., 2004). The exact underlying mechanisms of diabetic cardiomyopathy, however, are still incompletely understood.

1.3 Cardiac and hemodynamic changes in diabetes

As mentioned above, the cardiovascular complications in diabetes are vast and encompass a wide range of abnormalities. Diabetes is known to alter cardiac contractility, heart rate, cardiac output and vascular contractile function.

1.3.1 Cardiac contractility

There is evidence that cardiac contraction and relaxation (shortening and re-lengthening) are impaired in type 1 and type 2 diabetes. At the early stages of diabetes, left ventricular myocytes isolated from rats at 1 week after streptozotocin induction showed reductions in length and width, contributing to a 30% decrease in myocyte volume (Cagalinec et al., 2013). The authors also observed a significant decrease in myocyte shortening at a stimulation frequency of
0.5 Hz. Similarly, other studies have found depressed contractility in isolated cardiomyocytes from type 1 diabetic rats at 8-12 weeks following injection of streptozotocin (Ding et al., 2006; Wickley et al., 2006; Choi et al., 2002) as well as from type 2 diabetic db/db mice (Stolen et al., 2009; Kralik et al., 2005), which was likely associated with an attenuation of intracellular Ca\(^{2+}\) transient. Isolated Langendorff-perfused hearts from diabetic rats and mice exhibited lower maximal rates of increase (+dP/dt) and decrease (–dP/dt) of left ventricular pressure, reduced left ventricular systolic pressure (LVP) and left ventricular developed pressure (LVDP), and longer time to half-relaxation (Cao et al., 2012; Ligeti et al., 2006; Belke et al., 2004; El-Omar et al., 2003; Choi et al., 2002). These cardiac abnormalities have also been demonstrated in vivo. Indeed, there are reports of decreased ±dP/dt, LVP and LVDP, increased left ventricular end-diastolic pressure, diminished fractional shortening and ejection fraction, and slower isovolumic relaxation at rest in rat models of type 1 (Guo et al., 2014; Tappia et al., 2013; Bhatt and Veeranjaneyulu, 2012; Wang et al., 2011; Kain et al., 2011; Arozal et al., 2009; Bidasee et al., 2008; Borges et al., 2006; Cheng et al., 2004) and type 2 (Sen et al., 2012; Zhou et al., 2011) diabetes. Some studies, however, showed no changes in baseline systolic and diastolic function (Epp et al., 2013; Song et al., 2008a; Hutchings et al., 2005), which may be related to variability of the age of the animals, the type of animal model used or the severity of diabetes.

Cardiac contractile dysfunction is commonly observed in type 1 and type 2 diabetic patients (Korosoglou et al., 2012; Diamant et al., 2003; Schannwell et al., 2002; Danielsen et al., 1987), with diastolic abnormalities being more prominent. Around 40% of asymptomatic patients have impaired left ventricular diastolic function at the early stages of diabetes (Romano et al., 2010). Diastolic dysfunction can be influenced by changes in both intrinsic contractile
properties and extrinsic loading conditions (Lalande et al., 2010). In many patients, the systolic and diastolic impairment will eventually progress to heart failure with reduced ejection fraction. Multiple mechanisms, including alterations in Ca\(^{2+}\) signalling, myofilament Ca\(^{2+}\) sensitivity, actomyosin ATPase activity, adenylyl cyclase signalling and excitation-contraction coupling structures, are likely responsible for the depression of cardiac contractility in diabetes (Goyal and Mehta, 2013; Shpakov and Derkach, 2013; Choi et al., 2002). Excess accumulation of collagen (notably types I and III) from an imbalance of extracellular matrix homeostasis in the heart may also play a role by promoting myocardial fibrosis and subsequent decline in ventricular compliance (Wen et al., 2013; Li et al., 2012).

In addition, adrenoceptor-mediated inotropic responses are diminished in diabetic hearts. The cardiac contractile responses to isoproterenol, a non-selective β-adrenoceptor agonist, were significantly reduced in isolated cardiomyocytes (Tamada et al., 1998) and Langendorff-perfused hearts (Ligeti et al., 2006; op den Buijs et al., 2005) from rats at 4-6 weeks after injection of streptozotocin. In a more recent study, the cumulative concentration-response curve of LVDP to isoproterenol in isolated hearts from diabetic rats showed a significant rightward shift, with decreased potency (higher EC\(_{50}\)) and efficacy (maximum response) relative to age-matched controls (Bilginoglu et al., 2009). Furthermore, in vivo left ventricular contractility to isoproterenol, noradrenaline and dobutamine was depressed in streptozotocin-induced diabetic (Bidasee et al., 2008; Hutchings et al., 2005; Cheng et al., 2004) and Zucker diabetic fatty (Song et al., 2008a) rats. Downregulation of myocardial β-adrenoceptors, particularly the β\(_{1}\)-subtype (Bidasee et al., 2008; Altan et al., 2007; Matsuda et al., 1999; Gando et al., 1997), and defective Ca\(^{2+}\) handling (Ligeti et al., 2006; op den Buijs et al., 2005) may account for the decreased responsiveness to adrenoceptor activation.
1.3.2 Heart rate

Information regarding changes in heart rate in diabetes is less consistent. Many studies have shown an attenuated resting heart rate in streptozotocin-induced diabetic rats compared to control animals (Howarth et al., 2009; Bidasee et al., 2008; Borges et al., 2006; Hutchings et al., 2005; Cheng et al., 2004; Cheng and Pang, 2004; Dall’Ago et al., 2002; Fazan et al., 1999; Hicks et al., 1998; Kiff et al., 1991), while others have found no changes in db/db mice (Broderick et al., 2012; Kosugi et al., 2006; Bagi et al., 2005). The decrease in heart rate could be a result of depressed cardiac responsiveness to β-adrenoceptor stimulation (Dincer et al., 2001) and/or cardiac sympathetic denervation (Schnell et al., 2002; Stevens et al., 1998). Some type 1 and type 2 diabetic patients, on the other hand, have higher resting heart rates, most likely secondary to cardiac vagal neuropathy from autonomic dysfunction (Uusitupa et al., 1988; Danielsen et al., 1987). Resting heart rates of 90 to 100 beats/min with occasional increases up to 130 beats/min have been observed in diabetic patients with parasympathetic impairment (Vinik et al., 2013; Ewing et al., 1981). A relative predominance of sympathetic over parasympathetic tone can further aggravate the resting tachycardia (Pop-Busui, 2010).

Moreover, diabetic patients have lower heart rate variability compared to non-diabetic subjects (Kudat et al., 2006; Schroeder et al., 2005; Schannwell et al., 2002). Heart rate variability represents the variations in the beat-to-beat (RR) intervals, and is an index of autonomic nervous system function. It reflects the heart’s ability to adapt to changing internal and external conditions through the intricate interaction between the sympathetic and parasympathetic nervous systems. Reduced heart rate variability is strongly associated with a higher risk of coronary heart disease (Liao et al., 2002) and mortality (Maser et al., 2003) among individuals with diabetes. Abnormalities in heart rate variability are early signs of diabetic
cardiovascular autonomic neuropathy, which may lead to postural hypotension, impaired cardiovascular responses to exercise, and silent myocardial ischemia (Vinik et al., 2013; Pop-Busui, 2010; Schonauer et al., 2008).

1.3.3 Cardiac output

Cardiac output is dependent on heart rate, contractility, preload, and afterload (Vincent, 2008). Since these parameters are altered in diabetes, cardiac output is affected accordingly. There are some discrepancies in the literature concerning cardiac output in animal models of diabetes. A decrease in cardiac output has been previously reported in streptozotocin-treated rats at an early stage (6 days) of diabetes (Brands et al., 2000) and in 12- to 14-week-old db/db mice (Bagi et al., 2005). A lower cardiac output reduces the amount of blood that reaches organs and tissues, thus altering the distribution of blood flow. Indeed, regional blood flow in rats with streptozotocin-induced diabetes was elevated in the kidneys and intestines, but reduced in the skin and skeletal muscle (Hill and Larkins, 1989). Previous studies from our laboratory found no changes in baseline cardiac output and cardiac index between the control and diabetic rats (Song et al., 2008b; Cheng et al., 2004). In contrast, cardiac output was increased in rats at 2.5 months (Joffe et al., 1999) and 12 weeks (Carbonell et al., 1987) following injection of streptozotocin, as well as in 12- and 24-week-old db/db mice (Van den Bergh et al., 2006). Van den Bergh et al. (2006) suggested that a greater preload, together with a lower afterload, could help to maintain cardiac output despite impaired cardiac contractility. The changes in cardiac output appear to be dependent on the duration of diabetes, as cardiac output was similar between control and streptozotocin-treated rats at 1-, 2- and 4-weeks after induction of diabetes, but significantly decreased in the diabetic animals at 8 weeks (Hill and Larkins, 1989).
In clinical studies, cardiac output, measured by echocardiography, was markedly higher in patients with type 1 diabetes relative to non-diabetic controls (de Simone et al., 2000; Kimball et al., 1994). There were, however, no significant differences in baseline cardiac index between type 2 diabetic patients and non-diabetic subjects (Dalla Vestra et al., 2002).

1.3.4 Vascular contractile function

Vascular contractile responses have been extensively studied *in vitro* in arterial blood vessels of diabetic animals, but have produced variable results. The discrepancies may be attributable to differences in the animal model used, the stage of diabetes, and the type of blood vessel examined. In the aortae of streptozotocin-induced diabetic rats, decreased (Head et al., 1987; Ding et al., 1991), unaltered (Fulton et al., 1991; Head et al., 1987), and increased (Silan, 2008; Arun et al., 2004) contractile responses to noradrenaline and angiotensin II have been observed. The duration of diabetes ranged from 2-8 weeks in these studies. The efficacy (maximum response) of noradrenaline-induced contraction was markedly greater in the mesenteric arteries from streptozotocin-treated rats at 12-14 weeks after induction of diabetes, with no change in potency (EC$_{50}$) relative to age-matched controls (Bardell and MacLeod, 2001). In contrast, Van Buren et al. (1998) demonstrated an increased potency to noradrenaline in isolated mesenteric arteries after long-term diabetes (40 weeks). Moreover, contraction to phenylephrine was enhanced in the small mesenteric arteries from diabetic *db/db* mice compared to those from control mice (Xie et al., 2006; Pannirselvam et al., 2005; Guo et al., 2005), although no differences were documented in other studies (Belmadani et al., 2008; Howarth et al., 2006). Similar increases in phenylephrine-induced contractions have been found in the aortae (Tokutomi et al., 2011; Zhong et al., 2007) and renal arteries (Tian et al., 2011) of *db/db* mice.
Results from *in vivo* studies appear more consistent. It has been shown that arterial constrictor responses to different vasoactive agents, including noradrenaline, methoxamine, dopamine, angiotensin II and serotonin, were depressed in pithed diabetic rats compared to age-matched control animals (Hong et al., 2010; Heijnis et al., 1992; Lucas, 1985; Cavaliere and Taylor, 1981). Diminished noradrenaline- and angiotensin II-mediated pressor responses have also been reported in conscious (Cheng and Pang, 2004; Cheng et al., 2003; Yu and McNeill, 1992; Jackson and Carrier, 1983) and anesthetized (Hutchings et al., 2005; Cheng et al., 2004) rats with streptozotocin-induced diabetes. The *in vivo* vascular contractile impairment is likely dependant on the duration of diabetes, since pressor responses to noradrenaline were reduced at 5 weeks, but not at 2 weeks after injection of streptozotocin (Guillon et al., 1998). The noradrenaline-mediated vascular hyporesponsiveness in streptozotocin-induced diabetes could be explained by decreased expression of $\alpha_1$-adrenoceptors in the vasculature (Edith-Rodriguez et al., 2012; Park et al., 2011), in addition to impaired coupling of $\alpha_1$-adrenoceptors to G proteins (Weber and Macleod, 1997).

Despite the importance of the venous vasculature in regulating cardiac output and blood pressure, there is a paucity of research on venous function in diabetes. Changes in venous function may be implicated in some of the diabetes-associated cardiovascular complications. Reduced constriction of capacitance vessels in the legs and splanchnic region, for example, is a major factor contributing to orthostatic hypotension, which frequently occurs in diabetic patients with autonomic neuropathy (Pang, 2001). In a clinical study, forearm venous compliance was attenuated in long-term type 1 diabetic patients relative to healthy subjects, possibly due to the loss of viscoelastic properties of the venous vessel wall from structural changes such as irreversible cross-linking of glycated collagen fibres and other connective tissue proteins.
(Schaper et al., 1994). On the other hand, measurements of both venous compliance and mean circulatory filling pressure (MCFP; index of body venous tone) were found to be unchanged between control and streptozotocin-treated rats (Litwin et al., 1991). A previous study from our laboratory reported significantly lower MCFP responses to noradrenaline in streptozotocin-induced type 1 diabetic rats, suggesting depressed α-adrenoceptor-mediated constriction of capacitance vessels (Cheng et al., 2003). It remains to be determined if venous contractile function is altered in type 2 diabetes.

1.3.5 Potential mechanisms of cardiovascular complications

The pathogenesis of cardiovascular complications in diabetes is complex and multifactorial. During the last decade, the underlying mechanisms responsible for these complications have begun to unfold, allowing better understanding of the crucial link between diabetes and cardiovascular disease. Numerous factors, including, but not limited to, hyperglycemia, dyslipidemia, inflammation and oxidative stress, are involved in structural and functional abnormalities in the cardiovascular system of type 1 and type 2 diabetic patients. These factors do not act independently; instead, they interact concomitantly in an intricate manner to influence downstream pathways, ultimately causing cardiac and vascular impairments. There is evidence that hyperglycemia as well as dyslipidemia induce inflammation and/or oxidative stress, both of which may in turn modulate and intensify each other’s actions (refer to Sections 1.4 to 1.7). Since hyperglycemia, dyslipidemia, inflammation and oxidative stress are closely interconnected, together, they can create a vicious cycle to further exacerbate any diabetes-associated damage. The connection of these four factors to diabetic cardiovascular complications will be discussed in detail below and is summarized in Figure 1.1.
Figure 1.1 Potential mechanisms of cardiovascular complications in diabetes, which involves hyperglycemia, dyslipidemia, inflammation and oxidative stress. Details can be found in Sections 1.4 to 1.7. AGEs: advanced glycation end-products; COX-2: cyclooxygenase-2; FFAs: free fatty acids; HDL: high density lipoprotein; IL: interleukin; LDL: low density lipoprotein; NF-κB: nuclear factor-κB; PKC: protein kinase C; ROS: reactive oxygen species; TNF-α: tumor necrosis factor-α.
1.4 Hyperglycemia

Hyperglycemia is the hallmark and diagnostic criterion of diabetes. It is well established as an independent risk factor for cardiovascular disease (Aryangat and Gerich, 2010; Barr et al., 2009; Xu et al., 2005; Laakso, 1999). Indeed, a meta-analysis of observational studies showed an elevated cardiovascular risk with increasing levels of HbA1c in both type 1 and type 2 diabetic patients (Selvin et al., 2004). The association appeared to be stronger between HbA1c levels and peripheral artery disease as compared to either coronary heart disease or stroke. This is in agreement with a more recent meta-analysis, which reported relative risk values of 1.15 for coronary heart disease, 1.11 for heart failure, 1.11 for stroke, and 1.29 for peripheral artery disease with every 1% increase in HbA1c among patients with type 2 diabetes (Zhang et al., 2012). Furthermore, the impact of hyperglycemia on the risk of cardiovascular mortality has been investigated in middle-aged diabetic individuals. A one unit (%) increment of HbA1c increased cardiovascular mortality by 52.5% and 7.5% respectively in type 1 and type 2 diabetic subjects (Juutilainen et al., 2008), suggesting that hyperglycemia is a more important risk factor in type 1 than in type 2 diabetes.

1.4.1 Mechanisms of hyperglycemia-induced damage

Hyperglycemia induces damage to the heart and blood vessels at the tissue, cellular and biochemical level, hence leading to alterations in cardiovascular function (Ceriello, 2008). Acute increases in plasma glucose levels can produce left ventricular dysfunction (Ishihara et al., 2003), as well as adverse effects on hemodynamic parameters (Giugliano et al., 1997). Multiple hyperglycemia-induced changes associated with diabetic cardiovascular complications include defective glucose transport, structural impairment in the heart and vasculature (deposition of
extracellular matrix, cardiac fibrosis, myocyte hypertrophy, loss of endothelial cell junctions, vascular smooth muscle cell proliferation and migration, thickening of vessel walls), altered $\text{Ca}^{2+}$ metabolism, overproduction of reactive oxygen species (ROS), and reduced nitric oxide bioavailability (Iqbal et al., 2014; Spinetti et al., 2008). Glucose toxicity occurs through four major mechanisms highlighted below.

### 1.4.1.1 Polyol pathway

Hyperglycemia increases glucose flux through the polyol pathway. This pathway involves the enzyme aldose reductase, which reduces glucose to sorbitol using nicotinamide adenine dinucleotide phosphate (NADPH) as a co-factor. In the presence of hyperglycemia, NADPH consumption rises as excess glucose is diverted from glycolysis into the polyol pathway to be metabolized to sorbitol. Because NADPH is required to regenerate reduced glutathione (GSH; an intracellular antioxidant), the substantial depletion of NADPH will decrease the level of GSH to favour generation of ROS. The polyol pathway thus ultimately contributes to redox imbalance and oxidative stress, events that are associated with the pathogenesis of cardiovascular complications in diabetes (Iqbal et al., 2014; Brownlee, 2005).

### 1.4.1.2 Hexosamine pathway

Increased flux through the hexosamine pathway is another hyperglycemia-induced mechanism underlying diabetic cardiovascular complications. In this pathway, fructose-6-phosphate, an intermediate of glycolysis, is converted to glucosamine-6-phosphate by the enzyme glutamine:fructose-6-phosphate amidotransferase (GFAT), followed by metabolism to uridine diphosphate $N$-acetylglucosamine (UDP-GlcNAc). UDP-GlcNAc acts as a substrate for
O-linked \(N\)-acetylglucosamine transferase (OGT), which is a cytosolic and nuclear enzyme that catalyzes the transfer of \(N\)-acetylglucosamine in \(O\)-linkage to serine or threonine residues of intracellular proteins. Transcription factors become modified, often resulting in pathologic changes in gene expression. Under hyperglycemic conditions, activation of the hexosamine pathway increases the transcription of genes for transforming growth factor (TGF)-\(\beta1\) and plasminogen activator inhibitor-1 (PAI-1), as well as inflammatory cytokines such as tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and interleukin (IL)-6 (Buse, 2006; James et al., 2002). These genes are related to fibrosis, atherothrombosis and inflammation, and can consequently give rise to diabetic vascular complications. The \(O\)-linked \(N\)-acetylglucosamine modification of proteins in the hexosamine pathway has also been shown to play a role in hyperglycemia-induced cardiac contractile impairment, possibly attributable to abnormalities in \(Ca^{2+}\) cycling from reduced activity and expression of sarcoplasmic reticulum \(Ca^{2+}\)-ATPase (Clark et al., 2003).

1.4.1.3 Protein kinase C (PKC) pathway

The PKC family of serine/threonine kinases can phosphorylate a variety of proteins to modulate gene expression and signal transduction. In diabetes, elevated intracellular glucose increases the \textit{de novo} synthesis of diacylglycerol, subsequently activating the PKC-\(\beta\) and PKC-\(\delta\) isoforms. Activation of PKC is responsible for some of the deleterious effects on the cardiovascular system. It leads to alterations in vascular permeability, contractility, blood flow and coagulation by decreasing the expression of endothelial nitric oxide synthase (eNOS) while enhancing the expression of endothelin-1, vascular endothelial growth factor (VEGF) and PAI-1 (Das Evcimen and King, 2007). Structural abnormalities include thickening of basement membranes and accumulation of extracellular matrix components such as collagen and
fibronectin, which correlate with overexpression of TGF-β1 and connective tissue growth factor (Koya et al., 1997; Way et al., 2002). These PKC-associated changes are implicated in the development of vascular dysfunction, atherosclerosis, and diabetic cardiomyopathy (Geraldes and King, 2010).

1.4.1.4 Advanced glycation end-products (AGEs)

Another consequence of hyperglycemia is non-enzymatic glycation of free amino groups on proteins, lipids and nucleic acids resulting in the formation of AGEs. Indeed, higher serum concentrations of AGEs have been reported in diabetic patients relative to healthy subjects (Bansal et al., 2013; Aso et al., 2000). AGEs can accumulate in the heart and blood vessels, causing both structural and functional abnormalities (Singh et al., 2014). The adverse effects of AGEs are primarily mediated through cross-linking of intra- and extracellular proteins, as well as interaction with cell surface receptors for AGEs (RAGE). Increased cross-linking of collagen in the extracellular matrix, for example, contributes to diabetes-induced vascular and myocardial stiffness (Zieman and Kass, 2004; Candido et al., 2003). AGEs can initiate a number of downstream signalling pathways when bound to RAGE. Interaction of AGEs with RAGE has been shown to activate the transcription factor nuclear factor-κB (NF-κB), which in turn promotes upregulation of the pro-inflammatory genes encoding IL-1β, IL-6, TNF-α and cyclooxygenase-2 (Lorenzo et al., 2011; Neumann et al., 1999). This interaction can also stimulate NAD(P)H oxidase, xanthine oxidase and the mitochondrial respiratory chain to enhance the production of ROS (Basta et al., 2005; Wautier et al., 2001). Therefore, formation of AGEs in diabetic individuals may accelerate inflammation and oxidative stress, further aggravating their cardiovascular complications.
1.4.2 Targeting hyperglycemia

The beneficial effects of glucose control on microvascular complications have been documented in type 1 and type 2 diabetic patients, as evident from the Diabetes Control and Complications Trial (DCCT Research Group, 1993) and UK Prospective Diabetes Study (UKPDS Group, 1998), respectively. Strict glycemic control with insulin or sulphonylurea was able to decrease the incidence of end-organ complications and slow the progression of microvascular abnormalities, including retinopathy, nephropathy and neuropathy. There was a continuous reduction in microvascular complications as HbA1c levels decreased to normal range (Skyler et al., 2009). Recent trials also confirmed the benefits of intensive glycemic control on microvascular outcomes (Ismail-Beigi et al., 2010; ADVANCE Collaborative Group, 2008; DCCT/EDIC Research Group, 2011).

On the other hand, the potential of glucose lowering to reduce cardiovascular risk and macrovascular complications remains controversial. The Diabetes Control and Complications Trial (DCCT Research Group, 1993) did not find a significant decrease in macrovascular outcomes with intensive therapy in patients with type 1 diabetes (likely due to insufficient statistical power), but a follow-up study showed a delayed, long-term benefit (Nathan et al., 2005). During the 17 years of follow-up in these type 1 diabetic patients, intensive treatment aimed at achieving near normoglycemia reduced the risk of the first occurrence of non-fatal myocardial infarction, stroke or cardiovascular mortality by 57% relative to conventional treatment. In the UK Prospective Diabetes Study (UKPDS Group, 1998), despite a substantial 25% risk reduction in microvascular endpoints (retinopathy requiring photocoagulation, vitreous hemorrhage, renal failure), glycemic control did not significantly lower diabetes-related mortality or cardiovascular events (myocardial infarction, stroke) in newly diagnosed type 2
diabetic individuals. Three large randomized controlled trials — Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE; ADVANCE Collaborative Group, 2008), Action to Control Cardiovascular Risk in Diabetes (ACCORD; ACCORD Study Group, 2008), and Veterans Affairs Diabetes Trial (VADT; Duckworth et al., 2009) — suggest that achieving near-normal glycemic control in long-standing type 2 diabetic patients does not necessarily improve cardiovascular outcomes. In fact, aggressive control of blood glucose (HbA1c <6%) in the ACCORD study was significantly associated with increased mortality from cardiovascular causes (hazard ratio of 1.35) and a higher rate of severe hypoglycemia. Likewise, a recent meta-analysis of 13 studies involving type 2 diabetic subjects showed no benefits of strict glycemic control on all-cause as well as cardiovascular mortality relative to standard treatment (Boussageon et al., 2011). As appears from the aforementioned studies, intensive glucose lowering treatment is likely able to prevent and/or ameliorate macrovascular complications in patients with type 1 diabetes, but not in those with type 2 diabetes.

Although there is compelling evidence that hyperglycemia is associated with increased risk of cardiovascular morbidity and mortality in the diabetic population, the benefits of glucose lowering has not been equally convincing. Currently, it is still inconclusive whether insulin and oral hypoglycemic agents can reduce macrovascular events and cardiovascular mortality (Panicker et al., 2012; Kassem and Raz, 2009; Bolen et al., 2007). Hyperglycemia can lead to many long-term complications in the heart and blood vessels, but is certainly not the only factor involved. Non-glycemic related risk factors also play a significant role, particularly in patients with type 2 diabetes. In these patients, a multifactorial intervention consisting of other mechanism-based therapeutic strategies may be more beneficial (Gaede et al., 2008).
1.5 Dyslipidemia

Besides hyperglycemia, diabetic individuals frequently exhibit dyslipidemia. The characteristic features of diabetic dyslipidemia are increased plasma triglycerides, decreased high density lipoprotein (HDL) cholesterol, and increased small dense low density lipoprotein (LDL) cholesterol (Chehade et al., 2013). Dyslipidemia is generally more common in patients with type 2 than type 1 diabetes (Wadwa et al., 2005), and affects up to 97% of the diabetic population (Dokken, 2008). The Framingham Heart Study was the first to establish dyslipidemia as a constant feature in diabetes (Kannel, 1985; Mooradian, 2009). This study reported a significantly higher prevalence of hypertriglyceridemia (21% versus 12% in men and 25% versus 10% in women, respectively) and hypo-HDL cholesterolemia (19% versus 9% in men and 17% versus 8% in women, respectively) in diabetic patients relative to non-diabetic individuals. On the contrary, the prevalence of high LDL cholesterol levels in those with diabetes (9% in men and 15% in women) did not significantly differ from those without (11% in men and 16% in women). Similar findings were observed in the United States National Health and Nutrition Examination Survey 1999-2000 (Jacobs et al., 2005) and the UK Prospective Diabetes Study (UKPDS Group, 1997). Other investigators have reported elevated levels of LDL in type 1 and type 2 diabetic patients, accompanied by increased plasma triglycerides and reduced HDL (Martinez-Hervas et al., 2014; Siraj et al., 2006; Perez et al., 2000; Lopes-Virella et al., 1981). Taken together, these clinical studies confirm a strong association between diabetes and dyslipidemia, particularly high triglyceride and low HDL levels.

Dyslipidemia is one of the risk factors for cardiovascular disease in diabetes. Lower levels of HDL cholesterol together with higher levels of triglycerides and LDL cholesterol are associated with adverse cardiovascular events in type 1 (Weis et al., 2001; Laakso et al., 1986)
and type 2 (Fuller et al., 2001; Turner et al., 1998; Lehto et al., 1997; Laakso et al., 1993)
diabetic patients. These lipid abnormalities have been shown to escalate the risk of coronary
heart disease by 2- to 4-fold (Laakso et al., 1993). Analysis of data from diabetic individuals in
the Strong Heart Study found that every 10 mg/dL increment in LDL cholesterol or decrement in
HDL cholesterol corresponded to a 12% or 22% increase in cardiovascular disease risk,
respectively (Howard et al., 2000). Because dyslipidemia is highly correlated with
atherosclerosis, it can contribute to diabetic macrovascular complications (Farmer, 2007).
Moreover, the presence of subclinical atherosclerosis such as increased coronary artery calcium
deposition is a significant predictor of cardiovascular mortality in the diabetic population
(Agarwal et al., 2013).

1.5.1 Mechanisms of dyslipidemia-induced damage

In patients with diabetes, dyslipidemia is closely linked to impaired insulin signalling.
Both insulin deficiency and insulin resistance are associated with elevated levels of circulating
free fatty acids (FFAs), which can in turn activate PKC, diminish nitric oxide bioavailability, and
augment the production of ROS to cause cardiovascular abnormalities (Beckman, 2004; Creager
et al., 2003). Higher levels of FFAs promote the formation of small dense LDL cholesterol
(Sniderman et al., 2001). Compared to normal LDL particles, small dense LDL particles are
more atherogenic and more susceptible to oxidation and glycation. Oxidation of LDL stimulates
monocyte infiltration, vascular smooth muscle cell proliferation, and apoptosis in endothelial
cells (Mertens and Holvoet, 2001), while glycation of LDL lengthens its half-life (Rabbani et al.,
2010). These LDL particles can easily penetrate and form strong attachments to the blood vessel
wall, thereby increasing the risk of developing atherosclerosis (Sniderman et al., 2001). In
addition, they can cause greater production and secretion of PAI-1 (Festa et al., 1999) and thromboxane (Colas et al., 2011) in the vasculature, giving rise to a prothrombotic state. Elevated FFAs also triggers tissue inflammation through interaction with Toll-like receptors, subsequently activating NF-κB to upregulate inflammatory genes (Paneni et al., 2013).

Diabetes induces several alterations in lipoprotein metabolism that lead to hypertriglyceridemia, including increased flux of FFAs to the liver and reduced degradation of triglyceride-rich lipoproteins by lipoprotein lipase (LPL). Increased flux of FFAs to the liver promotes triglyceride production, which stimulates hepatic synthesis and secretion of very low density lipoproteins (VLDL). High levels of triglyceride-rich VLDL enable cholesteryl ester transfer protein (CETP) to facilitate transfer of triglycerides into HDL or LDL in exchange for cholesteryl esters. The HDL or LDL particles that are enriched with triglycerides undergo further hydrolysis via the enhanced action of hepatic lipase. These processes ultimately result in rapid clearance of HDL from the circulation and generation of smaller, denser LDL particles (Bardini et al., 2012; Mooradian, 2009; Adiels et al., 2008; Krauss, 2004). The other potential mechanism responsible for diabetes-associated hypertriglyceridemia is defective triglyceride catabolism by the enzyme LPL (Chen and Tseng, 2013). Decreased activity of LPL, which reduces the rate of triglyceride clearance, has been documented in diabetic individuals (Kashiwazaki et al., 1998; Pfeifer et al., 1983).

Lipid accumulation and abnormal energy metabolism in the heart may contribute to cardiomyopathy (Stratmann and Tschoepe, 2011; Ruberg, 2007). As shown in patients with type 1 (Herrero et al., 2006) and type 2 (McGill et al., 2011; Rijzewijk et al., 2009) diabetes, excessive utilization and oxidation of myocardial fatty acids is likely detrimental to cardiac function. In the diabetic heart, fatty acids tend to undergo incomplete β-oxidation, leading to
cardiac insulin resistance (Zhang et al., 2010a) and an overproduction of ROS that can exacerbate oxidative stress (Harmancey and Taegtmeyer, 2008).

1.5.2 Targeting dyslipidemia

Diabetic patients can achieve optimal lipid levels via effective management of dyslipidemia. Multiple randomized, placebo-controlled trials have demonstrated the benefits of 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors (statins) in preventing diabetes-associated cardiovascular events. Statins, a class of cholesterol lowering drugs, act primarily to decrease the levels of LDL cholesterol. The Collaborative Atorvastatin Diabetes Study (CARDS) found that statin treatment, relative to placebo, significantly reduced the incidence of acute coronary heart disease (by 36%), coronary revascularisations (by 31%), stroke (by 48%) and all-cause mortality (by 27%) in diabetic patients with no previous history of cardiovascular disease (Colhoun et al., 2004). Similar results, with 23%, 24% and 42% risk reduction in cardiovascular events, were respectively reported in the lipid-lowering arm of the Anglo-Scandinavian Cardiac Outcomes Trial (ASCOT-LLA; Sever et al., 2005), the Heart Protection Study (Collins et al., 2003), and the Scandinavian Simvastatin Survival Study (4S; Haffner et al., 1999). It is important to note that statins exert pleiotropic effects beyond correction of dyslipidemia, which may underlie their significant benefits on cardiovascular outcomes (Liao and Laufs, 2005). Some of these additional properties include improvements in endothelial function and blood flow, inhibition of thrombogenesis, stabilization of atherosclerotic plaques, as well as reduction of inflammation and oxidative stress.

Besides statins, fibrates and niacin (nicotinic acid) also confer favourable outcomes among the diabetic population. These drugs improve lipid metabolism by raising HDL
cholesterol and decreasing triglycerides. A few clinical trials support the use of fibrates or niacin to reduce cardiovascular risk in patients with diabetes, although the evidence is less extensive compared to statins (Hamilton and Watts, 2013; Shepherd et al., 2005). Fibrates appear to be most effective for those who exhibit marked hypertriglyceridemia, with or without a low HDL level (Scott et al., 2009). For some diabetic patients, combination therapy consisting of a statin plus a fibrate or niacin may further optimize their plasma lipid profile to yield additional cardiovascular benefits (Hamilton and Watts, 2013).

1.6 Inflammation

Inflammation is a normal immune response to tissue injury or infection. In diabetes, this response is dysregulated, creating a state of chronic, low-level inflammation. The inflammatory response, which involves the activation of monocytes and lymphocytes in addition to increased secretion of inflammatory cytokines, is initiated early in the pathophysiology of diabetes and is often present in the pre-diabetic state (Laakso, 2010; Badawi et al., 2010). Indeed, low-grade inflammation is associated with increased risk of diabetes, even in people without clinical evidence of cardiovascular disease (Isordia-Salas et al., 2014; Pitsavos et al., 2007).

Inflammatory markers are significantly increased in patients with type 1 (Devaraj et al., 2007; Gomes et al., 2003; Schalkwijk et al., 1999) and type 2 (Vinagre et al., 2014; Mirza et al., 2012; Mishra et al., 2011; Ford, 1999) diabetes. Common markers of systemic inflammation include C-reactive protein (CRP), IL-6 and TNF-α. Previous studies have shown that elevated levels of these inflammatory markers are associated with the progression of type 1 diabetes (Treszl et al., 2004) and development of type 2 diabetes (Hu et al., 2004; Spranger et al., 2003; Festa et al., 2002; Barzilay et al., 2001; Pradhan et al., 2001), with CRP being the most
consistent and strongest predictor. In diabetic patients, CRP has been recognized as an independent risk factor for fatal and non-fatal cardiovascular events (Vengen et al., 2009; Soinio et al., 2006; Coppola et al., 2006) as well as all-cause mortality (Cox et al., 2012; Kengne et al., 2012). Collectively, these findings suggest that inflammation is an important underlying factor in the pathogenesis of cardiovascular disease in diabetes.

1.6.1 Mechanisms of inflammation-induced damage

Inflammatory processes are implicated in the progression of type 1 and type 2 diabetes. Chronic, low-level inflammation has been found to suppress insulin signalling resulting in insulin resistance. Pro-inflammatory cytokines, such as TNF-α, IL-1β and IL-6, can decrease insulin sensitivity through the activation of Jun kinase (JNK) and IκB kinase-β/NF-κB pathways (Shoelson et al., 2006). These cytokines can further induce apoptosis of pancreatic β-cells, which ultimately reduces β-cell mass and insulin secretion (Donath et al., 2008). The autoimmune process, characterized by infiltration of macrophages and lymphocytes, can also evoke islet inflammation and cytokine-mediated β-cell destruction, especially in those with type 1 diabetes (Cernea and Dobreanu, 2013; Bending et al., 2012). In addition, generation of cytokines by inflammatory cells can upregulate the expression of adhesion molecules, increase the levels of fibrinogen and PAI-1, enhance monocyte and lymphocyte recruitment into atherosclerotic lesions, promote vascular smooth muscle cell proliferation and cardiac hypertrophy, stimulate deposition of extracellular matrix, and depress myocardial contractile function, thereby contributing to atherosclerosis, endothelial dysfunction and diabetic cardiomyopathy (Nunes et al., 2012; Savoia and Schiffrin, 2007; Tedgui and Mallat, 2006).
1.6.2 Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX), also known as prostaglandin-endoperoxide synthase, is an enzyme that catalyzes the conversion of arachidonic acid to various prostanoids. Two COX isoforms have been identified. COX-1 is constitutively expressed in most tissues and plays a critical role in normal physiological function, whereas COX-2 is the inducible isoform that is upregulated by cytokines, mitogens, hormones and growth factors (Botting, 2010; Rouzer and Marnett, 2009; Suleyman et al., 2007; Vane et al., 1998; Smith et al., 1996). Present in most tissues at low or undetectable levels under basal conditions, COX-2 can be rapidly induced in response to inflammatory stimuli. COX-2 is the dominant source of prostanoid synthesis during acute and chronic inflammation, thus is considered a pro-inflammatory mediator (Ricciotti and FitzGerald, 2011). Low-grade inflammation mediated by COX-2 may contribute to the pathogenesis of diabetic complications (Bagi et al., 2006; Helmersson et al., 2004). Overexpression of COX-2 has been detected in diabetic patients (Szerafin et al., 2006) and appears to be partially responsible for impaired vascular function as well as excessive ROS formation (Shi and Vanhoutte, 2008; Pannirselvam et al., 2005; Guo et al., 2005). Not surprisingly, COX-2 inhibitors have been shown to improve diabetic nephropathy (Cheng et al., 2002) and endothelial dysfunction (Abdelrahman and Al Suleimani, 2008) in experimental models of diabetes. However, it is unclear if inhibition of COX-2 has beneficial effects on in vivo cardiac and vascular contractile functions in diabetes.

1.6.3 Targeting inflammation

Anti-inflammatory drugs such as salicylates and aspirin have been extensively studied. These drugs can target inflammation by inhibiting the activity of NF-κB (Yin et al., 1998; Pierce
et al., 1996; Kopp and Ghosh, 1994) and/or COX (Bunimov and Laneuville, 2008; Vane, 1994). In a multicenter, randomized, placebo-controlled trial of patients with type 2 diabetes, salsalate, a prodrug of salicylate, improved glycemia and reduced inflammatory markers after 48 weeks of treatment (Goldfine et al., 2013). Aspirin, a prototypical non-steroidal anti-inflammatory drug, has been shown to increase insulin-stimulated glucose uptake and decrease hepatic glucose production in diabetic patients (Hundal et al., 2002), although conflicting results have been reported (Bratusch-Marrain et al., 1985). A recent meta-analysis indicated that aspirin appears to produce modest reductions in myocardial infarction and stroke in patients with diabetes, but current evidence regarding its use for primary prevention of cardiovascular events is still inconclusive (Pignone et al., 2010).

1.7 Oxidative stress

Oxidative stress arises as a result of an imbalance between ROS production and antioxidant capacity (Araki and Nishikawa, 2010). ROS are normal by-products of aerobic metabolism and include free radicals such as superoxide (\(\cdot \text{O}_2\)), hydroxyl (\(\cdot \text{OH}\)) and peroxyl (\(\cdot \text{RO}_2\)), as well as nonradical species such as hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and hydrochlorous acid (HOCl) (Matough et al., 2012; Johansen et al., 2005). Under physiological conditions, ROS are rapidly eliminated by various endogenous antioxidants comprising of superoxide dismutase (SOD), glutathione peroxidase (GPx), catalase (CAT), GSH, vitamin C (ascorbic acid), vitamin E (tocopherol) and \(\alpha\)-lipoic acid. However, during oxidative stress, the increased generation of ROS along with simultaneous deficiency in antioxidant defense mechanisms can lead to damage of cellular components (lipids, proteins, DNA), altering the structure and function of these biomolecules and ultimately causing cell death.
In both type 1 and type 2 diabetic patients, oxidative stress is increased, as evident by enhanced superoxide production (Hsu et al., 2006; Guzik et al., 2002). Moreover, indicators of lipid peroxidation, including thiobarbituric acid reactive substances and malondialdehyde, were found to be elevated in diabetic individuals (Shinde et al., 2011; Bhutia et al., 2011; Mahreen et al., 2010; Asicioglu et al., 2010; Indran et al., 2004; Ruiz et al., 1999; Griesmacher et al., 1995). These abnormalities are often accompanied by reduced antioxidant status. Lower levels of GSH and vitamin E, as well as decreased activities of SOD, GPx and CAT have been documented in patients with type 1 (Ramakrishna and Jailkhani, 2007; Dave and Kalia, 2007; Indran et al., 2004) and type 2 (Hisalkar et al., 2012; Shinde et al., 2011; Colak et al., 2005; Vericel et al., 2004) diabetes, although there appear to be some discrepancies, with a few studies reporting no differences or an increase in antioxidant levels and/or activities (Bandeira et al., 2012; Savu et al., 2012; Taheri et al., 2012; Seghieri et al., 2001; Willems et al., 1998). The conflicting data is likely due to differences in age, diabetes duration, prevalence of complications, and metabolic control of the diabetic subjects. Nevertheless, there is mounting evidence that oxidative stress plays a pivotal role in the pathogenesis of diabetic cardiovascular complications, particularly coronary artery disease and cardiomyopathy (Xu et al., 2014; Fiorentino et al., 2013; Matough et al., 2012; Giacco and Brownlee, 2010).

1.7.1 Mechanisms of oxidative stress-induced damage

Potential sources of ROS include NAD(P)H oxidase, xanthine oxidase, and mitochondrial enzymes (Son, 2012; Paravicini and Touyz, 2008), all of which are altered in diabetes via glucose dependent and independent mechanisms. NAD(P)H oxidase and xanthine oxidase are superoxide-generating enzymes that can be upregulated by hyperglycemia. In cultured
endothelial cells, exposure to high glucose led to marked increases in both superoxide production and expression of the NAD(P)H oxidase subunit p22phox (Ding et al., 2007; Weidig et al., 2004). Enhanced activity of NAD(P)H oxidase has been detected in animal models of diabetes (Li et al., 2010; Hink et al., 2001) and diabetic patients (Sorrentino et al., 2007; Guzik et al., 2002). There is evidence that inhibition of xanthine oxidase with allopurinol reduces oxidative stress in the heart and plasma of streptozotocin-induced diabetic rats and patients with type 1 diabetes, respectively (Desco et al., 2002), and improves endothelial function (Butler et al., 2000) and left ventricular hypertrophy (Szwejkowski et al., 2013) in patients with type 2 diabetes, highlighting the involvement of xanthine oxidase in diabetic cardiovascular complications. Hyperglycemia can also stimulate the production of ROS by direct glucose auto-oxidation (Wolff and Dean, 1987), excessive accumulation of AGEs and their subsequent interaction with RAGE (refer to Section 1.4.1.4), uncoupling of eNOS (Cai et al., 2005), and overflux of electron donors (NADH and FADH$_2$) into the mitochondrial electron transport chain (Yan, 2014). Furthermore, high glucose levels can interfere with the antioxidant system. For instance, decreased activity of antioxidant enzymes through enhanced protein glycation (Kedziora-Kornatowska et al., 1998), depletion of NADPH and GSH from overactivity of the polyol pathway (refer to Section 1.4.1.1), and loss of Cu$^{2+}$, an essential cofactor for SOD (Hamden et al., 2009), have been observed in hyperglycemic conditions. Reduced antioxidant capacity with concomitant overproduction of ROS would undoubtedly favour oxidative stress, an event associated with the development of diabetic complications, often accompanied by pancreatic β-cell dysfunction and insulin resistance (Pitocco et al., 2010).

Dyslipidemia (Puddu et al., 2009) and inflammation (Zhang et al., 2009) are also implicated in the generation of oxidative stress in diabetes. Oxidized LDL cholesterol, which is
elevated in diabetes, has been shown to stimulate superoxide production and subsequent apoptosis in human endothelial cells and rabbit aortae (Galle et al., 1999). In cultured aortic endothelial and smooth muscle cells, high levels of FFAs increased the formation of ROS through PKC-dependent activation of NAD(P)H oxidase (Inoguchi et al., 2000). In the diabetic heart, increased ROS from excessive β-oxidation of fatty acids can damage proteins, lipids and DNA, thereby inducing hypertrophy, contractile dysfunction, fibrosis and cell death (Lorenzo et al., 2013; van de Weijer et al., 2011). These aforementioned changes likely contribute to the acceleration of atherosclerosis and cardiomyopathy in patients with diabetes. Inflammation and oxidative stress are also closely linked. Accumulation of ROS can activate many pro-inflammatory signalling pathways, including the transcription factor NF-κB which regulates the expression of a large number of genes associated with diabetic complications, such as inducible nitric oxide synthase (iNOS), COX-2, NAD(P)H oxidase, VEGF and RAGE (Morgan and Liu, 2011; Gloire et al., 2006). Therefore, ROS-induced activation of NF-κB may cause impairment of cellular function. Furthermore, many of the NF-κB target genes can in turn promote the formation of ROS, creating a vicious cycle (Evans et al., 2003).

1.7.2 Targeting oxidative stress

Antioxidant therapies have shown some promise in experimental models of diabetes, but several large-scale clinical trials utilizing antioxidants appear to confer no significant benefits (Matough et al., 2012). The Heart Outcomes Prevention Evaluation (HOPE) Trial (Yusuf et al., 2000), the Heart Protection Study (Heart Protection Study Collaborative Group, 2002), the Prevention of Progression of Arterial Disease and Diabetes (POPADAD) Trial (Belch et al., 2008), the Women’s Antioxidant Cardiovascular Study (WACS; Song et al., 2009), and the
Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) Study (Kataja-Tuomola et al., 2010) all failed to demonstrate beneficial effects of antioxidant vitamins (vitamin C, vitamin E, β-carotene) on cardiovascular events and mortality in patients with diabetes. Insufficient dosages of the supplemented vitamins, inadequate monitoring of oxidative stress biomarkers, and short time frame of the clinical trials may have contributed to the negative findings.

1.8 Animal models

Numerous animal models have been developed to study the complications of diabetes and their underlying etiopathogenetic mechanisms. Although none of the currently available models completely resemble all aspects of human diabetes, each model displays certain characteristic features of diabetes and therefore serves as an invaluable tool to allow experimentation that would otherwise be impossible in humans. The most frequently used models are obtained spontaneously, or induced by chemicals or dietary manipulations. Spontaneous models of type 1 diabetes include the Bio-Breeding rat and non-obese diabetic mouse, while those of type 2 diabetes include the ob/ob mouse, db/db mouse, Zucker diabetic fatty rat, Otsuka Long Evans Tokushima Fatty rat, and Goto-Kakizaki rat (Wang et al., 2013b; Li and Sun, 2010). Many of these models have been produced through selective breeding or spontaneous mutations. Chemical agents are also widely used to induce diabetes, the most popular being alloxan and streptozotocin (Kumar et al., 2012). Animals treated with these chemicals exhibit hyperglycemia, glycosuria, polyphagia, polydipsia and ketosis, all of which are characteristics of diabetes. Streptozotocin is preferred over alloxan because it elicits sustained hyperglycemia for longer duration and has a lower incidence of mortality (Dontas et al., 2013). Diet-induced models involve feeding certain animals, such as the sand rat, C57BL/6J mouse and spiny mouse, a high-
fat or high-energy diet to render them diabetic (Srinivasan and Ramarao, 2007). These models typically require extended periods of dietary treatment, but are more closely associated with the natural progression of diabetes in the human population.

1.8.1 Streptozotocin-induced diabetic rats

Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosourea)-1-D-glucopyranose) is a naturally occurring glucose analogue and a broad spectrum antibiotic isolated from the bacterium *Streptomyces achromogenes* (Eleazu et al., 2013). It is transported into cells via glucose transporter-2 (GLUT2), thus is particularly toxic to pancreatic β-cells since these cells express relatively high levels of GLUT2 (Schnedl et al., 1994). The nitrosourea moiety of streptozotocin acts as an alkylating agent to break DNA strands, which in turn activates the nuclear enzyme poly(ADP-ribose) synthetase as part of the cell repair mechanism, resulting in substantial depletion of intracellular NAD⁺ and ATP (Yamamoto et al., 1981). By interfering with cellular energy metabolism, this process inhibits insulin biosynthesis and secretion, and ultimately causes β-cell death through necrosis (Lenzen, 2008; Saini et al., 1996). Besides its strong alkylating properties, there is also evidence that production of ROS (Takasu et al., 1991; Nukatsuka et al., 1988) as well as nitric oxide (Kroncke et al., 1995; Turk et al., 1993) contribute to the cytotoxic effects of streptozotocin in the pancreatic islets.

A single intravenous or intraperitoneal injection of streptozotocin can induce diabetes in different species, including various rodents (Eleazu et al., 2013). Its diabetogenic actions can generally be seen within 72 hours following administration. The severity of diabetes in rats ranges from mild hyperglycemia after a low dose of 35 mg/kg to fatal ketosis resulting in death after a high dose of 100 mg/kg (Junod et al., 1969). In adult rats, the most commonly
administered dose of streptozotocin is 60 mg/kg because it produces persistent, chronic hyperglycemia without requiring insulin supplementation for survival (Eddouks et al., 2012; Rodrigues et al., 1999; Tancrede et al., 1983). Rats injected with streptozotocin develop many well-characterized symptoms of type 1 diabetes, where selective destruction of the pancreatic β-cells with consequent insulin deficiency leads to hyperglycemia alongside glycosuria, polyuria, polyphagia, polydipsia and weight loss (Hebden et al., 1986). They also exhibit cardiovascular abnormalities similar to those in diabetic patients (refer to Section 1.3). In the experiments described in this thesis, streptozotocin-induced diabetic rats were used as a model of type 1 diabetes.

1.8.2 Fructose-fed rats

Fructose is a simple sugar found in many natural sources, most notably in fruits and honey. Commercially, it is produced in three forms: crystalline fructose, sucrose (table sugar), and high-fructose corn syrup (HFCS). Sucrose and HFCS have become major sources of fructose in the Western diet. Sucrose is a disaccharide comprising equimolar ratios of glucose and fructose, whereas HFCS is made by enzymatic isomerization of glucose (from corn starch) into a syrup mixture containing 42% or 55% fructose (Hanover and White, 1993). Because of its low cost and greater relative shelf-life and stability compared to sucrose, HFCS has been used extensively as a caloric sweetener in many processed foods and beverages (Vos et al., 2008; Bray et al., 2004). In the United States, the total caloric intake from HFCS rose dramatically between 1989 and 2000, from 77 to 199 kcal/person/day (Duffey and Popkin, 2008). The excessive consumption of fructose has been associated with the escalating rates of obesity (Bray et al., 2004) and type 2 diabetes (Johnson et al., 2009; Gross et al., 2004) observed in the
developed world over the past few decades. In fact, countries that use HFCS in their food supply were found to have a 20% higher prevalence of diabetes, independent of obesity and total sugar availability, compared to those that do not use HFCS (Goran et al., 2013). A recent meta-analysis of 11 prospective cohort studies suggested that greater consumption of sugar-sweetened beverages made with added sucrose or HFCS elevates the risk of metabolic syndrome and type 2 diabetes respectively by 20% and 26% (Malik et al., 2010).

Fructose, when consumed in excess, exerts a number of adverse metabolic effects. It has long been recognized that fructose is more lipogenic than glucose. A high flux of fructose to the liver bypasses the rate-limiting steps of glucose metabolism, resulting in de novo lipogenesis and ectopic fat deposition (Havel, 2005). Clinical studies have indeed shown a positive correlation between overconsumption of fructose and dyslipidemia, as evident from the elevated plasma triglyceride and LDL cholesterol levels in both healthy (Stanhope et al., 2011; Le et al., 2006; Bantle et al., 2000; Swanson et al., 1992) and obese (Stanhope et al., 2009) individuals after 2-10 weeks of a high-fructose diet. In addition, fructose can reduce satiety and contribute to increased food intake because it is not effective in stimulating insulin and leptin secretion (Teff et al., 2004). Fructose consumption also promotes insulin resistance (Aeberli et al., 2013; Stanhope et al., 2009; Beck-Nielsen et al., 1980), upregulation of pro-inflammatory mediators (Cox et al., 2011; Aeberli et al., 2011), and oxidative stress (Hokayem et al., 2013).

In terms of animal models, rats that are fed a high-fructose diet for 2-12 weeks exhibit insulin resistance, hyperinsulinemia, impaired glucose tolerance and hypertriglyceridemia (de Moura et al., 2009; Oron-Herman et al., 2008; Song et al., 2005; Hwang et al., 1987), all of which are features of the metabolic syndrome. These abnormalities induced by fructose have been implicated as important risk factors for the development of type 2 diabetes and/or
cardiovascular disease in humans (Tappy and Le, 2010; Johnson et al., 2007). The fructose-fed rats, however, do not become hyperglycemic and therefore cannot be used as a model of diabetes.

1.9 Research rationale and objectives

Diabetes mellitus is associated with vascular and cardiac contractile dysfunction. Diminished vasoconstrictor responses to sympathoneural stimulation (van Gurp et al., 2006), reduced β-adrenergic sensitivity of the heart to isoproterenol (Berlin et al., 1986), as well as left ventricular diastolic and systolic functional abnormalities (Elshahed et al., 2008; Raev, 1994) were observed in patients with type 1 diabetes. Previous experiments in our laboratory have shown that rats with streptozotocin-induced type 1 diabetes had impaired pressor response, venous constriction and cardiac contractility to various vasoactive or inotropic agents (Cheng et al., 2004; Cheng et al., 2003). However, it is unclear if in vivo cardiovascular function is altered in type 2 diabetes.

The cause of diabetes-associated cardiac and vascular contractile dysfunction remains elusive, although multiple factors are likely responsible. Hyperglycemia, the hallmark of diabetes, is known to play a pivotal role in the development and progression of microvascular complications, but its contribution to macrovascular complications is somewhat controversial (Duckworth, 2001), and attempts to improve cardiovascular outcomes through glucose control have failed to demonstrate significant therapeutic benefits in diabetic patients (refer to Section 1.4.2). Besides hyperglycemia, inflammation (King, 2008; Savoia and Schiffrin, 2007; Stehouwer et al., 2002) and oxidative stress (Xu et al., 2014; Fiorentino et al., 2013; Matough et al., 2012; Giacco and Brownlee, 2010) have recently emerged as important mechanisms.
underlying the pathogenesis of diabetes-related cardiovascular abnormalities. Targeting inflammation and oxidative stress with more effective agents may represent a promising strategy for the prevention and/or treatment of cardiovascular disease among the diabetic population.

The main objective of this thesis was to determine if hyperglycemia, inflammation and oxidative stress are implicated in cardiovascular dysfunction in two rat models of diabetes. Using pharmacological intervention, we hoped to elucidate the extent to which these factors mediate arterial, venous and cardiac contractile impairment in vivo, and whether certain drugs or therapies have beneficial effects in the management of diabetic cardiovascular complications.

Four studies were conducted to address the following specific aims:

1) To develop a rat model of type 2 diabetes that closely resembles the natural progression and metabolic characteristics of the disease in humans, through a combination of high-fructose feeding and subsequent streptozotocin injection.

2) To compare if there are differences in in vivo cardiovascular function between two animal models of diabetes, namely streptozotocin-induced type 1 diabetic rats and fructose-fed, streptozotocin-induced type 2 diabetic rats.

3) To determine if treatment with phlorizin, a glucose lowering agent, protects against the development of arterial and venous contractile dysfunction in two rat models of diabetes.

4) To examine if nimesulide, a selective COX-2 inhibitor with minimal risk of cardiovascular adverse events, has beneficial actions on cardiovascular responses to adrenaline in two rat models of diabetes.

5) To investigate if reducing oxidative stress with chronic administration of N-acetylcysteine (NAC), an antioxidant and GSH donor, restores cardiac and vascular contractile functions in two rat models of diabetes.
1.10 Hypotheses

We hypothesize that hyperglycemia is associated with vascular contractile dysfunction in both type 1 and type 2 diabetes. However, since the fructose-fed, streptozotocin-induced type 2 diabetic rats will have a greater number of risk factors such as dyslipidemia and insulin resistance, targeting hyperglycemia alone in this group may not confer significant benefits. The alteration of cardiovascular function in type 1 and type 2 diabetes also involves low-grade inflammation mediated by COX-2, as well as increased oxidative stress. Administration of a selective COX-2 inhibitor or an antioxidant will therefore improve arterial, venous and cardiac contractile responses to adrenergic agents in the diabetic but not control rats. Our findings will disclose the relative impact of hyperglycemia, inflammation and/or oxidative stress in the pathogenesis of vascular and cardiac contractile dysfunction in animal models of type 1 and type 2 diabetes.

The specific hypotheses are outlined below:

1) Diabetic rats induced through a combination of high-fructose feeding and subsequent injection of streptozotocin will exhibit hyperglycemia, hypertriglyceridemia and insulin resistance, all of which are features of type 2 diabetes. Arterial, venous and cardiac contractile functions will be depressed in these rats.

2) Due to some metabolic differences in the two animal models of diabetes, the fructose-fed, streptozotocin-induced type 2 diabetic rats will have greater impairment in cardiovascular function relative to the streptozotocin-induced type 1 diabetic rats.

3) Treatment with phlorizin will fully protect against the development of arterial and venous contractile dysfunction in streptozotocin-induced type 1 diabetic rats, but not in the fructose-fed, streptozotocin-induced type 2 diabetic rats.
4) Acute administration of nimesulide will improve, but not completely restore, cardiovascular responses to adrenaline in both rat models of diabetes.

5) Chronic administration of NAC will ameliorate the cardiac as well as vascular contractile abnormalities in both rat models of diabetes.
Chapter 2: A rat model of fructose-fed, streptozotocin-induced type 2 diabetes

2.1 Overview

Understanding of the pathogenesis, complications and genetic or environmental influences of type 2 diabetes requires the use of appropriate animal models of the disease. To date, numerous models of type 2 diabetes have been developed in rodents using genetic manipulation, chemical or dietary induction, or a combination thereof; however, most of the models do not sufficiently resemble the natural history and metabolic features of human type 2 diabetes. Genetic models, such as the \textit{db/db} or \textit{ob/ob} mice and Zucker diabetic fatty rats, are highly inbred and may not be applicable to the human population due to homogeneity of the animals’ genetic background (Srinivasan and Ramarao, 2007). These models are also expensive and limited in availability. Other animal models can be obtained through chemical induction with alloxan or streptozotocin to selectively destroy the pancreatic \(\beta\)-cells (Rees and Alcolado, 2005; Rodrigues et al., 1999), but the resulting insulin deficiency and hyperglycemia more closely resemble human type 1 rather than type 2 diabetes. Dietary induction alone has limited value in developing a diabetic model because the animals generally do not establish hyperglycemia (Huang et al., 2004; Chalkley et al., 2002). Nonetheless, these animals, if fed a high-fat diet for example, exhibit various other physiological abnormalities including insulin resistance, impaired glucose tolerance, hyperinsulinemia and dyslipidemia. Previous studies have taken advantage of these metabolic characteristics to develop a type 2 diabetes model in rats by a combination of high-fat diet and streptozotocin treatment (Srinivasan et al., 2005; Reed et al., 2000). The injection of streptozotocin leads to the inability to maintain glucose homeostasis, thus rendering the rats hyperglycemic.
Besides fat, refined carbohydrates, particularly fructose, are very predominant in the Western diet. Fructose has been used increasingly as a sweetener in many processed foods due to its low cost. As mentioned in the introduction, excessive intake of fructose contributes to the development of hypertriglyceridemia, insulin resistance, glucose intolerance, hyperinsulinemia and hypertension, all of which are symptoms of the metabolic syndrome (Tappy and Le, 2010; Elliott et al., 2002). Since the majority of dietary intake consists of carbohydrates and not fat (Gaesser, 2007; Briefel and Johnson, 2004), high fructose consumption may be one of the most important environmental factors associated with the growing incidence of type 2 diabetes in developed countries. Furthermore, there appear to be differences in metabolic features between the two diets. Rats fed a high-fructose diet, for example, have increased plasma triglyceride and insulin levels relative to those fed a high-fat diet (Huang et al., 2004).

The aim of this study was to develop an inexpensive, stable and long-term model of type 2 diabetes by means of combining high-fructose diet with subsequent streptozotocin induction in non-genetic, outbred rats. The resultant model should closely resemble the metabolic characteristics of human type 2 diabetes. As shown in previous studies (de Moura et al., 2009; Oron-Herman et al., 2008; Song et al., 2005; Hwang et al., 1987), feeding rats a high-fructose diet induces insulin resistance along with compensatory hyperinsulinemia, a condition similar to the prediabetic state in humans. In this study, the progression of fructose-fed rats to type 2 diabetes was achieved by injecting streptozotocin to initiate β-cell dysfunction such that insulin levels declined and the rats became unable to maintain glucose homeostasis, thereby rendering them hyperglycemic. We further investigated if vascular contractile responses to noradrenaline were altered in this rat model of type 2 diabetes via measurements of mean arterial pressure (MAP) and MCFP. MCFP is experimentally the equilibrium pressure in the circulation.
immediately after an abrupt cessation of blood flow (Guyton, 1963). It reflects the upstream venous pressure that drives venous return and can be used as an index of body venous tone (Pang, 2000).

2.2 Materials and methods

2.2.1 Experimental animals and induction of diabetes

Male Wistar rats (5 weeks old weighing approximately 175-225 g) were obtained from Charles River Canada. The rats were randomly assigned to one of four treatment groups — Control (CTL), Fructose (FRU), Streptozotocin (STZ) or Fructose-Streptozotocin (FRU-STZ). They were housed two per cage and maintained under ambient temperature (23 ± 2°C) with 12:12 h light:dark cycle (lights on from 7 a.m. to 7 p.m.). The animals were cared for in accordance with the Canadian Council on Animal Care Guidelines. Starting on Day 0, animals designated to the CTL and STZ groups were fed with standard laboratory diet (PMI Nutrition International, Brentwood, MO, USA) while those in the FRU and FRU-STZ groups were fed a high-fructose diet (60% w/w; Harlan Teklad, Madison, WI, USA). The compositions of the diets are listed in Table 2.1. Food and water were supplied ad libitum. Body weight, food and fluid intake, and resting blood glucose were measured on Days 0 and 10. Blood glucose concentrations were measured with the Accu-Chek Advantage glucometer (Roche Diagnostics, Mannheim, Germany) by sampling blood from the saphenous vein.

On Day 14, the animals were lightly anesthetized with halothane (Halocarbon Laboratories, River Edge, NJ, USA) for intravenous drug administration via the tail vein. Rats in the STZ and FRU-STZ groups were injected with streptozotocin (60 mg/kg, i.v.; Sigma, St.
Louis, MO, USA) whereas those in the CTL and FRU groups were administered an equal volume of vehicle (5% dextrose). The rats were considered to be diabetic and were used for the study if they developed hyperglycemia (> 16 mM) at 48 h after injection of streptozotocin. Body weight and food and fluid intake were measured on Day 35 and blood (0.5 ml) was collected from the left iliac artery of unfasted rats under light halothane anesthesia for measurements of plasma insulin and triglyceride. Blood samples were centrifuged at 10,000 RPM for 10 min at 4°C and the plasma was separated and kept frozen at -70°C until use.

Table 2.1  Diet composition (% w/w)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Regular Diet</th>
<th>High-fructose Diet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat</td>
<td>10.8</td>
<td>5.2</td>
</tr>
<tr>
<td>Protein</td>
<td>23.2</td>
<td>18.3</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Starch</td>
<td>39.5</td>
<td>0.4</td>
</tr>
<tr>
<td>Fructose</td>
<td>0.34</td>
<td>60.0</td>
</tr>
</tbody>
</table>

2.2.2  Surgical preparation

On Day 42, half of the rats from each group were allocated to either one of the following protocols: two-hour hyperinsulinemic euglycemic clamp to determine insulin sensitivity or a hemodynamic study to evaluate vascular function. To carry out the hyperinsulinemic euglycemic
clamp, the four groups of rats were surgically prepared under thiobutabarbital sodium (Inactin; Sigma, St. Louis, MO, USA) anesthesia (100 mg/kg, i.p.). The rats were tracheotomized and heparinized saline-filled (25 IU/ml) polyethylene cannulae (PE50) were inserted into one iliac artery, and both jugular veins. The arterial cannula was used to sample blood for measurement of glucose levels. Recombinant human insulin (Humulin R; Eli Lilly, Indianapolis, IN, USA) and dextrose (40% w/v) were infused into the left jugular vein via a three-way connector whereas the right jugular cannula was used for administering the anesthetic whenever necessary. The animals were allowed to stabilize for 1 h post-surgery before use.

The other half of the rats were prepared under halothane anesthesia for the hemodynamic study. Heparinized saline-filled (25 IU/ml) polyethylene cannulae (PE50) were inserted into the right carotid artery for continuous monitoring of MAP and heart rate, into the left jugular and right iliac veins for infusion of drugs, and into the inferior vena cava via the left iliac vein for the measurement of central venous pressure (CVP). All pressures were measured by P23DB pressure transducers (Gould Statham, CA, USA) and recorded using the BIOPAC computer system (MP150). A saline-filled, balloon-tipped catheter was also inserted into the right atrium via the right jugular vein. All cannulae were tunneled subcutaneously to the back of the neck and exteriorized. The rats were given at least 4 h to recover from anesthesia and surgery prior to the study.

### 2.2.3 Hyperinsulinemic euglycemic clamp

Euglycemic clamp was performed on unfasted rats in all groups (CTL, n=9; FRU, n=9; STZ, n=8; FRU-STZ, n=7). Blood glucose was measured with the Accu-Chek Advantage glucometer immediately before initiation of the clamp. As rats in both the STZ and FRU-STZ
groups developed severe hyperglycemia, i.v. bolus doses of insulin were administered to reduce blood glucose concentration to approximate 7.0 mM euglycemic level prior to commencement of the clamp study using the following experimentally-derived formula \([0.4 \text{ U} \times \text{kg} \times (x \text{ mM blood glucose} - 7.0 \text{ mM})]\). After 60 min, insulin infusion (6 mU/kg/min) was initiated (t = 0 min) and kept at a constant rate. Blood samples for glucose measurements were taken every 5 min. Dextrose (40% w/v) infusion began at t = 5 min at a variable rate and was adjusted as needed after each blood glucose measurement to maintain glucose concentrations at 7.0 ± 0.4 mM. The clamp was continued until t = 120 min whereby the experiment was terminated. The insulin sensitivity index was determined by taking the average glucose infusion rate (GIR) required for maintaining blood glucose constant at 7.0 mM from t = 90 min to t = 120 min.

2.2.4 Hemodynamic study

Rats in all four groups (CTL, n=8; FRU, n=7; STZ, n=8; FRU-STZ, n=9) were placed in a small cage and allowed to wander freely. After equilibration for 1 h, the rats were pretreated with propranolol \((8 \times 10^{-7} \text{ mol/kg i.v. bolus followed by continuous infusion at } 3.4 \times 10^{-7} \text{ mol/kg/min for the remainder of the study; Sigma, St. Louis, MO, USA}) to block β-adrenoceptors. Fifteen min after administration of propranolol, dose-response curves of intravenously infused noradrenaline \((1, 2, 4, 6, 8, 16 \text{ and } 24 \times 10^{-9} \text{ mol/kg/min; Sigma, St. Louis, MO, USA}) were constructed. Measurements of MAP and heart rate were taken before and at 15 min following the start of propranolol infusion, and at the plateau phase of each noradrenaline response. The rats were allowed to recover for 30 min before MCFP readings were taken at baseline, and at the plateau phase of response to ED_{40} and ED_{75} of the noradrenaline dose-MAP response curve. Each MCFP measurement was followed by a recovery period of 10 min.
2.2.5 Measurement of MCFP

The MCFP technique in rats has been described in detail (Pang, 2000; Tabrizchi and Pang, 1992). Briefly, the right atrial balloon was inflated by injection of saline to stop circulation transiently. Within 5 s, MAP decreased to a plateau value (final arterial pressure), while CVP increased to a plateau value (venous plateau pressure). MCFP was calculated using the following equation: MCFP = venous plateau pressure + 1/60 (final arterial pressure – venous plateau pressure), where 1/60 represents the ratio of arterial to venous compliance (Yamamoto et al., 1980).

2.2.6 Measurements of plasma insulin and triglyceride

Blood plasma samples collected on Day 35 were thawed on ice and prepared for assay of plasma insulin or triglyceride. Plasma insulin was measured by the Ultra Sensitive Rat Insulin ELISA method (Crystal Chem Inc., Downers Grove, IL, USA) and triglyceride was measured using an enzymatic technique (Infinity™ Triglycerides Liquid; Thermo Electron, Melbourne, Australia).

2.2.7 Statistical analyses

The results are presented as mean ± S.E.M. Dose-MAP response curves for noradrenaline were constructed by nonlinear regression, using the sigmoidal dose-response model with variable slope in GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego, CA, USA), to obtain ED$_{50}$ and $E_{\text{max}}$ values. The mean ED$_{50}$ and $E_{\text{max}}$ values were calculated from individual dose-response curves. Data for ED$_{50}$ were log-transformed prior to statistical analysis.
All data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey Test. A value of $P < 0.05$ was considered statistically significant.

2.3 Results

2.3.1 General characteristics of diabetes induction

There were no statistically significant differences in body weight and food and fluid intake among the four groups of rats at the commencement of the study and at Day 10 after feeding of the 60% high-fructose diet (results not shown). After injection of streptozotocin (60 mg/kg) or vehicle (5% dextrose) on Day 14, blood glucose measured on Day 16 in the STZ and FRU-STZ groups were significantly higher than those in the CTL and FRU groups (STZ 21.8 ± 1.1 mM versus CTL 6.6 ± 0.3; FRU-STZ 26.0 ± 1.7 mM versus FRU 6.5 ± 0.3 mM; $P < 0.05$), respectively, and blood glucose was also significantly higher in the FRU-STZ than the STZ group.

Measurements on Day 35 revealed marked differences in body weight and food and fluid intake between the groups (Table 2.2). Animals in the STZ and FRU-STZ groups had greater food and fluid intake compared to those in the CTL and FRU groups, respectively. In addition, the FRU-STZ group consumed significantly more water than the STZ group. By Day 35, body weights in both the STZ and FRU-STZ groups were significantly lower than those in the CTL and FRU groups. Blood glucose measurements in the four groups of unfasted rats taken on Day 42 were similar to those measured on Day 16, with blood glucose significantly higher in the STZ and FRU-STZ groups relative to the CTL and FRU groups (Table 2.2).
2.3.2 Plasma insulin and triglyceride

The two groups given streptozotocin had significantly reduced plasma insulin compared to the CTL and FRU groups (Table 2.2). Insulin levels between the STZ and FRU-STZ groups did not significantly differ from each other. Plasma triglyceride was markedly higher in the FRU-STZ than the FRU group, which in turn, was higher than levels in the CTL and STZ groups (Table 2.2).

2.3.3 Hyperinsulinemic euglycemic clamp

All rats in the STZ and FRU-STZ groups required bolus doses of insulin to reduce blood glucose to a normal physiological range prior to commencement of the euglycemic clamp. Blood glucose was maintained within 0.4 mM above or below the target level of 7.0 mM for the remainder of the experiment by adjusting, as needed, the GIR at 5 min intervals (Figure 2.1A). GIR values for the period $t = 90-120$ min were averaged to facilitate comparison among groups. The CTL group required significantly higher levels of glucose infusion to maintain euglycemia at 7.0 mM relative to the other two groups given streptozotocin (Figure 2.1). Differences between the CTL and FRU groups, however, were not statistically significant ($P = 0.075$), although the mean GIR value was slightly higher in the CTL group. In addition, mean GIR to maintain euglycemia in the FRU-STZ group was significantly less than that in the FRU group (Figure 2.1B).

2.3.4 Hemodynamic measurements

On Day 42, baseline MAP and heart rate were not significantly different between the four groups of rats, although heart rate was slightly lower in the STZ (356 ± 14 beats/min) and FRU-
STZ (355 ± 12 beats/min) groups relative to the CTL (406 ± 13 beats/min) and FRU (401 ± 21 beats/min) groups. Pretreatment with propranolol decreased heart rate in all the animals (CTL, −64 ± 8 beats/min; FRU, −64 ± 7 beats/min; STZ, −57 ± 4 beats/min; FRU-STZ, −48 ± 7 beats/min) at 15 min after the start of infusion, but did not alter MAP (results not shown). After treatment with propranolol, there were no significant differences in baseline values of MAP and MCFP between the four groups, but heart rate was significantly ($P = 0.041$) lower in the STZ than the CTL group (Table 2.3).

Noradrenaline produced dose-dependent increases in MAP and MCFP, as well as decrease in heart rate in all four groups of rats (Figure 2.2 and 2.3). Rats in both the STZ and FRU-STZ groups, relative to those in the CTL group, had significantly higher ED$_{50}$ (lower potency) of MAP response to noradrenaline; however, the E$_{max}$ (efficacy) values were not significantly different (Table 2.4). The ED$_{50}$ for the FRU-STZ group was slightly, but insignificantly, greater than for the STZ group (4.3 ± 0.3 versus 3.4 ± 0.6 nmol/kg/min). Furthermore, the FRU-STZ group showed significantly reduced MCFP responses to both the low and high doses of noradrenaline relative to the CTL and FRU groups, whereas the MCFP response to only the low dose of noradrenaline was decreased in the STZ group (Figure 2.3). Changes in heart rate to noradrenaline were similar amongst the four groups of rats (Figure 2.2B).
Table 2.2  Body weight, food/fluid intake, blood glucose, and plasma insulin and triglyceride in unfasted control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 5 or 6 weeks after initiation of dietary treatment

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>FRU</th>
<th>STZ</th>
<th>FRU-STZ</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>406 ± 11</td>
<td>385 ± 7</td>
<td>332 ± 17&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>335 ± 11&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>31 ± 1</td>
<td>31 ± 2</td>
<td>54 ± 3&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>60 ± 7&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fluid intake (ml)</td>
<td>54 ± 2</td>
<td>50 ± 3</td>
<td>211 ± 25&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>274 ± 25&lt;sup&gt;ab,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>6.2 ± 0.2</td>
<td>6.8 ± 0.2</td>
<td>25.2 ± 0.7&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>30.2 ± 1.3&lt;sup&gt;ab,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma insulin (ng/ml)</td>
<td>3.74 ± 0.35</td>
<td>4.02 ± 0.44</td>
<td>0.49 ± 0.06&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>0.67 ± 0.12&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plasma triglyceride (mM)</td>
<td>0.92 ± 0.12</td>
<td>2.43 ± 0.36&lt;sup&gt;ac&lt;/sup&gt;</td>
<td>1.18 ± 0.26</td>
<td>7.94 ± 1.20&lt;sup&gt;ab,c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 7-9 per group).

<sup>a</sup> Significantly different ($P < 0.05$) from CTL group.

<sup>b</sup> Significantly different ($P < 0.05$) from FRU group.

<sup>c</sup> Significantly different ($P < 0.05$) from STZ group.
Table 2.3  Baseline values of mean arterial pressure (MAP), heart rate (HR) and mean circulatory filling pressure (MCFP) in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose)

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>FRU</th>
<th>STZ</th>
<th>FRU-STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP (mmHg)</td>
<td>106 ± 2</td>
<td>104 ± 3</td>
<td>99 ± 3</td>
<td>101 ± 2</td>
</tr>
<tr>
<td>HR (beats/min)</td>
<td>336 ± 10</td>
<td>324 ± 15</td>
<td>295 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>302 ± 6</td>
</tr>
<tr>
<td>MCFP (mmHg)</td>
<td>6.4 ± 0.4</td>
<td>6.5 ± 0.6</td>
<td>5.5 ± 0.3</td>
<td>6.2 ± 0.3</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 7-9 per group). Data were obtained after pretreatment with propranolol.

<sup>a</sup>Significantly different (P < 0.05) from CTL group.
Table 2.4  ED\textsubscript{50} and E\textsubscript{max} values obtained from dose-response curves of changes in mean arterial pressure to noradrenaline in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose)

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>FRU</th>
<th>STZ</th>
<th>FRU-STZ</th>
</tr>
</thead>
<tbody>
<tr>
<td>ED\textsubscript{50} (nmol/kg/min)</td>
<td>1.9 + 0.2</td>
<td>2.4 + 0.5</td>
<td>3.4 + 0.6\textsuperscript{a}</td>
<td>4.3 + 0.3\textsuperscript{a,b}</td>
</tr>
<tr>
<td>E\textsubscript{max} (mmHg)</td>
<td>68 ± 4</td>
<td>77 ± 4</td>
<td>66 ± 6</td>
<td>71 ± 5</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 7-9 per group). Data were obtained after pretreatment with propranolol. All data of ED\textsubscript{50} were log-transformed prior to statistical analysis. Refer to Figure 2.2A for dose-response curves.

\textsuperscript{a}Significantly different (P < 0.05) from CTL group.

\textsuperscript{b}Significantly different (P < 0.05) from FRU group.
Figure 2.1  Two-hour hyperinsulinemic euglycemic clamp (A) and average steady-state glucose infusion rates (GIR; B) in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats. Dextrose (40% w/v) was infused at variable GIR to maintain blood glucose concentrations at 7.0 ± 0.4 mM. The average GIR (mean ± S.E.M., n = 7-9 per group) were calculated by averaging values from t = 90-120 min during the two-hour hyperinsulinemic euglycemic clamp. *Significantly different (P < 0.05) from CTL group. #Significantly different (P < 0.05) from FRU group.
Figure 2.2  Dose-response curves for the effects (mean ± S.E.M., n = 7-9 per group) of noradrenaline (1, 2, 4, 6, 8, 16 and 24×10^{-9} mol/kg/min) on mean arterial pressure (MAP; A) and heart rate (HR; B) in propranolol-treated control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose).
Figure 2.3  Effects (mean ± S.E.M., n = 7-9 per group) of the low (ED$_{40}$) and high (ED$_{75}$) doses of noradrenaline on the changes in mean circulatory filling pressure (MCFP) in propranolol-treated control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose). *Significantly different ($P < 0.05$) from corresponding responses in CTL group. #Significantly different ($P < 0.05$) from corresponding responses in FRU group.
2.4 Discussion

Our results show that rats fed with a high-fructose diet had slightly higher plasma insulin and were moderately insulin resistant, and frank hyperglycemia occurred following injection of streptozotocin at 2 weeks after initiation of the high-fructose diet. In this study, fructose feeding alone did not cause hyperglycemia. Similar to type 2 diabetic patients, conversion from prediabetes to frank hyperglycemia occurs only when the pancreatic β-cells can no longer compensate for the existing insulin resistance (Kahn, 2003). Injection of streptozotocin compromised the capacity of β-cells to secrete insulin, and rats in both the STZ and FRU-STZ groups became hyperglycemic. Rats in the FRU-STZ group, however, exhibited higher resting blood glucose than those in the STZ group, indicating that dietary intake of fructose in animals with impaired insulin secretion augments hyperglycemia which may intensify insulin resistance.

Rats in both the STZ and FRU-STZ groups had lower insulin levels relative to their respective controls, and all of them survived the 6-week study without insulin supplementation. Progression to hypoinsulinemia can be seen clinically in severe type 2 diabetic patients at a later stage of the disease. As hyperglycemia worsens over time, β-cell function deteriorates progressively, and these patients will eventually require insulin therapy to control their blood glucose (Mathis et al., 2001). Hypoinsulinemia is also observed in other animal models of type 2 diabetes, such as the desert gerbil Psammomys obesus and the spiny mouse Acomys cahirinus (Shafrir et al., 2006; Donath et al., 1999). Evidence suggests that β-cell apoptosis and necrosis can be induced by hyperglycemia during the advanced stages of diabetes.

Abnormality in lipid metabolism is evident in the FRU-STZ as well as the fructose-fed rats. Rats in the FRU-STZ group, however, had markedly higher plasma triglyceride which was 7-fold that of the CTL and STZ groups, and 3-fold that of the FRU group. Marked
hypertriglyceridemia may have been in part due to insulin deficiency, leading to decreased fat storage and hence pooling of triglycerides into the blood. In addition, fructose consumption can increase the synthesis of endogenous triglycerides in the liver (Elliott et al., 2002; Park et al., 1992). Because the metabolism of fructose bypasses the rate-controlling phosphofructokinase step in glycolysis, a large amount of triglycerides is produced.

To evaluate insulin action on glucose utilization, we used the hyperinsulinemic euglycemic clamp technique, the gold standard method for assessing insulin sensitivity in vivo. Similar to previous studies (Youn et al., 1994; Blondel et al., 1989), rats in the STZ group were insulin resistant relative to those in the CTL group, as they required a lower GIR to maintain blood glucose at 7.0 mM. This was perhaps secondary to glucotoxicity associated with prolonged hyperglycemia (Rossetti, 1995), as these rats were not treated with insulin. Diabetic rats in the FRU-STZ group, however, developed more pronounced insulin resistance than both the control and fructose-fed rats, which may have been due to greater hyperglycemia and/or hypertriglyceridemia. Elevated plasma triglyceride has been observed following high-fructose feeding in rats, and this was coupled with impaired insulin action in the liver and peripheral tissues (Thorburn et al., 1989). Hypertriglyceridemia has also been shown to decrease the number of insulin receptors (Bieger et al., 1984) and disrupt insulin transduction pathways (Gumbiner et al., 1996), thereby reducing insulin sensitivity.

Taken together, our results show that rats treated with fructose followed by streptozotocin had hyperglycemia, hypertriglyceridemia and insulin resistance, and these metabolic abnormalities closely resemble those of patients with advanced type 2 diabetes. High-fructose feeding in this study impaired insulin sensitivity and mildly increased insulin levels, reflecting the natural progression of human type 2 diabetes. Following streptozotocin injection, frank
hyperglycemia occurred likely due to β-cell dysfunction. Insulin secretion has been shown to progressively decrease in advanced type 2 diabetic patients, leading to insulin deficiency and reduced β-cell mass in pancreatic islets (Deng et al., 2004; Marchetti et al., 2004; Butler et al., 2003). Furthermore, diminished β-cell function has been observed in human subjects with normal or impaired glucose tolerance who subsequently became diabetic (Knowles et al., 2002; Weyer et al., 1999; Matthews et al., 1998), which suggests that in susceptible individuals, β-cell dysfunction may precede the onset of type 2 diabetes. Although streptozotocin is typically used to induce type 1 diabetes, its combination with high-fructose feeding in our rat model exacerbated the development of hyperglycemia, hypertriglyceridemia and insulin resistance. These pathological changes, especially the decline in insulin action and defective lipid metabolism, are observed widely in patients with type 2, but not type 1, diabetes.

At 6 weeks after initiation of dietary treatment, baseline hemodynamic variables were similar between all four groups of rats, with the exception of lower heart rate in the STZ group. This finding is in accordance with previous studies in streptozotocin-induced diabetic rats from this and other laboratories (Hutchings et al., 2005; Cheng et al., 2004; Cheng and Pang, 2004; Hicks et al., 1998; Kiff et al., 1991). Following 6 weeks of fructose consumption, baseline MAP in the FRU and FRU-STZ groups were similar relative to controls, although discrepancies in these values have been reported. Several studies indicated that hypertension could be present as early as 2 weeks after fructose feeding (Giacchetti et al., 2000; Hwang et al., 1987). On the contrary, some studies have shown no changes even after a more prolonged feeding period (D’Angelo et al., 2005; Kotchen et al., 1997). The inconsistencies observed could be due to differences in the methodologies used, namely, use of a higher percentage of dietary fructose and
indirect measurement of blood pressure by tail cuff plethysmography versus direct measurement of arterial pressure.

Rats in the STZ and FRU-STZ groups had reduced potency (higher ED$_{50}$) of MAP response to noradrenaline, but no differences in efficacy (E$_{max}$). Potency to noradrenaline was reduced slightly ($P > 0.05$) more in the FRU-STZ than the STZ group. These results are indicative of generalized hyposensitivity to $\alpha$-adrenoceptor-mediated vasoconstriction, since the chronotropic and inotropic ($\beta$-adrenoceptor-mediated) effects of noradrenaline were blocked by pretreatment with propranolol. The similar dose-dependent bradycardic responses to noradrenaline were likely due to reflex parasympathetic activation and not sympathetic withdrawal, as the rats were treated with propranolol. Although vascular contractile responses to vasoactive agents have been inconsistent in isolated arteries, most studies in conscious rats with streptozotocin-induced diabetes have shown reduced pressor response to noradrenaline (Cheng et al., 2003; Yu and McNeill, 1992; Jackson and Carrier, 1983). There is, however, a lack of information on $\alpha$-adrenoceptor-mediated vasoconstriction in animal models of type 2 diabetes in vivo. The present results show that the potency of pressor response to noradrenaline was attenuated in the fructose-streptozotocin-induced diabetic rats relative to the control and fructose-fed rats.

Besides reduced potency of MAP, animals in the FRU-STZ group also exhibited reduced MCFP responses to both the low and high doses of noradrenaline, indicating attenuated venoconstriction. This is, to our knowledge, the first study to demonstrate reduced venous constriction in vivo in type 2 diabetic rats. MCFP reflects total body venous tone, and is the driving force of venous return (Pang, 2000). Our results therefore indicate that the fructose-streptozotocin-induced diabetic rats had diminished constrictions of arterial resistance as well as
capacitance vessels. Rats in the STZ group had significantly reduced MCFP response to the low but not the high dose of noradrenaline. This observation suggests that combined treatment of fructose and streptozotocin causes relatively greater impairment of venous constriction to noradrenaline compared to streptozotocin alone, which may be secondary to differences in blood glucose concentrations, plasma triglyceride levels, and/or insulin sensitivity.

In conclusion, rats treated with streptozotocin alone or a combination of high dietary fructose and streptozotocin exhibited hyperglycemia, insulin resistance, and reduced potency of pressor as well as reduced MCFP responses to α-adrenoceptor activation. Hyperglycemia, insulin resistance and venous contractile dysfunction were more pronounced in the group given fructose and streptozotocin than that given streptozotocin alone. Plasma triglyceride was unchanged in the streptozotocin-induced diabetic rats, but markedly increased in rats fed with a high-fructose diet followed by injection of streptozotocin. The presence of marked hypertriglyceridemia, insulin resistance and vascular dysfunction makes the fructose-streptozotocin-induced diabetic rats a suitable model for study of metabolic and vascular abnormalities in advanced type 2 diabetes.
Chapter 3: Effects of phlorizin, a glucose lowering agent, on vascular function in two rat models of diabetes

3.1 Overview

Hyperglycemia causes a number of pathological changes in the cardiovascular system (Ceriello, 2008). It can initiate structural and functional abnormalities in endothelial and vascular smooth muscle cells of blood vessels (Orasanu and Plutzky, 2009), thereby increasing the risk of developing diabetic vascular complications. Potential mechanisms of hyperglycemia-induced damage include increased flux through the polyol and hexosamine pathways, abnormal activation of signalling cascades such as PKC, as well as formation of AGEs (refer to Section 1.4.1). Chronic hyperglycemia is the central initiating factor for diabetic microvascular complications in the retina, glomerulus and peripheral nerve (Ruderman et al., 1992). Several large prospective clinical trials have convincingly demonstrated the beneficial effects of glucose control on microvascular complications in patients with type 1 (DCCT/EDIC Research Group, 2011; DCCT Research Group, 1993) and type 2 (Ismail-Beigi et al., 2010; ADVANCE Collaborative Group, 2008; UKPDS Group, 1998) diabetes. On the other hand, the association between glucose levels and macrovascular complications among diabetic patients is not as strong (Libby and Plutzky, 2002; Laakso, 1999). Current evidence suggests that intensive glycemic control to near normoglycemia does not substantially reduce the incidence of macrovascular events in diabetic individuals, and may even be detrimental in the long run (Panicker et al., 2012; Kassem and Raz, 2009). Optimizing blood glucose levels thus appears to be insufficient as monotherapy for prevention of diabetic macrovascular complications, likely because the
Pathogenesis of these complications is multifactorial and not solely due to hyperglycemia (Duckworth, 2001). Given the inconsistent findings mentioned above, it is uncertain if lowering glucose can protect against the development of vascular contractile dysfunction in diabetes.

Phlorizin (1-[2-(beta-D-glucopyranosyloxy)-4,6-dihydroxyphenyl]-3-(4-hydroxyphenyl)-1-propanone), a naturally occurring glucoside isolated from the bark of apple trees, is a non-selective, competitive inhibitor of the sodium-glucose cotransporter (SGLT). Two main isoforms of this transporter, SGLT1 and SGLT2, are located primarily in the mucosa of the small intestine and proximal tubule of the kidney, respectively (Tahrani et al., 2013; Ehrenkranz et al., 2005). By blocking renal and intestinal glucose absorption through inhibition of SGLT, phlorizin can reduce levels of blood glucose and HbA1c. Rossetti et al. (1987) were the first to show that phlorizin can normalize blood glucose and insulin sensitivity in diabetic animals, independent of insulin levels. Phlorizin's ability to improve glucotoxicity without the risk of hypoglycemia makes it a potential therapeutic agent in the treatment of diabetes. Clinical development of phlorizin, however, has been limited by its poor oral bioavailability and some gastrointestinal adverse effects related to its lack of selectivity, including glucose-galactose malabsorption and severe diarrhea (DeFronzo et al., 2012; Mather and Pollock, 2010).

The purpose of this study was to investigate if reducing glucose with phlorizin ameliorates arterial and venous contractile responses to noradrenaline in two rat models of diabetes, induced through an injection of streptozotocin alone, or a combination of high-fructose feeding and subsequent injection of streptozotocin. Arterial and venous contractile functions were evaluated by measuring both MAP and MCFP in control and diabetic rats with or without phlorizin treatment. Phlorizin was used as a pharmacological tool to elucidate the role of hyperglycemia in the pathophysiology of diabetes-associated vascular contractile dysfunction.
3.2 Materials and methods

3.2.1 Experimental animals and induction of diabetes

Male Wistar rats (175-225 g) were obtained from Charles River Canada. The rats were randomly assigned to one of six treatment groups (n = 6-8 per group) — CTL, phlorizin-treated CTL, STZ, phlorizin-treated STZ, FRU-STZ or phlorizin-treated FRU-STZ. They were housed two per cage and maintained under ambient temperature (23 ± 2°C) with 12:12 h light:dark cycle (lights on from 7 a.m. to 7 p.m.). The animals were cared for in accordance with the Canadian Council on Animal Care Guidelines. Starting on Day 0, animals designated to the CTL and STZ groups were fed with standard laboratory diet (PMI Nutrition International, Brentwood, MO, USA) while those in the FRU-STZ groups were fed a high-fructose diet (60% w/w; Harlan Teklad, Madison, WI, USA), as previously described in Section 2.2.1. Food and water were supplied ad libitum. Body weight, food and fluid intake, and resting blood glucose were measured on Day 0. Blood glucose concentrations were measured with the Accu-Chek Aviva glucometer (Roche Diagnostics, Mannheim, Germany) by sampling blood from the saphenous vein.

On Day 14, rats in the STZ and FRU-STZ groups were injected with streptozotocin (60 mg/kg, i.v.; Sigma, St. Louis, MO, USA) whereas those in the CTL groups were administered an equal volume of vehicle (5% dextrose) via the tail vein under light isoflurane (Baxter, Mississauga, ON, Canada) anesthesia. The rats were considered to be diabetic and were used for the study if they developed hyperglycemia (> 16 mM) at 48 h after injection of streptozotocin. Food and fluid intake was measured again on Day 35.
3.2.2 Administration of phlorizin

On Day 28 (2 weeks after injection of streptozotocin or vehicle), half of the control and diabetic rats were administered phlorizin (60 mg/kg, s.c.; Sigma, St. Louis, MO, USA) twice a day for a duration of 2 weeks until the end of the study, while the other half received equal volumes of the vehicle (mixture of 6% sesame oil, 37% water, 38% Tween 80 and 19% propylene glycol by weight; similar to the microemulsion system ME4 in Chen et al., 2004). This dose of phlorizin was derived from pilot experiments and was found to reduce blood glucose in diabetic rats for at least 8 h after injection. Phlorizin or the vehicle was given at 12 h intervals (7 a.m. and 7 p.m. daily).

3.2.3 Surgical preparation

On Day 42, the rats were surgically prepared under isoflurane anesthesia. Body weight was measured prior to surgery. Heparinized saline-filled (25 IU/ml) polyethylene cannulae (PE50) were inserted into the right carotid artery for continuous monitoring of MAP and heart rate, into the left jugular and right iliac veins for infusion of drugs, and into the inferior vena cava for the measurement of CVP. All pressures were measured by P23DB pressure transducers (Gould Statham, CA, USA) and recorded using the BIOPAC computer system (MP150). A saline-filled, balloon-tipped catheter was also inserted into the right atrium via the right jugular vein. All cannulae were tunneled subcutaneously to the back of the neck and exteriorized. The rats were given at least 4 h to recover from anesthesia and surgery prior to the study.

3.2.4 Measurement of MCFP

MCFP was measured using the technique described in Section 2.2.5.
3.2.5 Experimental protocol

The rats were placed in a small cage and allowed to wander freely. After equilibration for 1 h, the rats were pretreated with propranolol ($8 \times 10^{-7} \, \text{mol/kg} \, \text{i.v.}$ bolus followed by continuous infusion at $3.4 \times 10^{-7} \, \text{mol/kg/min}$ for the remainder of the study; Sigma, St. Louis, MO, USA) to block $\beta$-adrenoceptors. Fifteen min after administration of propranolol, dose-response curves of intravenously infused noradrenaline ($1, 2, 4, 6, 8, 16$ and $24 \times 10^{-9} \, \text{mol/kg/min}$; Sigma, St. Louis, MO, USA) were constructed. Measurements of MAP and heart rate were taken before and at 15 min following the start of propranolol infusion, and at the plateau phase of each noradrenaline response. The rats were allowed to recover for 30 min before MCFP readings were taken at baseline, and at the plateau phase of response to $ED_{40}$ and $ED_{75}$ of the noradrenaline dose-MAP response curve. Each MCFP measurement was followed by a recovery period of 10 min. Before the rats were sacrificed, which was 8-10 h after the last injection of phlorizin or the vehicle, blood (1.5 ml) was collected for determination of plasma glucose, insulin and triglyceride levels. Blood samples were centrifuged at 10,000 RPM for 10 min at 4°C and the plasma was separated and kept frozen at -70°C until assayed.

3.2.6 Measurements of plasma glucose, insulin and triglyceride

Blood plasma samples collected after the experimental protocol were thawed on ice and prepared for assay of plasma glucose, insulin or triglyceride. Plasma glucose was measured by the hexokinase/glucose-6-phosphate dehydrogenase method (Infinity™ Glucose Hexokinase Reagent; Thermo Scientific, Middletown, VA, USA) and triglyceride was measured using an enzymatic technique (Infinity™ Triglycerides Reagent; Thermo Scientific, Middletown, VA, USA). Plasma insulin was measured as described in Section 2.2.6.
3.2.7 Statistical analyses

The results are presented as mean ± S.E.M. Dose-response curves of changes in MAP and heart rate to noradrenaline were constructed by nonlinear regression, using the sigmoidal dose-response model with variable slope in GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego, CA, USA), to obtain ED$_{50}$ and E$_{max}$ values. The mean ED$_{50}$ and E$_{max}$ values were calculated from individual dose-response curves. Data for ED$_{50}$ were log-transformed prior to statistical analysis. All data were analyzed by two-way ANOVA followed by the Tukey Test. A value of $P < 0.05$ was considered statistically significant.

3.3 Results

3.3.1 General characteristics of diabetes induction

There were no significant differences in body weight, food and fluid intake, and resting blood glucose among the six groups at the commencement of the study (results not shown). On Day 35, the intake of food and fluid was greater in the STZ and FRU-STZ groups compared to the CTL group (Table 3.1). In addition, the FRU-STZ group consumed significantly more food and water than the STZ group. By Day 42, rats in both the STZ and FRU-STZ groups had lower body weight, higher plasma glucose, and reduced plasma insulin relative to those in the CTL group (Table 3.1). Plasma triglyceride was markedly higher in the FRU-STZ than the CTL and STZ groups (Table 3.1).

Administration of phlorizin did not alter the body weight, food intake and plasma triglyceride in any group of rats, but significantly increased fluid intake in the CTL and STZ groups as well as decreased plasma insulin in the CTL group (Table 3.1). Furthermore, all the
rats treated with phlorizin had lower plasma glucose (by 50-70%) compared to their corresponding untreated groups (Table 3.1).

### 3.3.2 Baseline cardiovascular measurements

Pretreatment with propranolol decreased heart rate in all the animals (CTL, −70 ± 8 beats/min; phlorizin-treated CTL, −71 ± 7 beats/min; STZ, −56 ± 9 beats/min; phlorizin-treated STZ, −58 ± 10 beats/min; FRU-STZ, −64 ± 8 beats/min; phlorizin-treated FRU-STZ, −75 ± 9 beats/min) at 15 min after the start of infusion, but did not alter MAP (results not shown).

Baseline cardiovascular parameters in the six groups of rats on Day 42 are shown in Table 3.2. There were no significant differences in baseline values of heart rate, MAP and MCFP after treatment with propranolol, although heart rate was slightly ($P = 0.063$) lower in the STZ than the CTL and FRU-STZ groups. Administration of phlorizin did not significantly affect any of the baseline cardiovascular measurements in either the CTL or diabetic groups (Table 3.2).

### 3.3.3 Cardiovascular responses to noradrenaline

Noradrenaline produced dose-dependent increases in MAP and MCFP in all groups of rats; however, the increases were generally less in the diabetic groups (Figure 3.1A and 3.2). Relative to the CTL group, rats in the STZ and FRU-STZ groups had significantly higher ED$_{50}$ (lower potency) of MAP response to noradrenaline, but the $E_{\text{max}}$ (efficacy) values were not significantly different (Table 3.3). The ED$_{50}$ (4.0 ± 0.7 versus 5.4 ± 1.2 nmol/kg/min) and $E_{\text{max}}$ (54 ± 2 versus 61 ± 3 mmHg) were slightly, albeit insignificantly, lower in the STZ than the FRU-STZ group. Both the STZ and FRU-STZ groups also had reduced MCFP responses to the low and high doses of noradrenaline compared to the CTL group (Figure 3.2; open bars).
Moreover, in all groups of rats, heart rate was decreased by noradrenaline in a dose-dependent manner following pretreatment with propranolol (Figure 3.1B). The changes in heart rate, as reflected by their ED$_{50}$ and E$_{max}$ values, were similar amongst the CTL, STZ and FRU-STZ groups (Table 3.3).

### 3.3.4 Effect of phlorizin on cardiovascular responses to noradrenaline

Administration of phlorizin did not alter the vascular effects of noradrenaline in the CTL group, but significantly augmented the MCFP responses to both the low and high doses of noradrenaline in the STZ and FRU-STZ groups (Figure 3.2; shaded bars). The MAP responses were not significantly increased in the phlorizin-treated STZ and FRU-STZ groups (Figure 3.1A), although there was a small reduction in ED$_{50}$ relative to corresponding values in the untreated rats (Table 3.3). The noradrenaline-induced decreases in heart rate, however, were not altered by phlorizin in either the CTL or diabetic groups (Figure 3.1B).
Table 3.1  Body weight, food/fluid intake, plasma glucose, plasma insulin, and plasma triglyceride in control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 5 or 6 weeks after initiation of dietary treatment, either with or without administration of phlorizin.

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Food intake (g)</th>
<th>Fluid intake (ml)</th>
<th>Plasma glucose (mM)</th>
<th>Plasma insulin (ng/ml)</th>
<th>Plasma triglyceride (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>444 ± 12</td>
<td>24 ± 2</td>
<td>43 ± 2</td>
<td>8.7 ± 0.5</td>
<td>3.35 ± 0.25</td>
<td>0.76 ± 0.10</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>455 ± 6</td>
<td>29 ± 2</td>
<td>66 ± 3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.4 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.33 ± 0.11&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.72 ± 0.12</td>
</tr>
<tr>
<td><strong>STZ</strong></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>381 ± 19&lt;sup&gt;a&lt;/sup&gt;</td>
<td>44 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>180 ± 12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.3 ± 0.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.53 ± 0.20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.13 ± 0.19</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>420 ± 20</td>
<td>49 ± 2</td>
<td>220 ± 10&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.7 ± 0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.45 ± 0.11</td>
<td>1.30 ± 0.15</td>
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<tr>
<td><strong>FRU-STZ</strong></td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>360 ± 15&lt;sup&gt;a&lt;/sup&gt;</td>
<td>55 ± 4&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>244 ± 16&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>17.4 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.72 ± 0.28&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.28 ± 0.45&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>381 ± 7</td>
<td>52 ± 2</td>
<td>247 ± 17</td>
<td>6.2 ± 0.5&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.64 ± 0.23</td>
<td>4.48 ± 0.42</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 6-8 per group). Plasma samples were taken 8-10 h after the last injection of phlorizin or the vehicle.

<sup>a</sup> Significantly different (P < 0.05) from untreated CTL group.

<sup>b</sup> Significantly different (P < 0.05) from untreated STZ group.

<sup>c</sup> Significantly different (P < 0.05) from untreated rats within the same group.
Table 3.2 Baseline values of heart rate (HR), mean arterial pressure (MAP), and mean circulatory filling pressure (MCFP) in control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose), either with or without administration of phlorizin.

<table>
<thead>
<tr>
<th>Group</th>
<th>HR (beats/min)</th>
<th>MAP (mmHg)</th>
<th>MCFP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>336 ± 10</td>
<td>108 ± 3</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>347 ± 10</td>
<td>108 ± 2</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td><strong>STZ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>302 ± 15</td>
<td>103 ± 3</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>308 ± 12</td>
<td>103 ± 3</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td><strong>FRU-STZ</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>324 ± 6</td>
<td>107 ± 3</td>
<td>6.4 ± 0.2</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>325 ± 5</td>
<td>110 ± 2</td>
<td>6.3 ± 0.1</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 6-8 per group). Data were obtained after pretreatment with propranolol.
Table 3.3  ED_{50} and E_{max} values obtained from dose-response curves of changes in mean arterial pressure (MAP) and heart rate (HR) to noradrenaline in control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose), either with or without administration of phlorizin

<table>
<thead>
<tr>
<th></th>
<th>MAP</th>
<th>HR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED_{50} (nmol/kg/min)</td>
<td>E_{max} (mmHg)</td>
</tr>
<tr>
<td><strong>CTL</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>2.2 ± 0.2</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>2.5 ± 0.2</td>
<td>60 ± 2</td>
</tr>
<tr>
<td><strong>STZ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>4.0 ± 0.7^{a}</td>
<td>54 ± 2</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>3.4 ± 0.7</td>
<td>54 ± 2</td>
</tr>
<tr>
<td><strong>FRU-STZ</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>5.4 ± 1.2^{a}</td>
<td>61 ± 3</td>
</tr>
<tr>
<td>Phlorizin-treated</td>
<td>4.5 ± 0.6</td>
<td>63 ± 3</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 6-8 per group). Data were obtained after pretreatment with propranolol. All data of ED_{50} were log-transformed prior to statistical analysis. Refer to Figure 3.1 for dose-response curves.

^{a}Significantly different (P < 0.05) from untreated CTL group.
Figure 3.1  Dose-response curves for the effects (mean ± S.E.M., n = 6-8 per group) of noradrenaline (1, 2, 4, 6, 8, 16 and 24×10^{-9} mol/kg/min) on mean arterial pressure (MAP; A) and heart rate (HR; B) in propranolol-treated control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose), either with (solid lines) or without (dotted lines) administration of phlorizin.
**Figure 3.2** Effects (mean ± S.E.M., n = 6-8 per group) of the low (ED$_{40}$; A) and high (ED$_{75}$; B) doses of noradrenaline on the changes in mean circulatory filling pressure (MCFP) in propranolol-treated control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose), either with (shaded bars) or without (open bars) administration of phlorizin. *Significantly different ($P < 0.05$) from corresponding responses in untreated CTL group. #Significantly different ($P < 0.05$) from corresponding responses in untreated rats within the same group.
3.4 Discussion

Both groups of diabetic rats exhibited metabolic characteristics similar to the previous study, namely hyperglycemia and hypoinsulinemia in the STZ and FRU-STZ groups, in addition to hypertriglyceridemia in the FRU-STZ group. Baseline hemodynamic variables did not vary between the control and diabetic rats, although heart rate was slightly \( (P > 0.05) \) lower in the STZ group. To examine vascular contractile function in diabetes, the rats were challenged with noradrenaline. Propranolol-treated diabetic rats in the STZ and FRU-STZ groups had reduced potency (higher \( ED_{50} \)) of MAP response to noradrenaline compared to the control rats, but no changes in efficacy (\( E_{\text{max}} \)). These rats also had diminished MCFP responses to both the low and high doses of noradrenaline. Our results are consistent with those from the previous study, and reflect attenuated \( \alpha \)-adrenoceptor-mediated constrictions of arterial resistance as well as capacitance vessels in type 1 and type 2 diabetes. Moreover, following pretreatment with propranolol (a \( \beta \)-adrenoceptor antagonist), the rats showed dose-dependent bradycardic responses to noradrenaline, which were likely due to reflex parasympathetic activation induced by the pressor effect. The potency and efficacy of these heart rate responses were not significantly different between the control and diabetic rats, indicating no apparent impairment of cardiac parasympathetic reflexes in our rat models of diabetes.

Hyperglycemia, the hallmark of diabetes, is a major risk factor for developing diabetic vascular complications (Aryangat and Gerich, 2010; Xu et al., 2005). While glycemic control may reduce the risk of microvascular complications in diabetic patients, evidence that it reduces macrovascular complications is less compelling (Panicker et al., 2012; Kassem and Raz, 2009; Skyler et al., 2009; Bolen et al., 2007). We therefore sought to determine if phlorizin, a glucose lowering agent, protects against the development of arterial and venous contractile dysfunction.
in two rat models of diabetes. Phlorizin is a non-selective SGLT inhibitor that promotes urinary glucose excretion and thereby reverses hyperglycemia in an insulin-independent manner (Ehrenkranz et al., 2005). Our results demonstrate that phlorizin was able to significantly normalize hyperglycemia in both models of diabetes without changing body weight, food intake, or plasma insulin and triglyceride levels. This is in agreement with other studies conducted in partially pancreatectomized (Rossetti et al., 1987), alloxan-induced (Freitas et al., 2008) and streptozotocin-induced (Malatiali et al., 2008; Huang et al., 2007; Amessou et al., 1999) diabetic rats, and confirms that the glucose lowering action of phlorizin is independent of plasma insulin. We also observed greater fluid intake in the phlorizin-treated CTL and STZ groups, possibly as a compensatory response to increased osmotic diuresis from phlorizin-induced glycosuria (Wang et al., 2013a; Vallon et al., 2011). Rats in both the untreated and phlorizin-treated FRU-STZ groups, however, consumed similar amounts of water, likely because fluid intake had already reached maximal in the FRU-STZ group due to high severity of diabetes. Furthermore, phlorizin did not alter any of the vascular effects of noradrenaline in the control rats, but markedly augmented the venous contractile (MCFP) responses in the STZ and FRU-STZ groups. On the contrary, the pressor (MAP) responses were not significantly increased following phlorizin treatment. Normalization of plasma glucose by phlorizin therefore partially restored the α-adrenoceptor-mediated venous but not arterial constriction in our rat models of type 1 and type 2 diabetes, which suggests that hyperglycemia plays a greater role in depressing venous than arterial contractile function.

In line with evidence of diabetes-associated vascular abnormalities (Orasanu and Plutzky, 2009; Rahman et al., 2007), correcting hyperglycemia was hypothesized to prevent both arterial and venous contractile dysfunction. The reason for the lack of improvement in arterial
constriction relative to venous constriction in the phlorizin-treated diabetic rats is unclear, although one difference between arterioles and venules may explain our finding — venules possess less elastin but more collagen fibres than arterioles (Pang, 2001). In the presence of elevated glucose, collagen, a long-lived extracellular matrix molecule, is often subjected to non-enzymatic glycation, resulting in excessive accumulation of AGEs (Aronson, 2003). The degree of glycation is mainly influenced by the ambient glucose concentration and the duration of exposure to hyperglycemia. AGEs can form cross-links on collagen, which eventually contributes to alterations in stiffness or distensibility in the diabetic vasculature (Goldin et al., 2006; Zieman and Kass, 2004; Airaksinen et al., 1993). With a higher proportion of collagen fibres in venules relative to arterioles, these structural changes are likely more predominant in the venous vasculature. Phlorizin treatment might have, to a significant extent, reduced the glycation and cross-linking of collagen in the venules to ameliorate venous contractile function in the diabetic rats. Information on venous function in the literature is very limited, and more research is needed to confirm our speculation.

Results from the present study show that the action of elevated glucose alone does not fully account for all of the vascular impairments in our rat models of type 1 and type 2 diabetes, since their arterial and venous contractile responses were not completely restored by phlorizin. Factors other than hyperglycemia, such as inflammation and oxidative stress, may play a greater role in mediating venous and, in particular, arterial contractile dysfunction associated with diabetes. This idea is supported by evidence of vascular dysfunction in diabetes even after normalization of glucose (Paneni et al., 2012), in addition to the continuous persistence of oxidative stress despite optimized glycemic control (Xie et al., 2008; Kowluru et al., 2007). Also, once present, some of the hyperglycemia-induced abnormalities in diabetic vessels may not be
easily reversible (Takenaka et al., 2006). This phenomenon is termed 'metabolic memory' (Zhang and Wu, 2014), and explains the possibility of a sustained, long-lasting detrimental effect of hyperglycemia that is independent of the ensuing glucose level. Thus, exposure to high glucose for 2 weeks before initiation of phlorizin treatment may have contributed to the lack of improvement in arterial constriction to noradrenaline in both groups of diabetic rats. This is in concordance with another study where levels of PKC-\(\beta\), NAD(P)H oxidase subunit p47phox, and fibronectin (markers associated with diabetic vascular complications) remained significantly upregulated in human endothelial cells subjected to 1 week of glucose normalization subsequent to 2 weeks of continuous high glucose (Ihnat et al., 2007). More importantly, the advantages of early glycemic control on micro- and possibly macrovascular complications have been documented in type 1 as well as type 2 diabetic patients (Unnikrishnan et al., 2011).

A new class of antidiabetic drugs with a mechanism of action similar to that of phlorizin has recently been introduced into the market (Lau, 2014; Mather and Pollock, 2010). These inhibitors, developed based on the glucoside ring in phlorizin, are selective for SGLT2, an isoform of SGLT found almost exclusively in the S1 segment of the proximal renal tubule where 90% of glucose is reabsorbed (Kanai et al., 1994). Their efficacy in normalizing glycemia and reducing HbA1c has been confirmed by a number of clinical trials, and no serious adverse events have yet been reported (Fujita and Inagaki, 2014; Tahrani et al., 2013; DeFronzo et al., 2012). Long-term safety and cardiovascular outcomes with SGLT2 inhibitors are still unavailable and remains to be explored. Based on our results, however, these drugs may not offer significant benefits for diabetic vascular disease.

To summarize, rats with streptozotocin- and fructose-streptozotocin-induced diabetes had attenuated potency of pressor response and reduced venoconstriction to noradrenaline, indicating
functional impairment of arterial and venous constrictions in type 1 and type 2 diabetes.

Treatment with phlorizin, a glucose lowering agent, partially restored the venous but not arterial constriction in these rats. Overall, our study suggests that intensive glycemic control is insufficient as monotherapy for complete prevention of diabetes-associated vascular contractile dysfunction.
Chapter 4: Effects of nimesulide, a selective COX-2 inhibitor, on vascular and cardiac function in two rat models of diabetes

4.1 Overview

There are reports that inflammation is a key etiological factor in the pathogenesis of diabetic complications (Cao et al., 2011; Kellogg et al., 2009; King, 2008; Navarro and Mora, 2005). Indeed, chronic, low-level inflammation is present in diabetes, as evident by the upregulation of pro-inflammatory cytokines and enhanced expression of COX-2, the inducible isoform of COX (Savoia and Schiffrin, 2007; Bagi et al., 2006; Hu et al., 2004). Since activation of COX-2 can alter prostanoid synthesis and signalling, it may interfere with cardiovascular homeostasis (Sellers et al., 2010). Several studies have found that COX-2 is activated in both type 1 and type 2 diabetes. Increased expression of COX-2 was detected in the aorta of db/db (Bagi et al., 2005; Guo et al., 2005) and streptozotocin-induced diabetic (Watson et al., 2013; Nacci et al., 2009) mice, femoral artery of streptozotocin-induced diabetic rats (Shi and Vanhoutte, 2008), mesenteric artery of Zucker diabetic fatty (Retailleau et al., 2010) and Goto-Kakizaki (Ishida et al., 2011) rats, as well as in coronary arteriole of diabetic patients (Szerafin et al., 2006). COX-2 was also elevated at the mRNA and protein level in the myocardium of streptozotocin-induced diabetic mice (Rajesh et al., 2012) and rats (Guo et al., 2007).

It is unclear, however, if induction of COX-2 is responsible for cardiovascular abnormalities in diabetes. There is strong evidence from clinical trials that some selective COX-2 inhibitors, specifically the coxibs, are associated with increased cardiovascular adverse events (Farkouh and Greenberg, 2009; Grosser et al., 2006). In contrast, nimesulide (4-nitro-2-
phenoxy-methane-sulfonanilide), which is also a COX-2 inhibitor, has a safer cardiovascular risk profile and does not exert significant cardiotoxicity (Bjarnason et al., 2005; Rainsford et al., 2006; Suleyman et al., 2008). Nimesulide is a nonsteroidal anti-inflammatory drug of the sulfonanilide class with preferential COX-2 selectivity (Shah et al., 2001; Warner et al., 1999). Besides its primary pharmacological action as a COX-2 inhibitor, nimesulide can scavenge ROS (Kullich et al., 2007; Zheng et al., 2000) and suppress the expression of iNOS (Khanduja et al., 2006). These other mediators are involved in the inflammatory process and possibly implicated in the pathogenesis of diabetic cardiovascular complications (Song et al., 2008a; Cheng et al., 2005; Cheng et al., 2004; Cheng and Pang, 2004). The aim of this study was to investigate if nimesulide has beneficial actions on vascular and cardiac contractile responses to adrenaline in two rat models of diabetes, the streptozotocin-induced type 1 and fructose-fed, streptozotocin-induced type 2 diabetes. In this study, the dose-response effects of adrenaline on arterial and venous constriction, cardiac index and left ventricular contractile function were determined before and after i.v. injection of nimesulide in four groups of rats, namely, rats treated with (I) standard diet, (II) high fructose, (III) streptozotocin, and (IV) high fructose plus streptozotocin.

4.2 Materials and methods

4.2.1 Experimental animals and induction of diabetes

Male Wistar rats (175-225 g) were obtained from Charles River Canada. The rats were randomly assigned to one of four treatment groups (n = 8 per group) — CTL, FRU, STZ or FRU-STZ. They were housed two per cage and maintained under ambient temperature (23 ± 2°C) with 12:12 h light:dark cycle (lights on from 7 a.m. to 7 p.m.). The animals were cared for
in accordance with the Canadian Council on Animal Care Guidelines. Starting on Day 0, animals designated to the CTL and STZ groups were fed with standard laboratory diet (PMI Nutrition International, Brentwood, MO, USA) while those in the FRU and FRU-STZ groups were fed a high-fructose diet (60% w/w; Harlan Teklad, Madison, WI, USA), as previously described in Section 2.2.1. Food and water were supplied ad libitum. Body weight, food and fluid intake, and resting blood glucose were measured on Day 0. Blood glucose concentrations were measured with the Accu-Chek Aviva glucometer (Roche Diagnostics, Mannheim, Germany) by sampling blood from the saphenous vein.

On Day 14, rats in the STZ and FRU-STZ groups were injected with streptozotocin (60 mg/kg, i.v.; Sigma, St. Louis, MO, USA) whereas those in the CTL and FRU groups were administered an equal volume of vehicle (5% dextrose) via the tail vein under light halothane (Halocarbon Laboratories, River Edge, NJ, USA) anesthesia. The rats were considered to be diabetic and were used for the study if they developed hyperglycemia (> 16 mM) at 48 h after injection of streptozotocin. Food and fluid intake was measured again on Day 35.

4.2.2 Surgical preparation

On Day 42, the rats were surgically prepared under thiobutabarbitral sodium (Inactin; Sigma, St. Louis, MO, USA) anesthesia (100 mg/kg, i.p.). Body weight and resting blood glucose were measured prior to surgery. The rats were tracheotomized and allowed to breathe spontaneously in room air. Body temperature was maintained at 37°C with a rectal probe and a heat lamp attached to a temperature controller (Model 71; Yellow Spring Instruments, OH, USA). Heparinized saline-filled (25 IU/ml) polyethylene cannulae (PE50) were inserted into the left iliac artery for continuous monitoring of MAP and heart rate, into the left jugular and right
iliac veins for administration of drugs or radioactively-labelled red blood cells, and into the inferior vena cava for the measurement of CVP. A saline-filled, balloon-tipped catheter was also inserted into the right atrium via the right jugular vein. Additional cannulae were inserted into the left ventricle via the right carotid artery for the injection of radioactively-labelled microspheres and recording of LVP, and into the right iliac artery for the withdrawal of a reference blood sample required for the determination of blood volume and cardiac output. The $+\frac{dp}{dt}$ and $-\frac{dp}{dt}$ were also recorded. All pressures were measured by P23DB pressure transducers (Gould Statham, CA, USA) and recorded using the BIOPAC computer system (MP150).

4.2.3 Measurement of blood volume

Blood volume was determined by the $^{51}\text{Cr}$-labelled red blood cell technique (Palacios et al., 2002). Red blood cells (0.2 ml) labelled with radioactive $^{51}\text{Cr}$ (PerkinElmer, Woodbridge, ON, Canada) containing approximately 300,000 counts/min (cpm) were injected intravenously via the right iliac vein. At 5 min after injection, a reference blood sample (0.2 ml) was withdrawn from the right iliac artery. The injected $^{51}\text{Cr}$-labelled red blood cells and blood samples were counted for radioactivity at 240-400 keV using a Packard Cobra II automatic gamma counter (Ramsey, MN, USA).

4.2.4 Measurement of cardiac output

A well-stirred suspension (0.2 ml) of radioactively-labelled ($^{57}\text{Co}$) microspheres (15 μm diameter; PerkinElmer, Woodbridge, ON, Canada) was injected over 10 s into the left ventricle during simultaneous withdrawal of a reference blood sample at 0.35 ml/min for 45 s, as
previously described (Wang et al., 1995). The injected microspheres and blood samples were counted for radioactivity at 75-165 keV using a Packard Cobra II automatic gamma counter (Ramsey, MN, USA). The withdrawn blood was injected back into the rats immediately after counting of radioactivity.

4.2.5 Measurement of MCFP

MCFP was measured using the technique described in Section 2.2.5.

4.2.6 Experimental protocol

At 1 h after completion of surgery, resting blood volume was measured. Subsequently, readings of MAP, heart rate, LVP, ±dP/dt, cardiac output and MCFP were taken at baseline and at the plateau phase of response to infused adrenaline (6 and 16×10⁻⁹ mol/kg/min, i.v.; Sigma, St. Louis, MO, USA). The rats were allowed to recover for 10 min in between each set of cardiovascular measurements. At 10 min following cessation of the last adrenaline infusion, nimesulide (3 mg/kg, i.v.; Cayman Chemical, Ann Arbor, MI, USA) was injected over 90 s. This dose of nimesulide has previously been demonstrated to suppress COX-2 induced production of prostaglandin E₂ (Wallace et al., 1999; Nakatsugi et al., 1996; Harada et al., 1996), but did not alter COX-1 induced platelet aggregation (Baber et al., 2003). At 1 h after injection of nimesulide, the aforementioned cardiovascular variables were measured again at baseline and during each adrenaline infusion.
4.2.7 Calculations

Blood volume, cardiac output, arterial resistance \( R_A \) and venous resistance \( R_V \) were calculated using the following formulae:

血量 = \[ \frac{总注入放射性（cpm） \times 留出血液的体积}{流出血液的放射性（cpm）} \]

心输出量 = \[ \frac{流出血液的速率 \times 总注入放射性（cpm）}{流出血液的放射性（cpm）} \]

\[ R_A = \frac{MAP}{心输出量} \]

\[ R_V = \frac{MCFP - CVP}{心输出量} \]

In the \( R_V \) calculation, CVP rather than right atrial pressure was used to estimate pressure gradient to venous return (MCFP – right atrial pressure) because of technical difficulty in measuring right atrial pressure and MCFP concurrently. It has been shown that mean CVP is nearly identical to mean right atrial pressure (Rothe, 1993).

4.2.8 Statistical analyses

The results are presented as mean ± S.E.M. All cardiovascular data were analyzed by two-way repeated measures ANOVA followed by the Tukey Test for comparison of group means. One-way ANOVA was used to compare measurements of body weight, food and fluid intake, blood glucose and blood volume. A value of \( P < 0.05 \) was considered statistically significant.
4.3 Results

4.3.1 General characteristics of diabetes induction

There were no significant differences in body weight, food and fluid intake, and resting blood glucose among the four groups at the commencement of the study (results not shown). On Day 35, the intake of food and fluid was greater in the STZ and FRU-STZ groups compared to the CTL and FRU groups (Table 4.1). In addition, the FRU-STZ group consumed significantly more water than the STZ group. By Day 42, rats in both the STZ and FRU-STZ groups had significantly lower body weight than those in the CTL and FRU groups (Table 4.1). Blood glucose measurements taken on Day 42 were significantly higher in the STZ and FRU-STZ groups relative to the CTL and FRU groups (Table 4.1). Furthermore, rats in the FRU-STZ group had lower body weight and higher blood glucose than those in the STZ group. Measurements of resting blood volume, however, were not significantly different between the four groups of rats (Table 4.1).

4.3.2 Baseline cardiovascular measurements

Baseline cardiovascular parameters in the four groups of rats on Day 42 are shown in Table 4.2. There were no significant differences in baseline cardiovascular measurements between the CTL and FRU groups. Before treatment with nimesulide, baseline values of MCFP, RV and cardiac output were similar between the four groups, but heart rate and −dP/dt were significantly lower in the STZ than the CTL and FRU groups. Rats in the STZ group also had lower baseline MAP, RA, LVP and +dP/dt compared to those in the CTL group, while in the FRU-STZ group, only baseline −dP/dt was decreased compared to CTL. Baseline values of
cardiac index were significantly higher in both the STZ and FRU-STZ groups relative to the CTL and FRU groups. Nimesulide did not significantly affect any of the baseline cardiovascular measurements in the four groups of rats (Table 4.2).

4.3.3 Cardiovascular responses to adrenaline

Adrenaline produced dose-dependent increases in MAP, R_A, MCFP, and R_V in all four groups of rats; however, the increases were generally greater in the CTL and FRU groups than the STZ and FRU-STZ groups (Figure 4.1 and 4.2; open bars). Both the STZ and FRU-STZ groups showed reduced MAP responses to the low dose of adrenaline relative to the CTL and FRU groups, whereas a reduced MAP response to the high dose of adrenaline was only observed in the STZ group (Figure 4.1). The R_A responses to the low and high doses of adrenaline were attenuated in the STZ and FRU-STZ groups compared to CTL; R_A responses were also significantly lower in the STZ than the Fructose group (Figure 4.1). Furthermore, rats in the STZ and FRU-STZ groups, relative to the CTL and FRU groups, had significantly lower MCFP and R_V responses to both the low and high doses of adrenaline (Figure 4.2).

Adrenaline dose-dependently increased heart rate, LVP, +dP/dt and −dP/dt in the four groups of rats, but did not significantly alter cardiac index (Figure 4.3 and 4.4; open bars). Changes in heart rate and cardiac index to adrenaline were similar amongst the four groups (Figure 4.3). On the other hand, rats in the STZ and FRU-STZ groups had significantly lower LVP, +dP/dt and −dP/dt responses to the low dose of adrenaline, as well as lower LVP responses to the high dose of adrenaline than the CTL and FRU groups (Figure 4.4). Changes in +dP/dt and −dP/dt to the high dose of adrenaline were not significantly different except for the FRU-STZ group, which showed a reduced +dP/dt response relative to CTL (Figure 4.4).
4.3.4 Effect of nimesulide on cardiovascular responses to adrenaline

Administration of nimesulide did not alter the vascular effects of adrenaline in the CTL and FRU groups, but significantly augmented the MCFP as well as $R_V$ responses to both the low and high doses of adrenaline in the STZ and FRU-STZ groups (Figure 4.1 and 4.2; shaded bars). The MAP and $R_A$ responses were slightly, but insignificantly, increased in the diabetic rats after treatment with nimesulide. The cardiac responses to adrenaline, however, were not altered by nimesulide in any of the four groups of rats (Figure 4.3 and 4.4; shaded bars).
Table 4.1  Body weight, food/fluid intake, blood glucose, and resting blood volume in unfasted control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 5 or 6 weeks after initiation of dietary treatment

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>FRU</th>
<th>STZ</th>
<th>FRU-STZ</th>
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</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>463 ± 7</td>
<td>469 ± 11</td>
<td>415 ± 7&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>377 ± 11&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Food intake (g)</td>
<td>30 ± 1</td>
<td>29 ± 1</td>
<td>56 ± 2&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>59 ± 1&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Fluid intake (ml)</td>
<td>71 ± 4</td>
<td>65 ± 3</td>
<td>251 ± 10&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>368 ± 18&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>7.6 ± 0.2</td>
<td>8.3 ± 0.2</td>
<td>23.6 ± 0.6&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>29.7 ± 1.0&lt;sup&gt;a,b,c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blood volume (ml)</td>
<td>21.7 ± 0.4</td>
<td>22.0 ± 0.7</td>
<td>22.0 ± 0.5</td>
<td>21.5 ± 0.4</td>
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</tbody>
</table>

All values are mean ± S.E.M. (n = 8 per group).

<sup>a</sup> Significantly different (P < 0.05) from CTL group.

<sup>b</sup> Significantly different (P < 0.05) from FRU group.

<sup>c</sup> Significantly different (P < 0.05) from STZ group.
Table 4.2  Baseline values of mean arterial pressure (MAP), arterial resistance ($R_A$), mean circulatory filling pressure (MCFP), venous resistance ($R_V$), heart rate (HR), left ventricular systolic pressure (LVP), maximal rates of increase (+dP/dt) and decrease (–dP/dt) of left ventricular pressure, cardiac output (CO), and cardiac index (CI) before and after administration of nimesulide in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (60 mg/kg) or the vehicle (5% dextrose)

<table>
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<tr>
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<th>CTL</th>
<th>FRU</th>
<th>STZ</th>
<th>FRU-STZ</th>
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<tr>
<td><strong>MAP (mmHg)</strong></td>
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<td></td>
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<tr>
<td>Before nimesulide</td>
<td>112 ± 3</td>
<td>112 ± 4</td>
<td>100 ± 2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>105 ± 4</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>111 ± 2</td>
<td>111 ± 4</td>
<td>99 ± 3</td>
<td>104 ± 4</td>
</tr>
<tr>
<td><strong>$R_A$ (mmHg min/ml)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Before nimesulide</td>
<td>1.57 ± 0.05</td>
<td>1.52 ± 0.10</td>
<td>1.23 ± 0.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37 ± 0.05</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>1.62 ± 0.03</td>
<td>1.50 ± 0.05</td>
<td>1.32 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.48 ± 0.05</td>
</tr>
<tr>
<td><strong>MCFP (mmHg)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Before nimesulide</td>
<td>4.7 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>5.3 ± 0.2</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>4.7 ± 0.2</td>
<td>4.8 ± 0.2</td>
<td>5.2 ± 0.2</td>
<td>4.6 ± 0.2</td>
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<tr>
<td><strong>$R_V$ (mmHg min/ml)</strong></td>
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<tr>
<td>Before nimesulide</td>
<td>0.056 ± 0.003</td>
<td>0.049 ± 0.004</td>
<td>0.057 ± 0.003</td>
<td>0.053 ± 0.003</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>0.059 ± 0.003</td>
<td>0.051 ± 0.003</td>
<td>0.061 ± 0.002</td>
<td>0.056 ± 0.003</td>
</tr>
<tr>
<td><strong>HR (beats/min)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Before nimesulide</td>
<td>342 ± 9</td>
<td>346 ± 7</td>
<td>297 ± 9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>320 ± 11</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>345 ± 9</td>
<td>345 ± 8</td>
<td>300 ± 12&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>319 ± 14</td>
</tr>
<tr>
<td><strong>LVP (mmHg)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before nimesulide</td>
<td>149 ± 3</td>
<td>144 ± 4</td>
<td>134 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>137 ± 5</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>148 ± 3</td>
<td>142 ± 4</td>
<td>133 ± 3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>136 ± 5</td>
</tr>
<tr>
<td><strong>+dP/dt (mmHg/s)</strong></td>
<td></td>
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<tr>
<td>Before nimesulide</td>
<td>3625 ± 57</td>
<td>3487 ± 118</td>
<td>3142 ± 65&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3481 ± 168</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>3666 ± 77</td>
<td>3494 ± 92</td>
<td>3151 ± 85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3446 ± 163</td>
</tr>
</tbody>
</table>
Table 4.2 (continued)

<table>
<thead>
<tr>
<th></th>
<th>CTL</th>
<th>FRU</th>
<th>STZ</th>
<th>FRU-STZ</th>
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<tr>
<td>( -dP/dt ) (mmHg/s)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Before nimesulide</td>
<td>3709 ± 63</td>
<td>3498 ± 106</td>
<td>2933 ± 79(^{a,b})</td>
<td>3288 ± 159(^a)</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>3646 ± 86</td>
<td>3426 ± 88</td>
<td>2916 ± 101(^{a,b})</td>
<td>3213 ± 168(^a)</td>
</tr>
<tr>
<td>( CO ) (ml/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before nimesulide</td>
<td>72 ± 4</td>
<td>76 ± 4</td>
<td>80 ± 5</td>
<td>75 ± 3</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>67 ± 2</td>
<td>75 ± 2</td>
<td>76 ± 3</td>
<td>68 ± 3</td>
</tr>
<tr>
<td>( CI ) (ml/min/kg)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before nimesulide</td>
<td>156 ± 6</td>
<td>162 ± 7</td>
<td>194 ± 15(^{a,b})</td>
<td>199 ± 8(^{a,b})</td>
</tr>
<tr>
<td>After nimesulide</td>
<td>145 ± 4</td>
<td>159 ± 3</td>
<td>183 ± 8(^a)</td>
<td>181 ± 7(^a)</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 8 per group). Values were obtained at 10 min prior to the start of adrenaline infusion, both before and after the administration of nimesulide (3 mg/kg, \textit{i.v.}).

\(^a\) Significantly different \((P < 0.05)\) from CTL group.

\(^b\) Significantly different \((P < 0.05)\) from FRU group.
Figure 4.1  Effects (mean ± S.E.M., n = 8 per group) of the low (6×10^{-9} mol/kg/min; left column) and high (16×10^{-9} mol/kg/min; right column) doses of adrenaline on the changes in mean arterial pressure (MAP; A, B) and arterial resistance (R_A; C, D) from baseline before (open bars) and after (shaded bars) administration of nimesulide (3 mg/kg, i.v.) in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats. \(^a\)Significantly different (\(P < 0.05\)) from corresponding responses in CTL group. \(^b\)Significantly different (\(P < 0.05\)) from corresponding responses in FRU group.
**Figure 4.2** Effects (mean ± S.E.M., n = 8 per group) of the low (6×10⁻⁹ mol/kg/min; left column) and high (16×10⁻⁹ mol/kg/min; right column) doses of adrenaline on the changes in mean circulatory filling pressure (MCFP; A, B) and venous resistance (Rᵥ; C, D) from baseline before (open bars) and after (shaded bars) administration of nimesulide (3 mg/kg, i.v.) in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats. *a*Significantly different (P < 0.05) from corresponding responses in CTL group. *b*Significantly different (P < 0.05) from corresponding responses in FRU group. #Significantly different (P < 0.05) from responses before the administration of nimesulide within the same group.
**Figure 4.3**  Effects (mean ± S.E.M., n = 8 per group) of the low (6×10⁻⁹ mol/kg/min; left column) and high (16×10⁻⁹ mol/kg/min; right column) doses of adrenaline on the changes in heart rate (HR; A, B) and cardiac index (CI; C, D) from baseline before (open bars) and after (shaded bars) administration of nimesulide (3 mg/kg, i.v.) in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats.
Figure 4.4  Effects (mean ± S.E.M., n = 8 per group) of the low (6×10^{-9} mol/kg/min; left column) and high (16×10^{-9} mol/kg/min; right column) doses of adrenaline on the changes in left ventricular systolic pressure (LVP; A, B), and maximal rates of increase (+dP/dt; C, D) and decrease (−dP/dt; E, F) of left ventricular pressure from baseline before (open bars) and after (shaded bars) administration of nimesulide (3 mg/kg, i.v.) in control (CTL), fructose-fed (FRU), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats. aSignificantly different (P < 0.05) from corresponding responses in CTL group. bSignificantly different (P < 0.05) from corresponding responses in FRU group.
4.4 Discussion

At 6 weeks after initiation of dietary treatment, there were no significant differences in baseline cardiovascular variables between all four groups of rats, with the exception of lower MAP, $R_A$, heart rate, LVP and $\pm dP/dt$ in the STZ group and lower $-dP/dt$ in the FRU-STZ group. These findings are in agreement with reports of reduced MAP, heart rate, as well as cardiac contractile function in streptozotocin-induced diabetic rats from this and other laboratories (Tappia et al., 2013; Bhatt and Veeranjaneyulu, 2012; Kain et al., 2011; Arozal et al., 2009; Cheng et al., 2004; Yu and McNeill, 1992). Furthermore, the four groups of rats had similar baseline cardiac output measurements, but cardiac index (cardiac output normalized by body weight) was increased in the two groups of diabetic rats due to reductions in resistance to blood flow ($R_A$).

Upon challenge with exogenous adrenaline, both the vascular (MAP, MCFP) and cardiac (LVP, $\pm dP/dt$) contractile responses were generally attenuated in the diabetic but not control rats. Diabetic rats in the STZ and FRU-STZ groups had reduced MAP, MCFP, $R_A$ and $R_V$ responses to adrenaline. The decrease in pressor response was likely due to decreased $R_A$ as cardiac output was not changed. Blood volume measurements were similar between the four groups of rats. In the absence of a change in blood volume, a reduced MCFP response denotes an increase in venous compliance, which should theoretically decrease the driving force of venous return and thus cardiac output (Pang, 2000). The responses of cardiac output to adrenaline were, however, not significantly altered in both groups of diabetic rats. This was likely due to decreases in flow resistances ($R_A$ and $R_V$, which facilitate venous return) opposing the reduction in MCFP (which attenuates venous return). LVP and $\pm dP/dt$ responses to adrenaline were also reduced in both groups of diabetic rats, indicating compromised cardiac contractile function when imposed with
increased workload. Attenuated pressor, MCFP, LVP and ±dP/dt responses to vasoactive agents, including noradrenaline, have been reported in rat models of streptozotocin-induced type 1 diabetes in vivo (Hong et al., 2010; Hutchings et al., 2005; Cheng et al., 2004; Cheng et al., 2003; Yu and McNeill, 1992; Jackson and Carrier, 1983). Similar to the studies in the previous two chapters, our findings show that type 1 as well as type 2 diabetes are associated with generalized depression of contractile function in the cardiovascular system.

The mechanisms responsible for the reduced vascular and cardiac contractile responses to exogenous adrenaline in type 1 or type 2 diabetes have yet to be fully elucidated. Recent studies have suggested that chronic, low-grade inflammation in diabetes is associated with cardiovascular abnormalities (Savoia and Schiffrin, 2007; Stehouwer et al., 2002), which may be partly due to the activation of COX-2 (Bagi et al., 2006). Increased expression of COX-2 has been observed in the vasculature (Watson et al., 2013; Nacci et al., 2009; Shi and Vanhoutte, 2008; Szerafin et al., 2006; Bagi et al., 2005; Guo et al., 2005) and heart (Rajesh et al., 2012; Guo et al., 2007) of diabetic animals as well as patients. Therefore, we investigated if nimesulide, a selective inhibitor of COX-2, would improve cardiovascular function in two animal models of diabetes. Nimesulide was chosen among other selective COX-2 inhibitors because unlike the coxibs, it has a low risk of cardiovascular adverse events such as myocardial infarction and hypertension (Bjarnason et al., 2005; Rainsford et al., 2006). Nimesulide belongs to a group of nonsteroidal anti-inflammatory drugs that has 5 to 50 times greater selectivity for COX-2 over COX-1 (Warner et al., 1999), with a COX-1/COX-2 IC₅₀ ratio of 17.69 when assessed in whole blood assays in vitro (Patrignani, 2000). More importantly, the selectivity of nimesulide for COX-2 has been demonstrated in rats (Baber et al., 2003; Taniguchi et al., 1997; Nakatsugi et al., 1996) and humans (Shah et al., 2001; Cullen et al., 1998). In rats, the ED₅₀
values for inhibition of carrageenan-induced paw edema and adjuvant-induced arthritis, an index of anti-inflammatory activity, were found to be 1.25 and 2.4 mg/kg respectively in two separate studies (Swingle et al., 1976; Tanaka et al., 1992). There is evidence that the dose of nimesulide used in this study (3 mg/kg) can reduce COX-2 induced formation of prostaglandin E\(_2\) in in vivo rat models of acute inflammation (Wallace et al., 1999; Nakatsugi et al., 1996; Harada et al., 1996) without significantly altering COX-1 mediated platelet aggregation (Baber et al., 2003), which indeed confirms its selectivity for COX-2.

In the present study, acute treatment with nimesulide had no observable effects on baseline hemodynamic variables in the four groups of rats. In both groups of diabetic rats, nimesulide markedly augmented adrenaline-induced increases in venous tone (MCFP; by 20-40%) and resistance (R\(_V\); by 40-60%), and also slightly (\(P > 0.05\)) increased arterial constriction (MAP; by 10-20%) and resistance (R\(_A\); by 20-40%), suggesting that activation of COX-2 may be implicated in diabetes-associated vascular contractile impairment and that nimesulide appears to augment constriction of venules to a greater extent than arterioles. Our results are in agreement with those of Bishop-Bailey et al. (1998), which showed that venous smooth muscle cells, when stimulated with a mixture of inflammatory cytokines, expressed more COX-2 protein and released greater amounts of prostaglandin E\(_2\) compared to arterial smooth muscle cells. Since these cytokines, notably TNF-\(\alpha\) and IL-1\(\beta\), are known to be upregulated in diabetes (Devaraj et al., 2007; Navarro and Mora, 2005; Spranger et al., 2003), it is reasonable to speculate that the contribution of COX-2 to vascular impairment may differ between arterial and venous vessels. The ability of nimesulide to selectively improve vascular responses to adrenaline in the diabetic but not control rats demonstrates that activation of COX-2 plays a greater role in depressing venous than arterial contractile function in type 1 and type 2 diabetes.
In contrast, nimesulide did not affect cardiac contractile (LVP, ±dP/dt), heart rate nor cardiac index responses to adrenaline in either the control or diabetic rats. Both groups of diabetic rats had reduced +dP/dt and –dP/dt responses to adrenaline, reflecting systolic and diastolic dysfunction respectively. Systolic and, in particular, diastolic abnormalities are prominent features of diabetic cardiomyopathy (Goyal and Mehta, 2013) and can occur as early as 4-6 weeks after induction of diabetes in animal models (Yu et al., 2007; Zhong et al., 2001; Hoit et al., 1999). Accumulating evidence suggests that inflammation is one of the factors implicated in the pathogenesis of diabetic cardiomyopathy (Guleria et al., 2013; Jadhav et al., 2013; Rajesh et al., 2012; Lorenzo et al., 2011). Indeed, increased expression of the inflammatory markers TNF-α, IL-1β, IL-6 and COX-2 has been found in the myocardium of streptozotocin-induced diabetic mice (Rajesh et al., 2012) and rats (Guo et al., 2007). It has been hypothesized that advanced cardiac structural alterations may not be reversible with any form of treatment once they are present in the diabetic heart (Picano, 2003). Since early therapeutic intervention is critical to improve the prognosis of diabetic cardiomyopathy, future studies involving chronic administration of nimesulide will shed more light on the role of COX-2 in cardiac contractile impairment associated with diabetes.

Nimesulide is different from other selective COX-2 inhibitors, as it exhibits multifactorial actions with regards to its anti-inflammatory activity. Besides selectively inhibiting COX-2, nimesulide can also scavenge ROS (Kullich et al., 2007; Zheng et al., 2000) and suppress the expression of iNOS (Khanduja et al., 2006). These properties of nimesulide may, in part, contribute to the improved arterial and venous constrictions observed in the present study, since oxidative stress (Cheng et al., 2005) and activation of iNOS (Song et al., 2008a; Cheng et al.,
2004; Cheng and Pang, 2004) have been implicated in diabetes-induced cardiovascular abnormalities \textit{in vivo}.

In conclusion, our results show that rats with streptozotocin- and fructose-streptozotocin-induced diabetes had reduced arterial, venous and cardiac contractile responses to adrenaline. The depression of cardiovascular contractile function in these models of diabetes might involve alterations in prostanoid synthesis due to the activation of COX-2, as acute administration of nimesulide, a selective COX-2 inhibitor, augmented the venous and, to a less extent, arterial constriction to adrenaline. Our findings demonstrate a link between the role of COX-2 and attenuated cardiovascular function \textit{in vivo} in rat models of type 1 and type 2 diabetes.
Chapter 5: Effects of NAC, an antioxidant, on vascular and cardiac function in two rat models of diabetes

5.1 Overview

As mentioned in the introduction, oxidative stress is increased in patients with type 1 and type 2 diabetes (McGrowder et al., 2013; Singh et al., 2009), and there is accumulating evidence that oxidative stress plays a pivotal role in the pathogenesis of diabetic cardiovascular complications (Xu et al., 2014; Fiorentino et al., 2013; Matough et al., 2012; Giacco and Brownlee, 2010). Indeed, studies have shown an enhanced production of ROS in diabetic individuals (Hsu et al., 2006; Guzik et al., 2002), as well as in animal models of diabetes (Serpillon et al., 2009; Matsumoto et al., 2003; Ohkuwa et al., 1995), which can consequently initiate lipid peroxidation, protein oxidation and DNA damage. Higher serum levels of malondialdehyde, an indicator of lipid peroxidation, were detected in type 2 diabetic patients with micro- and/or macrovascular complications (Shinde et al., 2011; Bhutia et al., 2011; Mahreen et al., 2010). Various markers of oxidative damage were also elevated in the heart (Saddala et al., 2013; Cao et al., 2012; Lei et al., 2012; Aragno et al., 2008) and blood vessels (Pari et al., 2012; Shi and Vanhoutte, 2008; Hink et al., 2001) of streptozotocin-induced diabetic rats. Furthermore, decreased levels and/or activities of endogenous antioxidant enzymes such as SOD and GPx have been reported in type 1 (Ramakrishna and Jaiikhani, 2007; Dave and Kalia, 2007) and type 2 (Hisalkar et al., 2012; Shinde et al., 2011) diabetic patients. Taken together, these findings support a contribution of oxidative stress to the development of cardiovascular abnormalities in diabetes.
In recent years, there has been growing interest in the therapeutic use of antioxidants for prevention of oxidative stress and management of diabetic complications (Rahimi et al., 2005). Antioxidants can reduce oxidative stress by scavenging, removing, and/or neutralizing free radicals, thus minimizing oxidative damage to lipids, proteins and DNA. Previous studies have demonstrated beneficial therapeutic effects of antioxidants, such as selenium, vitamin E, ascorbic acid and CAT, in ameliorating cardiac dysfunction in streptozotocin-induced diabetic mice and rats (Kumar et al., 2013; Aydemir-Koksoy et al., 2010; Turdi et al., 2007). Very little, however, is known regarding the effects of antioxidant treatment on arterial versus venous constrictions in vivo. In addition, it is unclear if oxidative stress alters cardiovascular function in type 1 and type 2 diabetes to the same extent.

Even though conventional antioxidant vitamins do not appear to improve cardiovascular outcomes in clinical trials (refer to Section 1.7.2), it is plausible that other antioxidants have a protective role against the development of diabetic cardiovascular complications. One potential therapeutic agent is NAC, an acetylated cysteine residue that acts both as a precursor of the endogenous antioxidant GSH and as a direct scavenger of ROS (Rushworth and Megson, 2014; Kelly, 1998; Aruoma et al., 1989). The aim of this study was to investigate if reducing oxidative stress with chronic administration of NAC restores in vivo cardiac and vascular contractile functions in two rat models of diabetes, induced through an injection of streptozotocin alone, or a combination of high-fructose feeding and subsequent injection of streptozotocin. In this study, the dose-response effects of dobutamine on heart rate and left ventricular contractile function, as well as those of noradrenaline on arterial and venous constrictions were determined in control, streptozotocin-induced diabetic and fructose-streptozotocin-induced diabetic rats in the absence or presence of treatment with NAC.
5.2 Materials and methods

5.2.1 Experimental animals and induction of diabetes

Male Wistar rats (200-250 g) were obtained from Charles River Canada. The rats were randomly assigned to one of six treatment groups (n = 7-9 per group) — CTL, NAC-treated CTL, STZ, NAC-treated STZ, FRU-STZ or NAC-treated FRU-STZ. They were housed two per cage and maintained under ambient temperature (23 ± 2°C) with 12:12 h light:dark cycle (lights on from 7 a.m. to 7 p.m.). The animals were cared for in accordance with the Canadian Council on Animal Care Guidelines. Starting on Day 0, animals designated to the CTL and STZ groups were fed with standard laboratory diet (PMI Nutrition International, Brentwood, MO, USA) while those in the FRU-STZ groups were fed a high-fructose diet (60% w/w; Harlan Teklad, Madison, WI, USA), as previously described in Section 2.2.1. Food and water were supplied ad libitum. Body weight and resting blood glucose were measured on Day 0. Blood glucose concentrations were measured with the Accu-Chek Aviva glucometer (Roche Diagnostics, Mannheim, Germany) by sampling blood from the saphenous vein.

On Day 14, rats in the STZ and FRU-STZ groups were injected with streptozotocin (50 mg/kg, i.v.; Sigma, St. Louis, MO, USA) whereas those in the CTL groups were administered an equal volume of vehicle (5% dextrose) via the tail vein under light isoflurane (Baxter, Mississauga, ON, Canada) anesthesia. The rats were considered to be diabetic and were used for the study if they developed hyperglycemia (> 16 mM) at 48 h after injection of streptozotocin.
5.2.2 Administration of NAC

On Day 21 (1 week after injection of streptozotocin or vehicle), half of the control and diabetic rats were administered NAC (1.5 g/kg/day, p.o.; Sigma, St. Louis, MO, USA) in their drinking water for a total duration of 3 weeks. Fluid consumption was monitored every 2 days, and the concentration of NAC in the water was adjusted accordingly. The amount of NAC consumed during the study (measured between Days 36 and 42) was 1.5 ± 0.1 g/kg/day. This dose of NAC has been shown to inhibit oxidative stress in diabetic (Lei et al., 2012; Kamboj et al., 2010; Xia et al., 2006) and fructose-fed (Song et al., 2005) rats, as measured by the reduced levels of malondialdehyde and 15-F_{2t}-isoprostane, in addition to improvement in the activity of antioxidant enzymes.

5.2.3 Surgical preparation

On Day 42, the rats were surgically prepared under isoflurane anesthesia. Body weight was measured prior to surgery. Heparinized saline-filled (25 IU/ml) polyethylene cannulae (PE50) were inserted into the left iliac artery for continuous monitoring of MAP and heart rate, into the left jugular and right iliac veins for infusion of drugs, and into the inferior vena cava for the measurement of CVP. An additional cannula was inserted into the left ventricle via the right carotid artery for the measurement of LVP, +dP/dt and –dP/dt. All pressures were measured by P23DB pressure transducers (Gould Statham, CA, USA) and recorded using the BIOPAC computer system (MP150). In addition, a saline-filled, balloon-tipped catheter was inserted into the right atrium via the right jugular vein. All cannulae were tunneled subcutaneously to the back or the neck and exteriorized. The rats were given at least 4 h to recover from anesthesia and surgery prior to the study.
5.2.4 Measurement of MCFP

MCFP was measured using the technique described in Section 2.2.5.

5.2.5 Experimental protocol

The rats were placed in a small cage and allowed to wander freely. After equilibration for 1 h, increasing doses of dobutamine (0.3, 1, 3, 10 and 30 μg/kg/min, i.v.; Sigma, St. Louis, MO, USA) were administered to construct dose-response curves for heart rate, LVP and ±dP/dt. Each dose of dobutamine was infused until a plateau response was attained (2-8 min duration). The rats were allowed to recover for 30 min before pretreatment with propranolol (8×10^{-7} mol/kg i.v. bolus followed by continuous infusion at 3.4×10^{-7} mol/kg/min for the remainder of the study; Sigma, St. Louis, MO, USA) to block β-adrenoceptors. At 15 min after administration of propranolol, measurements of MAP and MCFP were taken at baseline, and at the plateau phase of response to infused noradrenaline (3 and 8×10^{-9} mol/kg/min, i.v.; Sigma, St. Louis, MO, USA). Each set of MAP and MCFP measurements was followed by a recovery period of 10 min. Before the rats were sacrificed, blood (1.5 ml) was collected for determination of plasma glucose and triglyceride levels. Blood samples were centrifuged at 10,000 RPM for 10 min at 4°C and the plasma was separated and kept frozen at -70°C until assayed.

5.2.6 Measurements of plasma glucose and triglyceride

Blood plasma samples collected after the experimental protocol were thawed on ice and prepared for measurement of plasma glucose or triglyceride using the techniques described in Section 3.2.6.
5.2.7 Statistical analyses

The results are presented as mean ± S.E.M. Dose-response curves for dobutamine were constructed by nonlinear regression, using the sigmoidal dose-response model with variable slope in GraphPad Prism (version 5.00 for Windows; GraphPad Software, San Diego, CA, USA), to obtain $E_{\text{max}}$ values. The mean $E_{\text{max}}$ values were calculated from individual dose-response curves. All data, including baseline values, $E_{\text{max}}$ values for heart rate, LVP and $\pm dP/dt$, and changes in MAP and MCFP responses, were analyzed by two-way ANOVA followed by the Tukey Test. A value of $P < 0.05$ was considered statistically significant.

5.3 Results

5.3.1 General characteristics of diabetes induction

There were no significant differences in body weight and resting blood glucose among the six groups at the commencement of the study (results not shown). On Day 42, rats in both the STZ and FRU-STZ groups had significantly lower body weight and higher plasma glucose than those in the CTL group (Table 5.1). In addition, plasma triglyceride was markedly elevated in the FRU-STZ group relative to the CTL and STZ groups. Chronic administration of NAC did not alter the body weight and plasma glucose in any group of rats, but significantly reduced plasma triglyceride by 58% in the FRU-STZ group (Table 5.1).

5.3.2 Baseline cardiovascular measurements

Baseline cardiovascular parameters in the six groups of rats on Day 42 are shown in Table 5.2. There were no significant differences in baseline values of heart rate, LVP, $\pm dP/dt$,
MAP and MCFP between the CTL, STZ and FRU-STZ groups. Treatment with NAC did not significantly affect any of the baseline cardiovascular measurements in these groups of rats (Table 5.2).

5.3.3 Cardiac responses to dobutamine

Dobutamine dose-dependently increased heart rate, LVP, +dP/dt and −dP/dt in the six groups of rats; however, the increases were markedly greater in the CTL than the STZ and FRU-STZ groups (Figure 5.1; dotted lines). Relative to the CTL group, rats in both the STZ and FRU-STZ groups had significantly lower $E_{\text{max}}$ values of heart rate (35-55% of CTL), LVP (40-60%), +dP/dt (35-45%) and −dP/dt (50-60%) responses to dobutamine. $E_{\text{max}}$ values between the STZ and FRU-STZ groups, however, did not differ (Table 5.3).

Chronic administration of NAC did not alter the cardiac effects of dobutamine in the CTL group (Figure 5.1). On the other hand, these responses were in general slightly, but insignificantly, increased in the diabetic groups treated with NAC, except for the dobutamine-induced increases in heart rate and +dP/dt, which were significantly augmented in the NAC-treated FRU-STZ group (Figure 5.1; solid lines). Although the $E_{\text{max}}$ values of all the cardiac parameters in both diabetic groups were somewhat increased by dobutamine after treatment with NAC, only those pertaining to changes in heart rate and +dP/dt in the NAC-treated FRU-STZ group were significantly higher (by respectively 2.1- and 1.8-fold relative to the untreated FRU-STZ group; Table 5.3).
5.3.4 Vascular responses to noradrenaline

Noradrenaline produced dose-dependent increases in MAP and MCFP in all six groups of rats (Figure 5.2). The increases were markedly less in the diabetic groups. Rats in the STZ and FRU-STZ groups had significantly reduced MAP (60-80% of CTL) and MCFP (30-45%) responses to both the low and high doses of noradrenaline relative to those in the CTL group (Figure 5.2; open bars).

Treatment with NAC did not alter the vascular effects of noradrenaline in the CTL group, but significantly augmented the MAP as well as MCFP responses to both the low and high doses of noradrenaline in the STZ and FRU-STZ groups (Figure 5.2; shaded bars). In both of the NAC-treated diabetic groups, MAP responses were restored to values similar to corresponding responses in the CTL group. On the other hand, MCFP responses were only partially restored in these rats.
Table 5.1  Body weight, plasma glucose, and plasma triglyceride in control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 6 weeks after initiation of dietary treatment, either with or without administration of N-acetylcysteine (NAC)

<table>
<thead>
<tr>
<th></th>
<th>Body weight (g)</th>
<th>Plasma glucose (mM)</th>
<th>Plasma triglyceride (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTL</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>424 ± 8</td>
<td>8.8 ± 0.6</td>
<td>1.00 ± 0.13</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>399 ± 9</td>
<td>8.2 ± 0.5</td>
<td>0.71 ± 0.08</td>
</tr>
<tr>
<td><strong>STZ</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Untreated</td>
<td>348 ± 10</td>
<td>21.7 ± 1.1</td>
<td>1.64 ± 0.18</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>337 ± 11</td>
<td>20.5 ± 0.8</td>
<td>1.23 ± 0.20</td>
</tr>
<tr>
<td><strong>FRU-STZ</strong></td>
<td></td>
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</tr>
<tr>
<td>Untreated</td>
<td>312 ± 7</td>
<td>22.1 ± 0.8</td>
<td>4.93 ± 0.77</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>322 ± 14</td>
<td>21.6 ± 0.9</td>
<td>2.05 ± 0.44</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 7-9 per group).

\(^{a}\)Significantly different (P < 0.05) from untreated CTL group.

\(^{b}\)Significantly different (P < 0.05) from untreated STZ group.

\(^{c}\)Significantly different (P < 0.05) from untreated rats within the same group.
Table 5.2  Baseline values of heart rate (HR), left ventricular systolic pressure (LVP), maximal rates of increase (+dP/dt) and decrease (‒dP/dt) of left ventricular pressure, mean arterial pressure (MAP), and mean circulatory filling pressure (MCFP) in control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following *i.v.* injection of streptozotocin (50 mg/kg) or the vehicle (5% dextrose), either with or without administration of *N*-acetylcysteine (NAC).

<table>
<thead>
<tr>
<th></th>
<th>HR (beats/min)</th>
<th>LVP (mmHg)</th>
<th>+dP/dt (mmHg/s)</th>
<th>‒dP/dt (mmHg/s)</th>
<th>MAP (mmHg)</th>
<th>MCFP (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTL</strong></td>
<td></td>
<td></td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>380 ± 15</td>
<td>142 ± 3</td>
<td>3750 ± 131</td>
<td>3888 ± 136</td>
<td>107 ± 3</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>377 ± 12</td>
<td>144 ± 2</td>
<td>3857 ± 127</td>
<td>4018 ± 128</td>
<td>105 ± 1</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td><strong>STZ</strong></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>385 ± 12</td>
<td>138 ± 5</td>
<td>3855 ± 179</td>
<td>3755 ± 271</td>
<td>97 ± 4</td>
<td>6.2 ± 0.2</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>380 ± 15</td>
<td>145 ± 4</td>
<td>3882 ± 121</td>
<td>3742 ± 145</td>
<td>103 ± 4</td>
<td>6.1 ± 0.1</td>
</tr>
<tr>
<td><strong>FRU-STZ</strong></td>
<td></td>
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<tr>
<td>Untreated</td>
<td>397 ± 7</td>
<td>142 ± 2</td>
<td>4037 ± 83</td>
<td>3930 ± 141</td>
<td>99 ± 1</td>
<td>6.2 ± 0.1</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>366 ± 9</td>
<td>141 ± 5</td>
<td>3790 ± 132</td>
<td>3782 ± 127</td>
<td>101 ± 3</td>
<td>6.0 ± 0.1</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (*n* = 7-9 per group). Data for MAP and MCFP were taken at 15 min after propranolol pretreatment.
Table 5.3  \( E_{\text{max}} \) values obtained from dose-response curves of changes in heart rate (HR), left ventricular systolic pressure (LVP), and maximal rates of increase (+dP/dt) and decrease (‒dP/dt) of left ventricular pressure to dobutamine in control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats at 4 weeks following i.v. injection of streptozotocin (50 mg/kg) or the vehicle (5% dextrose), either with or without administration of \( N \)-acetylcysteine (NAC).

<table>
<thead>
<tr>
<th></th>
<th>( \Delta \text{HR} ) (beats/min)</th>
<th>( \Delta \text{LVP} ) (mmHg)</th>
<th>( \Delta +\text{dP/dt} ) (mmHg/s)</th>
<th>( \Delta -\text{dP/dt} ) (mmHg/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CTL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>130 ± 12</td>
<td>37 ± 5</td>
<td>1573 ± 110</td>
<td>1805 ± 204</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>126 ± 14</td>
<td>37 ± 5</td>
<td>1579 ± 111</td>
<td>1892 ± 248</td>
</tr>
<tr>
<td><strong>STZ</strong></td>
<td></td>
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</tr>
<tr>
<td>Untreated</td>
<td>70 ± 10\textsuperscript{a}</td>
<td>16 ± 3\textsuperscript{a}</td>
<td>634 ± 67\textsuperscript{a}</td>
<td>948 ± 162\textsuperscript{a}</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>80 ± 16</td>
<td>22 ± 2</td>
<td>832 ± 111</td>
<td>1213 ± 94</td>
</tr>
<tr>
<td><strong>FRU-STZ</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>47 ± 7\textsuperscript{a}</td>
<td>21 ± 3\textsuperscript{a}</td>
<td>621 ± 104\textsuperscript{a}</td>
<td>1056 ± 134\textsuperscript{a}</td>
</tr>
<tr>
<td>NAC-treated</td>
<td>100 ± 11\textsuperscript{b}</td>
<td>29 ± 3</td>
<td>1126 ± 112\textsuperscript{b}</td>
<td>1329 ± 141</td>
</tr>
</tbody>
</table>

All values are mean ± S.E.M. (n = 7-9 per group). Refer to Figure 5.1 for dose-response curves.

\textsuperscript{a} Significantly different (\( P < 0.05 \)) from untreated CTL group.

\textsuperscript{b} Significantly different (\( P < 0.05 \)) from untreated rats within the same group.
Figure 5.1  Dose-response curves for the effects (mean ± S.E.M., n = 7-9 per group) of dobutamine (0.3, 1, 3, 10 and 30 μg/kg/min) on heart rate (HR; A), left ventricular systolic pressure (LVP; B), and maximal rates of increase (+dP/dt; C) and decrease (‒dP/dt; D) of left ventricular pressure in control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats, either with (solid lines) or without (dotted lines) administration of N-acetylcysteine (NAC). *Significantly different (P < 0.05) from corresponding responses in untreated CTL group. #Significantly different (P < 0.05) from corresponding responses in untreated rats within the same group.
Figure 5.2  Effects (mean ± S.E.M., n = 7-9 per group) of the low (3×10⁻⁹ mol/kg/min; left column) and high (8×10⁻⁹ mol/kg/min; right column) doses of noradrenaline on the changes in mean arterial pressure (MAP; A, B) and mean circulatory filling pressure (MCFP; C, D) from baseline in propranolol-treated control (CTL), streptozotocin-induced diabetic (STZ) and fructose-streptozotocin-induced diabetic (FRU-STZ) rats, either with (shaded bars) or without (open bars) administration of N-acetylcysteine (NAC). *Significantly different (P < 0.05) from corresponding responses in untreated CTL group. #Significantly different (P < 0.05) from corresponding responses in untreated rats within the same group.
5.4 Discussion

At 6 weeks after initiation of dietary treatment, diabetic rats in the STZ and FRU-STZ groups had reduced body weight and higher plasma glucose, consistent with our previous results. In addition, there was marked increase in plasma triglyceride in the FRU-STZ group, which suggests impairment of lipid metabolism following high fructose consumption.

Baseline cardiovascular parameters were similar between all groups of rats. However, upon challenge with exogenous dobutamine (a selective β₁-adrenoceptor agonist), attenuations of heart rate as well as cardiac contractile responses (LVP, ±dP/dt) were observed in the diabetic relative to the control rats, exemplified by significant decreases in the efficacy (E_{max}) of heart rate, left ventricular pressure, and contractility (±dP/dt). Diabetic rats in the STZ and FRU-STZ groups also had reduced MAP and MCFP responses to noradrenaline. Since the rats were pretreated with propranolol (a β-adrenoceptor antagonist), the impaired vascular contractile responses reflect diminished constrictions of arterial resistance as well as capacitance vessels to α-adrenoceptor activation, as discussed in the previous chapters. Our results indicate that both type 1 and type 2 diabetes are associated with depressions of β-adrenoceptor-mediated cardiac contractile function, in addition to α-adrenoceptor-mediated arterial and venous constrictions.

The cause of diabetes-induced cardiac and vascular contractile dysfunction remains elusive, although multiple factors are likely responsible. Oxidative stress has recently emerged as an important mechanism in the pathogenesis of diabetic cardiovascular complications (Xu et al., 2014; Fiorentino et al., 2013; Matough et al., 2012; Giacco and Brownlee, 2010). Indeed, there is evidence that ROS, such as superoxide and hydroxyl radical, are elevated in diabetic patients (Hsu et al., 2006; Guzik et al., 2002) and animal models of diabetes (Serpillon et al., 2009; Matsumoto et al., 2003; Ohkuwa et al., 1995). The overproduction of ROS can damage cell
structures and disrupt cellular signalling, which ultimately interferes with physiological functions. Markers of oxidative damage have been detected in the heart (Saddala et al., 2013; Cao et al., 2012; Lei et al., 2012; Aragno et al., 2008) and blood vessels (Pari et al., 2012; Shi and Vanhoutte, 2008; Hink et al., 2001) of streptozotocin-induced diabetic rats, as well as in the serum of patients with type 2 diabetes (Shinde et al., 2011; Bhutia et al., 2011; Mahreen et al., 2010). Furthermore, endogenous antioxidants are shown to be downregulated in diabetic patients (Hisalkar et al., 2012; Shinde et al., 2011; Ramakrishna and Jailkhani, 2007; Dave and Kalia, 2007). These findings demonstrate that in type 1 and type 2 diabetes, increased generation of ROS along with reduced levels or activities of antioxidant defenses contribute to oxidative stress, a known culprit in the development of cardiovascular abnormalities.

Since oxidative stress is implicated in diabetes, use of antioxidants may be beneficial in ameliorating diabetic complications and slowing disease progression. Selenium, vitamin E, ascorbic acid and CAT, for example, appeared to exert therapeutic potential for diabetic cardiomyopathy in animal models (Kumar et al., 2013; Aydemir-Koksoy et al., 2010; Turdi et al., 2007), while pretreatment with vitamin E and α-lipoic acid respectively prevented structural alterations in the heart (Rosen et al., 1995) and aorta (Balkis Budin et al., 2009) of streptozotocin-induced diabetic rats. Moreover, α-lipoic acid treatment was shown to improve endothelium-dependent vasodilation in type 2 diabetic patients (Heinisch et al., 2010) and experimental models of diabetes (Okudan et al., 2011; Sena et al., 2008).

Another potentially therapeutic antioxidant is NAC, which acts both as a precursor of GSH and a direct scavenger of ROS (Kelly, 1998; Aruoma et al., 1989). In this study, we examined if chronic administration of NAC would restore in vivo cardiac and vascular contractile functions in two rat models of diabetes. Treatment with NAC did not affect any of the
cardiovascular responses in the control rats, but markedly augmented the maximum heart rate and left ventricular contractility (+dP/dt) responses to dobutamine in the FRU-STZ group, as well as the noradrenaline-induced increases in MAP and venous tone (MCFP) by similar extents in both groups of diabetic rats. These results suggest that depressions of cardiac and vascular contractile functions in type 1 and type 2 diabetes are mediated, at least in part, by oxidative stress, and can be ameliorated using the antioxidant NAC. Furthermore, oxidative stress appears to alter vascular constriction in type 1 and type 2 diabetes to a similar extent. NAC is known to reduce oxidative stress through modulation of antioxidative status and has recently been investigated for a wide range of disorders, including diabetes (Rushworth and Megson, 2014). It was found to decrease lipid peroxidation in the heart and plasma of diabetic animals (Xu et al., 2013; Lei et al., 2012; Xia et al., 2006; Cheng et al., 2005), which is likely associated with restoration of antioxidant activity, repletion of GSH and inhibition of apoptosis (Kamboj et al., 2010). Clinical studies have reported significant improvements in endothelial function of type 2 diabetic patients following long- (Martina et al., 2008) and short-term (Masha et al., 2009) administration of NAC. In addition, treatment with NAC for 3 months limited the loss of cardiomyocytes from apoptosis and completely reversed compensatory hypertrophy in the heart of streptozotocin-induced diabetic rats (Fiordaliso et al., 2004). Based on these findings, it is reasonable to speculate that improved cardiovascular function by NAC in the diabetic rats is due to attenuation of oxidative stress.

Although there have been some conflicting results in the literature concerning the beneficial effects of NAC on the diabetic heart (Xu et al., 2013; Yildirim et al., 2013; Xia et al., 2006; Cheng et al., 2005), we interestingly noticed that the cardiac responses to dobutamine following NAC treatment were significantly ($P < 0.05$) augmented in the FRU-STZ group, but
only slightly \( P > 0.05 \) increased in the STZ group. A greater restoration of cardiac function in the FRU-STZ group relative to the STZ group may be due to differences between the metabolic profiles of the two diabetes models. As mentioned earlier, the fructose-streptozotocin-induced type 2 diabetic rats exhibited marked increase in plasma triglyceride, which was not observed in the streptozotocin-induced type 1 diabetic rats. This elevated plasma triglyceride was significantly normalized after chronic NAC administration. Similar results regarding the lipid lowering actions of NAC have been documented in rats fed a high-fructose (Song et al., 2005), high-sucrose (Souza et al., 2011; Novelli et al., 2009) or high-fat (Yang et al., 2006) diet. NAC has also been shown to prevent insulin resistance associated with oxidative stress (Blouet et al., 2007; Haber et al., 2003). In type 2 diabetes, alterations in the diabetic heart, including increased lipid oxidation, intramyocardial accumulation of triglycerides as well as cardiac insulin resistance, can promote systolic and diastolic functional abnormalities (Gray and Kim, 2011; van de Weijer et al., 2011). It is possible that NAC, by conferring protection against oxidative stress with concomitant reversal of hypertriglyceridemia, may synergistically enhance \textit{in vivo} cardiac function in our rat model of fructose-fed, streptozotocin-induced diabetes. Further research is needed to elucidate the mechanism involved.

In summary, our results show that rats with streptozotocin- and fructose-streptozotocin-induced diabetes had reduced arterial and venous constrictions to noradrenaline, as well as attenuated cardiac contractility to dobutamine. The impaired cardiovascular contractile function in these models of diabetes might involve increased oxidative stress, as chronic administration of NAC, an antioxidant, improved the cardiac and vascular contractile responses in the diabetic rats. Findings from this study reinforce the benefits of antioxidant supplementation in the management of cardiac and vascular contractile dysfunction in type 1 and type 2 diabetes.
Chapter 6: General discussion and conclusions

6.1 A rat model of fructose-fed, streptozotocin-induced type 2 diabetes

One of our aims was to develop an inexpensive, reproducible animal model of advanced type 2 diabetes mellitus with metabolic characteristics similar to those of humans. This was achieved through a combination of high-fructose feeding and subsequent injection of streptozotocin in non-genetic, outbred rats. Fructose was chosen to induce type 2 diabetes because of its abundance in the Western diet and its increasing use as a sweetener in many processed foods (Tappy and Le, 2010; Vos et al., 2008). In fact, refined carbohydrate high in fructose, but not protein or fat, is significantly correlated with the prevalence of type 2 diabetes after accounting for total energy intake (Gross et al., 2004). Overconsumption of fructose therefore seems to closely reflect the natural history of human type 2 diabetes in the general population. In our study, fructose feeding alone for 6 weeks did not significantly elevate blood glucose. Hence, an injection of streptozotocin was required to render the rats hyperglycemic, enabling us to generate an appropriate model of diabetes. Our results show that rats treated with a combination of high dietary fructose and streptozotocin exhibited the profile of advanced type 2 diabetes, as characterized by the presence of hyperglycemia, marked hypertriglyceridemia, insulin resistance and hypoinsulinemia. Impairment of lipid metabolism was particularly evident in these rats, resembling a prevalent feature in type 2 diabetic patients (Chehade et al., 2013). The hypertriglyceridemia is undoubtedly a consequence of high fructose consumption, since it was not observed in rats treated only with streptozotocin. The fructose-fed, streptozotocin-induced model of diabetes thus can be used for further investigation into the pathophysiological...
mechanisms related to diabetic hypertriglyceridemia and the efficacy of pharmacological interventions to normalize this condition.

Our fructose-streptozotocin-induced model of type 2 diabetes does come with some limitations, as it does not display all the features of the disease in humans. These diabetic rats were not obese and had relatively low levels of plasma insulin. It has been previously shown that high-fructose feeding in rats for a period longer than 6 weeks does not produce any significant changes in body weight (de Moura et al., 2009; Song et al., 2005). The fructose-streptozotocin combination was therefore not expected to result in weight gain. However, even in the absence of obesity, the fructose-fed, streptozotocin-induced type 2 diabetic rats in our studies still exhibited marked disturbances in glucose and lipid metabolism similar to obese diabetic patients (Tappy and Le, 2010). The hypoinsulinemia in our rat model of type 2 diabetes is likely due to the high dose (50 or 60 mg/kg) of streptozotocin used. This abnormality reflects a later stage of type 2 diabetes, when β-cell function progressively deteriorates as hyperglycemia continues to worsen. In fact, insulin therapy often becomes necessary in patients with severe type 2 diabetes who are insulin deficient and non-responsive to other treatments (Mathis et al., 2001). Although there are differences between the fructose-streptozotocin-induced rat model and human type 2 diabetes, the model resembles many well-defined characteristics of the disease, which allows us to study the pathophysiological changes associated with advanced type 2 diabetes.

### 6.2 Cardiovascular function in two rat models of diabetes

*In vivo* cardiovascular function was assessed in the streptozotocin-induced type 1 and fructose-streptozotocin-induced type 2 diabetic rats via measurements of blood pressure, venous tone, vascular resistance, heart rate, cardiac contractility and cardiac index. Baseline
cardiovascular variables were generally similar between control and diabetic rats, although heart rate appeared to be lower in the STZ group. Diabetic rats in the STZ and FRU-STZ groups did not display considerable abnormalities at rest until challenged with exogenously administered adrenoceptor agonists, namely, noradrenaline, adrenaline and dobutamine. Our results are relevant to diabetic patients, many of whom have a high risk for cardiovascular disease even in an asymptomatic state (Harris and White, 2007). These patients are often screened or tested with stressors such as exercise or a pharmacological agent like dobutamine to evaluate their cardiovascular risk (Chopra and Peter, 2012; Hanzal and Ducharme, 2006). In our studies, both the streptozotocin- and fructose-streptozotocin-induced diabetic rats had reduced arterial and venous constrictions to α-adrenoceptor activation, as well as reduced left ventricular contractile responses to β-adrenoceptor activation. Taken together, our findings suggest that generalized depressions of in vivo vascular and cardiac contractile functions occur not only in type 1 but also in type 2 diabetes. The fructose-streptozotocin-induced rat model is hence suitable for study of cardiovascular abnormalities in chronic type 2 diabetes. More importantly, using this model, we have established an association between type 2 diabetes and venous contractile dysfunction, an area of research that is lacking and remains to be explored.

In all four studies, there were no significant differences in vascular and cardiac contractile responses to adrenoceptor agonists between the STZ and FRU-STZ groups, even though the responses were on the whole slightly attenuated in the latter. This suggests that the presence of hypertriglyceridemia does not significantly worsen in vivo cardiovascular function in our fructose-fed, streptozotocin-induced rat model of type 2 diabetes. In contrast to humans, rats are less prone to developing atherosclerosis, an event highly correlated with dyslipidemia and macrovascular complications (Karimi, 2012; Russell and Proctor, 2006). As a result, 6 weeks of
fructose feeding followed by streptozotocin injection may not be long enough to disclose any substantial hypertriglyceridemia-associated cardiovascular abnormalities. Lengthening the duration of diabetes may give us more insights on the possible differences in contractile function between the two rat models of diabetes.

6.3 Possible mechanisms of cardiovascular contractile dysfunction in diabetes

The mechanisms responsible for the attenuated vascular and cardiac contractile responses to exogenous noradrenaline, adrenaline and/or dobutamine in type 1 and type 2 diabetes are unclear, but likely involve multiple factors. In our studies, administration of phlorizin (a glucose lowering agent), nimesulide (a selective COX-2 inhibitor) or NAC (an antioxidant) was able to improve some aspects of cardiovascular dysfunction in the streptozotocin- and fructose-streptozotocin-induced diabetic rats, which demonstrates that diabetes-associated depressions of vascular and cardiac contractile functions are mediated, at least in part, by hyperglycemia, COX-2-induced inflammation and oxidative stress, respectively. These factors are possibly implicated in the downregulation of α- (Edith-Rodriguez et al., 2012; Park et al., 2011) and β-adrenoceptors (Bilginoglu et al., 2009; Altan et al., 2007), in addition to altered myocardial Ca\(^{2+}\) handling (Ligeti et al., 2006; op den Buijs et al., 2005) and impaired adenylyl cyclase signalling cascade (Shpakov and Derkach, 2013), thereby resulting in cardiovascular hyporesponsiveness to adrenoceptor agonists.

Normalization of plasma glucose by phlorizin partially restored the α-adrenoceptor-mediated venous but not arterial constriction in our rat models of diabetes. Similarly, in both groups of diabetic rats, acute administration of nimesulide augmented venous constriction to a greater extent than arterial constriction. Our findings indicate that hyperglycemia and activation
of COX-2 play a more substantial role in depressing venous than arterial contractile function in type 1 and type 2 diabetes. Differences between arterioles and venules, such as higher collagen content and greater COX-2 expression in the latter, may explain these observations. Chronic treatment with NAC, on the other hand, appears to offer extensive protection against vascular abnormalities, since it significantly improved both arterial and venous constrictions in the streptozotocin- and fructose-streptozotocin-induced diabetic rats. Oxidative stress is thought to be the "final common pathway" of hyperglycemia-induced vascular complications (Pitocco et al., 2013), thus it is not surprising that NAC prevented considerably the progression of vascular contractile dysfunction in our rat models of diabetes.

The lack of improvement in arterial constriction following phlorizin treatment, albeit unexpected, is supported by an emerging concept known as 'metabolic memory'. Indeed, a persistence of diabetic vascular complications despite reversal of hyperglycemia has been reported in experimental and clinical studies (Zhang and Wu, 2014). Oxidative stress is believed to play a central role in the occurrence of 'metabolic memory'. There is evidence that ROS-mediated cellular damage continues to persist (Xie et al., 2008) and total antioxidant capacity remains reduced (Kowluru et al., 2007) even after glucose normalization. Simultaneous, but not separate, control of glycemia and oxidative stress was found to inhibit the deleterious effects from previous exposure to high glucose in endothelial cells (Ihnat et al., 2007) and completely normalize endothelial function in type 1 diabetic patients (Ceriello et al., 2007), confirming the importance of early glycemic control to avoid future diabetic complications and the benefits of combination therapy to mitigate the risk of vascular abnormalities. Initiating phlorizin treatment earlier in our rat models of diabetes may shed more light on the complex interplay between 'metabolic memory' and cardiovascular dysfunction in diabetes.
Overall, results from our studies suggest that cardiovascular contractile abnormalities in type 1 and type 2 diabetes are, at least in part, associated with hyperglycemia, COX-2-induced inflammation and oxidative stress. Intensive glycemic control is insufficient as monotherapy for complete prevention of vascular contractile dysfunction in diabetes, so other strategies that target dyslipidemia, inflammation and/or oxidative stress should be incorporated into the treatment plan to achieve optimal outcomes. Decreasing oxidative stress with the antioxidant NAC appears to have the added benefit of reversing hypertriglyceridemia, which may concomitantly lead to significant ameliorations of vascular as well as cardiac contractile functions.

The most effective strategy for managing cardiovascular risk in diabetes will involve reducing not only glucose levels, but also other risk factors such as inflammation and oxidative stress. Physicians can consider the use of combination therapy to better control the multitude of cardiovascular complications. Combination therapy with a glucose lowering agent, an anti-inflammatory drug and an antioxidant may confer additive or synergistic effects (Koh et al., 2009) that will protect against cardiovascular contractile dysfunction in the diabetic population. Treatment should also be implemented early in the course of the disease to maximize the benefits (Pozzilli et al., 2014).

6.4 Final summary

The key findings from our four studies are summarized below:

1) Diabetic rats induced through a combination of high-fructose feeding and subsequent injection of streptozotocin exhibited hyperglycemia, marked hypertriglyceridemia, insulin resistance and hypoinsulinemia, all of which resemble features of advanced type 2 diabetes.
Arterial and venous constrictions to α-adrenoceptor activation and cardiac contractile responses to β-adrenoceptor activation were depressed in these rats.

2) The fructose-streptozotocin-induced type 2 diabetic rats did not have greater impairment in vascular and cardiac contractile responses relative to the streptozotocin-induced type 1 diabetic rats, suggesting that the presence of hypertriglyceridemia does not significantly worsen in vivo cardiovascular function in our rat model of type 2 diabetes.

3) Treatment with phlorizin, a glucose lowering agent, partially restored the venous but not arterial constriction in both rat models of diabetes.

4) Acute administration of nimesulide, a selective COX-2 inhibitor, did not alter cardiac contractile responses, but improved the venous and, to a less extent, arterial constriction in both rat models of diabetes.

5) Chronic administration of NAC, an antioxidant, ameliorated the cardiac as well as vascular contractile responses in both rat models of diabetes.

6.5 Study limitations

Our studies focused primarily on the changes in functional cardiovascular parameters in vivo, and did not explore changes at the structural, biochemical and/or molecular levels. Therefore, we were only able to provide indirect evidence of attenuated inflammation and oxidative stress in the diabetic animals following administration of nimesulide or NAC. Our results suggest, but do not prove, that inflammation and oxidative stress contribute, at least in part, to cardiovascular contractile dysfunction in diabetes. Furthermore, the use of rats may limit the generalization of our findings to the human population. No animal models to date fully represent the etiology and complexity of human diabetes. Nevertheless, they depict many clinical
features of the disease, and are essential tools for drug development and understanding of the pathogenesis of diabetic complications.

6.6 Future directions

The research in this thesis has provided insights into several treatment options for cardiovascular contractile dysfunction associated with diabetes. However, we have not addressed whether different durations of treatment with phlorizin, nimesulide or NAC produce similar or variable outcomes. In particular, it will be interesting to investigate how early chronic treatment with a glucose lowering agent (started before hyperglycemia develops) affects in vivo cardiovascular function in our models of diabetes to substantiate the concept of 'metabolic memory'. Moreover, differences in the underlying pathophysiology between arterioles and venules, such as structural and biochemical alterations, remain to be elucidated. Since diabetes, particularly type 2 diabetes, is a heterogeneous disease that encompasses a diverse spectrum of pathophysiological processes, multifactorial intervention, although challenging, will likely be the most effective therapy in the long run (Gaede et al., 2008). In this regard, future studies should evaluate if the concomitant control of glycemia, inflammation and oxidative stress can abolish diabetic cardiovascular complications. Such knowledge will disclose important strategies for the management of diabetes, ultimately reducing the risk of cardiovascular disease and mortality in the diabetic population.
References


