Regional heterogeneity of the vascular dysfunction in \textit{db/db} mice: Role of reactive oxygen species

by

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Abstract

**Background:** The high mortality and morbidity rates associated with diabetes are mainly attributed to its cardiovascular complications. It remains questionable whether diabetes has a general deleterious effect on the vasculature, or if different arteries exhibit differential vulnerability to the diabetic milieu. This thesis compared the functional adaptation of three arteries: the aorta, carotid and femoral arteries, to the diabetic milieu present in *db/db* mice, and elucidated the mechanisms underlying the arteries' differential adaptation. Additionally, the functional and molecular alterations in the aorta and femoral artery in response to moderate-intensity exercise training were compared.

**Methods:** Vasodilatory and contractile responses were examined in isolated aortae, carotid and femoral arteries from *db/db* and control mice to assess the endothelium and vascular smooth muscles' functions. Additionally, the protein expressions of endothelial nitric oxide synthase (eNOS), Akt, cyclooxygenase and superoxide dismutase (SOD) isoforms were examined. In parallel, plasma markers of glycemia, oxidative stress, inflammation, and dyslipidemia were measured. Thereafter, a correlation analysis was performed to estimate the strength of association between plasma variables and vascular responses.

**Results:** The aortae of *db/db* mice exhibited a progressive impairment in endothelial and vascular smooth muscle functions. The carotid artery was the most resilient and maintained unaltered functional responses in *db/db* mice, likely because the carotid artery, in contrast to the aorta, relaxes in response to superoxide anion or peroxynitrite. The femoral arteries of *db/db* mice showed reduced endothelium-dependent hyperpolarizing factor-mediated vasodilatation and attenuated contractile responses, probably due to the lack of expression of extracellular SOD.
in the femoral artery. The benefits of exercise training were confined to nitric oxide-mediated vasodilatation in the aortae and femoral arteries of \( db/db \) mice, and were associated with increased eNOS/Akt and SOD expressions and reduced cardiovascular risk factors.

**Conclusions:** Substantial heterogeneity exists between the aorta, carotid and femoral arteries both at functional (signalling pathways) and molecular levels (protein expression) under physiological and diabetic conditions. Understanding regional differences in vasomotor control, coupled with advanced drug delivery systems will open new venues for developing therapies that target specific vascular beds with minimal systemic side effects.
Preface
The animals care and experimental protocols described in this thesis were approved by the Animal Care Committee at the University of British Columbia (Animal Care Certificate A06-0308).

A portion of the work presented in this thesis was published as two articles. Specifically, the correlation analysis of the vascular responses with plasma variables, presented in tables (3-3) and (3-4), was the basis of the following publication:


I was responsible for designing the study, conducting the vascular functional experiments and measuring the biochemical parameters, except for the plasma lipids which were analysed at Vancouver General Hospital. Additionally, I wrote the manuscript. Dr Anat Fisher was responsible for the statistical analysis including the correlation analysis and stepwise model selection. Dr Saeid Golbidi helped with the cleaning of the aortae.

Also, a small part of the third study was published as below:


I was responsible for the study design, data collection and analysis. Dr Khazaei wrote the manuscript for the above paper. The third study included in this thesis was based on a previous study conducted by my colleague, Dr Farzad Moien-Afshari.

As for the rest of the work presented in this thesis, I was responsible for the experimental design, data collection, analysis and presentation in addition to the writing. Dr Baohua Wang kindly taught me the basics of western blot and helped with troubleshooting.
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<th>Description</th>
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<tbody>
<tr>
<td>4-AP</td>
<td>4-aminpyridine</td>
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<tr>
<td>5-HT</td>
<td>serotonin</td>
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<tr>
<td>8-isoprostane</td>
<td>8-<em>iso</em>PGF$_{2\alpha}$</td>
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<td>ACh</td>
<td>acetylcholine</td>
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<td>Ang II</td>
<td>angiotensin II</td>
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<td>AUC</td>
<td>area under the curve</td>
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<td>BH$_{(4)}$</td>
<td>tetrahydrobiotin</td>
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<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
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<td>cGMP</td>
<td>cyclic guanosine monophosphate</td>
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<td>CHTX</td>
<td>charybdotoxin</td>
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<td>COX</td>
<td>cyclooxgenase</td>
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<tr>
<td>CPI-17</td>
<td>PKC-potentiated myosin phosphatase inhibitor of 17 kDa</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>CYP-450</td>
<td>cytochrome P-450</td>
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<td>diacyl glycerol</td>
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<td>EDHF</td>
<td>endothelium-ependent hyperpolarizing factor</td>
</tr>
<tr>
<td>EGFR</td>
<td>endothelium growth factor receptor</td>
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<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
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<td>Ex</td>
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<td>GTT</td>
<td>glucose tolerance test</td>
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<td>HDL</td>
<td>high density lipoprotein</td>
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<td>LDL</td>
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<td>PPAR</td>
<td>peroxisome proliferator-activated receptor</td>
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<td>PSS</td>
<td>physiologic salt solution</td>
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<td>RhoGEF</td>
<td>Rho guanine nucleotide exchange factor</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<td>Sed</td>
<td>sedentary</td>
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<tr>
<td>SERCA</td>
<td>sarco(endo)plasmic reticulum Ca(^{2+})-transporting ATPase</td>
</tr>
<tr>
<td>S.E.</td>
<td>standard error</td>
</tr>
<tr>
<td>SNP</td>
<td>sodium nitroprusside</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>TNF(_{\alpha})</td>
<td>tumor necrosis factor-alpha</td>
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<tr>
<td>TP</td>
<td>thromboxane prostanoids receptor</td>
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<td>VGCC</td>
<td>voltage gated calcium channel</td>
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<td>VSMs</td>
<td>vascular smooth muscles</td>
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<td>Wk</td>
<td>weeks</td>
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<tr>
<td>X</td>
<td>xanthine</td>
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<td>XO</td>
<td>xanthine oxidase</td>
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Acknowledgments

It has been a long journey, not an easy one; but definitely a journey that was full of learning opportunities that I hope I made the most of.

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Last but not least, thanks to my parents and my children. Without you, nothing could have been accomplished or would have meant anything…
To my girls

To the future

For a better future
1- Introduction

1-1 Diabetes mellitus: the epidemic and impact

Diabetes mellitus is a heterogeneous group of disorders characterized by chronic hyperglycemia and an increased risk for cardiovascular diseases due to defective insulin secretion and/or action. Symptoms of marked hyperglycemia include polyuria, polydipsia, weight loss, sometimes with polyphagia, and blurred vision. The long-term effects of diabetes include damage and failure of various organs. Many metabolic abnormalities typically co-exist in diabetic patients but hyperglycemia remains the hallmark and the diagnostic criteria of diabetes. In 1997, the first Expert Committee on the Diagnosis and Classification of Diabetes Mellitus lowered the fasting plasma glucose cut-off to 7.0 mM from 7.8 mM and confirmed the long-standing 2 hours post-glucose load diagnostic value of 11.1 mM. These diagnostic values are associated with an inflection point for the prevalence of retinopathy (American Diabetes Association, 2011).

Diabetes is one of the oldest known diseases and was described by several civilizations more than two thousand years ago (Farmer, 1952, Christopoulou-Aletra and Papavramidou, 2008). What is currently making the headlines is the alarming increase in the global prevalence of diabetes. The report released by the World Health Organization in 1998 estimated the prevalence of diabetes in adults (20 years and older) to be 4.0% in 1995, and was projected to rise to 5.4% by 2025, affecting over 300 million patients and possibly presenting the biggest epidemic the world may ever know (King et al., 1998). The more recent studies have revealed more shocking figures. Shaw et al. (2010) assessed the global prevalence of diabetes among adults to have already reached 6.4%, affecting 285 million adults in 2010, and they have projected that it will increase to 7.7% and 439 million adults by 2030. Another recent report assessed the number of
adult diabetics to be about 347 million in 2008, with North America recording the largest rise in fasting blood glucose— 0.18 mM per decade for men and 0.14 mM per decade for women (Danaei et al., 2011). In Canada, the prevalence of diabetes was 4.2% in 2000, but is projected to reach 9.9% in 2020, probably due to rising obesity rates, sedentary lifestyles, an aging population, and new immigration waves (The Canadian Diabetes Association, 2009).

The growing burden of diabetes on global health and national productivity is overwhelming in developed countries and catastrophic for developing countries. Diabetes is responsible for 6.8% and 15.7% of global and North American mortality, respectively (Roglic and Unwin, 2010). Life expectancy may be shortened by 15 years for type 1 diabetic patients and by 5 to 10 years for type 2 diabetics (The Canadian Diabetes Association, 2009).

Globally, 12% of health expenditure is anticipated to be spent on diabetes. At least 376 billion dollars US were spent in 2010 and this is expected to rise to 490 billion dollars US in 2030 (Zhang et al., 2010). The figures are similarly gloomy for Canada. The cost of diabetes was estimated to be 12.2 billion dollars in 2010, nearly double the amount spent in 2000, and is expected to rise by another 4.7 billion dollars by 2020 (The Canadian Diabetes Association, 2009).

**1-2 Diabetes mellitus classification**

The concept of classifying diabetes mellitus into subtypes was first introduced in 1951 but was not accepted until 1980 by the World Health Organization (Gale, 2006, WHO expert committee on diabetes mellitus, 1980), and it has been controversial since then as there is a lack of clear criteria for distinguishing the subtypes of diabetes, and many diabetic patients do not easily fit into a single class (Gale, 2006).

**Type 1 diabetes** (previously encompassed by the terms insulin-dependent diabetes, type 1 diabetes or juvenile-onset diabetes), accounts for 5–10% of diabetic patients and is caused by pancreatic beta-cell destruction which is either immune-cells mediated or idiopathic. Type 1 diabetic patients usually suffer from absolute insulin deficiency and therefore are dependent on insulin for survival.

**Type 2 diabetes** (previously encompassed by the terms non-insulin-dependent diabetes, or adult-onset diabetes), accounts for 90–95% of diabetic patients. Type 2 diabetic patients have relative rather than absolute insulin deficiency, and they do not need insulin for survival but mostly for control of their glucose levels. The aetiology of type 2 diabetes is not known but it is established that its risk increases with age, obesity and physical inactivity. The hyperglycemia develops slowly and the patients present with no abrupt symptoms of diabetes; therefore they are underdiagnosed and are at increased risk of developing macro and microvascular complications.

**Other specific types** include genetic defects of the beta-cell function, genetic defects in insulin action, diseases of the exocrine pancreas, endocrinopathies, drug- or chemically-induced diabetes, infections, specific forms of immune-mediated diabetes and other genetic syndromes sometimes associated with diabetes.

**Gestational diabetes mellitus** defined as any degree of glucose intolerance which is first recognized during pregnancy.
1-3 Vascular complications of diabetes mellitus

Diabetes mellitus was invariably defined as a metabolic disorder and was always classified under endocrinopathies; however, now it is regarded as a cardiovascular disease equivalent. With the availability of insulin treatment and glucose monitoring devices, the risk of the acute metabolic complications of diabetes (hypo- and hyper-glycemic comas) has subsided, but has left in place the more chronic vascular complications. About 65% of deaths in individuals with type 2 diabetes are related to heart diseases or strokes (The Canadian Diabetes Association, 2009, Reasner, 2008, Winer and Sowers, 2004). The vulnerability of specific cell types, particularly vascular cells, to diabetic complications may reflect the failure of those cells to down-regulate their uptake of glucose when extracellular glucose concentrations are elevated for long durations (Kaiser et al., 1993).

The risk of cardiovascular death is at least 3-fold higher for diabetic patients relative to non-diabetics after adjustment for age, race and other cardiovascular risks (Wei et al., 1998, Stamler et al., 1993, Juutilainen et al., 2008). Indeed, the risk of dying from coronary heart diseases for type 2 diabetic patients is comparable to that for non-diabetic patients who have had a myocardial infarction previously (Haffner et al., 1998). A collaborative meta-analysis of 102 studies showed that diabetes increases the hazard ratios for coronary heart disease by 183%-219%, for ischaemic stroke by 195%-265%, for haemorrhagic stroke by 119%-205%, for unclassified stroke by 159%-213% and for other vascular diseases by 151%-198%, independently from other conventional risk factors (Sarwar et al., 2010). In Canada, the vascular complications of diabetes are responsible for 80% of deaths in diabetic patients and are the major causes of blindness and non-traumatic limb amputations (The Canadian Diabetes Association, 2009).
The increased risk of cardiovascular mortality and morbidity in diabetic patients are largely due to accelerated atherosclerosis and increased thrombosis, possibly triggered by endothelial dysfunction (Feener and King, 1997). It is estimated that diabetes ages the vasculature by about 15 years (Booth et al., 2006).

Diabetic microvascular complications mainly affect the vasculature of the eye, kidney and peripheral nerves that in turn lead to retinopathy, nephropathy and neuropathy, while the macrovascular complications are manifested in the coronary, cerebral, and lower-limb arteries (Feener and King, 1997). Our understanding of the differential vulnerability of specific vascular beds to either micro- or macro-vascular complications in diabetic subjects is quite limited.

1-4 Altered vascular reactivity in diabetes

Vascular reactivity studies encompass investigating the responses of intact or isolated blood vessels to contractile or vasodilatory stimuli. Vascular reactivity in clinical and experimental diabetes has been the subject of numerous studies. The vasodilatory responses have been more intensely investigated, but have yielded variable results. Although the majority of studies reported some degree of impairment either in the endothelial- (O'Driscoll et al., 1999, Ting et al., 1996, De Vriese et al., 2000, Brunner et al., 2005), or vascular smooth muscles (VSMs)- (Caballero et al., 1999, Williams et al., 1996, Lam et al., 2006) mediated vasodilatation, some reports demonstrated unaltered responses (Bagi et al., 2005, Fortes et al., 1983, Granstam and Granstam, 2003), whereas other studies even reported enhanced vasodilatation (Szerafin et al., 2006, Skyrme-Jones et al., 2000a, Skyrme-Jones et al., 2000b). The discrepancies of these results are likely due to differences in the stage of diabetes, the anatomical origin of the blood vessels examined and the signalling pathways of the vasoactive agents used.
Mounting evidence indicates that endothelial dysfunction is implicated in the development of diabetic vascular complications as endothelial integrity is crucial in preventing atherosclerosis and thrombosis (Brunner et al., 2005, Widlansky et al., 2003). Accordingly, endothelial dysfunction, estimated biochemically by measuring the plasma levels of endothelium-derived regulatory proteins e.g. von Willebrand factor, or estimated functionally by examining the endothelium-dependent vasodilatation in the coronary or peripheral circulation, has been shown to be an independent predictor of cardiovascular events and death in diabetic patients (Stehouwer et al., 2002, Jager et al., 1999, Jager et al., 2001, Suwaidi et al., 2000, Schachinger et al., 2000, Lerman and Zeiher, 2005). Among important mediators synthesized by endothelial cells, nitric oxide (NO) has received special attention because of its vasodilator, anti-thrombotic, anti-proliferative, and anti-inflammatory properties (Kawashima, 2004). Different pathomechanisms have been proposed to interfere with NO signalling under diabetic conditions including impaired receptor transduction (Miike et al., 2008), reduced substrate or cofactors availability (Pannirselvam et al., 2002, Pieper and Peltier, 1995), downregulated Akt/endothelial nitric oxide synthase (eNOS) (Zhong et al., 2007, Fukuda et al., 2010b), increased destruction of NO by reactive oxygen species (ROS) (San Martin et al., 2007, Wong et al., 2010), upregulated caveolin-1 (Lam et al., 2006) and/or decreased response of the VSMs to NO (McVeigh et al., 1992, Watts et al., 1996, van Etten et al., 2002).

In the present thesis, I used acetylcholine (ACH), the muscarinic agonist, to examine the endothelial function. Acetylcholine, by acting on the muscarinic receptors in the endothelium, induces a transient rise in intracellular Ca\(^{2+}\) activating calmodulin which in turn interacts with caveolin and eliminate caveolin’s inhibitory effect on eNOS. Following calmodulin activation, heat shock protein 90 binds to eNOS favouring the recruitment of Akt leading to the
phosphorylation of eNOS, production of NO, and vasodilation (Balligand et al., 2009, Thorin and Thorin-Trescases, 2009).

To assess the VSMs function, I used sodium nitroprusside (SNP), and isoprenaline. Sodium nitroprusside spontaneously releases NO in aqueous solutions which directly activates soluble guanylyl cyclase (sGC) in VSMs triggering vasodilatation, therefore bypassing the role of endothelium. Isoprenaline acts on the β-adrenoceptors in the VSMs leading to the activation of adenylate cyclase pathway and increasing cyclic adenosine monophosphate (cAMP) level (Harden, 1983). Isoprenaline can activate, through cAMP-dependent and independent mechanisms, calcium activated potassium channels (K_{Ca}) and voltage activated potassium channels (K_{v}) leading to hyperpolarization and vasodilatation (Matsushita et al., 2006).

To examine the contractile responses, I used phenylephrine (PE), high extracellular KCl solution, the thromboxane A2 analogue (U-46619), and the calcium ionophore (A23187) in the presence of a NOS inhibitor. Phenylephrine acts on G-protein coupled α1-adrenoceptors to activate phospholipase C (PLC) releasing inositol triphosphate (IP₃) and diacylglycerol (DAG) from the cell membrane phosphatidylinositol bisphosphate (PIP₂). In turn, IP₃ releases calcium from the sarcoplasmic reticulum, whereas DAG stimulates protein kinase C (PKC) increasing the sensitivity of the contractile apparatus to cytosolic calcium (Chen and Rembold, 1995). High extracellular KCl concentration stops the efflux of intracellular K⁺, causing cell depolarization with subsequent activation of voltage gated calcium channels (VGCC) and influx of extracellular calcium (Chen and Rembold, 1995). U-46619 acts on the thromboxane prostanoids receptor (TP) leading to the activation of two pathways: the PLC pathway and the Rho guanine nucleotide exchange factor (RhoGEF) pathway (Feletou et al., 2010). A23187 increases the cell membrane permeability to calcium leading to the influx of extracellular calcium (Shi et al., 2007). The
increase in cytosolic calcium activates myosin light chain kinase (MLCK) which phosphorylates myosin light chain triggering contraction (Nishimura et al., 1989). These signalling pathways are presented in illustration (1-1).

Illustration 1-1: Simplified schematic diagram showing signalling pathways for phenylephrine, KCl, U-46619 and A23187

DAG= diacyl glycerol, IP$_3$= inositol triphosphate, MLCK= myosin light chain kinase, MLCP= myosin light chain phosphatase, PE= phenylephrine, PIP$_2$= phosphatidylinositol bisphosphate, PLC= phospholipase C, PKC= protein kinase C, RhoGEF= Rho guanine nucleotide exchange factor, TP= thromboxane prostanoids receptor, VGCC= voltage gated calcium channels
1-5 The diabetic milieu

In addition to hyperglycemia, which is the hallmark and the diagnostic criterion of diabetes, a cluster of metabolic abnormalities typically co-exists in type 2 diabetic patients, who usually exhibit obesity, insulin resistance, dyslipidemia, systemic chronic inflammation and oxidative stress. Diabetic vasculopathies are probably not caused by a single offender such as hyperglycaemia, but rather involve an amalgam of mechanisms and several key players. To design effective preventative and therapeutic therapies, a better understanding of the mechanisms underlying diabetic vasculopathies is needed. Below, I outline four of the major metabolic abnormalities present in type 2 diabetic patients, namely, hyperglycemia and dyslipidemia, which represent classical cardiovascular risk factors, as well as inflammation and oxidative stress which are considered novel risk factors. Evidence linking each of these risk factors to cardiovascular diseases, along with the proposed mechanisms for their detrimental effects are presented. In addition, both supporting and opposing evidence for the benefits of therapies that specifically target each risk factor are briefly discussed.

1-5-1 Hyperglycemia

Several studies including large scale prospective clinical studies have provided evidence that hyperglycemia is associated with an increased risk for cardiovascular events and mortality, independent of other risk factors in individuals with type 1 or type 2 diabetes (Stratton et al., 2000, Klein et al., 1994, Laakso and Kuusisto, 1996, Hanefeld et al., 1996, Selvin et al., 2004). Even at the prediabetic stage, a slightly elevated fasting blood glucose level is associated with modest increases in the risk for cardiovascular disease (Sarwar et al., 2010, Ford et al., 2010).
Acute hyperglycemia induced by oral-glucose loading suppressed endothelium-dependent vasodilatation in normal (Williams et al., 1998), and diabetic patients (Kawano et al., 1999). In vitro incubation with high glucose concentrations, although it cannot completely reflect the complex effects of chronic hyperglycemia present in diabetic patients, was shown to alter the expressions of pro-oxidant and antioxidant enzymes, as well as eNOS and Akt activity in cultured endothelial cells (Weidig et al., 2004, Srinivasan et al., 2004, Du et al., 2001, Song et al., 2007) and can ultimately lead to endothelial cells apoptosis (Tamareille et al., 2006). Additionally, high glucose levels have been shown to reduce the function of K_v (Li et al., 2003, Liu et al., 2001, Rainbow et al., 2006), ATP-sensitive potassium channels (K_ATP) and the large conductance calcium-activated potassium-channels (BK_Ca) in VSMs (Kinoshita et al., 2004, Lu et al., 2006) leading to impaired vasodilatation. There are several proposed mechanisms that could link hyperglycemia to vascular injury including activation of the polyol pathway, glucose auto-oxidation, increased formation of diacylglycerol with subsequent activation of protein kinase C, nonenzymatic formation of advanced glycation end products, increased oxidative stress, and alterations in genes function (Feener and King, 1997, De Vriese et al., 2000, Ding and Triggle, 2010).

1-5-1-1 Targeting hyperglycemia
In both type 1 and type 2 diabetic patients, intensive glycemic control reduced microvascular complications, and in longer-term follow-up reduced macrovascular events (The Diabetes Control and Complications Trial Research Group, 1993, UK Prospective Diabetes Study (UKPDS) Group, 1998). The positive effects of intensive treatment on the risk of cardiovascular disease were mostly attributed to decreases in glycosylated haemoglobin (Nathan et al., 2005, Stettler et al., 2006). Each 1% reduction in glycosylated haemoglobin was associated with a 37% decrease in the risk for microvascular complications, a 21% decrease in the risk for any diabetes-
related death and a 14% decrease in the risk for myocardial infarction (Stratton et al., 2000). Additionally, the use of specific glucose lowering agents e.g. metformin, acarbose and pioglitazone was also associated with reduced risks of cardiovascular events and mortality (UK Prospective Diabetes Study (UKPDS) Group, 1998, Dormandy et al., 2005, Hanefeld et al., 2004).

On the other hand, a recent systematic review that examined 216 studies which used oral hypoglycaemic agents concluded that the evidence of their benefits in reducing cardiovascular mortality is still inconclusive (Bolen et al., 2007). Surprisingly, metformin showed more benefits in the cardiovascular outcomes than two sulfonylureas and insulin in type 2 diabetic patients although its glucose-lowering potency is not greater than the other treatments (UK Prospective Diabetes Study (UKPDS) Group, 1998, Turner et al., 1999), suggesting that there were other important effects of metformin aside from lowering blood glucose that contributed to its beneficial effects. Furthermore, the Action to Control Cardiovascular Risk in Diabetes study showed that aggressive reduction of hyperglycemia to nearly normal levels in long-term type 2 diabetic patients did not reduce further the cardiovascular events but was rather associated with increased risks for hypoglycaemia and mortality (Ismail-Beigi et al., 2010).

Therefore, there is solid evidence that hyperglycemia is associated with increased risks for cardiovascular events and mortality; however, the evidence that targeting hyperglycemia only will reduce the cardiovascular complications of diabetes seems less robust.

1-5-2 Dyslipidemia

Type 2 diabetic patients often suffer from dyslipidemia that is characterized by increased triglyceride-rich lipoproteins, reduced high density lipoprotein (HDL) levels, and a relative
increase in small dense low density lipoprotein (LDL) particles (Falko et al., 2005). Several studies showed that the plasma triglycerides level can predict both cardiovascular diseases and death (Hanefeld et al., 1996, Hokanson and Austin, 1996, Bansal et al., 2007), while other studies highlighted LDL as the strongest predictor of cardiovascular diseases in diabetic individuals. They reported that each 10 mg/dl increase in LDL level was associated with a 12% increase in cardiovascular diseases risk (Turner et al., 1998, Howard et al., 2000, Laatikainen et al., 2005). The association between reduced HDL levels and increased risk of cardiovascular diseases is well established independently of other risk factors in both diabetic and non-diabetic patients (Turner et al., 1998, Goldbourt et al., 1997, Ting et al., 2010, Abbott et al., 1988).

Several studies indicate a significant role for dyslipidemia in the endothelial dysfunction evident in diabetic patients (Watts et al., 1996, O'Brien et al., 1997, Tan et al., 1999). Oxidized LDL and lysophosphatidylcholine, which is formed during the oxidation of LDL (McIntyre et al., 1999), can reduce eNOS expression and activity (Liao et al., 1995, Vergnani et al., 2000), inhibit endothelium-dependent hyperpolarizing factor (EDHF)-mediated vasodilatation (Matsumoto et al., 2006, Fukao et al., 1995), activate PKC (Ohara et al., 1994), enhance the release of endothelin-1 (Boulanger et al., 1992), and increase endothelial cells apoptosis (Dimmeler et al., 1997, Guerci et al., 2001).

Levels of HDL have been shown to be positively associated with endothelium-dependent vasodilatation in several studies (Ford et al., 2009, Higashi et al., 2010, Tsuchiya et al., 2009, Bosevski et al., 2007). In addition to the role of HDL particles in the uptake of peripheral cholesterol to the liver, they exhibit anti-inflammatory, antithrombotic and antioxidant properties (Bitzur et al., 2009). HDL particles restore endothelium-dependent vasodilatation (Bisoendial et al., 2003, Spieker et al., 2002), stimulate endothelial NO release (Nofer et al., 2004, Yuhanna et
al., 2001), promote endothelial cells repair (Sumi et al., 2007, Tso et al., 2006) and downregulate angiotensin II type 1 receptors (Van Linthout et al., 2009). Hypertriglyceridemia can enhance monocytes binding to endothelial cells (Hoogerbrugge et al., 1996). However, the relationship of elevated plasma triglycerides to vascular injury remains more obscure than those of LDL or HDL (Ryden et al., 2007).

1-5-2-1 Targeting dyslipidemia
Several clinical trials support the use of statins to reduce cardiovascular events in type 2 diabetic patients. The Heart Protection Study, which included 5963 diabetic patients, showed that simvastatin reduced by approximately 25% the risk of major coronary events, stroke, and revascularization compared with placebo (Collins et al., 2003). In the Scandinavian Simvastatin Survival Study, simvastatin treatment led to a 35% decrease in LDL, along with a 42% decrease in the incidence of non-fatal myocardial infarction and cardiovascular mortality (Haffner et al., 1999). Similar beneficial outcomes were achieved by atorvastatin and pravastatin in the Collaborative Atorvastatin Diabetes Study (Colhoun et al., 2004), Cholesterol and Recurrent Events study, and Long-term Intervention with Pravastatin in Ischemic Disease Study (Goldberg et al., 1998, Sacks et al., 2002). Diabetic Chinese patients treated with statins had a 40-50% risk reduction in cardiovascular diseases compared to non-users (Ting et al., 2010). Patients with impaired fasting glucose levels, but not fully diabetic, were also reported to benefit from simvastatin with significant decreases in total mortality, major coronary events, and revascularizations (Haffner et al., 1999). A meta-analysis which examined 76 randomized controlled studies involving 170,255 participants concluded that statin therapy reduced all-cause mortality, cardiovascular diseases mortality, myocardial infarction, revascularization and strokes (Mills et al., 2011). Examining the Steno-2 trial, which applied a multiple risk factors reduction strategy, Gaede and Pedersen concluded that lipid-lowering rather than glycemic control
accounted for the major reduction of cardiovascular events in type 2 diabetic patients (Gaede and Pedersen, 2004).

Therapies that target HDL also confer beneficial results. Both fibrates and niacin, which are used to elevate HDL levels, have been associated with reduction in all-cause and cardiovascular mortality (Rubins et al., 1996, Ali et al., 2008). A recent study revealed that an increase in HDL levels and not a reduction of glycosylated haemoglobin is responsible for pioglitazone's favourable influence on cardiovascular outcomes in the PROactive study (Dormandy et al., 2005, Ferrannini et al., 2011). A Chinese study found that every 1 mM increase in HDL level was associated with a 41% reduction in the risk of cardiovascular diseases (Ting et al., 2010). The combined effects of lowering LDL and raising HDL were also associated with better cardiovascular outcomes; administration of simvastatin and niacin reduced the frequency of coronary diseases from 24% to 3% compared with placebo in the HDL-Atherosclerosis Treatment Study (Brown et al., 2001).

However, several studies showed that statins have multiple effects, aside from reducing LDL levels, including antioxidant and anti inflammatory actions, as well as a direct effect on NO bioavailability (Davignon, 2004, Antoniades et al., 2011, Beckman and Creager, 2006), which may underlie statins’ beneficial effects on the cardiovascular outcomes. Indeed, atorvastatin was shown to improve endothelial function in smokers, hypertensive and diabetic patients with normal plasma cholesterol levels (Beckman et al., 2004). A recent study claimed that patients who adhere to statins are generally more health-seeking and that could be a confounding factor in the ‘too good to be true’ effects of statins (Dormuth et al., 2009). Alarmingly, a recent study that examined 17 randomized controlled studies, reported an increase (9%) in the incidence of diabetes in statins consumers (Mills et al., 2011). The addition of niacin to statin therapy in a 36-
month study did not show clinical benefits despite significant improvements in HDL and triglyceride levels in patients with atherosclerotic cardiovascular disease (AIM-HIGH Investigators *et al.*, 2011). Also the addition of torcetrapib, an inhibitor of cholesteryl ester transfer protein leading to an increase in HDL level and a decrease in LDL level, resulted in an increased risk of mortality and cardiovascular morbidity in 15,067 patients at high cardiovascular risk (Barter *et al.*, 2007).

**1-5-3 Inflammation**

Despite tight glucose control, some diabetic patients continue to develop vascular complications (Zimmerman and Flores, 2009). It has been proposed that a dysregulated immune response is responsible for the vascular injury in diabetes as it is for pancreatic beta-cell damage. This immune response leads to inflammation that predates the onset of frank hyperglycemia. In support of this are the findings that diabetic patients have increased levels of inflammatory cytokines e.g. C-reactive protein (CRP) (Schalkwijk *et al.*, 1999, Coppola *et al.*, 2006), indicating chronic inflammation. Indeed, atherosclerosis, which is the leading cause of death in diabetic patients, is currently recognized as an inflammatory disease (van den Oever *et al.*, 2010). The obesity often present in type 2 diabetic patients may underlie the state of chronic inflammation in diabetic patients (Aldhahi and Hamdy, 2003, Balistreri *et al.*, 2010, Brooks *et al.*, 2010).

In my studies, plasma CRP level was measured as a marker of systemic inflammation. CRP is an acute phase protein that is mainly generated by the liver in response to trauma, infection, stress, and inflammation as a component of the nonspecific innate immune response (Cray *et al.*, 2009). Differences in the components of the acute-phase response exist among species, but CRP has been shown to be a vital component of this response in humans and mice (Cray *et al.*, 2009,
The Level of plasma CRP has been shown to predict the risk of cardiovascular events in diabetic and non-diabetic populations (Coppola et al., 2006, Cesari et al., 2003) and to predict the risk of death in diabetic patients (Linnemann et al., 2006). In addition, increased levels of CRP can predict the risk of developing type 2 diabetes (Mugabo et al., 2010). There is evidence that CRP, besides its predictive role, can directly trigger vascular dysfunction (Sprague and Khalil, 2009) via reducing eNOS expression and bioactivity (Venugopal et al., 2002, Qamirani et al., 2005), altering calcium channels expression and activity (Tiwari et al., 2006), upregulating Rho-kinase function (Hiroki et al., 2004), increasing the production of ROS (Zhang et al., 2008) and/or enhancing cyclooxygenase (COX) expression (Mitchell et al., 1995). Human atherosclerotic plaques express CRP (Mugabo et al., 2010) and there is evolving evidence that CRP plays an active role in atherogenesis by promoting monocyte chemotaxis and adhesion, and increasing the uptake of oxidized LDL (Venugopal et al., 2005).

1-5-3-1 Targeting inflammation
A recent study showed that salsalate, a prodrug form of salicylate, improved in vivo glucose and lipid homeostasis in diabetic patients (Goldfine et al., 2008). However, three meta-analyses studies indicate that aspirin appears to produce a modest-sized reduction in myocardial infarction and stroke in patients with diabetes, but the current evidence is not conclusive (Pignone et al., 2010, Zhang et al., 2010, De Berardis et al., 2009).

1-5-4 Oxidative stress
Oxidative stress occurs when the rate of production of ROS exceeds the capacity of the antioxidant defence mechanisms to detoxify them, leading to potential damage of proteins, lipids and nucleic acids. A growing body of evidence indicates that hyperglycemia, dyslipidemia and
inflammation can induce oxidative stress through multiple pathways (Ohara et al., 1994, Dimmeler et al., 1997, Chandel et al., 2001, Zhang et al., 2009, Ding et al., 2007, Mugge et al., 1994, Rueckschloss et al., 2001). Indeed, increased levels of oxidative stress markers have been demonstrated in type 2 diabetic patients (Mezzetti et al., 2000, Davi et al., 1999, Basu, 2008), and in several animal models of diabetes (Shi and Vanhoutte, 2008, Matsumoto et al., 2007, Elmi et al., 2008).

There is almost a consensus regarding the role of oxidative stress in the progression of diabetic vascular complications (Ding and Triggle, 2010, Feletou and Vanhoutte, 2006, Giacco and Brownlee, 2010). There are several potential sources of ROS in the vasculature including nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase), COX, uncoupled eNOS, arachidonic acid metabolism and mitochondrial respiratory chain enzymes (Feletou and Vanhoutte, 2006, Wolin, 1996, Brandes and Kreuzer, 2005).

**1-5-4-1 ROS and vasodilatation**
The three major endothelium-dependent vasodilatation pathways: NO, prostacyclin, and EDHF can be inhibited by ROS (Feletou and Vanhoutte, 2006). Superoxide anion (O$_2^-$) can quench NO to form peroxynitrite radical (ONOO’) in a reaction that is 3 to 4 times faster than the dismutation of O$_2^-$ by superoxide dismutase (SOD) (Beckman, 1996). In turn, ONOO’ can uncouple eNOS (Zou et al., 2002, Milstien and Katusic, 1999), inhibit Akt (Song et al., 2007), soluble guanylyl cyclase (Weber et al., 2001), prostacyclin synthase (Beckman, 1996, Zou et al., 1999) and SOD (Alvarez et al., 2004). All of O$_2^-$, ONOO’ and hydrogen peroxide (H$_2$O$_2$) have been shown to modulate intracellular calcium regulation (Lounsbury et al., 2000) and potassium channel activity (Guterman et al., 2005), and therefore can interfere with EDHF-mediated vasodilatation (Liu et al., 2006). Hydrogen peroxide has also been shown to inhibit myoendothelial communication via gap junctions (Lin and Takemoto, 2005).
1-5-4-2 ROS and vasoconstriction

The contractile function can also be altered by ROS via multiple pathways. For example, ROS can directly depolarize cell membranes (Kourie, 1998), inhibit potassium channels (Gutterman et al., 2005), modulate the mobilization of calcium from intracellular stores or extracellular fluids (Lounsbury et al., 2000, Forman et al., 2002, Amberg et al., 2010) and/or enhance the formation of isoprostanes from membrane lipids which are potent vasoconstrictors (Cracowski et al., 2002). Additionally, ROS can stimulate proinflammatory cytokines production (Naik and Dixit, 2011) and hence further propagate vascular inflammation. Also, ROS can cause long-lasting epigenetic changes (Giacco and Brownlee, 2010).

Despite the mounting evidence that oxidative stress contributes to diabetic vasculopathy, there is a lack of evidence that markers of oxidative stress are significantly associated with cardiovascular diseases in diabetic patients as is the case for markers of hyperglycemia, dyslipidemia or inflammation (Al-Benna et al., 2006, Huraux et al., 1999). These findings may reflect a deficiency in reliable oxidative stress markers rather than a misunderstanding of the role of oxidative stress.

There are different approaches to assess oxidative stress. Direct measurement of ROS levels is possible but complicated due to their high reactivity, short half-lives and diverse nature. Alternatively, measurements of the more stable end products of the reaction of ROS with the cell lipids, proteins, nucleic acids and carbohydrates are more feasible and easier to quantify as markers of oxidative stress. Polyunsaturated fatty acids, such as arachidonic acid, are particularly subject to oxidation because of the instability of their hydrogen bonds. Therefore, lipid peroxidation is a faster reaction than oxidation of proteins, nucleic acids, or carbohydrates (Basu, 2008).
Isoprostanes are prostaglandin-like compounds that are formed \textit{in vivo} by non-enzymatic, free radical-catalyzed peroxidation of arachidonic acid. Isoprostanes are biologically active, particularly $8$-\textit{iso PGF$_{2a}$} (8-isoprostone), which is one of the major isoprostanes formed \textit{in vivo} with potent vasoconstrictive and inflammatory properties. The level of $8$-\textit{iso PGF$_{2a}$} can be increased under physiological conditions such as human pregnancy, but mounting evidence associates its increase with diseases e.g. diabetes, atherosclerosis and asthma. An inverse correlation has been reported between plasma levels of $8$-\textit{isoprostane} and both endothelium-dependent and independent vasodilatation in healthy women (Sarabi \textit{et al.}, 1999). Currently, isoprostanes are considered reliable markers of \textit{in vivo} lipid peroxidation and oxidative stress in humans (Basu, 2008) and animals (Kadiiska \textit{et al.}, 2005), being more sensitive than malondialdehyde as a measure of lipid peroxidation (Longmire \textit{et al.}, 1994) and not affected by the lipid content of the diet (Basu, 2008). Therefore, I used the level of plasma $8$-\textit{isoprostone} as a marker of oxidative stress in the current study.

\textbf{1-5-4-3 Targeting oxidative stress}

Although several \textit{in vitro} and \textit{in vivo} studies showed that antioxidants were capable of ameliorating vascular function in experimental and clinical diabetes (Tesfamariam and Cohen, 1992, Pieper \textit{et al.}, 1996, Hattori \textit{et al.}, 1991, Pieper \textit{et al.}, 1997, Pieper and Siebeneich, 1998, Keegan \textit{et al.}, 1995, Rosen \textit{et al.}, 1996), the long-term benefits of traditional antioxidants e.g. vitamin E for diabetic patients in large-scale clinical trials have not been demonstrated (Belch \textit{et al.}, 2008, Ble-Castillo \textit{et al.}, 2005, Darko \textit{et al.}, 2002, Heart Protection Study Collaborative Group, 2002, Kataja-Tuomola \textit{et al.}, 2010, Lonn \textit{et al.}, 2005, Lu \textit{et al.}, 2005, Song \textit{et al.}, 2009). Furthermore, some studies revealed that antioxidants may exert harmful effects (Brown \textit{et al.}, 2001, Bjelakovic \textit{et al.}, 2007). These surprising findings could be due to the fact that ROS play important physiological and not only pathological roles in the cell, a concept referred to as the
double agent theory (Lane, 2003). Therefore, any interference with ROS signalling may have
diverse and potentially opposing actions on the cell function and integrity.

1-5-4-4 Physiological roles of ROS in the vasculature
The presence of numerous sources of ROS in virtually all cell types (Feletou and Vanhoutte,
2006) implies that ROS are essential components that the cell strives to generate and regulate
and not just to detoxify and eliminate. In addition to their well-established role in mediating the
phagocytic cells’ attack against invading organisms (Finkel, 2011), ROS are increasingly
recognized as important physiological regulators of cellular signaling pathways (Finkel, 2011,
Zinkevich and Gutterman, 2011, Wolin, 2009). In the vasculature, ROS have been shown to
mediate the responses to physiological stimuli via their actions on calcium channels (Amberg et
al., 2010, Trebak et al., 2010), some protein kinases (Knock and Ward, 2011), and intracellular
and compartmental control of ROS production and disposal maintain vascular homeostasis and
provide specificity in signalling (Amberg et al., 2010).

1-5-4-4-1 ROS as mediators of vasoconstriction
Current evidence indicates that ROS, generated mostly by NADPH oxidase and the
mitochondria, mediate the contractile responses to hypoxia (Wolin, 2009, Wang and Zheng,
2010), mechanical stretch (Oeckler et al., 2003, Birukov, 2009), pressure (Ungvari et al., 2003)
and some hormones such as angiotensin II (Seshiah et al., 2002).

1-5-4-4-2 ROS as mediators of vasodilatation
Endothelium-dependent vasodilatation has been shown to be mediated by O$_2^-$ or its metabolite(s)
in rat basilar arteries (Hong et al., 1989), by H$_2$O$_2$ in rat aortae and femoral arteries (Karasu,
2000) and femoral arteries (Leung et al., 2006) and mouse cerebral arteries (Drouin and Thorin,
2009), and by nitrosothiols in rat carotid arteries (Leo et al., 2010). Hydrogen peroxide is a well-
recognized endothelium-derived hyperpolarizing factor in animals and humans and plays important roles in coronary metabolic vasodilatation (Shimokawa, 2010). Also, exogenously-introduced ROS released NO from intracellular stores in human umbilical vein endothelial cells (Ng et al., 2007) and triggered potassium channels-mediated vasodilatation in cerebral arteries of anesthetised cats (Wei et al., 1996). Peroxynitrite, directly by stimulating cyclic guanosine monophosphate synthesis (cGMP) synthesis (Tarpey et al., 1995), activating prostaglandin endoperoxide H2 synthase (Schildknecht and Ullrich, 2009), activating potassium channels, enhancing myosin light chain phosphatase activity and/or interfering with calcium movement (Li et al., 2004b) or indirectly via S-nitrosothiols (Wu et al., 1994, Mayer et al., 1995) with subsequent NO regeneration (Villa et al., 1994) induced vasodilatation in several species: rats (Villa et al., 1994, Benkusky et al., 1998, Nossaman et al., 2004), dogs (Liu et al., 1994, Li et al., 2004a), cows (Wu et al., 1994), rabbits (Ohashi et al., 2005, Pagano et al., 1999) and cats (Wei et al., 1996), and in different blood vessels such as skeletal (Benkusky et al., 1998, Nossaman et al., 2004), mesenteric (Benkusky et al., 1998), pulmonary (Wu et al., 1994), coronary (Villa et al., 1994, Liu et al., 1994), carotid (Ohashi et al., 2005, Pagano et al., 1999) and cerebral arteries (Wei et al., 1996, Li et al., 2004a).

In parallel with ROS acting as mediators of vasodilatation, antioxidants have been shown to reduce vasodilatations in several studies. An oral antioxidant cocktail (vitamins C, E, and alpha-lipoic acid) reduced exercise-induced vasodilatation in the brachial arteries in healthy subjects (Richardson et al., 2007). Ebselen (a ONOO⁻ scavenger) was shown to attenuate ACh depressor response in anesthetised rats (Dabisch et al., 2008). Also, vascular endothelial growth factor-induced coronary vasodilatation was impaired in NADPH oxidase-knockdown mice which had reduced ROS levels (Feng et al., 2010).
1-6 Multifactorial aetiology and treatment of diabetic vascular complications

It is clear from the results of several clinical studies and as discussed above that hyperglycemia, dyslipidemia, inflammation and oxidative stress all contribute to the progression of diabetic vascular complications (Turner et al., 1998). Therefore, a corresponding multifactorial intervention, targeting hyperglycemia and the other modifiable cardiovascular risk factors, is probably required to effectively reduce cardiovascular risks in diabetic patients (Gaede et al., 2008).

Indeed, the Steno-2 study indicates that intensive integrated therapy, targeting hyperglycaemia, hypertension, dyslipidemia, and microalbuminuria in type 2 diabetic patients was associated with a 50% lower risk of cardiovascular events compared with conventional treatment (Gaede et al., 1999, Gaede et al., 2003). It is proposed that integrated therapy offers more benefits than single-factor intervention strategies, although a direct comparison is hindered by differences in population demographics, duration of the interventions, and the end points examined in the different clinical trials (Gaede and Pedersen, 2004). Accordingly, current guidelines including those of the American Diabetes Association and the International Diabetes Federation advocate aggressive management of blood glucose, lipid profile, and blood pressure to minimize complications in patients with type 2 diabetes (Reasner, 2008).

However, an integrated multifactorial therapy necessarily requires greater exposure to different drugs, resulting in potentially more side effects and reduced patients’ convenience and compliance. Realistically, fewer than 10% of diabetic patients achieve their targets of glycemia and lipid levels (Saydah et al., 2004), and consequently the outcomes of diabetic patients are consistently worse than non-diabetics in terms of cardiovascular events and mortality (Haffner et
al., 1998, Sarwar et al., 2010, Hanefeld et al., 2009), thus emphasizing the urgent need for new therapeutic strategies.

1-7 Exercise training in diabetes

Exercise is a cornerstone in the prevention and management of type 2 diabetes; it has been associated not only with reduced cardiovascular risk factors (Walker et al., 1999, Lehmann et al., 1997, Yeater et al., 1990, Lehmann et al., 1995), but also with reduced cardiovascular diseases (Hu et al., 2001) and total mortality (Hu et al., 2005, Trichopoulou et al., 2006, Gregg et al., 2003). The ability of exercise to exert multifaceted effects on the vasculature leading to restoration of function, while causing no major adverse effects, underlie the favourable outcomes of exercise. Exercise has been shown to affect glycemia (Sigal et al., 2006), dyslipidemia (Chen et al., 2009), inflammation (Kasapis and Thompson, 2005) and oxidative stress (Ji, 2008). Also, exercise has been shown to modulate many aspects of vascular homeostasis: NO bioavailability (Maiorana et al., 2003), potassium channels activity (Bowles, 2000, Chen et al., 2001, Bowles et al., 1998) and calcium signalling (Bowles, 2000, Witczak et al., 2006, Witczak and Sturek, 2004). Functionally, exercise has repeatedly been shown to improve vasodilation in diabetic patients (Maiorana et al., 2001, Cohen et al., 2008, Okada et al., 2010) and several animal models of diabetes (Sakamoto et al., 1998, Mayhan et al., 2011, Xiang et al., 2005, Moien-Afshari et al., 2008); however, the effects of exercise on the vascular contractile function are less consistent (Setty et al., 2004, Claudino et al., 2011, Esser et al., 2007, Mokelke et al., 2003).

Our understanding of how all these actions of exercise are linked together is limited, but important to explore. The design of a pharmaceutical agent acting on the same pathways that exercise targets would have obvious benefits for diabetic patients, particularly because they have
a lower capacity to exercise (Joshi et al., 2010, Albright et al., 2000, Kelley et al., 2002), a problem that can be exacerbated by aging or other comorbidities.

1-8 Animal models of type 2 diabetes

None of the currently available animal models is exactly equivalent to human diabetes; however, some models exhibit many characteristic features of the human disease and allow invasive experimentation that would be otherwise impossible in humans (Srinivasan and Ramarao, 2007). In the present study, I used (BKS.Cg-m\(^{+/+}\) Lepr\(^{db}\)/J) mice conveniently referred to as \(db/db\) mice as a genetic model of type 2 diabetes. This mice strain harbors a spontaneous diabetes mutation (\(db\)) in the leptin receptor (Lepr) gene. Mice, homozygous for the mutation (\(db/db\)), are hyperphagic, obese and hyperinsulinemic at 2 to 4 weeks of age and hyperglycemic 2 to 4 weeks later. Therefore, \(db/db\) mice share many of the phenotypic characteristics (obesity, glucose intolerance, hyperglycemia, hyperinsulinemia and hyperlipidemia) of type 2 diabetes, except for the level of HDL which is elevated in \(db/db\) mice, while mostly reduced in type 2 diabetic patients. Nishina et al. (1994) attributed the elevated HDL to the concurrent hyperinsulinemia present in \(db/db\) mice. Hyperinsulinemia can stimulate peripheral cholesterogenesis thus increasing the need for HDL for reverse cholesterol transport. It is also possible that the lack of cholesterol ester transfer protein in \(db/db\) mice delays cholesterol plasma clearance and contributes to the elevated HDL levels observed (Nishina et al., 1994).

Hummel study was the first to report the (\(db\)) mutation in 1966 (Hummel et al., 1966), and since then \(db/db\) mice have been extensively used as a model of type 2 diabetes with 3651 entries in Pubmed up till Dec 2011. This model offers the advantage of presenting the three phases of clinical diabetes: progressive loss of beta-cell function with subsequent postprandial hyperglycemia (phase I), then fasting hyperglycemia (phase II), and finally pancreatic islets
atrophy (phase III) over a short time period (Like and Chick, 1970a, Like and Chick, 1970b). Thus, the use of this animal model reduces the cost of longitudinal studies.

However, human type 2 diabetes results from complex interactions between environmental and genetic factors, of which leptin receptor defects represent rare cases (Farooqi and O'Rahilly, 2006). Consequently, db/db mice cannot fully represent the complexity of human diabetes. Other polygenic animal models of type 2 diabetes are available but they do not necessarily represent the same aetiopathogenic mechanisms of human diabetes.

1-8-1 Altered vascular reactivity in db/db mice

Several studies have examined the vasomotor function in db/db mice as summarized in table (1-1) for vasodilatory responses, and table (1-2) for contractile responses. There is solid evidence indicating altered vascular reactivity in db/db mice. However, studies examining different arteries reported varying results. Endothelium-dependent ACh induced vasodilatation was reported to be impaired in the aorta (Yamamoto et al., 2010), small mesenteric arteries (Lagaud et al., 2001), coronary artery (Belmadani et al., 2008) and coronary arterioles (Park et al., 2008), but not in gracilis muscle arterioles (Bagi et al., 2005) (Table 1-1). The EDHF component of the endothelium-dependent vasodilatation was preserved in small mesenteric arteries (Pannirselvam et al., 2002), but reduced in the coronary arterioles (Park et al., 2008). Similarly, VSMs response to direct NO donors or cAMP elevating agents was either unaltered or reduced in different arteries (Lam et al., 2006, Miike et al., 2008, Yamamoto et al., 2010, Seto et al., 2007, Nuno et al., 2009). Changes in the vascular contractile responses (Table1-2) in db/db mice were also variable. The myogenic tone was increased in small mesenteric arteries (Belmadani et al., 2008), gracilis muscle arterioles (Bagi et al., 2005), coronary arteries (Belmadani et al., 2008), but not in coronary arterioles (Bagi et al., 2003). Also, the constrictor response to PE was not uniformly
changed under diabetic conditions (Belmadani et al., 2008, Xie et al., 2006). Clearly, differences in age (Zhang et al., 2008, Lagaud et al., 2001) and sex (Nuno et al., 2009) of the mice used in these studies are some of the factors contributing to these diverse results. However, studies that used mice of similar sex and ages reported varying results in different blood vessels.

Furthermore, different pathomechanisms have been proposed by these studies to contribute to the vascular dysfunction in db/db mice including receptor dysfunction (Miike et al., 2008), increased ROS production (San Martin et al., 2007, Wong et al., 2010), downregulation of Akt and eNOS (Pannirselvam et al., 2002, Zhong et al., 2007, Fukuda et al., 2010b), upregulation of caveolin-1 (Lam et al., 2006), increased production of endogenous vasoconstrictor prostanoids (Lagaud et al., 2001) and increased inflammatory cytokines (Zhang et al., 2008, Park et al., 2011).

On the other hand, several interventions using different strategies including calcium channels blockers (Yamamoto et al., 2010), peroxisome proliferator-activated receptor gamma (PPARgamma) agonists (Miike et al., 2008, Howarth et al., 2006), antioxidants (San Martin et al., 2007), Rho kinase inhibitors (Nuno et al., 2009, Xie et al., 2006), COX inhibitors (Bagi et al., 2005, Guo et al., 2005, Kanie and Kamata, 2000), apelin (Zhong et al., 2007), a cholesterol absorption inhibitor (Fukuda et al., 2010b), inhibitors of the renin angiotensin axis (Wong et al., 2010), folic acid (Seto et al., 2010), an endothelial growth factor inhibitor (Belmadani et al., 2008), anti-interleukin 6 (Park et al., 2008), tumor necrosis factor-alpha (TNFalpha) neutralizing antibodies (Zhang et al., 2008), and exercise training (Moien-Afshari et al., 2008, Esser et al., 2007) have ameliorated some aspects of the vascular dysfunction in db/db mice. These findings provide hope for developing effective treatments for diabetic patients, but at the same time reflect the complexity of the puzzle that we have yet to unravel.
Table 1-1: Vasodilatory responses in *db/db* mice compared with normoglycemic control mice

<table>
<thead>
<tr>
<th>Blood vessel</th>
<th>Sex and age of the mice</th>
<th>Vascular responses</th>
<th>Suggested pathomechanisms</th>
<th>Interventions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aorta</strong></td>
<td>Male 10 wk</td>
<td>ACh ↓, SNP ↔</td>
<td>Uncoupling of eNOS</td>
<td>Nifedipine improved ACh dilatation by inhibiting eNOS uncoupling</td>
<td>(Yamamoto <em>et al.</em>, 2010)</td>
</tr>
<tr>
<td></td>
<td>Male 11 wk</td>
<td>ACh ↓</td>
<td></td>
<td>Combination of pioglitazone and candesartan improved eNOS phosphorylation and ACh dilatation</td>
<td>(Fukuda <em>et al.</em>, 2010a)</td>
</tr>
<tr>
<td></td>
<td>Male 11 wk</td>
<td>ACh ↓, SNP ↓, ADP ↔, A-23187 ↔ NaF ↔</td>
<td>Dysfunction of ACh receptors and/or receptor-G protein coupling</td>
<td>A PPAR&lt;sub&gt;gamma&lt;/sub&gt; agonist improved ACh vasodilatation</td>
<td>(Miike <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td></td>
<td>12 wk</td>
<td>ACh ↓, Nitroglycerin ↔</td>
<td>NADPH oxidase increases ROS production</td>
<td><em>In vivo</em> tempol for 8 weeks or <em>in vitro</em> apocynin incubation improved ACh dilatation</td>
<td>(San Martin <em>et al.</em>, 2007)</td>
</tr>
<tr>
<td></td>
<td>Male 12 wk</td>
<td>ACh ↓, SNP ↔</td>
<td></td>
<td></td>
<td>(Kanie and Kamata, 2000)</td>
</tr>
<tr>
<td></td>
<td>Female 12 wk</td>
<td>Carbachol ↓, SNP ↔</td>
<td>The lumen of the aortae under resting tension was greater in diabetic mice</td>
<td></td>
<td>(Piercy and Taylor, 1998)</td>
</tr>
<tr>
<td></td>
<td>Male 15 wk</td>
<td>ACh ↓</td>
<td>Reduced expression of apelin receptor and reduced phosphorylation of Akt and eNOS</td>
<td>Apelin treatment ameliorated the response to ACh by potentiating the phosphorylation of Akt and eNOS</td>
<td>(Zhong <em>et al.</em>, 2007)</td>
</tr>
</tbody>
</table>
Table 1-1: Vasodilatory responses in db/db mice compared with normoglycemic control mice (continued)

<table>
<thead>
<tr>
<th>Blood vessel</th>
<th>Sex and age of the mice</th>
<th>Vascular responses</th>
<th>Suggested pathomechanisms</th>
<th>Interventions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Male 15 wk (fed western diet)</td>
<td>ACh ↓↓</td>
<td>Reduced Akt and eNOS phosphorylation and downregulation of SOD</td>
<td>Ezetimibe, a cholesterol absorption inhibitor, reduced oxidative stress and inflammation and improved eNOS function</td>
<td>(Fukuda et al., 2010b)</td>
</tr>
<tr>
<td></td>
<td>Male 18 wk</td>
<td>ACH ↓ SNP ↔</td>
<td>Ang II type 1 receptor/NADPH oxidase dependent ROS mediate endothelial dysfunction</td>
<td>valsartan or enalapril for 6 weeks, or acute incubation with apocynin, tempol, or losartan improved the vasodilatation</td>
<td>(Wong et al., 2010)</td>
</tr>
<tr>
<td>Female 6 months</td>
<td>ACh ↓ SNP ↓ Isoprenaline ↔ Cromakalim ↔</td>
<td>Increased caveolin-1 mRNA and protein expression</td>
<td></td>
<td></td>
<td>(Lam et al., 2006)</td>
</tr>
<tr>
<td>Female 6 months</td>
<td>Carbachol ↓ PPAR-gamma agonist ↓ forskolin ↓</td>
<td></td>
<td></td>
<td></td>
<td>(Seto et al., 2007)</td>
</tr>
<tr>
<td>Female 6 months</td>
<td>ACh ↓</td>
<td></td>
<td>Folic acid consumption improved ACh dilatation via enhancement of PI3K/eNOS/Akt cascade</td>
<td></td>
<td>(Seto et al., 2010)</td>
</tr>
<tr>
<td>Male or female</td>
<td>ACh↓ SNP ↓ only in males</td>
<td></td>
<td></td>
<td></td>
<td>(Nuno et al., 2009)</td>
</tr>
</tbody>
</table>
Table 1-1: Vasodilatory responses in *db/db* mice compared with normoglycemic control mice (continued)

<table>
<thead>
<tr>
<th>Blood vessel</th>
<th>Sex and age of the mice</th>
<th>Vascular responses</th>
<th>Suggested pathomechanisms</th>
<th>Interventions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small mesenteric arteries</td>
<td>Male 12 and 16 wk</td>
<td>basal and stimulated NO ↓</td>
<td>Vasodilatation may be countered by an endogenous vasoconstrictor prostanoids</td>
<td></td>
<td>(Lagaud <em>et al</em>., 2001)</td>
</tr>
<tr>
<td></td>
<td>Male 12-16 wk</td>
<td>ACh ↓ EDHF ↔ SNP ↔</td>
<td>Increased superoxide anion and decreased BH₄, result in uncoupling of eNOS</td>
<td></td>
<td>(Pannirselvam <em>et al</em>., 2002)</td>
</tr>
<tr>
<td></td>
<td>Male 16 wk</td>
<td>ACh ↓</td>
<td></td>
<td>PPARγ agonist improved ACh</td>
<td>(Howarth <em>et al</em>., 2006)</td>
</tr>
<tr>
<td></td>
<td>Male 10-14 wk</td>
<td>shear stress ↓ ACh ↓ NO donor ↔</td>
<td>Reduced eNOS expression and phosphorylation</td>
<td>Treatment with EGFR inhibitor for 2 wks improved shear stress and ACh vasodilatation</td>
<td>(Belmadani <em>et al</em>., 2008)</td>
</tr>
<tr>
<td>Gracilis muscle arterioles</td>
<td>Male 12-14 wk</td>
<td>ACh ↔</td>
<td></td>
<td></td>
<td>(Bagi <em>et al</em>., 2005)</td>
</tr>
<tr>
<td>Coronary artery</td>
<td>Male 10-14 wk</td>
<td>shear stress ↓ ACh ↓ NO donor ↔</td>
<td></td>
<td>Treatment with EGFR inhibitor for 2 weeks improved shear stress and ACh vasodilatation</td>
<td>(Belmadani <em>et al</em>., 2008)</td>
</tr>
<tr>
<td></td>
<td>Male 13 wk</td>
<td>ACh ↓ SNP ↔</td>
<td>Oxidative stress induces endothelial dysfunction</td>
<td>Exercise training improved ACh vasodilatation</td>
<td>(Moien-Afshari <em>et al</em>., 2008)</td>
</tr>
</tbody>
</table>
Table 1-1: Vasodilatory responses in db/db mice compared with normoglycemic control mice (continued)

<table>
<thead>
<tr>
<th>Blood vessel</th>
<th>Sex and age of the mice</th>
<th>Vascular responses</th>
<th>Suggested pathomechanisms</th>
<th>Interventions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary arterioles</td>
<td>Male 12 wk</td>
<td>Flow ↓, ACh ↓, NO donor ↓, adenosine ↔</td>
<td>NADPH oxidase produced ROS reduce NO bioavailability</td>
<td>PPARγ agonist for 7 days or preincubation with SOD or apocynin improved vasodilatation</td>
<td>(Bagi et al., 2003, Bagi et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Male or female 24-30 wk</td>
<td>ACh/NO ↓, ACh/EDHF ↓, SNP ↔</td>
<td>IL-6 induces endothelial dysfunction</td>
<td>Anti-IL-6 restored ACh/EDHF vasodilatation</td>
<td>(Park et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Male or female 12–16 wk</td>
<td>ACh ↓, Flow ↓, SNP ↔</td>
<td>PAR2 induces endothelial dysfunction by up-regulating TNFα and activating NADPH oxidase</td>
<td>Treatment with PAR2 antagonist improved the vasodilatation</td>
<td>(Park et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>Male or female 12, 18 and 24 wk</td>
<td>ACh ↓, Flow ↓</td>
<td>TNFα increases the production of superoxide via NADPH oxidase and/or mitochondria respiratory chain</td>
<td>TNFα neutralizing antibodies ameliorated endothelial dysfunction</td>
<td>(Zhang et al., 2008)</td>
</tr>
</tbody>
</table>

↓= decrease; ↑= increase; ↔= not altered

ACh= acetylcholine; Ang II= angiotensin II; BH4= tetrahydrobiopterin; EDHF= endothelium dependent hyperpolarizing factor; EGFR= endothelium growth factor receptor; eNOS= endothelial nitric oxide synthase; IL-6= interleukin-6; NADPH oxidase= nicotinamide adenine dinucleotide phosphate oxidase; NO= nitric oxide; PAR2= protease-activated receptor-2; PPARγ= peroxisome proliferator-activated receptor- gamma; SNP= sodium nitroprusside; SOD= superoxide dismutase; TNFα= tumor necrosis factor-alpha, wk= weeks
### Table 1-2: Contractile responses in db/db compared with normoglycemic control mice

<table>
<thead>
<tr>
<th>Blood vessel</th>
<th>Sex and age of the mice</th>
<th>Vascular responses</th>
<th>Suggested pathomechanisms</th>
<th>Interventions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aorta</td>
<td>Female 8-14 wk</td>
<td>High KCl ↑ Ang II ↑ PE ↑ 5-HT ↑ in denuded muscle strips</td>
<td>COX-2 mRNA and protein are increased</td>
<td>Inhibition of COX-2 alleviated agonist but not potassium induced contraction</td>
<td>(Guo et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>10 wk</td>
<td>5-HT ↑ Ang II ↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Voluntary exercise alleviated the hypercontractility in association with reduced cholesterol and free fatty acids</td>
<td>(Esser et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Male 11 wk</td>
<td>PGF₂α-alpha ↑</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male 12 wk</td>
<td>NE ↑</td>
<td>Superoxide anion, via vasoconstrictor prostanoids enhanced NE contraction</td>
<td>NE contraction was attenuated by indomethacin and SOD.</td>
<td>(Kanie and Kamata, 2000)</td>
</tr>
<tr>
<td></td>
<td>Female 12 wk</td>
<td>PE ↑</td>
<td>Hyperglycemia and /or insulin resistance</td>
<td></td>
<td>(Piercy and Taylor, 1998)</td>
</tr>
<tr>
<td></td>
<td>Male 15 wk</td>
<td>High KCl ↑ Ang II ↑ PE ↑ U-46619 ↔</td>
<td>Reduced expression of apelin receptor and phosphorylation of Akt and eNOS</td>
<td>Apelin treatment ameliorated the response to Ang II by potentiating the phosphorylation of Akt and eNOS</td>
<td>(Zhong et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Male or female 5-HT ↑</td>
<td>Increased Rho kinase function</td>
<td>Inhibition of Rho kinase reduced 5-HT contraction</td>
<td>(Nuno et al., 2009)</td>
<td></td>
</tr>
</tbody>
</table>
Table 1-2: Contractile responses in db/db mice compared with normoglycemic control mice (continued)

<table>
<thead>
<tr>
<th>Blood vessel</th>
<th>Sex and age of the mice</th>
<th>Vascular responses</th>
<th>Suggested pathomechanisms</th>
<th>Interventions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small mesenteric arteries</td>
<td>Male 10-14 wk</td>
<td>Myogenic tone ↑ PE ↔</td>
<td>Downregulation of eNOS expression and phosphorylation</td>
<td>Treatment with a EGFR inhibitor for 2 weeks reduced the myogenic tone</td>
<td>(Belmadani et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Male 12-14 wk</td>
<td>PE ↑ High KCl ↑</td>
<td>Upregulation of rho kinase and CPI-17</td>
<td>Inhibiting Rho kinase alleviated the contractile hyperreactivity</td>
<td>(Xie et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Male 16 wk</td>
<td>PE ↑</td>
<td>Increased ROS, enhanced COX activity and thromboxane synthesis, but no changes in the eNOS/NO pathway</td>
<td></td>
<td>(Pannirselvam et al., 2005)</td>
</tr>
<tr>
<td>Gracilis muscle arterioles</td>
<td>Male 12-14 wk</td>
<td>Myogenic tone ↑ PE ↔</td>
<td>Upregulation of COX-2</td>
<td>An inhibitor of COX-2 reduced the myogenic tone</td>
<td>(Bagi et al., 2005)</td>
</tr>
<tr>
<td></td>
<td>Male 12-14 wk</td>
<td>Myogenic tone ↑ U-46619 ↔</td>
<td>Production of H₂O₂ was enhanced leading to increased synthesis of thromboxane A₂</td>
<td>Myogenic tone reduced back to control level by catalase</td>
<td>(Erdei et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>Male 12 wk</td>
<td>Myogenic tone ↑ Ang II ↑</td>
<td>Activation of prostaglandin E2 EP1 receptors increases arteriolar tone</td>
<td>EP1 receptor antagonist reduced the arteriolar tone</td>
<td>(Rutkai et al., 2009)</td>
</tr>
</tbody>
</table>
Table 1-2: Contractile responses in *db/db mice* compared with normoglycemic control mice
(continued)

<table>
<thead>
<tr>
<th>Blood vessel</th>
<th>Sex and age of the mice</th>
<th>Vascular responses</th>
<th>Suggested pathomechanisms</th>
<th>Interventions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary artery</td>
<td>Male 10-14 wk</td>
<td>Myogenic tone ↑</td>
<td>Treatment with EGFR inhibitor for 2 weeks reduced the myogenic tone</td>
<td>(Belmadani <em>et al.</em>, 2008)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Male 13 wk</td>
<td>U-46619 ↔</td>
<td></td>
<td>(Moien-Afshari <em>et al.</em>, 2008)</td>
<td></td>
</tr>
<tr>
<td>Coronary arterioles</td>
<td>Male 12-14 wk</td>
<td>Myogenic tone ↔</td>
<td></td>
<td>(Bagi <em>et al.</em>, 2003)</td>
<td></td>
</tr>
</tbody>
</table>

↓ = decrease; ↑ = increase; ↔ = not altered

Ang II = angiotensin II; COX = cyclooxygenase; CPI-17 = protein kinase C-potentiatiated phosphatase inhibitor of 17 kDa; EGFR = endothelial growth factor receptor; eNOS = endothelial nitric oxide synthase; 5-HT = serotonin; NE = norepinephrine; PE = phenylephrine; PGF$_2$-alpha = prostaglandin F$_2$-alpha; ROS = reactive oxygen species; SOD = superoxide dismutase, wk = weeks
1-9 Regional heterogeneity of vascular responsiveness to pathophysiological conditions

It is plausible that different vascular beds subjected to distinct local environments and serving specific functions via diverse signalling pathways behave differently under physiological conditions and probably respond in distinct ways to pathophysiological stimuli. Indeed, an expanding body of evidence demonstrates the differential adaptations of different arteries to aging, hypoxia, hypercholesterolemia and streptozotocin induced diabetes (Tables 1-3 and 1-4). In addition to differential changes in vasodilatory and contractile responses, opposing changes in compliance have been observed in regionally different arteries. In atherosclerotic mice, the compliance of the carotid artery was decreased whereas the compliance of the cerebral arteries was increased (Bolduc et al., 2011). However, the factors giving rise to such heterogeneity are largely unknown.

Understanding that both the endothelial and the VSM cells from regionally distinct vascular beds, and in some cases from the same vascular bed, show marked heterogeneity with respect to their phenotypes (Frid et al., 1997, Nakamura et al., 1998, Yu et al., 1997), receptors subtypes (Lewis et al., 2005, Zheng et al., 2008, Mueed et al., 2004), potassium channels subtypes and density (Cheong et al., 2001, Michelakis et al., 1997, Cox, 2005), growth rates (Thorin et al., 1997), components of second messenger cascades e.g. sGC (D'Angelis et al., 1998) or PKC (Mueed et al., 2004), and subcellular organelles e.g. mitochondria (Michelakis et al., 2002) may help explain the divergent responses of different arteries to a common stimulus. Nevertheless, it is clear that there is much more to explore. Understanding the mechanisms underlying vascular heterogeneity can provide important future directions for therapies aimed at preventing vascular diseases.
Table 1-3: Regional heterogeneity in the vascular responsiveness to vasodilatory stimuli under pathophysiological conditions

<table>
<thead>
<tr>
<th>Pathological condition</th>
<th>Blood vessels</th>
<th>Vascular responsiveness</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aging</td>
<td>Aorta and pulmonary artery of normal rats</td>
<td>Endothelium-dependent vasodilatation was impaired in the aorta but not the pulmonary artery</td>
<td>(Tschudi et al., 1996)</td>
</tr>
<tr>
<td></td>
<td>Aorta, coronary, basilar and renal arteries of Watanabe heritable hyperlipidaemic rabbits</td>
<td>Endothelium-dependent vasodilatation to ACh and A23187 was reduced in the aorta and coronary but not basilar or renal arteries</td>
<td>(Kitagawa et al., 1994)</td>
</tr>
<tr>
<td>Cholesterol-rich diet</td>
<td>Aorta, carotid and basilar arteries of rabbits</td>
<td>Endothelium-dependent relaxation was more severely impaired in the aortae than the carotid but preserved in basilar artery</td>
<td>(Kanamaru et al., 1989)</td>
</tr>
<tr>
<td></td>
<td>Aorta, cerebral, femoral and mesenteric small arteries of rabbits</td>
<td>The response to ACh was impaired in the aorta but not other arteries</td>
<td>(Simonsen et al., 1991)</td>
</tr>
<tr>
<td>High fat diet</td>
<td>Carotid and femoral arteries of mice</td>
<td>In the carotid, but not in the femoral artery, ROS-induced dilation increased with increasing dietary fat intake but ACh-mediated relaxation was enhanced only in the femoral artery</td>
<td>(Bhattacharya et al., 2008)</td>
</tr>
<tr>
<td>Obesity</td>
<td>Aorta and mesenteric artery of obese Zucker rats</td>
<td>ACh vasorelaxation was impaired in the mesenteric artery but slightly enhanced in the aorta</td>
<td>(Subramanian and MacLeod, 2003)</td>
</tr>
<tr>
<td></td>
<td>Aorta, coronary and mesenteric arteries of diabetic rats</td>
<td>Response to iloprost was reduced in both coronary and mesenteric arteries, but not the aorta</td>
<td>(Bouchard et al., 1999)</td>
</tr>
<tr>
<td></td>
<td>Carotid, femoral and mesenteric arteries of diabetic rats</td>
<td>NO-mediated vasorelaxation was reduced in the carotid and femoral arteries while EDHF component was augmented in the mesenteric arteries</td>
<td>(Shi et al., 2006)</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>Systemic and pulmonary arteries of rats</td>
<td>Renal arteries dilate in response to hypoxia, whereas the pulmonary arteries constrict</td>
<td>(Michelakis et al., 2002, Waypa and Schumacker, 2010)</td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>Renal, mesenteric and hindquarters vasculature of diabetic rats</td>
<td>Bradykinin-mediated vasodilatation was depressed in the hindquarters vasculature, but was normal in the renal and mesenteric vascular beds</td>
<td>(Kiff et al., 1991)</td>
<td></td>
</tr>
</tbody>
</table>

ACh= acetylcholine; EDHF= endothelium dependent hyperpolarizing factor; NO= nitric oxide
Table 1-4: Regional heterogeneity in the vascular responsiveness to contractile stimuli under pathophysiological conditions

<table>
<thead>
<tr>
<th>Pathological condition</th>
<th>Blood vessels</th>
<th>Vascular responsiveness</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Streptozotocin-induced diabetes</td>
<td>Renal and superior mesenteric artery of diabetic rats</td>
<td>Enhanced contractile response to U46619 in renal but not superior mesenteric artery</td>
<td>(Arikawa et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Basilar, coronary, renal and tail arteries of diabetic rats</td>
<td>The vasopressin-induced contractions were reduced in basilar and coronary arteries, increased in renal arteries, and not modified in tail arteries</td>
<td>(Garcia-Villalon et al., 2003)</td>
</tr>
<tr>
<td></td>
<td>Aorta and basilar artery of diabetic rats</td>
<td>Responses to NE and 5-HT were enhanced in the aorta but unchanged in the basilar artery</td>
<td>(Abiru et al., 1991)</td>
</tr>
<tr>
<td></td>
<td>Aorta and mesenteric artery of diabetic rats</td>
<td>Contractile responses of the mesenteric artery but not aorta to a PKC activator were increased</td>
<td>(Abebe and MacLeod, 1990)</td>
</tr>
<tr>
<td></td>
<td>Renal and superior mesenteric artery of diabetic mice</td>
<td>Renal artery but not superior mesenteric artery exhibited an enhanced contractile response to U46619</td>
<td>(Arikawa et al., 2006)</td>
</tr>
<tr>
<td>Obesity</td>
<td>Aorta and mesenteric artery of obese Zucker rats</td>
<td>Contractile responses to NE, ET-1 and KCl were depressed in the mesenteric artery but unchanged in the aorta</td>
<td>(Subramanian and MacLeod, 2003)</td>
</tr>
<tr>
<td>High cholesterol diet</td>
<td>Femoral and cerebral small arteries of cholesterol-fed rabbits</td>
<td>Sensitivity to NE was decreased in the femoral arteries but increased in cerebral small arteries</td>
<td>(Simonsen et al., 1991)</td>
</tr>
<tr>
<td>High fat diet</td>
<td>Carotid and femoral arteries of mice</td>
<td>In the carotid artery Ang II-induced contraction was attenuated, whereas ET-1-induced vasoconstriction was significantly increased</td>
<td>(Bhattacharya et al., 2008)</td>
</tr>
<tr>
<td>Coronary ligation</td>
<td>The aorta and pulmonary artery of ovariectomized female rats</td>
<td>Reduced response to L-NAME in the aorta but not pulmonary artery indicating reduced basal NO</td>
<td>(Nekooeian et al., 1998)</td>
</tr>
</tbody>
</table>

Ang II= angiotensin II; ET-1= endothelin-1; NE= norepinephrine; 5-HT= serotonin
1-10 Research rationale, objectives and experimental design

Diabetic chronic vascular complications are still challenging the medical community worldwide, posing a huge burden on diabetic patients and harvesting their lives. The macrovascular complications of diabetes are mostly defined as strokes, coronary, and peripheral arterial diseases. Whether diabetes has a general deleterious effect on the vasculature, or if there is differential vulnerability of specific arteries to the diabetic milieu, has yet to be disclosed.

The main objectives of my thesis was to compare the functional adaptation of the aorta, carotid and femoral arteries to hyperglycemia, dyslipidemia, oxidative stress and inflammation present in db/db mice, a genetic model of type 2 diabetes, and to elucidate the mechanisms underlying the differential adaptation of the three arteries at biochemical, molecular and functional levels. Additionally, I examined if exercise training could reverse the functional dysfunction in the aortae and femoral arteries of db/db mice.

The experiments explored the functional changes in the conduit arteries of db/db mice, and tried to explain them in light of concurrent biochemical and molecular alterations (Illustration 1-2). I examined both the vasodilatory and contractile responses to different vasoactive agents to test the endothelial and VSMs function in the aortae, carotid and femoral arteries of db/db and control mice. To elucidate potential mechanisms underlying the results of the functional experiments, I measured the protein expression of some enzymes that play key roles in vascular homestasis, namely eNOS, Akt, COX-1, COX-2, SOD-1, SOD-2 and SOD-3. In parallel, markers of hyperglycemia (plasma glucose), systemic oxidative stress (plasma 8-isoprostane) and inflammation (plasma CRP), and dyslipidemia (plasma triglycerides and cholesterol) were measured. Thereafter, a correlation analysis was performed to estimate the strength of association between plasma variables and vascular responses.
Illustration 1-2: General framework of the present thesis

COX= cyclooxygenase, CRP= C-reactive protein, eNOS= endothelial nitric oxide synthase,
SOD= superoxide dismutase
To achieve these goals, three studies were conducted as described below:

**First study:** Regional heterogeneity of the endothelium-dependent and -independent vasodilatation in *db/db* mice

**Specific goals:**

1- To compare endothelium-dependent and -independent vasodilatation in the aortae, carotid and femoral arteries from *db/db* and control mice at three ages

2- To elucidate the mechanisms underlying the differential responses of the three arteries to the diabetic milieu, particularly with respect to the role of ROS

3- To evaluate the relative contribution of obesity, hyperglycemia, oxidative stress, dyslipidemia and inflammation to the impairment of vasodilatation in *db/db* mice

**Illustration 1-3: General framework of the first study in the present thesis**

cAMP = cyclic adenosine monophosphate, cGMP = cyclic guanosine monophosphate, CRP = C-reactive protein, EDHF = endothelium dependent hyperpolarizing factor, NO = nitric oxide
**Second study:** Regional heterogeneity of the vascular contractile responses in *db/db* mice and their controls

**Specific goals:**

1- To compare the contractile responses of the aortae, carotid and femoral arteries of *db/db* and control mice

2- To elucidate the mechanisms underlying the differential adaptation of the three arteries to the diabetic milieu

3- To evaluate the relative contribution of hyperglycemia, oxidative stress, dyslipidemia and inflammation to the contractile dysfunction in the aortae of *db/db* mice

**Illustration 1-4:** General framework of the second study in the present thesis

SOD= superoxide dismutase, CRP= C-reactive protein
**Third study: Effects of exercise on the vasomotor function of the aortae and femoral arteries in db/db mice: Mechanisms beyond weight loss**

**Specific goals:**

1- To examine the functional, molecular and biochemical adaptations to short- and long-term exercise training in the aortae and femoral arteries of *db/db* mice and their controls

2- To elucidate the mechanisms underlying the beneficial effects of exercise on NO-mediated vasodilatation

**Illustration 1-5: General framework of the third study in the present thesis**

COX= cyclooxygenase, CRP= C-reactive protein, eNOS= endothelial nitric oxide synthase, SOD= superoxide dismutase
2-Materials and Methods

2-1 Animals

Five-week old male db/db mice (BKS.Cg-m +/+ Lepr<sup>db</sup>/J) and their age- and gender-matched normoglycemic heterozygous littermates db/m (BKS.Cg-m<sup>+/+</sup> Lepr<sup>db/+</sup>/J), conveniently referred to as controls in the current study, were purchased from Jackson Laboratory (Bar Harbor, Maine, USA). The mice were housed under standardized conditions in the animal facility of the Department of Anaesthesiology, Pharmacology & Therapeutics, University of British Columbia. The mice received standard rodent chow and had free access to water. The animals care and experimental protocols were approved by the Animal Care Committee of the University of British Columbia. A total of 160 mice were used for the experiments described in this thesis over three consecutive years. Mice were acclimatized to the housing conditions for at least one week before the start of experiments at the age of 6 weeks. Body weight and fasting blood glucose were recorded weekly.

2-2 Exercise training protocol

Six-week old db/db and control mice (db/m) were randomly assigned to either sedentary (sed) or exercise (ex) groups to yield four groups: db/db sed, db/db ex, control sed and control ex. Mice assigned to the exercise groups underwent moderate intensity exercise on a motorized wheel system (Lafayette Instrument Co, Indiana, USA) for an hour a day, five days per week at a set time each day. The exercise regimen continued for either four or eight weeks including an initial two weeks of acclimatization during which exercise speed was gradually increased from 2.5 meters/minute to a target value of 5.2 meters/minute, which represents a daily forced exercise of 312 meters. The speed used in our protocol was less than 25% of the maximum speed attained by
C57Bl/6J mice which is 25 meters/minute (Massett and Berk, 2005). The effectiveness of the exercise program was examined by comparing the heart-to-body weight ratio and skeletal muscle citrate synthase activity.

2-3 Measurement of citrate synthase activity

There are numerous methods used for the assessment of physical activity (LaPorte et al., 1985, Melanson and Freedson, 1996, Schutz et al., 2001). These physical activity measures can be classified into four main categories: 1) measurements based on energy expenditure or oxygen uptake, 2) measurements based on biochemical or physiological markers such as heart rate and skeletal muscles citrate synthase activity, 3) measurements based on motion sensing e.g. accelerometers and 4) questionnaires and surveys which are considered less objective but non invasive. No single technique meets all the desired criteria of being valid, reliable and cost effective, so the choice of the method depends on the goal and design of the study.

Citrate synthase is the initial enzyme of the tricarboxylic acid cycle involved in cellular respiration in the mitochondria. Citrate synthase activity is used to assess oxidative capacity and mitochondrial density in skeletal muscles and has been extensively applied to validate the effectiveness of exercise training particularly in experimental animals (Leek et al., 2001, Spina et al., 1996, Hoppeler, 1986). However, citrate synthase activity assay can be affected by several factors (Leek et al., 2001) leading to variable results. Therefore, I used both citrate synthase activity assay and the heart to body weight ratio to validate the exercise protocol used in my study.

Frozen adductor muscles were homogenized in ice-cold homogenization buffer CelLytic MT (Sigma, Missouri, USA), then centrifuged at 4°C for 30 minutes at 16000g. The supernatant was assayed
for protein content using Coomassie Plus (Bradford) Protein Assay (Pierce-Thermo Fisher Scientific, Illinois, USA). Ten micrograms of protein from each adductor muscle was analyzed for oxidative capacity using a citrate synthase assay kit (Sigma, Missouri, USA) according to the manufacturer’s instructions.

2-4 Intraperitoneal glucose tolerance test
Mice were fasted overnight (from 9 pm to 9 am). To obviate the short-term effects of exercise, the mice were not exercised 24 hours before undergoing the glucose tolerance test (GTT). In the morning, mice were given a 40% glucose solution (2.0 g glucose/kg) by intraperitoneal (i.p.) injection. Blood samples were collected from the tail vein at 0, 15, 30, 60, and 120 minutes post glucose administration. Plasma was separated by centrifugation and stored at -76°C for later analyses of glucose by Glucose Assay kit (Sigma, Missouri, USA), and insulin by Insulin Mouse ELISA (ALPCO, New Hampshire, USA) according to the manufacturers’ instructions. The area under the curve (AUC) for plasma glucose or insulin levels was calculated by GraphPad Prism (version 5). Insulin sensitivity index was calculated based on the formula of Matsuda and DeFronzo (Matsuda and DeFronzo, 1999) with a minor modification (I used 100 instead of 10,000 as a constant to yield more convenient numbers).

Insulin sensitivity index =

\[
\frac{100}{\sqrt{fasting\ blood\ glucose\ (mM) \times fasting\ blood\ insulin\ (\frac{ng}{ml}) \times mean\ GTT\ glucose\ (mM) \times mean\ GTT\ insulin\ (\frac{ng}{ml})}}
\]

2-5 Plasma and tissues sample collection
Mice were anesthetised with pentobarbital (50 mg/kg, i.p.) combined with heparin (50 U/kg). Blood samples were withdrawn slowly from the inferior vena cava and collected into two
Eppendorf tubes, one of which contained 0.005 % butylated hydroxy toluene (an antioxidant to inhibit in vitro formation of 8-isoprostane) for later measurement of plasma 8-isoprostane. The blood was immediately centrifuged (10 min at 4°C, 1000g) to separate the plasma, which was stored in aliquots at -76°C for later analyses. The animals were euthanized by removing the heart after blood collection. The hearts were weighed after being plotted dry with filter papers. The thoracic aortae, carotid and femoral arteries were dissected, and placed in ice-cold physiologic salt solution (PSS). With the aid of a dissecting microscope and microsurgery instruments, the arteries were cleared of the adherent connective tissues. The dissected arteries were either snap-frozen in liquid nitrogen and stored at -76°C for western blot analysis, or mounted in wire-myograph chambers for isometric force measurement. A part of the thigh adductor muscle was snap frozen in liquid nitrogen and stored at -76°C for citrate synthase analysis.

2-6 Measurement of plasma variables
Plasma glucose, insulin, 8-isoprostane and CRP levels were measured using glucose oxidase assay (Sigma, Missouri, USA), mouse insulin enzyme immunoassay (Alpco diagnostic, New Hampshire, USA), 8-isoprostane enzyme immunoassay (Cayman Chemical, Michigan, USA) and mouse CRP ELISA (Alpco diagnostic, New Hampshire, USA), respectively, according to the manufacturers’ instructions. Some of the plasma samples from db/db mice were diluted two or three times to fall within the range of the standard curves of the kits. Plasma triglycerides, cholesterol, and HDL were measured spectrophotometrically by Dade Behring RxL Max at Vancouver General Hospital.
2-7 Assessment of vascular function

The dissected arteries were cut into 2 mm long rings and mounted in wire myograph chambers (Danish Myotechnology, Aarhus, Denmark) for isometric force measurement (Mulvany and Halpern, 1977). Each vessel chamber was filled with 5 ml PSS continually gassed with carbogen (95% O₂ + 5% CO₂) and maintained at pH 7.4 and 37°C. The aortic, carotid and femoral rings were gradually stretched to their optimal resting tensions (5.5, 4 or 3 mN, respectively). The optimal resting tension was determined in preliminary experiments as the resting tension that elicited maximum force in response to 60 mM KCl. During the steps of adjusting the resting tension and equilibration, the PSS was replaced at 20 minutes intervals. The arterial rings were allowed to equilibrate for at least 30 minutes after reaching their optimum basal tension, and then were challenged twice with 80 mM KCl before constructing the dose response curves.

2-7-1 Assessment of endothelium-dependent and-independent vasodilatation

The aortic or carotid rings were constricted with a submaximal dose (producing 60%-80% of the maximum response) of the α₁-adrenoceptor agonist PE. For the femoral rings, the thromboxane A₂ analogue U-46619 (10⁻⁸ to 10⁻⁷ M) was used because PE does not yield stable contractions in the femoral arteries, probably due to increased outwards potassium currents. After a stable contraction was obtained, ACh (10⁻⁹ to 10⁻⁵ M) was added at half-log increments in a cumulative manner to examine endothelium-dependent vasodilatation. After a 30-minute washout period, the arterial rings were reconstricted with PE or U-46619, and sodium nitroprusside (SNP) a direct NO donor (10⁻¹⁰ to 10⁻⁵ M at half-log increments in a cumulative manner), or isoprenaline (10⁻⁹ to 10⁻⁵ M at half-log increments in a cumulative manner) was added to examine endothelium-independent vasodilatation. Responses to vasodilators (ACh, SNP or isoprenaline) were calculated as the percent decrease in force with respect to the initial PE or U-46619 induced constriction (% relaxation).
2-7-2 Assessment of the relative contributions of NOS, COX, or (NOS and COX) independent pathways to ACh-induced vasodilatation

The arterial rings were preincubated with a NOS inhibitor (N\textsubscript{ω}-Nitro-L-arginine methyl ester (L-NAME), 10\textsuperscript{-4} M), a COX inhibitor (indomethacin, 10\textsuperscript{-5} M), the combination of L-NAME (10\textsuperscript{-4} M) and indomethacin (10\textsuperscript{-5} M) for 30 minutes, or a depolarizing concentration of KCl (20 mM) before the construction of ACh concentration response curves.

2-7-3 Assessment of the effects of certain vasoactive drugs on ACh-induced vasodilatation

To elucidate the signalling pathway of ACh in the aortae, carotid, or femoral arteries, I preincubated the arterial rings for 30 minutes with the drugs below before ACh concentration response curves were constructed. The doses have been selected based on pilot experiments and literature review.

- Apocynin (a NADPH oxidase inhibitor; 10\textsuperscript{-4} M)
- Tempol (a SOD mimetic; 10\textsuperscript{-3} M)
- Xanthine (10\textsuperscript{-4} M) and xanthine oxidase (0.01 U/ml) as an exogenous source of O\textsubscript{2}^-
- Diethylthiocarbamate (an inhibitor of SOD; 3 x 10\textsuperscript{-3} M)
- Ebselen (a ONOO\textsuperscript{-} scavenger; 3 x 10\textsuperscript{-5}M)
- 4-Aminopyridine (4-AP) (a K\textsubscript{v} blocker; 10\textsuperscript{-3} M)
- The combination of ebselen (3 x 10\textsuperscript{-5} M) and 4-AP (10\textsuperscript{-3} M)
- BaCl\textsubscript{2} (an inward rectifier potassium channels (K\textsubscript{ir}) blocker; 3 x 10\textsuperscript{-5} M)
- Iberitoxin (a BK\textsubscript{ca} blocker; 10\textsuperscript{-7} M)
- Charybdotoxin (a BK\textsubscript{ca} and intermediate conductance calcium activated potassium channel blocker; 10\textsuperscript{-7} M)
- Glybenclamide (a K\textsubscript{ATP} inhibitor; 10\textsuperscript{-5} M)
• Apamin (a small conductance calcium activated potassium channels blocker; 10^{-7} M)

• Catalase (an enzyme that catalyzes the decomposition of H_2O_2 into water and oxygen; 1200 U/ml)

or

• Sulfaphenazole (a cytochrome P 450 mono-oxygenase (CYP-450) inhibitor; 10^{-5} M)

2-7-4 Assessment of the effect of exogenous O2^- or ONOO^- on partially constricted aortae and carotid arteries

Aortic and carotid rings were constricted with a submaximal dose of PE then exposed to a combination of xanthine (10^{-4} M) and xanthine oxidase (0.01U/ml) with or without a low concentration of SNP (10^{-9} M) to generate O_2^- or ONOO^-, respectively (Ohashi et al., 2005). To examine potential non-specific actions of xanthine or xanthine oxidase, we added each of them alone and recorded their effects on vascular tone. In a subset of experiments, 4-AP (10^{-3} M) was added before PE to examine the role of K_v in mediating the effect of O_2^- or ONOO^-.

2-7-5 Assessment of basal NO

Phenylephrine concentration response curves were constructed in the absence and the presence of L-NAME (10^{-4} M). By inhibiting NOS, L-NAME decreases basal NO production and thus eliminates its background vasorelaxant effect. Therefore, L-NAME is expected to increase PE-induced contractions under physiological conditions. The AUC for PE concentration response curve obtained in the presence of L-NAME was calculated, and divided by the AUC obtained in the absence of L-NAME. The increase in AUC due to the presence of L-NAME is calculated and used as a measure of basal NO release. The higher the level of basal NO, the larger the increase in PE contractions induced by L-NAME.
2-7-6 Assessment of the contractile responses

The aortae: Concentration response curves to different contractile agents: KCl (30 and 80 mM) and either PE (10⁻⁹ M to 10⁻⁵ M), U-46619 (10⁻⁹ M to 10⁻⁶ M) or calcium ionophore A23187 (10⁻⁹ M to 10⁻⁵ M) were constructed in the aortae of db/db and control mice. Responses to PE were examined in the presence or the absence of L-NAME (10⁻⁴ M), apocynin (10⁻⁴ M), sulfaphenazole (10⁻⁵ M), indomethacin (10⁻⁵ M), nifedipine (10⁻⁵ M) or cyclopiazonic acid (10⁻⁵ M).

The carotid arteries: responses to KCl (30 and 80 mM) and PE (10⁻⁹ M to 10⁻⁵ M) were examined.

The femoral arteries: responses to KCl (30 and 80 mM) and PE (10⁻⁸ M to 10⁻⁴ M) were examined. Concentration response curves to PE were constructed in the presence or the absence of L-NAME (10⁻⁴ M), apocynin (10⁻⁴ M), sulfaphenazole (10⁻⁵ M), indomethacin (10⁻⁵ M), xanthine (10⁻⁴ M) + xanthine oxidase (0.01 U/ml), nifedipine (10⁻⁵ M) cyclopiazonic acid (10⁻⁵ M) or 4-AP (1 mM).

The contractile responses are presented either as the net force generated (mN) or as the percentage of KCl (80 mM) induced contraction.
2-8 Western blot

Arteries from 3-5 mice from the same anatomical origin (aorta, carotid or femoral arteries), and animal group were pooled to yield enough protein for western blot analysis. Frozen arteries were homogenized in ice-cold RIPA buffer (Santa Cruz Biotechnology, California, USA). The homogenates were centrifuged at 10,000 g for 30 minutes at 4°C. The protein content of the supernatants was determined by the Coomassie Plus (Bradford) Protein Assay (Pierce-Thermo Fisher Scientific, Illinois, USA). Protein samples (30-40 μg of total protein) were prepared in Laemmli sample buffer, separated by 12% or 8-10% sodium dodecyl sulphate -polyacrylamide gel electrophoresis and then transferred overnight (at 4°C, 40 V) to nitrocellulose membranes. Afterwards, the membranes were blocked for 1 hour in 5% skim milk in Tris buffered saline containing 0.1% tween 20, washed (3 x 15 minutes), and incubated overnight at 4°C with primary antibodies against SOD-1, SOD-2, SOD-3, Akt, COX-1, COX-2 (Santa Cruz Biotechnology, California, USA), eNOS (cell signaling, Massachusetts, USA) or β-actin (BD Transduction Labs, Ontario, Canada). Membranes were washed (3 x 15 minutes), and then incubated with their corresponding horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, California, USA) for 2 hours. After washing (3 x 15 minutes), the membranes were visualized using an enhanced chemiluminescent detection kit (Pierce-Thermo Fisher Scientific, Illinois, USA) and ChemiDoc XRS (BioRad Laboratories, Ontario, Canada). Volume analyses of the protein bands were performed by Quantity One software (BioRad Laboratories, Ontario, Canada). To control for any differences in protein loading, band intensity of the specified protein was related to the band intensity of a housekeeping protein (β-actin).

2-9 Statistical analysis

Data are displayed as mean ± standard error (S.E.). The sample size of each group is specified as (n) in the footnote for each table or figure. Vascular function data were recorded and analyzed by
Powerlab 4/25 and Labchart 7 reader (AD instruments, Australia). Non-linear regression, maximum response (Emax), sensitivity (EC50) and AUC were calculated using Prism version 5.0 (GraphPad software, California, USA). Statistical analysis was performed using Prism version 5.0 (GraphPad software, California, USA) using different statistical tests as indicated below each table or figure. The statistical significant difference was set at $p<0.05$.

To estimate the relative strength of association between plasma variables and vascular responses, SAS 9.1.3 software (SAS institute INC, NC, USA) was used. First, we calculated the Pearson regression coefficients and corrected for multiple comparisons using the False Discovery Rate of Benjamini and Hochberg (Benjamini and Hochberg, 1995). Second, linear regression and stepwise model selection were performed to select the best model that fits the data. Each of the independent variable was evaluated according to the significance level of its association with the vascular response when added to the existing model (starting with a model that included intercept only). The most significant variable was included in the model if it met the statistical criterion for entry (significant level of 0.1 or smaller); otherwise, the analysis was stopped. After the first independent variable was included, the program re-evaluated all the non-included predictors similarly to the previous step. Additionally, at each step the independent variables in the model were evaluated for removal based on the significance level for exclusion (0.3 or higher). Due to sample size, we stopped the procedure once two variables were included in the model. The chosen significance levels (0.1 and 0.3) ensured that all important variables were considered in the model; it was shown that when the conventional significance level (0.05) was used some important confounders were likely not to end up in the final model (Greenland, 1983, Mickey and Greenland, 1989).
2-10 Drugs, reagents and solutions

All drugs were obtained from Sigma-Aldrich (Missouri, USA) except U-46619 which was purchased from Cayman Chemical (Michigan, USA). Western blot reagents were supplied by BioRad Laboratories (Ontario, Canada). Primary and secondary antibodies were purchased from Santa Cruz Biotechnology (California, USA), cell signaling (Massachusetts, USA) and BD Transduction Labs (Ontario, Canada). PSS was prepared according to the following composition (in mM): NaCl (119), KCl (4.7), KH₂PO₄ (1.18), MgSO₄ (1.17), NaHCO₃ (24.9), EDTA (0.023), CaCl₂ (1.6) and dextrose (11.1). High KCl solution was prepared by equimolar substitution of NaCl in PSS.
3-Results

3-1 Regional heterogeneity of the endothelium-dependent and -independent vasodilatation in db/db mice

3-1-1 Age-related changes in body weight, fasting plasma glucose, plasma 8-isoprostane, CRP and lipid profile in db/db and normoglycemic control mice

Table (3-1) shows that at the age of 6-8 weeks old, the fasting plasma glucose level of db/db mice was not significantly different from control mice. However, db/db mice weighed more, exhibited higher systemic inflammation as indicated by plasma CRP level and had higher plasma triglycerides, cholesterol, LDL and HDL levels compared with their age-matched controls. Meanwhile, their plasma 8-isoprostane level was not significantly different from control mice.

By 10 to 14 weeks of age, db/db developed significant hyperglycemia and systemic oxidative stress as revealed by higher plasma 8-isoprostane level. Also, db/db (10-14 weeks old) mice maintained higher body weight, plasma CRP, triglycerides, cholesterol and HDL levels compared with their age-matched control mice. Similarly, at 14-20 weeks of age db/db mice showed higher body weights, fasting plasma glucose, plasma 8-isoprostane, CRP and triglycerides compared to their age matched controls.

There were age-related increases (from 6-8 to 14-20 weeks old) in body weight, fasting plasma glucose, plasma 8-isoprostane, CRP and triglycerides in db/db mice indicating the progression of the metabolic abnormalities associated with diabetes. In contrast, control (db/m) mice showed an expected age-dependent increase in body weight, but otherwise maintained steady values for all the other parameters measured.
Table 3-1: Age-related changes in body weight, fasting plasma glucose, plasma 8-isoprostanes, CRP and lipid profile in db/db and normoglycemic control mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th></th>
<th>db/db</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6-8 wk</td>
<td>10-14 wk</td>
<td>14-20 wk</td>
<td>6-8 wk</td>
<td>10-14 wk</td>
<td>14-20 wk</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>20.67 + 0.34</td>
<td>27.96 ± 0.29β</td>
<td>31.90 ± 0.36φ</td>
<td>30.74 ± 0.37*</td>
<td>45.87±0.65 *#</td>
<td>48.86 ±1.22* # @</td>
</tr>
<tr>
<td>Fasting plasma glucose (mM)</td>
<td>4.36 ± 0.15</td>
<td>5.03 ± 0.23</td>
<td>5.90 ± 0.50</td>
<td>5.68 ± 0.31</td>
<td>31.54±1.33 *#</td>
<td>54.66 ±1.53 *#@</td>
</tr>
<tr>
<td>Plasma 8-isoprostane (pg/ml)</td>
<td>43.30 ± 10.85</td>
<td>36.92± 6.18</td>
<td>69.93±15.89</td>
<td>65.85±12.90</td>
<td>210.9±27.07 *#</td>
<td>283.1 ± 80.99 *#</td>
</tr>
<tr>
<td>Plasma CRP (ng/ml)</td>
<td>1.83 ± 0.30</td>
<td>2.57 ± 0.32</td>
<td>2.51 ± 0.36</td>
<td>3.30 ± 0.31</td>
<td>4.05 ±0.22 *</td>
<td>5.12 ± 0.25 *#</td>
</tr>
<tr>
<td><strong>Plasma lipid profile</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.69 ± 0.09</td>
<td>0.72 ± 0.07</td>
<td>1.36 ± 0.12 *</td>
<td>1.93 ± 0.32 *#</td>
<td>2.82 ± 0.24 *# @</td>
<td></td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>2.43 ± 0.09</td>
<td>2.47 ± 0.14</td>
<td>3.98 ± 0.16 *</td>
<td>3.77 ± 0.16 *</td>
<td>2.82 ±0.15 # @</td>
<td></td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>0.91 ± 0.07</td>
<td>0.95 ± 0.09</td>
<td>1.66 ± 0.11 *</td>
<td>1.36 ± 0.12</td>
<td>0.6 ± 0.28 # @</td>
<td></td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>1.2 ± 0.04</td>
<td>1.19 ± 0.14</td>
<td>1.69 +0.06 *</td>
<td>1.82 ± 0.08 *</td>
<td>1.4 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

Values are displayed as mean ± S.E. and represent n= 5-11 except for body weight n= 12-20. Statistical analysis was done using two-way ANOVA followed by Bonferroni post-test

* denotes p<0.05 vs. age matched control, # denotes p<0.05 vs. db/db (6-8 wk), @ denotes p<0.05 vs. db/db (10-14 wk), β denotes p<0.05 vs. control (6-8 wk), φ denotes p<0.05 vs. control (10-14 wk)

CRP= C-reactive protein, HDL= high density lipoprotein, LDL= Low density lipoprotein

For lipid profile variables, values for control (10-14 wk) and control (14-20 wk) mice were pooled
3-1-2 Intraperitoneal glucose tolerance test in db/db and control mice

Figure (3-1) and table (3-2) show that the glucose tolerance of db/db mice (6 weeks old) was markedly impaired although their fasting plasma glucose was not significantly different from control mice. Upon challenging the mice with a glucose load (2 gm/kg), db/db mice showed elevated plasma glucose levels at 30, 60 and 120 minutes after glucose injection. The AUC for plasma glucose levels (AUC$_{\text{glucose}}$) in db/db mice was 213.8% of their age-matched control mice, despite the secretion of larger amounts of insulin (AUC$_{\text{insulin}}$ for db/db was 540 % that of control mice). Therefore, db/db mice at that age (6 weeks old) were glucose-intolerant but not diabetics.

At the subsequent age points (10 weeks) and (14 weeks), db/db mice showed marked fasting hyperglycemia (at time zero) and glucose intolerance at all time points following glucose administration. Similarly, their plasma insulin levels remained higher than their age-matched controls. Thus, db/db mice at 10 and 14 weeks of age were considered diabetic. As shown in table (3-2), there was an age-related deterioration of glycemic status in db/db mice as AUC$_{\text{glucose}}$ for db/db increased from 213.8% to 477% to 546% of the corresponding values in the age-matched control group. Although AUC$_{\text{insulin}}$ for db/db remained higher than those for control mice at all ages examined, the ability of db/db mice to secrete insulin was decreasing with age from 540% to 305% to 178.1% of the age-matched control group.
Figure 3-1: Intraperitoneal glucose tolerance test for db/db and control mice at 6, 10 or 14 weeks of age

Panels A-1, B-1 & C-1 show plasma glucose values while panels A-2, B-2 & C-2 show plasma insulin levels after glucose injection.

Values are displayed as mean ± S.E. and represent n= 6-10. Area under the curve was calculated by Prism software version 5 and compared by two-way ANOVA followed by Bonferroni post-test.

* denotes p<0.05 vs. age matched controls.
Table 3-2: Area under the curve for i.p. GTT undertaken by db/db and control mice

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th></th>
<th></th>
<th>db/db</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 wk</td>
<td>10 wk</td>
<td>14 wk</td>
<td>6 wk</td>
<td>10 wk</td>
<td>14 wk</td>
</tr>
<tr>
<td><strong>AUC (glucose mM. min)</strong></td>
<td>838.5 ±</td>
<td>1234 ±</td>
<td>1842 ±</td>
<td>1793 ±</td>
<td>5892 ±</td>
<td>10059 ±</td>
</tr>
<tr>
<td></td>
<td>44.92</td>
<td>57.30</td>
<td>132.0</td>
<td>141.0 *</td>
<td>161.5* #</td>
<td>270.9* # @</td>
</tr>
<tr>
<td></td>
<td>(213 %)</td>
<td>(477 %)</td>
<td>(546 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>AUC (insulin ng. min)</strong></td>
<td>136 ± 9.85</td>
<td>129.4 ±</td>
<td>147.7 ±</td>
<td>735.6 ±</td>
<td>394.8 ±</td>
<td>263.1 ±</td>
</tr>
<tr>
<td></td>
<td>9.23</td>
<td>8.79</td>
<td></td>
<td>53.84 *</td>
<td>43.54 * #</td>
<td>21.02 *# @</td>
</tr>
<tr>
<td></td>
<td>(540 %)</td>
<td>(305 %)</td>
<td>(178.1 %)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are displayed as mean ± S.E. and represent n= 6-8
Area under the curve was calculated by Prism software version 5 and compared by two-way ANOVA followed by Bonferroni post-test. The percentage written is relative to the age-matched control group
* denotes p<0.05 vs. age-matched control, # denotes p<0.05 vs. db/db (6 wk), @ denotes p<0.05 vs. db/db (10 wk)
AUC= area under the curve
3-1-3 Impaired endothelium-dependent and -independent vasorelaxation in the aortae of \textit{db/db} mice

Figure (3-2) shows that at 6-8 weeks of age, there was a small but significant impairment in the responses to ACh in the aortae of \textit{db/db} mice relative to their age-matched controls. However, SNP relaxed the aortae to the same magnitude in \textit{db/db} and control mice.

Figure (3-3) demonstrates that at the successive age points (10-14 weeks old) and (14-20 weeks old), the aortae of \textit{db/db} mice showed progressive deterioration in the responses to ACh (Emax: \textit{db/db} (6-8 wk) = 71.59 ± 1.51\%, \textit{db/db} (10-14 wk) = 53.40 ± 2.06\%, and \textit{db/db} (14-20 wk) = 24.34 ± 1.84\%). Starting from 10-14 weeks of age, \textit{db/db} mice exhibited attenuated SNP-induced vasodilatation (Emax: \textit{db/db} (6-8 wk) = 86.95 ± 1.72\%, \textit{db/db} (10-14 wk) = 74.98 ± 1.474\% vs. \textit{db/db} (14-20 wk) = 71 ± 1.25\%).
Figure 3-2: Cumulative concentration response curves of ACh and SNP in phenylephrine constricted aortae of db/db and control mice at 6-8 weeks, 10-14 weeks or 14-20 weeks of age

Panels A-1, B-1 & C-1 show the response to ACh while panels A-2, B-2 & C-2 show response to SNP, at 6-8 weeks (panels A-1 and A-2), 10-14 weeks (panels B-1 and B-2) and 14-20 weeks (panels C-1 and C-2) of age. Values are displayed as mean ± S.E. and represent n= 6-10 mice. Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test, * denotes p<0.05 vs. age matched control. ACh= acetylcholine, SNP= sodium nitroprusside
Figure 3-3: Cumulative concentration response curves of ACh (upper panel) and SNP (lower panel) in phenylephrine constricted aortae of db/db mice at 6-8 weeks, 10-14 weeks and 14-20 weeks of age

Values are displayed as mean ± S.E. and represent n= 6-10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes $p<0.05$ vs. db/db aorta (6-8 wk), # denotes $p<0.05$ vs. db/db aorta (10-14 wk)
ACh= acetylcholine, SNP= sodium nitroprusside
3-1-3-1 Correlation analysis between plasma variables and ACh maximum response in the aortae of db/db and control mice

To understand the factors contributing to the vascular dysfunction in the aortae of db/db mice, we calculated the Pearson’s correlation coefficient between ACh maximum response (Emax) and body weight, plasma glucose, 8-isoprostane, CRP, triglycerides, LDL and HDL, representing the major perturbations (obesity, hyperglycemia, oxidative stress, inflammation and dyslipidemia, respectively) associated with type 2 diabetes. Maximum relaxation to ACh was negatively correlated with: body weight, plasma glucose and plasma 8-isoprostane as shown in Table (3-3). After adjusting for the type of mice (db/db or control), body weight was the determinant of ACh induced vasorelaxation. Next, we adjusted for weight to find that HDL was the only determinant of ACh-induced vasorelaxation in the aortae of db/db and control mice. Stepwise model selection demonstrated that body weight was the key determinant of ACh-induced vasorelaxation.
Table 3-3: Correlation analysis between some metabolic variables and ACh maximum response in the aortae of *db/db* and normoglycemic control mice

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s correlation coefficient</th>
<th>Statistical significance</th>
<th>Number of data pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>-0.64</td>
<td><em>p</em>&lt;0.0001</td>
<td>37</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>-0.61</td>
<td><em>p</em>&lt;0.0001</td>
<td>36</td>
</tr>
<tr>
<td>Plasma 8-isoprostane</td>
<td>-0.54</td>
<td><em>p</em>&lt;0.05</td>
<td>36</td>
</tr>
<tr>
<td>Plasma CRP</td>
<td>-0.37</td>
<td><em>p</em>&gt;0.05</td>
<td>35</td>
</tr>
<tr>
<td>Plasma triglycerides</td>
<td>-0.35</td>
<td><em>p</em>&gt;0.05</td>
<td>32</td>
</tr>
<tr>
<td>Plasma HDL</td>
<td>-0.16</td>
<td><em>p</em>&gt;0.05</td>
<td>32</td>
</tr>
<tr>
<td>Plasma LDL</td>
<td>-0.09</td>
<td><em>p</em>&gt;0.05</td>
<td>31</td>
</tr>
</tbody>
</table>

Correction for multiple comparisons was performed using the False Discovery Rate of Benjamini and Hochberg

CRP= C- reactive protein, HDL= high density lipoprotein, LDL= low density lipoprotein

A version of this table was published in Sallam *et al.* (2011)
3-1-3-2 Relative contributions of NOS, COX or (NOS and COX) independent pathways to ACh-induced vasorelaxation in the aortae of db/db and control mice

Preincubation of the aortic rings with L-NAME (10^{-4} M), a non specific NOS inhibitor, for 30 minutes completely abolished ACh-induced vasodilatation (data not shown) in both db/db and control mice, indicating that vasodilatory effect of ACh was totally NOS-mediated and that the contributions of COX or EDHF to ACh-induced vasorelaxation were negligible in the aortae.

3-1-3-3 Effect of L-NAME, indomethacin, apocynin and tempol on impaired ACh-induced vasorelaxation in the aortae of db/db mice (14-20 wk)

Plasma 8-isoprostane level was negatively correlated with ACh Emax (Table 3-3) suggesting that oxidative stress plays a role in the impaired vasodilatation associated with diabetes. In vascular tissues, there are many potential sources of ROS including NADPH oxidase, COX, uncoupled NOS, arachidonic acid metabolites and mitochondrial respiratory chain enzymes. We anticipated that inhibiting the enzymatic system with the largest input of ROS in the aortae would produce the most marked improvement in ACh-induced relaxation. I preincubated the aortic rings of db/db (14-20 weeks old) mice with L-NAME (a NOS inhibitor; 10^{-4} M), indomethacin (a COX inhibitor; 10^{-5} M), apocynin (a NADPH oxidase inhibitor; 10^{-4} M) or tempol (a cell permeable SOD mimetic; 10^{-3} M) for 30 minutes before ACh concentration response curves were constructed. None of these chemicals could improve ACh-induced vasodilatation in the aortae of db/db mice and L-NAME completely abolished the vasodilatation (Fig 3-4).
Figure 3-4: Effect of apocynin, indomethacin, L-NAME and tempol on ACh-induced vasodilatation in the aortae of db/db mice (14-20 weeks)

Values are displayed as mean ± S.E. and represent n= 5-10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. db/db aorta
3-1-3-4 Impaired isoprenaline-induced vasodilatation in the aortae of db/db mice (14-20 weeks)

To examine if the vascular dysfunction observed in aortae of db/db was confined to NO-mediated pathway, I examined the response to isoprenaline (a β-adrenoceptor agonist acting via the cAMP pathway (Harden, 1983)). A diminished response to isoprenaline was observed in the aortae of db/db mice (Emax: db/db= 69.19±2.01 % vs. control= 87.21± 1.83 %, p<0.05) (Fig 3-5). To elucidate the signalling pathway of isoprenaline, we preincubated the aortic rings with 4-AP (a Kv blocker) or iberitoxin (a BKCa blocker) for 30 minutes before isoprenaline dose responses were constructed. The action of isoprenaline was totally abolished by 4-AP (n= 3, p<0.05, while iberitoxin had an insignificant effect (n= 3), indicating that isoprenaline-induced vasodilatation is mediated via Kv and not BKCa in the aortae.
Figure 3-5: Cumulative concentration response curves of isoprenaline in phenylephrine constricted aortae of db/db (14-20 weeks old) and normoglycemic control mice

Values are displayed as mean ± S.E. and represent n=12-20 rings from 5 mice each
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. control aorta (14-20 wk)
3-1-4 Preserved ACh- and SNP-induced vasodilatation in the carotid arteries of db/db mice

Despite the array of metabolic abnormalities (hyperglycemia, dyslipidemia, systemic inflammation and oxidative stress) evident in db/db mice, responses to ACh and SNP were preserved in the carotid arteries of db/db mice compared with normoglycemic controls across the three age points examined (Fig 3-6), markedly different from the adaptation of the aortae to the diabetic milieu. There can be few possible explanations for the discrepancy between the aorta and carotid artery. First, the local generation of ROS in the carotid artery of db/db mice may not be elevated under diabetic conditions in contrast to the aorta. Alternatively, the carotid artery may be able to upregulate its antioxidant defence mechanisms to counterbalance any increase in local ROS production, and thus is capable of maintaining a physiological local redox status. The third possibility is that the signalling pathway mediating ACh-induced vasodilatation in the carotid artery is different from that in the aorta, and can accommodate any increase in ROS.
Figure 3-6: Cumulative concentration response curves of ACh (upper panel) and SNP (lower panel) in phenylephrine constricted carotid arteries of db/db and normoglycemic control mice

Values are displayed as mean ± S.E. and represent n= 6-10 mice in each group. Statistical analysis was done using two-way repeated measures ANOVA

ACh= acetylcholine, SNP= sodium nitroprusside
3-1-4-1 The carotid arteries maintain full vasodilatation to ACh in the presence of induced oxidative stress

To test if the preserved vasorelaxant effect of ACh in the carotid arteries of db/db mice can be attributed to unchanged production of ROS in the carotid arteries, I challenged the carotid arteries and aortae of control mice with an exogenous source of ROS: xanthine (10^-4 M) and xanthine oxidase (0.01 U/ml) for 30 minutes before the construction of ACh concentration response curves. ACh-induced vasorelaxation was significantly impaired in the aortic rings by this intervention; meanwhile, the carotid arteries exhibited full relaxation to ACh (Fig 3-7).

To test if the carotid arteries possess higher antioxidant capacity than the aortae enabling them to maintain a physiological redox status despite increased ROS generation. I preincubated both the aortic and carotid rings with a SOD blocker: diethyldithiocarbamate (3 x 10^-3 M) for 30 minutes. ACh-induced vasorelaxation was impaired in the aortic but not carotid rings (Fig 3-7). To check if diethyldithiocarbamate impaired ACh-induced vasorelaxation in the aortae due to a non-specific action, I examined its effect on SNP (10^-6 M) induced vasodilatation; diethyldithiocarbamate impaired the response to ACh but not SNP in the aortae.

Therefore, in the presence of an increased oxidative burden induced either exogenously (using xanthine and xanthine oxidase system), or endogenously (blocking SOD by diethyldithiocarbamate), the carotid arteries but not the aortae maintained full ACh-induced vasodilatation. The plausible explanation left is that the signalling pathway mediating the vasorelaxant effect of ACh in the carotid arteries is different from that in the aortae and can accommodate the increase in oxidative burden.
Figure 3-7: Cumulative concentration response curves of ACh in phenylephrine constricted carotid arteries (upper panel) or aortae (lower panel) of normoglycemic control mice (14-20 weeks old) in the presence or the absence of xanthine and xanthine oxidase or diethyldithiocarbamate

Values are displayed as mean ± S.E. and represent n= 5-7 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. control aorta
ACh= acetylcholine, DCC= diethyldithiocarbamate, X= xanthine, XO= xanthine oxidase
3-1-4-2 Elucidation of the signalling pathway for ACh-induced vasodilatation in the carotid arteries

3-1-4-2-1 Relative contributions of NOS, COX and (NOS and COX) independent pathways to ACh-induced vasorelaxation in the carotid arteries

Preincubation of the carotid rings with L-NAME (10^-4 M) for 30 minutes abolished ACh-induced vasodilatation in both db/db (n= 15) and control mice (n=9) at the three age points examined signifying that ACh-induced vasorelaxation was totally mediated by NOS. However, this does not necessarily mean that the vasodilatation was only NO-mediated as NOS can produce products other than NO (Rabelink and Luscher, 2006, Ketonen et al., 2010, Sabri et al., 2010, Schmidt and Alp, 2007).

3-1-4-2-2 The carotid artery and the aorta show different sensitivity and efficacy for ACh

Figure (3-8) shows that the carotid arteries were more sensitive to ACh (-log EC50: carotid= 8.49 ± 0.05 vs. aorta= 7.54 ± 0.06, p<0.05) and relaxed to a higher magnitude (Emax: carotid= 90.25 ± 1.04 % vs. aorta=75.52 ± 1.4 %, p<0.05) compared with the aortae. Meanwhile, there was no significant difference between the carotid artery and the aorta with respect to SNP Emax or log EC59. These findings suggest that the signalling pathways for ACh in the carotid artery and the aorta are different, and probably the vasoactive factor(s) mediating ACh-induced vasorelaxation in the carotid artery is different from the NO species released by SNP.
Figure 3-8: Cumulative concentration response curves of ACh (upper panel) and SNP (lower panel) in phenylephrine constricted carotid arteries and aortae of normoglycemic control mice (14-20 weeks old)

Values are displayed as mean ± S.E. and represent n= 6-10 mice in each group
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. control carotid
ACh= acetylcholine, SNP= sodium nitroprusside
3-1-4-2-3 Effect of catalase, tempol, ebselen on ACh-induced vasorelaxation in the carotid arteries

We examined the possibility that the species mediating ACh-induced vasorelaxation in the carotid arteries may be $O_2^-$, or $H_2O_2$ the more stable product of $O_2^-$, or ONOO$^-$ the product of the reaction of NO with $O_2^-$. I incubated the carotid rings with a superoxide anion scavenger (tempol, $10^{-3}$ M), an enzyme that catalyzes the decomposing of $H_2O_2$ (catalase, 1200 U/ml), or a ONOO$^-$ scavenger (ebselen, $3 \times 10^{-5}$ M) for 30 minutes before constructing ACh concentration response curves. Ebselen and to a lesser extent tempol blunted ACh-induced vasorelaxation in the carotid arteries of control and $db/db$ mice, suggesting that ONOO$^-$ plays a key role in mediating ACh-induced vasorelaxation in the carotid arteries (Fig 3-9). These findings may explain how the carotid arteries of $db/db$ mice maintained full relaxation to ACh under increased oxidative stress as the product(s) of NO with $O_2^-$ can still relax the carotid arteries. The question that was left to answer was how ONOO$^-$ relaxed the carotid arteries.
Figure 3-9: Cumulative concentration response curves of ACh in phenylephrine constricted carotid arteries of normoglycemic control mice (14-20 weeks old) in the presence or the absence of tempol, catalase or ebselen

Values are displayed as mean ± S.E. and represent n= 6-20 rings from 4-10 mice in each group. Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test.

* denotes p<0.05 vs. control carotid

ACh= acetylcholine
3-1-4-2-4 Effect of potassium channels blockers on ACh-induced vasorelaxation in the carotid arteries

Earlier studies have reported that ROS can modulate vascular tone through an array of mechanisms including direct interactions with potassium channels. There are four types of potassium channels expressed in the vasculature: $K_v$, $K_{ATP}$, $K_{ir}$ and $K_{Ca}$. I examined the roles of these channels in ACh-induced vasorelaxation in the carotid arteries by using their respective blockers: 4-AP ($3 \times 10^{-3} \text{ M}$), glybenclamide ($10^{-5} \text{ M}$), barium chloride ($3 \times 10^{-5} \text{ M}$) and the combination of charybdotoxin ($10^{-7} \text{ M}$) and apamin ($10^{-7} \text{ M}$). Only 4-AP could impair ACh-induced vasorelaxation in the carotid arteries, indicating that $K_v$ plays a key role in mediating ACh-induced vasorelaxation in the carotid arteries of control mice (Fig 3-10). Similar experiments were performed on the carotid arteries of $db/db$ mice and yielded similar results (data not shown).

3-1-4-2-5 Ebselen and 4-AP act on the same pathway in the carotid arteries

To check if $K_v$ is the target of ONOO$^-$ in the carotid arteries, I compared the effects of 4-AP, ebselen or their combination on ACh concentration response curves. Ebselen, 4-AP and their combination all reduced ACh maximum response to the same extent ($E_{max}$: $53.59 \pm 2.67\%$, $47.09 \pm 3.57\%$, $48.34 \pm 5.06\%$ respectively, $p>0.05$) (Fig 3-11). There was no additive effect with respect to ACh Emax suggesting that ONOO$^-$ and $K_v$ are probably involved in the same pathway.
Figure 3-10: Cumulative concentration response curves of ACh in phenylephrine constricted carotid arteries of normoglycemic control mice (14-20 weeks old) in the presence or the absence of 4-aminopyridine, glybenclamide, barium or the combination of charybdotoxin and apamin

Values are displayed as mean ± S.E. and represent n= 5-10 mice in each group
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. control carotid
ACh= acetylcholine, 4-AP= 4-aminopyridine, Ba++= Barium salt, CHTX= charybdotoxin
Figure 3-11: Cumulative concentration response curves of ACh in phenylephrine constricted carotid arteries of normoglycemic control mice (14-20 weeks old) in the presence or the absence of 4-aminopyridine, ebselen, or the combination of ebselen and 4-aminopyridine

Values are displayed as mean ± S.E. and represent n= 5-10 mice in each group
ACh Emax was compared by one way ANOVA
ACh= acetylcholine, 4-AP= 4-aminopyridine
3-1-4-2-6 The carotid arteries but not the aortae relax in response to exogenous superoxide anion or peroxynitrite generation

To confirm that ONOO\(^{-}\) plays a key role in relaxing the carotid arteries, I exposed constricted carotid arteries to an exogenous ONOO\(^{-}\) generating system [xanthine (10\(^{-4}\) M) + xanthine oxidase (0.01U/ml) + a low concentration of SNP (10\(^{-9}\) M)]. Adding (xanthine + xanthine oxidase) with or without SNP, i.e. O\(_2^{-}\) or ONOO\(^{-}\), relaxed the carotid arteries (51.78 ± 6.72\%, n=16 rings from 6 mice). This relaxation was not noticed after adding either xanthine or xanthine oxidase alone indicating that O\(_2^{-}\) is the moiety that relaxes the carotid arteries. Superoxide anion induced relaxation in the carotid arteries was inhibited by 4-AP (n=4), implying a role for K\(_v\) in mediating O\(_2^{-}\) vasorelaxant action in agreement with our previous finding that 4-AP inhibit ACh induced vasorelaxation in the carotid arteries. Meanwhile, the same treatment [xanthine (10\(^{-4}\)M) + xanthine oxidase (0.01U/ml)] could not relax the aortae (n=9) but ACh could relax the aortae. Representative traces are shown in figures (3-12) and (3-13).
Figure 3-12: Representative traces showing the effect of superoxide generating system (xanthine/xanthine oxidase) on phenylephrine constricted carotid artery (panel A) and aorta (panel B) of normoglycemic control mice (14-20 weeks old)

ACh= acetylcholine, PE=phenylephrine, X= xanthine, XO= xanthine oxidase
Figure 3-13: Representative trace showing the effect of superoxide generating system (xanthine/xanthine oxidase) on phenylephrine constricted carotid artery of normoglycemic control mice (14-20 weeks old) in the presence of 4-aminopyridine

ACh= acetylcholine, 4-AP= 4-aminopyridine, PE=phenylephrine, X= xanthine, XO= xanthine oxidase
3-1-5 Reduced ACh-induced relaxation in the femoral arteries of db/db mice

Similar to the aortae, ACh-induced vasorelaxation was impaired in the femoral arteries of db/db mice starting at the age of 6-8 weeks compared with their normoglycemic controls (Emax: \( db/db = 64.18 \pm 2.34\% \) vs. control= 84.79 \( \pm 2.24\% \), \( p<0.05 \)) (Fig 3-14). I used indomethacin (10\(^{-5}\) M), L-NAME (10\(^{-4}\) M), the combination of L-NAME and indomethacin, a depolarizing concentration of KCl (20 mM), or the combination of L-NAME, indomethacin and KCl to elucidate the relative contributions of NOS, COX and (COX & NOS) independent pathway to the vasodilatation in the femoral arteries. Indomethacin alone did not have any significant effect on ACh-induced vasorelaxation in either \( db/db \) or control mice indicating that prostacyclin did not contribute to ACh vasorelaxation (data not shown). Preincubation with L-NAME and indomethacin, reduced the vasorelaxation in the femoral arteries significantly but did not completely abolished it, in contrast to the case in the aortae and carotid arteries, where L-NAME abolished ACh-induced vasorelaxation. This residual vasodilatation was abolished by KCl (20 mM) or the combination of charybdotoxin (10\(^{-7}\) M) and apamin (10\(^{-7}\) M), suggesting that this component of ACh-induced vasodilatation has a hyperpolarizing nature and is commonly referred to as EDHF.

Figure (3-14) shows that both NOS- and EDHF-mediated pathways were impaired in \( db/db \) mice. However, the contribution of EDHF was more markedly reduced in \( db/db \) mice compared with NOS-dependent pathway, as reflected by both reduced net AUC\(_{EDHF} \): \( db/db = 24.72 \pm 10.89 \) area unit vs. control= 89.22 \( \pm 7.65 \) area unit (\( p<0.05 \)), and reduced relative contribution to total ACh-induced vasorelaxation (\( db/db = 19.04 \% \) vs. control= 40.79\%). As indomethacin alone did not significantly change ACh-induced vasorelaxation in the femoral arteries, the residual AUC after subtracting the portion representing EDHF should reflect the NOS-dependent pathway; this component was also reduced in \( db/db \) mice (residual AUC\(_{NOS} \) \( db/db = 105.08 \) vs. residual
However, the relative contribution of NOS-dependent pathway to total ACh-induced vasorelaxation was increased in \( db/db \) relative to control mice (\( db/db = 80.96\% \) vs. control 59.21%).

I tried to isolate the NOS-dependent pathway by using a depolarizing concentration of KCl to eliminate the hyperpolarization-dependent pathway. The depolarization resistant pathway was also significantly reduced in \( db/db \) mice (AUC\(_{KCl \ resistant}\) \( db/db = 50.12 \pm 5.84 \) vs. control = 108.21 \( \pm 7.73, p<0.05 \)). Interestingly, the sum of the AUCs yielded by the depolarization-resistant pathway and the (NOS and COX)-resistant pathway is smaller than the total AUC of ACh-induced vasorelaxation. This difference was more pronounced in \( db/db \) mice (AUC\(_{KCl \ resistant} \) (50.12 \( \pm 5.84 \)) + AUC\(_{L-NAME \ and \ indomethacin \ resistant} \) (24.72 \( \pm 10.89 \)) < AUC\(_{ACh} \) (129.8 \( \pm 11.84 \)) suggesting that NO exerts a hyperpolarizing action or that NO and EDHF have synergistic actions.

In contrast to the age-related impairment of ACh in the aortae of \( db/db \) mice, ACh-induced vasorelaxation in the femoral arteries of \( db/db \) mice did not deteriorate further with age (Emax: \( db/db(6-8 \ wk) = 64.18 \pm 2.34\%, db/db(10-14 \ wk) = 56.75 \pm 1.77 \% \), \( db/db(14-20 \ wk) = 66.96 \pm 1.2\%, p>0.05 \)); neither did the relative contribution of EDHF to total ACh-induced vasorelaxation change with age (data not shown).

3-1-5-1 Identity of EDHF in the femoral arteries

The COX-and NOS-independent ACh vasorelaxation in the femoral arteries was abolished by 20 mM KCl (n=4), or the combination of charybdotoxin (10\(^{-7}\)M) and apamin (10\(^{-7}\)M) n= 5, but not by Ba\(^{2+}\) (3 x 10\(^{-5}\)M, n= 4) or sulfaphenazole (10\(^{-5}\) M, n=10).
Figure 3-14: Cumulative concentration response curves of ACh in U-46619 constricted femoral arteries of db/db (6-8 weeks old) and normoglycemic control mice in the presence or the absence of L-NAME (10^{-4} M) plus indomethacin (10^{-5} M) or KCl (20 mM)

Values are displayed as mean ± S.E. and represent n= 7-10 mice in each group
Area under the curve was compared by two-way ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. control mice
ACh= acetylcholine, COX= cyclooxygenase, IND= indomethacin, L-N= Nω-Nitro-L-arginine methyl ester, KCl= potassium chloride, NOS= nitric oxide synthase, ⊥= dependent
3-1-5-2 Basal NO release in the femoral arteries

Figure (3-15) shows that L-NAME augmented PE contractions more effectively in the femoral arteries of control mice (182.2% of the contraction in the absence of L-NAME) than in db/db mice (157.7% of the contraction in the absence of L-NAME) indicating that basal NO production was reduced in the femoral arteries of db/db mice.

![Cumulative concentration response curves of phenylephrine in the presence or the absence of L-NAME (10^{-4} M) in the femoral arteries of db/db (10-14 weeks old) and normoglycemic control mice](image)

**Figure 3-15:** Cumulative concentration response curves of phenylephrine in the presence or the absence of L-NAME (10^{-4} M) in the femoral arteries of db/db (10-14 weeks old) and normoglycemic control mice

Values are displayed as mean ± S.E. and represent n= 6-10 mice in each group
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test. Area under the curve was calculated using Prism version 5.0 (GraphPad software)
* denotes p<0.05 vs. db/db femoral (10-14 wk) + L-NAME
L-NAME= Nω-Nitro-L-arginine methyl ester
3-1-5.3 Impaired endothelium-independent SNP-induced vasodilatation in the femoral arteries of db/db mice

Starting from the age of 14 weeks old, femoral arteries from db/db mice showed reduced sensitivity to SNP suggesting a dysfunction at the level of VSMs (Fig 3-16).

![Graph showing cumulative concentration response curves of SNP in U-46619 constricted femoral arteries of db/db (14-20 weeks old) and normoglycemic control mice.](image)

**Figure 3-16: Cumulative concentration response curves of SNP in U-46619 constricted femoral arteries of db/db (14-20 weeks old) and normoglycemic control mice**

Values are displayed as mean ± S.E. and represent n= 6-10 mice in each group
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. control femoral (14-20 wk)
SNP= sodium nitroprusside
3-1-6 Summary of the results

The results of the first study in the present thesis are summarized in the illustration below (3-1).

Illustration 3-1: Summary of the results of the first study in the present thesis

Data displayed are from *db/db* mice relative to their age matched controls

ACh= acetylcholine, EDHF= endothelium dependent hyperpolarizing factor, NO= nitric oxide, SNP= sodium nitroprusside

\[\text{decreased, increased, unchanged}\]
3-2 Regional heterogeneity of the vascular contractile responses in \textit{db/db} and normoglycemic control mice

3-2-1 Enhanced contractile responses in the aortae of \textit{db/db} mice

The aortae of \textit{db/db} (10-14 weeks old) mice showed enhanced responses to both PE and KCl compared with their normoglycemic controls (Fig 3-17). After normalizing the PE induced contractions to maximum KCl (80 mM) contraction, an enhanced sensitivity to PE was still observed (log EC50: control= -6.95 ± 0.11 vs. \textit{db/db}= -7.23 ± 0.07, \textit{p}<0.05, unpaired t test). The augmented PE contractions in \textit{db/db} aortae (Emax= 13.28 ± 0.5 mN) were not attenuated after 30 minutes preincubation with L-NAME (10^{-4} M) (Emax= 16.01 ± 0.52 mN), apocynin (10^{-4} M) (Emax= 11.42 ± 0.32 mN) or sulfaphenazole (10^{-5} M) (Emax= 12.22 ± 0.49 mN). However, preincubation with indomethacin (10^{-5} M) for 30 min alleviated the enhanced PE contractions in \textit{db/db} mice without affecting the contractions in control mice (Fig 3-17), suggesting the involvement of prostanoid(s) in the hypercontractility observed in \textit{db/db} mice.

To further elucidate the role of prostanoids, I examined the responses to U-46619 (a thromboxane A\textsubscript{2} analogue) and found an increased sensitivity to U-46619 in \textit{db/db} aortae (log EC50: control= -7.42 ± 0.04 vs. \textit{db/db}= -7.84 ± 0.05, \textit{p}<0.05, unpaired t test). Next, I examined the responses to the calcium ionophore A23197 in the presence of L-NAME (10^{-4} M); A23197 yielded larger contractions in \textit{db/db} mice compared with their age-matched controls (Emax: control= 5.51 ± 0.38 mN vs. \textit{db/db}= 8.44 ± 0.22 mN, \textit{p}<0.05) (Fig 3-18).
Figure 3-17: Contractile responses to KCl (upper panel) and phenylephrine (lower panel) in the aortae of db/db and control mice at 10-14 weeks of age

Values are displayed as mean ± S.E. and represent n= 6-10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 compared to control aorta (10-14 wks), # denotes p<0.05 compared to db/db aorta (10-14 wks)
Ind= indomethacin
Figure 3-18: Cumulative concentration response curves of U-46619 (upper panel) and calcium ionophore A23187 (lower panel) in the aortae of db/db and normoglycemic control mice (14-20 weeks old)

Values are displayed as mean ± S.E. and represent n=7-9 mice for U-46619 and n=4-7 mice for A23187 concentration response curves.
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test.
* denotes $p<0.05$ vs. control aorta (14-20 wk)
3-2-1-1 Correlation analysis between plasma variables and PE maximum response in the aortae of db/db and control mice

To understand the factors contributing to the vascular dysfunction in the aortae of db/db mice, we calculated the Pearson’s correlation coefficient between PE maximum response (Emax) and body weight, plasma glucose, 8-isoprostane, CRP, triglycerides, LDL and HDL. Maximum contraction to PE was positively correlated with body weight, plasma glucose, plasma 8-isoprostane and plasma CRP as shown in Table (3-4). Stepwise model selection demonstrated that plasma CRP ($p=0.008$) and plasma glucose ($p=0.03$) were the key determinants of PE induced contractions in the aortae.

Table 3-4: Correlation analysis between some metabolic variables and PE maximum response in the aortae of db/db and normoglycemic control mice

<table>
<thead>
<tr>
<th></th>
<th>Pearson’s correlation coefficient</th>
<th>Statistical significance</th>
<th>Number of data pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight</td>
<td>0.73</td>
<td>$p&lt;0.0001$</td>
<td>34</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>0.71</td>
<td>$p&lt;0.0001$</td>
<td>33</td>
</tr>
<tr>
<td>Plasma 8-isoprostane</td>
<td>0.66</td>
<td>$p&lt;0.0001$</td>
<td>33</td>
</tr>
<tr>
<td>Plasma CRP</td>
<td>0.66</td>
<td>$p&lt;0.0001$</td>
<td>33</td>
</tr>
<tr>
<td>Plasma triglycerides</td>
<td>0.48</td>
<td>$p&gt;0.05$</td>
<td>29</td>
</tr>
<tr>
<td>Plasma HDL</td>
<td>0.43</td>
<td>$p&gt;0.05$</td>
<td>30</td>
</tr>
<tr>
<td>Plasma LDL</td>
<td>0.07</td>
<td>$p&gt;0.05$</td>
<td>29</td>
</tr>
</tbody>
</table>

Correction for multiple comparisons was performed using the False Discovery Rate of Benjamini and Hochberg
CRP= C- reactive protein, HDL= high density lipoprotein, LDL= low density lipoprotein
A version of this table was published in Sallam et al. (2011)
3-2-2 Unaltered contractile responses in the carotid arteries of \textit{db/db} mice

Similar to the vasorelaxation responses, the carotid arteries of \textit{db/db} \textit{(10-14 weeks old)} mice contracted by the same magnitude in response to PE and KCl as their age-matched control mice (Fig 3-19).

![Graph showing contractile responses to KCl and phenylephrine](image)

**Figure 3-19:** Contractile responses to KCl (upper panel) and phenylephrine (lower panel) in the carotid arteries of \textit{db/db} and control mice at 10-14 weeks of age.

Values are displayed as mean ± S.E. and represent \( n = 10 \) mice

Statistical analysis was done using two-way repeated measures ANOVA
3-2-3 Diminished contractile responses in the femoral arteries of db/db mice
In contrast to the aortae, the femoral arteries of db/db (10–20 weeks old) mice showed diminished contractile responses to PE and KCl compared with control mice (Fig 3-20). However, after normalizing PE-induced contractions to maximum KCl (80 mM) contraction, the differences between db/db and control mice were insignificant. The attenuated PE-induced contractions in db/db mice were not ameliorated after 30 minutes incubation with L-NAME (10^-4 M) as shown in figure (3-15), or indomethacin (10^-5 M) (Emax= 25.05 ± 1.92 % KCl 80 mM contraction).
Figure 3-20: Contractile responses to KCl (upper panel) and phenylephrine (lower panel) in the femoral arteries of db/db and control mice at 10-20 weeks of age

Values are displayed as mean ± S.E. and represent n= 10-15 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 compared to control femoral (10-20 wk)
3-2-3-1 Effects of xanthine/xanthine oxidase on the contractile responses in the femoral arteries

Although oxidative stress has been repeatedly linked to augmented contractile responses in diabetes and hypertension, it was intriguing to examine if the attenuated contractions in the femoral arteries of db/db mice could also be attributed to oxidative stress. Challenging the femoral arteries of control mice with an exogenous source of $O_2^-$: xanthine ($10^{-4}$ M) + xanthine oxidase (0.01 U/ml) for 30 minutes, significantly suppressed the contractile responses to both KCl (50 mM) and PE in the femoral arteries (Fig 3-21), raising questions about the target(s) of ROS in the femoral artery and the aorta, and whether acting on distinct targets in different arteries could explain the diverse functional outcomes.
Figure 3-21: Contractile responses to KCl (upper panel) and phenylephrine (lower panel) in the femoral arteries of control mice (14-20 weeks old) before and after xanthine/xanthine oxidase challenge

Values are displayed as mean ± S.E. and represent n= 6-20 rings from 3-6 mice
Statistical analysis was done using paired t test for KCl responses and two-way repeated measures ANOVA followed by Bonferroni post-test for phenylephrine responses
# denotes p<0.05 compared to KCl (50 mM), * denotes p<0.05 compared to control femoral (14-20 wk)
X= xanthine, XO= xanthine oxidase
3-2-3-2 Source(s) of intracellular calcium for PE-induced contractions in the aortae and femoral arteries
The characteristics of PE concentration response curves were different in the aorta compared with the femoral artery. Maximum response to PE in the aortae of control mice was 378.5 ± 12.84 % of max KCl contraction; whereas, in the femoral artery it equaled 70.07 ± 3.41% of max KCl contraction (p<0.05). Similarly, sensitivity to PE was different in the two arteries (logEC50: aorta= -6.73 ± 0.08 vs. femoral= -5.99 ± 0.13, p<0.05) suggesting that the mechanisms underlying the contractions in the two arteries may be different.

I examined the source(s) of calcium mediating PE-induced contractions in both arteries by using nifedipine as a blocker of voltage gated calcium channels (VGCC) (the main route for extracellular calcium influx), and cyclopiazonic acid as a blocker of SERCA to deplete the sarcoplasmic reticulum (the main intracellular calcium store). In the aortae, both nifedipine and cyclopiazonic acid almost abolished the contractions, indicating that both extracellular and intracellular calcium are required for PE-induced contractions. In the femoral arteries, nifedipine but not cyclopiazonic acid suppressed the contractions (Fig 3-22), suggesting that the influx of extracellular calcium via VGCC is the main source of intracellular calcium needed to elicit contractions. Consistent with this finding, the maximum PE response was comparable (70.07 ± 3.41 %) to maximum KCl-induced contraction in the femoral arteries as both agents relay on the same pathway (influx of extracellular calcium via VGCC). However, PE-induced contractions in the aortae were 378.5 ± 12.84 % of maximum KCl contraction, suggesting that there is another source of calcium in addition to extracellular calcium influx.
Figure 3-22: Cumulative concentration response curves of phenylephrine in the aortae (upper panel) and the femoral arteries (lower panel) of control mice in the presence or the absence of nifedipine or cyclopiazonic acid

Values are displayed as mean ± S.E. and represent n= 4-12 rings from 3-5 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
# denotes p<0.05 vs. control aorta, * denotes p<0.05 vs. control femoral
3-2-3-3 The role of Kv in the attenuated contractile responses in the femoral arteries of \textit{db/db} mice

Potassium channels are abundant in vascular tissues. Potassium ion efflux is the main hyperpolarizing influence that buffers depolarization and vasoconstriction. I investigated whether the attenuated contractions in the femoral arteries of \textit{db/db} mice could be attributed to increased K\(^+\) channels function. I examined the effects of 4-AP as a blocker of K\(v\) on basal tension and PE-induced contractions. An increased constriction to 4-AP was observed in \textit{db/db} mice (13.81 ± 2.76 % maximum KCl contraction) vs. control mice (5.41 ± 0.7 % maximum KCl contraction, \(p<0.05\)), indicating an enhanced function of K\(v\) under resting conditions in \textit{db/db} mice. However, 4-AP shifted PE concentration response curves to the left to the same extend in \textit{db/db} and control mice, and did not increase the maximum contractions in \textit{db/db} mice (Fig 3-23). Therefore, the attenuated contractile responses in the femoral arteries of \textit{db/db} mice could not be attributed to an increased K\(v\) background activity despite their enhanced function under resting conditions.

3-2-4 Measurements of the internal diameters of the aortae, carotid and femoral arteries in \textit{db/db} and control mice

There were no significant differences in the internal diameters of the aortae, carotid or femoral arteries between \textit{db/db} (0.68 ± 0.02 mm, 0.29 ± 0.01 mm, 0.24 ± 0.01 mm) and control mice (0.64 ± 0.03 mm, 0.29 ± 0.01 mm, 0.25 ± 0.01 mm), respectively.
Figure 3-23: Effects of 4-aminopyridine on basal tension (upper panel) and on phenylephrine cumulative concentration response curves (lower panel) in the femoral arteries of db/db and control mice (14-20 weeks old)

Values are displayed as mean ± S.E. and represent n= 9-11 mice
Statistical analysis was done using unpaired t test for 4-AP induced contractions, and two-way repeated measures ANOVA for phenylephrine concentration response curves
* denotes p<0.05 compared to control femoral
3-2-5 Protein expression of SOD-1, SOD-2 and SOD-3 in the aortae, carotid and femoral arteries of db/db and control mice

To elucidate the potential mechanisms underlying the results of the functional experiments, the protein expressions of the three isoforms of SOD: cytosolic (SOD-1), mitochondrial (SOD-2) and extracellular (SOD-3) in the aortae, carotid and femoral arteries were examined. As shown in figure (3-24), the femoral arteries of control and db/db mice strikingly lack the expression of SOD-3 in contrast to the aortae and carotid arteries.
Figure 3-24: Representative western blot of superoxide dismutase isoforms in the aortae, carotid and femoral arteries of db/db and control mice (10-20 weeks old)

n= 4-5 independent experiments
SOD= superoxide dismutase
3-2-6 Summary of the results

The present study revealed marked heterogeneity in the adaptation of the aorta, carotid and femoral arteries to the diabetic milieu. The aortae of db/db mice showed augmented contractile responses and increased sensitivity to constrictor prostanoid(s). The carotid arteries maintained unaltered contractions. On the other hand, the femoral arteries of db/db mice showed attenuated contractile responses coinciding with lack of expression of SOD-3. The results are summarized in illustration (3-2).

**Illustration 3-2: Summary of the results of the second study in the present thesis**

Data displayed are from db/db mice relative to their age matched controls

PE= phenylephrine, SOD= superoxide dismutase

↓ decreased, ↑ increased, ↔ unchanged
3-3 Effect of exercise training on vascular reactivity in the aortae and femoral arteries of \(db/db\) mice

3-3-1 Effects of exercise on the metabolic state

When \(db/db\) mice were 10-14 weeks old, they had heavier body weights, elevated fasting plasma glucose levels, lower insulin sensitivity, increased plasma 8-isoprostane and CRP, and dyslipidemia compared with age-matched controls. Four weeks of exercise reduced plasma 8-isoprostane and triglycerides levels in \(db/db\) mice relative to the sedentary \(db/db\) mice, whereas exercise increased insulin sensitivity and elevated plasma CRP in the normoglycemic control mice compared with sedentary controls (Table 3-5).

Longer term exercise (8 weeks) reduced the body weights of \(db/db\) mice. Body weight reduction was associated with improved glycemic control, as reflected by lower fasting plasma glucose levels and improved glucose tolerance, as well as reduced plasma CRP in \(db/db\) mice. Similar to the effects observed after short-term exercise (4 weeks), plasma 8-isoprostane and triglycerides levels of \(db/db\) mice were reduced by 8 weeks of exercise (Table 3-6).
Table 3-5: Effects of exercise on body weights and plasma variables of db/db (10-14 weeks old) and normoglycemic control mice

<table>
<thead>
<tr>
<th></th>
<th>Control (10-14 wk)</th>
<th>db/db (10-14 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sedentary</td>
<td>Exercise</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>27.96 ± 0.29</td>
<td>25.8 ± 0.4</td>
</tr>
<tr>
<td><strong>Fasting blood glucose (mM)</strong></td>
<td>5.03 ± 0.23</td>
<td>3.34 ± 0.17</td>
</tr>
<tr>
<td><strong>GTT&lt;sub&gt;AUC&lt;/sub&gt; glucose (mM. min)</strong></td>
<td>1234 ± 57.30</td>
<td>1231 ± 22.65</td>
</tr>
<tr>
<td><strong>GTT&lt;sub&gt;AUC&lt;/sub&gt; insulin (ng. min/ml)</strong></td>
<td>129.4 ± 9.23</td>
<td>68.56 ± 6.75</td>
</tr>
<tr>
<td><strong>Insulin sensitivity index</strong></td>
<td>23.66 ± 2.92</td>
<td>45.75 ± 4.15</td>
</tr>
<tr>
<td><strong>Plasma 8-isoprostane (pg/ml)</strong></td>
<td>36.92 ± 6.18</td>
<td>17.03 ± 2.79</td>
</tr>
<tr>
<td><strong>Plasma CRP (ng/ml)</strong></td>
<td>2.57 ± 0.32</td>
<td>3.81 ± 0.23</td>
</tr>
<tr>
<td><strong>Plasma lipid profile</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Triglycerides (mM)</strong></td>
<td>0.72 ± 0.07</td>
<td>0.76 ± 0.08</td>
</tr>
<tr>
<td><strong>Cholesterol (mM)</strong></td>
<td>2.47 ± 0.14</td>
<td>2.38 ± 0.09</td>
</tr>
<tr>
<td><strong>LDL (mM)</strong></td>
<td>0.95 ± 0.09</td>
<td>0.79 ± 0.05</td>
</tr>
<tr>
<td><strong>HDL (mM)</strong></td>
<td>1.19 ± 0.14</td>
<td>1.23 ± 0.07</td>
</tr>
</tbody>
</table>

Values are displayed as mean ± S.E. and represent n= 5-11 except for body weight n= 12-20
Statistical analysis was done using one-way ANOVA followed by Bonferroni post-test
* denotes p<0.05 vs. sedentary control, # denotes p<0.05 vs. exercise control, $ denotes p<0.05 vs. sedentary db/db.
GTT AUC= area under the curve for the glucose tolerance curve, CRP= C-reactive protein, HDL= high density lipoprotein, LDL= Low density lipoprotein.
Table 3-6: Effect of exercise on body weights and plasma variables of *db/db* (14-20 weeks old) and normoglycemic control mice

<table>
<thead>
<tr>
<th></th>
<th>Control (14-20 wk)</th>
<th>db/db (14-20 wk)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sedentary</td>
<td>Exercise</td>
</tr>
<tr>
<td><strong>Body weight (g)</strong></td>
<td>31.9 ± 0.36</td>
<td>28.32 ± 0.58</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*#</td>
</tr>
<tr>
<td><strong>Fasting blood glucose (mM)</strong></td>
<td>5.9 ± 0.50</td>
<td>5.79 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*#</td>
</tr>
<tr>
<td><strong>GTT AUC glucose (mM. min)</strong></td>
<td>1842 ± 132.0</td>
<td>1785 ± 47.87</td>
</tr>
<tr>
<td></td>
<td>*#</td>
<td>*# $</td>
</tr>
<tr>
<td><strong>GTT AUC insulin (ng. min/ml)</strong></td>
<td>147.7 ± 8.79</td>
<td>109.1 ± 5.57</td>
</tr>
<tr>
<td></td>
<td>*#</td>
<td>*#</td>
</tr>
<tr>
<td><strong>Insulin sensitivity index</strong></td>
<td>17.53 ± 1.55</td>
<td>23.70 ± 1.84</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*#</td>
</tr>
<tr>
<td><strong>Plasma 8-isoprostane (pg/ml)</strong></td>
<td>69.93 ± 15.89</td>
<td>57.26 ± 6.83</td>
</tr>
<tr>
<td></td>
<td>*#</td>
<td>*#</td>
</tr>
<tr>
<td><strong>Plasma CRP (ng/ml)</strong></td>
<td>2.51 ± 0.36</td>
<td>4.51 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td><strong>Plasma lipid profile</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mM)</td>
<td>0.72 ± 0.07</td>
<td>0.78 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>*#</td>
<td>$</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>2.47 ± 0.14</td>
<td>2.40 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>#</td>
<td>#</td>
</tr>
<tr>
<td>LDL (mM)</td>
<td>0.95 ± 0.09</td>
<td>0.78 ± 0.05</td>
</tr>
<tr>
<td>HDL (mM)</td>
<td>1.19 ± 0.14</td>
<td>1.28 ± 0.06</td>
</tr>
</tbody>
</table>

Values are displayed as mean ± S.E. and represent n= 5-11 except for body weight n= 12-20
Statistical analysis was done using one-way ANOVA followed by Bonferroni post-test
* denotes $p<0.05$ vs. sedentary control, # denotes $p<0.05$ vs. exercise control, $\$$ denotes $p<0.05$ vs. sedentary *db/db*
GTT AUC= area under the curve for the glucose tolerance curve, CRP= C-reactive protein, HDL= high density lipoprotein, LDL= Low density lipoprotein.
3-3-2 Effects of exercise on the vasomotor function

3-3-2-1 Effects of short-term exercise on the vascular reactivity of the aortae

Short-term exercise restored ACh-induced relaxation in db/db mice (Emax: db/db ex= 76.25 ± 1.32 % vs. db/db sed= 57.58 ± 2.04 %, p<0.05). Preincubation with L-NAME (10^{-4} M) abolished ACh vasorelaxation in both sedentary and exercise groups. Similarly, exercise improved SNP-induced relaxation (Emax: db/db ex= 88.05 ± 1.36 % vs. db/db sed= 74.98 ± 1.474 %, p<0.05) (Fig 3-25).

However, exercise further increased the enhanced contractions in the aortae of db/db mice in response to both 80 mM KCl (db/db ex= 7.83 ± 0.34 mN vs. db/db sed= 6.62 ± 0.48 mN, p<0.05) and PE (Emax: db/db ex= 14.28 ± 0.21 mN vs. db/db sed= 12.71± 0.22 mN, p<0.05) as shown in figure (3-26). Preincubation with indomethacin (10^{-5} M) reduced PE contractions in the aortae of both exercised and sedentary db/db mice and abolished the difference between the two groups (Fig 3-26).
Figure 3-25: Cumulative concentration response curves of acetylcholine (upper panel) and sodium nitroprusside (lower panel) in phenylephrine constricted aortae of sedentary and exercise db/db and control mice at 10-14 weeks of age

Values are displayed as mean ± S.E. and represent n= 6-10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 compared to db/db sed aorta (10-14 wk)
ex= exercise, sed= sedentary
Figure 3-26: Contractile responses to KCl (upper panel) and phenylephrine (middle and lower panel) in the aortae of sedentary and exercise *db/db* and control mice at 10-14 weeks of age

Values are displayed as mean ± S.E. and represent n= 10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes $p<0.05$ compared to control sed aorta (10-14 wk), # denotes $p<0.05$ compared to control ex aorta (10-14 wk), $@$ denotes $p<0.05$ compared to *db/db* sed aorta (10-14 wk), @ denotes $p<0.05$ compared to *db/db* ex aorta (10-14 wk)
ex= exercise, Ind= indomethacin, sed= sedentary
3-3-2-2 Effect of short-term exercise on the vascular reactivity of the femoral arteries

Short-term exercise enhanced ACh-induced vasorelaxation in the femoral arteries from db/db mice (Emax: db/db ex= 71.54 ± 1.41 % vs. db/db sed= 60.79 ± 1.74 %, p<0.05). Preincubation with L-NAME (10^{-4} M) and indomethacin (10^{-5} M) nullified exercise-induced improvement in db/db mice, but did not abolish the vasorelaxation in control mice in contrast to the effect of L-NAME in the aortae (Fig 3-27).

Exercise augmented KCl (80 mM) (Emax: db/db ex= 4.14 ± 0.25 mN vs. db/db sed= 3.02 ± 0.22 mN, p<0.05) and PE (Emax: db/db ex= 3.13 ± 0.16 mN vs. db/db sed= 1.94 ± 0.1 mN, p<0.05) contractions in the femoral arteries of db/db mice. Exercise also enhanced KCl contractions in normoglycemic control mice (Emax: control ex=5.7± 0.78 mN vs. control sed= 4.53±0.78 mN, p<0.05) (Fig 3-28).
Figure 3-27: Cumulative concentration response curves of acetylcholine in the absence (upper panel) or the presence (lower panel) of the combination of L-NAME (10^{-4} M) and indomethacin (10^{-5} M) in U-46619 constricted femoral arteries of sedentary and exercise db/db and control mice at 10-14 weeks of age

Values are displayed as mean ± S.E. and represent n= 6-10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 compared to control sed femoral (10-14 wks), # denotes p<0.05 compared to control ex femoral (10-14 wks), $ denotes p<0.05 compared to db/db sed femoral (10-14 wks)
ex= exercise, L-NAME= N_ω-Nitro-L-arginine methyl ester, sed= sedentary
Figure 3-28: Contractile responses to KCl (upper panel) and phenylephrine (lower panel) in the femoral arteries of sedentary and exercise db/db and control mice at 10-14 weeks of age.

Values are displayed as mean ± S.E. and represent n= 6-10 mice.

Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test.

* denotes $p<0.05$ compared to control sed femoral (10-14 wks), # denotes $p<0.05$ compared to control ex femoral (10-14 wks), $\$$ denotes $p<0.05$ compared to db/db sed femoral (10-14 wks)

sed= sedentary, ex= exercise
3-3-2-3 Effect of long-term exercise on the vascular reactivity of the aortae and femoral arteries

Long-term exercise (8 weeks) improved ACh-induced, NO-mediated vasorelaxation in the aortae and femoral arteries of \( db/db \) mice, but did not restore the L-NAME and indomethacin resistant component in the femoral arteries (Fig 3-29 and 3-30). Also, long-term exercise enhanced SNP-induced vasorelaxation in the aortae of \( db/db \) mice (Fig 3-29).

Unlike the effect of short-term exercise, long-term exercise (8 weeks) did not augment the contractions in response to KCl (80 mM) and PE in the aortae of \( db/db \) mice, but did not reduce the enhanced aortic contractions in \( db/db \) mice to the magnitude observed in normoglycemic control mice (Fig 3-31).

Long-term exercise augmented KCl induced contractions in the femoral arteries of \( db/db \) mice (Emax: \( db/db \) ex= 5.1 ± 0.32 mN vs. \( db/db \) sed= 3.78 ± 0.28 mN, \( p<0.05 \)), without altering PE induced contractions, whereas long-term exercise reduced PE-induced contractions in normoglycemic control mice (Fig 3-32).
Figure 3-29: Cumulative concentration response curves of acetylcholine (upper panel) and sodium nitroprusside (lower panel) in phenylephrine constricted aortae of sedentary and exercise db/db and control mice at 14-20 weeks of age

Values are displayed as mean ± S.E. and represent n= 6-10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 compared to control sed aorta (14-20 wk), # denotes p<0.05 compared to control ex aorta (14-20 wk), @ denotes p<0.05 compared to db/db ex aorta (14-20 wk)
ex= exercise, sed= sedentary
Figure 3-30: Cumulative concentration response curves of acetylcholine in the absence (upper panel) or the presence (lower panel) of the combination of L-NAME (10^{-4} M) and indomethacin (10^{-5} M) in U-46619 constricted femoral arteries of sedentary and exercise db/db and control mice at 14-20 weeks of age

Values are displayed as mean ± S.E. and represent n= 6-10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
$ denotes p<0.05 compared with db/db sed aorta (14-20 wks)
ex= exercise, L-NAME= Nω-Nitro-L-arginine methyl ester, sed= sedentary
Figure 3-31: Contractile responses to KCl (upper panel) and phenylephrine (lower panel) in the aortae of sedentary and exercise db/db and control mice at 14-20 weeks of age

Values are displayed as mean ± S.E. and represent n= 10 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes $p<0.05$ compared to control sed aorta (14-20 wk), # denotes $p<0.05$ compared with control ex aorta (14-20 wk)
sed= sedentary, ex= exercise
Figure 3-32: Contractile responses to KCl (upper panel) and phenylephrine (lower panel) in the femoral arteries of sedentary and exercise db/db and control mice at 14-20 weeks of age

Values are displayed as mean ± S.E. and represent n= 6-9 mice
Statistical analysis was done using two-way repeated measures ANOVA followed by Bonferroni post-test
* denotes p<0.05 compared with control sed femoral (14-20 wk), # denotes p<0.05 compared with control ex femoral (14-20 wk), $ denotes p<0.05 compared with db/db sed femoral (14-20 wk)

sed= sedentary, ex= exercise
3-3-3 Exercise and aortic proteins expression

Four weeks of exercise increased the protein expressions of SOD-2 and SOD-3 in the aortae of \(db/db\) mice compared with sedentary \(db/db\). Long-term exercise (8 weeks) increased the expression of SOD-1 in the aortae of \(db/db\) mice compared to sedentary \(db/db\) (Fig 3-33).

Additionally, short-term exercise elevated the protein content of eNOS and Akt in the aortae of normoglycemic control mice compared with the sedentary controls. However, longer duration of exercise (8 weeks) was required to increase eNOS and Akt expression in the aortae of \(db/db\) mice (Fig 3-34).

Exercise did not alter the aortic protein content of COX-1. However, levels of COX-2 were increased by short (4 weeks) but not long (8 weeks) term exercise in the aortae of \(db/db\) and control compared with the sedentary groups (Fig 3-35).
Figure 3-33: Representative western blot and bar graph of superoxide dismutase isoforms in the aortae of sedentary and exercise db/db and control mice

Values are displayed as mean ± S.E. and represent n= 4-5 experiments
Statistical analysis was done using two-way ANOVA
$ denotes $p<0.05$ compared with db/db sed, @ denotes $p<0.05$ compared with db/db ex.
ex= exercise, sed= sedentary, SOD= superoxide dismutase.
Figure 3-34: Representative western blot and bar graph of eNOS and Akt in the aortae of sedentary and exercise db/db and control mice

Values are displayed as mean ± S.E. and represent n= 4-5 experiments

Statistical analysis was done using two-way ANOVA

$ denotes p<0.05 compared with db/db sed, @ denotes p<0.05 compared with db/db ex, α denotes p<0.05 compared with control sed

eNOS= endothelial nitric oxide synthase, ex= exercise, sed= sedentary
Figure 3-35: Representative western blot and bar graph of COX-1 and COX-2 in the aortae of sedentary and exercise db/db and control mice

Values are displayed as mean ± S.E. and represent n= 4 experiments
Statistical analysis was done using two-way ANOVA
$ denotes p<0.05 compared with db/db sed
COX=cyclooxygenase, ex= exercise, sed= sedentary
3-3-4 Summary of the effects of exercise on the metabolic status, vascular reactivity and aortic enzymes expression in db/db mice

Short-term exercise enhanced NO-mediated vasorelaxation in the aortae and femoral arteries of db/db mice, in parallel with increased SOD-2 and SOD-3 protein expressions, reduced plasma oxidative stress and triglycerides, but independent of weight loss, glycemia, or inflammation. However, exercise could not restore EDHF-mediated vasodilatation in the femoral arteries or mitigate the hypercontractility in the aortae of db/db mice. Longer duration of exercise reduced body weight, glucose intolerance and systemic inflammation, and increased the aortic Akt and eNOS expression, but these changes did not reflect into further improvements in the vascular function compared with short-term exercise (Illustration 3-3).
Illustration 3-3: Summary of the results of the third study in the present thesis

8-IP = 8-isoprostane, ACh = acetylcholine, COX = cyclooxygenase, CRP = C-reactive protein, eNOS = endothelial nitric oxide synthase, NO = nitric oxide, PE = phenylephrine, SNP = sodium nitroprusside, SOD = superoxide dismutase, TC = triglycerides
3-3-5 Correlation analysis between some metabolic variables and ACh maximum response in the aortae of exercise and sedentary \textit{db/db} and control mice

Exercise restored ACh-induced vasodilatation in the aortae of \textit{db/db} mice in parallel with reduced blood glucose, markers of systemic oxidative stress, inflammation and triglycerides. However, the relative contribution of these actions is unclear. When including all four groups: sedentary and exercise \textit{db/db} and normoglycemic mice, ACh maximum response was negatively associated with body weight, plasma glucose, 8-isoprostane and triglycerides. However, when looking specifically at the exercise groups including \textit{db/db} and control mice, or \textit{db/db} only, we did not find a significant correlation between ACh response and any of the metabolic variables examined (Table 3-7).

Table 3-7: Correlation analysis between some metabolic variables and ACh maximum response in the aortae of exercise and sedentary \textit{db/db} and normoglycemic control mice

<table>
<thead>
<tr>
<th></th>
<th>Sedentary and exercise \textit{db/db} and control</th>
<th>Exercise \textit{db/db} and control</th>
<th>Exercise \textit{db/db}</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight</strong></td>
<td>R= 0.49 *, \textit{p}&lt;0.0001 \textit{n}=58</td>
<td>R= -0.24, \textit{p}&gt;0.05 \textit{n}=21</td>
<td>R= -0.01, \textit{p}&gt;0.05 \textit{n}=15</td>
</tr>
<tr>
<td><strong>Plasma glucose</strong></td>
<td>R= -0.45 *, \textit{p}= 0.0004 \textit{n}=56</td>
<td>R= -0.20, \textit{p}&gt;0.05 \textit{n}=21</td>
<td>R= 0.08, \textit{p}&gt;0.05 \textit{n}=15</td>
</tr>
<tr>
<td><strong>Plasma 8-isoprostane</strong></td>
<td>R= -0.41*, \textit{p}= 0.0017 \textit{n}=57</td>
<td>R= 0.1, \textit{p}&gt;0.05 \textit{n}=21</td>
<td>R= 0.37, \textit{p}&gt;0.05 \textit{n}=15</td>
</tr>
<tr>
<td><strong>Plasma CRP</strong></td>
<td>R= -0.19, \textit{p}&gt;0.05 \textit{n}=54</td>
<td>R= 0.17, \textit{p}&gt;0.05 \textit{n}=19</td>
<td>R= 0.19, \textit{p}&gt;0.05 \textit{n}=13</td>
</tr>
<tr>
<td><strong>Plasma triglycerides</strong></td>
<td>R= -0.38 *, \textit{p}= 0.005 \textit{n}=53</td>
<td>R= -0.26, \textit{p}&gt;0.05 \textit{n}=21</td>
<td>R= -0.1, \textit{p}&gt;0.05 \textit{n}=15</td>
</tr>
</tbody>
</table>

CRP= C-reactive protein, \textit{n}= number of data pairs, \textit{p}= statistical significance level, R= Pearson correlation coefficient.
3-3-6 Efficacy of the exercise program

The effectiveness of the exercise program was confirmed by increases in skeletal muscle oxidative capacity, as indicated by citrate synthase activity (control ex$_{(10-14 \text{ weeks old})}$ = 5.0±0.06 μmole.min$^{-1}$.ml$^{-1}$ vs. control sed$_{(10-14 \text{ weeks old})}$ = 4.7±0.053 μmole.min$^{-1}$.ml$^{-1}$, p< 0.05; db/db ex$_{(10-14 \text{ weeks old})}$ = 3.9±0.04 μmole.min$^{-1}$.ml$^{-1}$ vs. db/db sed$_{(10-14 \text{ weeks old})}$ = 3.6±0.06 μmole.min$^{-1}$.ml$^{-1}$, p<0.05; control ex$_{(14-20 \text{ weeks old})}$ = 6.61 ± 0.54 μmole.min$^{-1}$.ml$^{-1}$ vs. control sed$_{(14-20 \text{ weeks old})}$ = 4.21±0.32 μmole.min$^{-1}$.ml$^{-1}$, p<0.05).

The heart/body weight ratio of control ex (0.43± 0.01%) was higher than control sed (0.39 ± 0.01%, p<0.05). Similarly, db/db ex (0.26±0.01%) had higher heart/body weight ratios than db/db sed (0.23±0.01%, p<0.05).
4-Discussion

4-1 Regional heterogeneity of the endothelium-dependent and independent vasodilatation in db/db mice

4-1-1 Progression of the metabolic status in db/db mice

At the age of 6-8 weeks, db/db mice were obese, hyperinsulinemic, glucose intolerant, and hyperlipidemic, and exhibited higher levels of systemic inflammation compared with their age-matched controls, but their fasting plasma glucose levels were not significantly higher. These results are consistent with previous reports (Miike et al., 2008, San Martin et al., 2007) showing that db/db mice at that age are hyperinsulinemic and hyperlipidemic, but normoglycemic and thus they are considered prediabetics. It is interesting to note that systemic inflammation (indicated by the plasma CRP level) was observed in db/db mice at an earlier stage (6-8 weeks old) than systemic oxidative stress (indicated by the plasma 8-isoprostane level). This finding supports the notion that chronic inflammation is an early process in the pathogenesis of diabetes that predates oxidative injury (Qamirani et al., 2005, Festa et al., 2003, Sattar, 2004, Busija et al., 2004, Helmersson et al., 2004, Andersen and Pedersen, 2008).

By the age of 10-14 weeks old, db/db mice were fully diabetic. Their blood glucose level was 6-fold higher than their age-matched controls, and they showed significantly elevated systemic oxidative stress as indicated by the level of plasma 8-isoprostane. At that stage, db/db mice shared many of the perturbations: hyperglycemia, hyperinsulinemia and hyperlipidemia that are observed in type 2 diabetic patients. The glucose tolerance test showed a progressive deterioration of glycemic control in db/db mice with age (from 6-8 to 14-20 weeks). Similarly, there were age-related increases in body weight, plasma 8-isoprostane, CRP and triglycerides
and a decrease in glucose tolerance in db/db mice indicating the progression of the diabetic milieu.

4-1-2 Impaired endothelium-dependent and independent vasorelaxation in the aortae of db/db mice

4-1-2-1 Endothelial dysfunction
Functional experiments showed that aortae of db/db (6-8 weeks old) exhibited a slightly but significantly impaired response to ACh (an endothelium-dependent vasodilator), but not to SNP indicating endothelial dysfunction. Endothelial dysfunction has been shown to precede the onset of frank hyperglycemia in both experimental animals (Viswanad et al., 2006, Miller et al., 1999, Erdos et al., 2002) and clinical studies (Caballero et al., 1999, Andersen and Pedersen, 2008). However, these results disagree with the results of Miike et al. (2008) study which showed that maximal ACh-induced relaxation was not reduced in the aortae of prediabetic db/db mice (6 weeks old). The discrepancy could be because of an age difference as I examined db/db mice at a slightly older age (6-8 weeks), or because of methodological differences including the composition of the working solution (PSS vs. Krebs–Henseleit solution), the nature of the background constrictor tone (PE vs. PGF\(_{2\alpha}\)) and/or the method used to calculate vasorelaxation (percent relaxation with respect to the initial PE constriction vs. percentage of papaverine induced relaxation).

The aortae of db/db mice showed a progressive deterioration in the response to ACh with age. Impaired ACh-induced vasorelaxation was reported in db/db aortae (Lam et al., 2006, Miike et al., 2008, Zhong et al., 2007, San Martin et al., 2007, Wong et al., 2010, Kanie and Kamata, 2000), mesenteric arteries (Pannirselvam et al., 2002, Lagaud et al., 2001) and coronaries (Park et al., 2008, Bagi et al., 2003, Bagi et al., 2004). The obesity, inflammation, dyslipidemia and insulin resistance evident in db/db mice could all be contributing to the observed endothelial
dysfunction via an array of mechanisms proposed, examined, and reviewed previously (Guerci et al., 2001, Sprague and Khalil, 2009, Feletou and Vanhoutte, 2006, Busija et al., 2004, Hsueh et al., 2004, Kim et al., 2006, Ritchie et al., 2004, Cersosimo and DeFronzo, 2006, Gillham et al., 2008, Woodman et al., 2005, Okon et al., 2007). The correlation analysis showed that ACh maximum response was negatively correlated with body weight, plasma glucose and plasma 8-isoprostane. The stepwise model selection identified body weight as the major determinant of ACh vasodilatation in the aortae of db/db and control mice.

4-1-2-2 Vascular smooth muscles dysfunction
The aortae started to exhibit attenuated SNP-induced vasorelaxation at 14-20 weeks of age indicating a dysfunction at the level of VSMs. These results suggest that the endothelium is vulnerable to the metabolic abnormalities associated with diabetes at an earlier stage than VSMs; however, with the progression of the disease, VSMs are inevitably affected. Previous studies have reported reduced responses to NO donors in db/db aortae (Lam et al., 2006) and coronaries (Bagi et al., 2004), as well as in other animal models of diabetes (Lam et al., 2006, Bagi et al., 2004, Bitar et al., 2005, Majithiya and Balaraman, 2005, Frisbee, 2001) and diabetic patients (Caballero et al., 1999).

4-1-2-3 Relative contributions of NADPH oxidase, COX, NOS to the impaired vasodilatation in the aortae of db/db mice
Although the role of oxidative stress in the diabetic vasculopathy is not disputed, the source(s) of ROS and their relative contribution to the vascular injury are not well characterized. There are several potential sources of ROS in the vasculature including NADPH oxidase, COX, uncoupled NOS, arachidonic acid metabolites and the mitochondrial respiratory chain enzymes (Feletou and Vanhoutte, 2006, Wolin, 1996, Brandes and Kreuzer, 2005). Overproduced ROS can interfere with ACh signalling at multiple sites: receptor binding, eNOS activation and signalling,
NO degradation, activation of soluble guanyl cyclase and/or relaxation of the contractile proteins (De Vriese et al., 2000, Feletou and Vanhoutte, 2006, Lounsbury et al., 2000, Pannirselvam et al., 2006).

I anticipated that inhibiting the enzymatic system with the largest input of ROS in the aortae of db/db mice would cause the most marked improvement in ACh-induced relaxation. My results show that ACh-induced vasorelaxation in db/db(14-20 wk) mice was not improved by indomethacin, apocynin, or L-NAME. To rule out the possibility that there are sources of ROS other than NADPH oxidase, eNOS or COX contributing to vascular dysfunction, I used tempol, a cell-permeable SOD mimetic, at a dose that caused maximum suppression of the O$_2^-$ level in cultured VSMs (Luo et al., 2009). Similarly, tempol failed to ameliorate ACh-induced vasorelaxation in the aortic rings of db/db mice. It is possible that the vascular dysfunction is irreversible at this age/stage (14-20 weeks) in db/db mice, or at least it could not be ameliorated by an acute (30 minutes) in vitro incubation, particularly given that there are other metabolic abnormalities associated with diabetes besides oxidative stress, namely inflammation and dyslipidemia which were evident in db/db mice. Similarly, incubation with SOD could not restore the bioavailability of NO in the aortae from old rats (Tschudi et al., 1996) and catechin, an antioxidant polyphenol, could not improved endothelial function in aged atherosclerotic mice (Gendron et al., 2010). The multifactorial nature of diabetic vasculopathy has been noted in previous studies (Woodman et al., 2005, Okon et al., 2007) and is further supported by the finding that the responses to all ACh, SNP and isoprenaline were impaired at that age, implying that the dysfunction probably goes beyond scavenging of NO by ROS.

My results are consistent with those of Miike et al. (2008), which showed that ACh-induced vasorelaxation in the aortae of db/db mice (11 weeks old) was not improved by ozagrel, a thromboxane A$_2$ synthetase inhibitor, or acetylsalicylic acid, a COX inhibitor, indicating no
involvement of prostanoid(s) in the impaired vasorelaxation. However, the current results disagree with the San Martin et al. (2007) study which showed that incubation with apocynin (5 x 10^{-4} M) for 30 minutes was able to restore ACh-induced vasorelaxation in the aortae of db/db_{(12 weeks old)} mice. Also, another study showed that apocynin (10^{-4} M) improved ACh-induced vasorelaxation in the coronary arteries of db/db_{(12 weeks old)} mice (Bagi et al., 2004). The discrepancy could be attributed to the difference in the age of the mice used (12 weeks vs. 14-20 weeks), which may reflect different stages of diabetes and possibly different mechanisms underlying the vascular dysfunction. Similarly, a previous study showed that curcumin treatment attenuated the vascular dysfunction in the aortae of STZ-induced diabetic rats at the early stage but not the medium and late stages of diabetes (Majithiya and Balaraman, 2005). Also, Okon et al. (2007) showed that the endothelial dysfunction in ob/ob mice was correlated with the increase in plasma levels of glucose, insulin, and lipids during the early stage but not the later stage in this animal model. My results disagree with (Wong et al., 2010) who showed that preincubation with apocynin (10^{-4} M) or tempol (10^{-4} M) for 30 minutes restored ACh-induced vasorelaxation in the aortae of db/db_{(18 weeks old)} mice; the reasons for this discrepancy are not fully known, but different animal sources and experimental conditions (working solutions and resting tension 3 mN vs. 5.5 mN) may play a role. Additionally, there has been some dispute about the mechanism of action of apocynin and whether it acts as a NADPH oxidase inhibitor in the vasculature or not (Touyz, 2008).

4-1-2-4 Generalized vascular dysfunction in the aortae of db/db mice
To investigate if the vascular dysfunction observed in the aortae of db/db is confined to the NO-mediated pathway, I examined the responses to isoprenaline: a β-adrenoceptor agonist that acts mainly via the cAMP pathway, and K_{Ca} and/or BK_{Ca} (Harden, 1983, Matsushita et al., 2006, Li et al., 2003, Gutterman et al., 2005, Chai et al., 2005, Eichhorn and Dobrev, 2007). Aortae of
*db/db* (14-20 weeks old) mice showed impaired responses to isoprenaline. These findings are in agreement with previous studies showing reduced responses to isoprenaline under diabetic conditions (Chai *et al.*, 2005, Mayhan, 1994a, Pelligrino *et al.*, 1994, Bubolz *et al.*, 2005). The dysfunction was mostly attributed to reduced $K_v$ expression or function (Bubolz *et al.*, 2005, Chai *et al.*, 2007). The fact that the aortae of *db/db* mice showed impaired responses to all three vasodilators: ACh, SNP and isoprenaline, suggests a general dysfunction in the VSMs that may not be limited to the cGMP or cAMP pathways. Furthermore, the contractile responses of the aortae to several contractile agents acting via different pathways were augmented (described later), further reinforcing the suggestion of a general dysfunction in VSMs that possibly involves intracellular Ca$^{2+}$ dysregulation. Reactive oxygen species are known to alter vascular Ca$^{2+}$ regulation including sarcoplasmic reticulum Ca$^{2+}$-transporting ATPase (SERCA) inhibition, intracellular Ca$^{2+}$ stores mobilization, extracellular Ca$^{2+}$ influx and/or contractile proteins sensitization (Jin *et al.*, 1991). Also, some inflammatory cytokines have been reported to affect Ca$^{2+}$ channels expression and activity (Sprague and Khalil, 2009).

However, other investigators have reported non-generalized impairment of vasorelaxation in some diabetic models: the response to SNP but not to isoprenaline or cromakalim was reduced in the thoracic aortae of *db/db* mice (Lam *et al.*, 2006), the response to isoprenaline but not to nitroglycerin or forskolin was impaired in the pial arterioles of streptozotocin-induced diabetic rats (Mayhan, 1994b), and attenuated responses to ACh and SNP but not to forskolin or aprikalim were reported in the gracilis arteries of obese Zucker rats (Frisbee, 2001).

**4.1-3 Carotid arteries of *db/db* mice maintain full relaxation to ACh and SNP**

In contrast to the progressive impairment of vasorelaxation in the aortae, the carotid arteries of *db/db* mice showed unaltered vasodilatation in response to ACh and SNP throughout the three ages examined. These findings add to an expanding body of evidence demonstrating the
differential adaptation of various arteries to different pathological conditions such as aging, hypoxia, hypercholesterolemia and streptozotocin induced diabetes (Tables 1-3 and 1-4).

4-1-3-1 Mechanisms underlying vascular heterogeneity

Although vascular heterogeneity is a well-accepted and fairly-documented concept, very few studies have attempted to delineate the mechanisms underlying such heterogeneity. In the present study, the difference between the aorta and carotid artery could be attributed to either different local environments in the aorta and carotid artery or different signalling cascades. The first possible explanation is that the local redox status is different in the aorta compared with the carotid artery. The local redox status depends on the magnitude of ROS production and ROS removal by the local antioxidant defence mechanisms. Therefore, it is possible that the local production of ROS in the carotid arteries is not upregulated in db/db mice. Alternatively, the antioxidant defence mechanisms in the carotid arteries may be upregulated to counterbalance the increase in local production of ROS, thus maintaining normal redox status in the carotid arteries of db/db. To address these possibilities, I induced oxidative stress by using the xanthine/xanthine oxidase system, or by inhibiting SOD, the key antioxidant enzyme, in both the carotid arteries and aortae of control mice. Through both interventions, the carotid arteries but not the aortae fully relaxed in response to ACh, indicating that the carotid artery but not the aorta can resist oxidative stress. Although I did not directly measure the level of ROS in the carotid arteries, two other studies (Bagi et al., 2003, Bagi et al., 2004) showed enhanced $\text{O}_2^-$ production (detected by dihydroethydine staining and lucigenin enhanced chemiluminescence) in the carotid arteries of db/db (12 weeks old) mice relative to their age-matched controls. All together, these results indicate that the carotid artery can resist oxidative stress more than the aorta, and suggest that the signalling cascade rather than the local environment is different between the two arteries. Differences in signalling cascade can arise from different receptor subtypes that are activated by
ACh in the aorta and carotid artery. In agreement with my results, a previous study showed that the rabbit carotid arteries but not aortae resisted the in vitro induction of oxidative stress and maintained a normal response to ACh (Pagano et al., 1999) and several studies showed that different arteries express distinct receptor subtypes (Lewis et al., 2005, Zheng et al., 2008, Mueed et al., 2004).

4-1-3-2 ROS mediate vasorelaxation in the carotid arteries
ACh-induced vasorelaxation was abolished by L-NAME preincubation in both the aortae and carotid arteries of db/db and control mice, signifying that ACh-vasorelaxation was totally mediated by NOS in both arteries. However, the carotid artery showed different maximum response and sensitivity to ACh compared with the aorta; whereas both the aorta and carotid artery show similar maximum response and sensitivity to SNP, suggesting that ACh-signalling pathway in the carotid artery is different from that in the aorta and that the vasoactive factor(s) mediating ACh vasorelaxation in carotid is/are different from the NO species released by SNP, albeit this/these vasomediator(s) is/are NOS dependent. Similarly, a recent study (Leo et al., 2010) demonstrated that the carotid artery in rats maintained normal responses to ACh despite a concurrent diabetes-related decrease in NO release and an increase in O$_2^-$ level due to the emergence of a new vasodilator component in diabetic arteries that is NOS independent, but interestingly still involves NO or NO derivative(s).

4-1-3-3 ROS as Vasodilators
While ROS are notorious for their detrimental actions on various cellular targets and vascular functions (De Vriese et al., 2000, Feletou and Vanhoutte, 2006, Lounsbury et al., 2000, Woodman et al., 2005); ROS are also increasingly recognized as physiologically important mediators that play key roles in cellular signalling (Wolin, 1996, Gutterman et al., 2005, Faraci, 2006, Liaudet et al., 2009). Indeed, ACh induced vasorelaxation was shown to be mediated by
O$_2^-$ or its metabolite(s) in the rat basilar artery (Hong et al., 1989), by H$_2$O$_2$ in the rat aorta (Karasu, 2000) and femoral arteries (Leung et al., 2006), and by nitrosothiols in the rat carotid artery (Leo et al., 2010). Also, exogenously-introduced ROS released NO from intracellular stores in human umbilical vein endothelial cells (Ng et al., 2007).

4-1-3-4 Dual effects of antioxidants
On the other hand, antioxidants are known for their ability to restore endothelium-dependent vasorelaxation under certain pathological conditions, e.g. diabetes and hypertension (Frisbee, 2001, MacKenzie and Martin, 1998, Ling et al., 2005, Rodriguez-Manas et al., 2003, Rosen et al., 1995). However, antioxidants are also shown to reduce exercise-induced brachial artery vasodilatation in healthy volunteers (Richardson et al., 2007), and ebselen (a ONOO$^-$ scavenger) was shown to attenuate ACh-depressor response in anesthetised rats (Dabisch et al., 2008).

4-1-3-5 Peroxynitrite as a vasodilator
These previous findings open the possibility that ACh-induced vasorelaxation in the carotid arteries is mediated by a NOS-generated ROS. Previous studies have shown that NOS can produce products other than NO, e.g. O$_2^-$ (Feletou and Vanhoutte, 2006, Leo et al., 2010, Rabelink and Luscher, 2006, Sabri et al., 2010, Schmidt and Alp, 2007) and H$_2$O$_2$ (Leung et al., 2006, Drouin and Thorin, 2009, Matoba et al., 2000). If this is the case, then the response to ACh may be preserved in the carotid artery even under conditions of oxidative stress associated with diabetes. To examine this possibility, I preincubated the carotid arteries of control mice with tempol to scavenge O$_2^-$, catalase to decompose H$_2$O$_2$, or ebselen to scavenge ONOO$^-$ before constructing ACh concentration response curves. Ebselen, and to a lesser extent tempol, blunted ACh-induced vasorelaxation suggesting, that ONOO$^-$ plays a key role in mediating ACh-induced vasorelaxation in the carotid arteries. These results are consistent with previous studies that
demonstrated the ability of ONOO\textsuperscript{–} to induce vasodilatation in several species and arteries (Wei et al., 1996, Wu et al., 1994, Villa et al., 1994, Benkusky et al., 1998, Ohashi et al., 2005).

4-1-3-6 Signalling pathway of ONOO\textsuperscript{–} as a vasodilator

Earlier studies have reported that ROS can modulate the vascular tone through an array of mechanisms including direct interactions with potassium channels (Gutterman et al., 2005, Liu and Gutterman, 2002). Only 4-AP but not glybenclamide, BaCl\textsubscript{2}, or the combination of charybdotoxin with apamin could attenuate ACh-induced vasorelaxation in the carotid arteries indicating that K\textsubscript{v} mediates (at least partially) the vasorelaxant action of ACh in the carotid arteries. The combination of ebselen and 4-AP did not inhibit ACh induced vasorelaxation any further than either of the two agents individually, suggesting that ONOO\textsuperscript{–} and K\textsubscript{v} act on the same pathway and that K\textsubscript{v} may be the target of ONOO\textsuperscript{–} in the carotid artery.

My results are in agreement with earlier studies which showed that ONOO\textsuperscript{–}-induced vasorelaxation via activation of potassium channels (Wei et al., 1996, Li et al., 2004a, Ohashi et al., 2005). However, none of the previous studies suggested that ONOO\textsuperscript{–} act at K\textsubscript{v}. Actually, another study showed that ONOO\textsuperscript{–} inhibited K\textsubscript{v} in isolated rat coronary arteries (Li et al., 2004a). However, the interactions of potassium channels with ROS are particularly complex and our knowledge is still limited (Gutterman et al., 2005, Liu and Gutterman, 2002). It is possible that ONOO\textsuperscript{–} interacts differently with distinct subtypes of K\textsubscript{v}. Other studies have suggested that ONOO\textsuperscript{–} induces vasorelaxation via interacting with the thiol groups in some cellular components, e.g. glutathione to form s-nitrosothiols, which can directly activate guanylyl cyclase or release NO causing vasodilatation (Wu et al., 1994, Mayer et al., 1995, Li et al., 2004a, Ronson et al., 1999). However, the present study did not explore the role of nitrosothiols and their participation in the signalling cascade should not be ruled out.
4-1-3-7 Differential response of the carotid artery and aorta to exogenously-produced ROS

To confirm that ONOO⁻ mediates vasorelaxation in the carotid artery via the activation of Kᵥ, I exposed the carotid artery to exogenously-produced ONOO⁻ [xanthine + xanthine oxidase + a low concentration of SNP] in the presence or the absence of 4-AP. I found that the addition of xanthine/xanthine oxidase with or without SNP to constricted carotid arteries induces vasodilatation that was abolished by 4-AP. Contrary to the carotid, the aorta contracts in response to xanthine/xanthine oxidase but still relaxes in response to ACh. Several studies have showed that the effects of ROS and particularly ONOO⁻ are site-dependent. Peroxynitrite induced vasodilatation in the rabbit internal carotid artery, but not in the common carotid artery (Ohashi et al., 2005). Similarly, ONOO⁻ induced vasodilatation in the rat hindquarters but not the pulmonary vasculature (Nossaman et al., 2004), and reduced hindquarter and mesenteric but not renal vascular resistances (Benkusky et al., 1998). Furthermore, ONOO⁻ impaired isoprenaline-induced relaxation in hindquarters and renal but not mesenteric vascular beds (Lewis et al., 2005). Also, a recent study showed that O₂⁻ causes Rho kinase-dependent Ca²⁺ sensitization in the pulmonary artery, resulting in constriction; whilst O₂⁻ opens Kᵥ and relaxes the mesenteric artery (Snetkov et al., 2011). The differential effects of ROS may be attributed to different spatial arrangements of ROS and other cellular targets which can provide specificity in signalling (Forman et al., 2002).

Altogether, my results suggest that ACh-induced vasorelaxation in the carotid artery via stimulation of NOS which releases NO; in turn, NO reacts with O₂⁻ (formed by NOS or another source) to form ONOO⁻ which diffuses to VSMs and activates Kᵥ causing relaxation. This pathway may explain how the carotid arteries can accommodate oxidative stress and maintain
full relaxation in response to ACh under diabetic conditions since the product(s) of NO under oxidative stress can still relax the carotid arteries.

4-1-4 Impaired endothelium-dependent vasorelaxation in the femoral arteries of \textit{db/db} mice

Similar to the aortae, and contrary to the carotid arteries, the femoral arteries exhibited impaired ACh-induced vasorelaxation from the age of 6-8 weeks old. However, ACh-induced vasorelaxation in the femoral arteries was only partially inhibited by the combination of L-NAME and indomethacin, indicating the contribution of a (NOS and COX) independent pathway that was abolished by high extracellular KCl or the combination of charybdoxotoxin and apamin indicating the hyperpolarizing nature of his component, commonly refered to as EDHF. This finding adds to an expanding body of evidence demonstrating that EDHF plays a more pronounced role in smaller vessels (De Vriese \textit{et al.}, 2000, Shi \textit{et al.}, 2006, Nagao \textit{et al.}, 1992, Cowan \textit{et al.}, 1993, Hill \textit{et al.}, 2001). The EDHF-mediated component was markedly impaired in \textit{db/db} mice at the earliest age examined (6-8 weeks old); this finding is consistent with previous studies (Miller \textit{et al.}, 1999, Katakam \textit{et al.}, 2000, Katakam \textit{et al.}, 1999, Young \textit{et al.}, 2008), which showed that EDHF-mediated vasodilatation was impaired in insulin-resistant rats.

Impaired EDHF could be attributed to defects in K$^{+}$ channel function (Erdos \textit{et al.}, 2002, Matsumoto \textit{et al.}, 2006, Burnham \textit{et al.}, 2006, Ding \textit{et al.}, 2005) and/or gap junctions (Young \textit{et al.}, 2008, Ding \textit{et al.}, 2005, Ding \textit{et al.}, 2005). Inflammation (Gillham \textit{et al.}, 2008), oxidative stress (Feletou and Vanhoutte, 2006, Kusama \textit{et al.}, 2005) and hyperlipidemia (Matsumoto \textit{et al.}, 2006) have all been suggested as contributing to EDHF impairment, although the exact mechanisms are largely unknown.
### 4-1-4-1 Identity of EDHF in the femoral arteries

There are many candidates for EDHF (Feletou and Vanhoutte, 2009) and the list has not been finalized yet. In the femoral arteries, the EDHF component was abolished by a depolarizing concentration of KCl or the combination of charybdotoxin and apamin, but not by BaCl$_2$ or sulfaphenazole, indicating the involvement of $K_{Ca}$ but not $K_{IR}$ or cytochrome P450. The role of cytochrome P450 in mediating EDHF was evident in some arteries (Pannirselvam et al., 2006, Katakam et al., 2000, Hungerford et al., 2000, Nishikawa et al., 1999, Vanheel and Van de Voorde, 1997, Widmann et al., 1998) but not in others (Dabisch et al., 2008, Eichler et al., 2003, Coleman et al., 2001a, Coleman et al., 2001b, de Wit et al., 1999, Eckman et al., 1998).

In $db/db$ mice, EDHF was preserved in small mesenteric arteries (Pannirselvam et al., 2002) where it was mediated by cytochrome P450 isoenzyme and BK$_{Ca}$ (Pannirselvam et al., 2006), whereas in the coronary arterioles, EDHF was attenuated but its relative contribution to ACh-induced vasodilatation increased (Park et al., 2008).

### 4-1-4-2 Impaired endothelium-independent vasorelaxation in the femoral arteries of $db/db$ mice

At 14-20 weeks old, $db/db$ mice showed reduced the sensitivity to SNP in the femoral arteries, suggesting a dysfunction at the level of VSMs. These results are in parallel with the findings in the aortae indicating that the endothelium shows earlier vulnerability to the noxious milieu associated with diabetes; however, with the progression of the disease, the VSMs are eventually affected.

Altogether, the present findings demonstrate a substantial heterogeneity between the aorta, carotid and femoral arteries in $db/db$ mice. The aorta exhibited a progressive impairment in endothelium-dependent and independent vasorelaxation, suggesting a generalized dysfunction in the VSMs. On the other hand, the carotid artery resisted the oxidative stress and maintained full
relaxation to ACh since O$_2^-$ and ONOO$^-$ can induce vasodilatation in the carotid artery possibly by activating K$_v$. Only in the femoral artery but not the aorta or the carotid did the EDHF pathway contribute significantly to ACh-induced vasodilatation. This contribution was reduced in $db/db$ mice at an early stage and did not further deteriorate at later ages examined. Illustration (4-1) presents the mechanisms proposed to explain vascular heterogeneity in $db/db$ and control mice.
Illustration 4-1: Schematic diagram illustrating the proposed mechanisms underlying the differential responses of the aorta, carotid and femoral artery to ACh and SNP under physiological and diabetic conditions

ACh = acetylcholine, L-Arg = L-arginine, sGC = soluble guanylyl cyclase, cGMP = cyclic guanosine monophosphate, GTP = guanosine triphosphate, M = muscarinic receptors, NO = nitric oxide, eNOS = endothelial nitric oxide synthase, SNP = sodium nitroprusside, VSM = vascular smooth muscle, ONOO\(^{-}\) = peroxynitrite, O\(_{2}^{-}\) = superoxide anion
4-2 Regional heterogeneity of the vascular contractile responses in 
*db/db* mice and their controls

This study was undertaken to compare the contractile responses of the aortae, carotid and 
femoral arteries from *db/db* and control mice, and to explore the mechanisms underlying the 
arteries’ altered responses under diabetic conditions. A marked diversity exists between these 
arteries. The aortae showed augmented contractile responses, the carotid maintained unaltered 
contractions; meanwhile, the femoral arteries exhibited attenuated responses. These findings 
suggest that although oxidative stress is a common denominator underlying vascular dysfunction, 
its final functional outcome depends on many factors, including the species of ROS involved and 
their respective targets in the vasculature, which can be different in various arteries.

4-2-1 Enhanced contractile responses in the aortae of *db/db* mice

The aortae of *db/db* mice showed enhanced responses to both receptor-dependent agonists (PE 
and U-46619) and receptor-independent agents (KCl and A23197) compared to their 
normoglycemic controls. These findings are in agreement with previous studies showing 
enhanced contractions in the aortae (Miike *et al.*, 2008, Zhong *et al.*, 2007, Kanie and Kamata, 
2000, Piercy and Taylor, 1998) and small mesenteric arteries of *db/db* mice (Pannirselvam *et al.*, 
2002, Xie *et al.*, 2006, Guo *et al.*, 2005, Pannirselvam *et al.*, 2005), as well as in other models of 
Enhancement of responses to all the contractile agents used (PE, U-46619, high K⁺ and A23197), 
which utilize different signalling pathways to increase intracellular calcium to generate force 
(Illustration 1-1), suggests that the dysfunction occurred at a common step, probably at the level 
of cytosolic free calcium regulation and /or the sensitization of the contractile proteins to 
cytosolic calcium.
Indeed, previous studies showed that the gene expression of SERCA was reduced in the aortae of 
\textit{db/db} (Guo \textit{et al.}, 2005). If lower gene expression coincides with reduced protein function, 
higher cytosolic calcium can accumulate and result in hypercontractility. Additionally, increased 
protein expressions of the activated form of CPI-17 and RhoA (Xie \textit{et al.}, 2006), and increased 
activity of Rho kinase (Nuno \textit{et al.}, 2009) were reported in the aortae of \textit{db/db} mice, possibly 
leading to increased calcium sensitization.

\textbf{4-2-1-1 Oxidative stress and vascular hypercontractility}

The role of oxidative stress in the vascular hypercontractility observed in some diabetic models is not disputed (Shi \textit{et al.}, 2007, Feletou and Vanhoutte, 2006, Kanie and Kamata, 2000, Pannirselvam \textit{et al.}, 2005, Erdei \textit{et al.}, 2007, Shi \textit{et al.}, 2007, Ozcelikay \textit{et al.}, 2000, Matsumoto \textit{et al.}, 2007). In particular, Kanie and Kamata (2000) showed that the enhanced norepinephrine contractions in the aortae of \textit{db/db} were reduced by SOD, whereas the contractile responses of the aortae from control mice to norepinephrine were enhanced by diethyldithiocarbamate (an inhibitor of Cu/Zn SOD), confirming the role of oxidative stress in vascular hypercontractility.

\textbf{4-2-1-2 Sources of ROS in the aorta}

The sources of ROS in individual arteries/vascular beds are not fully characterized. Several enzyme systems present in the vasculature (eNOS, CYP450 monoxygenase, COX, and NADPH oxidase) are capable of generating ROS (Feletou and Vanhoutte, 2006, Brandes and Kreuzer, 2005). Hence, I examined the relative contributions of these enzymes to the oxidative burden and hypercontractility in the aortae of \textit{db/db} mice by using their specific inhibitors, namely L-NAME, sulfaphenazole, indomethacin or apocynin, respectively. Only indomethacin could significantly, although partially, alleviate the enhanced PE contractions in the aortae of \textit{db/db} mice indicating a key role for COX and prostanoids.
4-2-1-3 Cyclooxygenase and hypercontractility

Several reports suggested that COX play a role in the augmented vascular contractions associated with diabetes (Bagi et al., 2005, Shi and Vanhoutte, 2008, Guo et al., 2005, Kanie and Kamata, 2000, Pannirselvam et al., 2005, Erdei et al., 2007, Okon et al., 2003, Matsumoto et al., 2007, Tesfamariam et al., 1990). However, this study, to the best of my knowledge, is the first to compare a COX inhibitor with other potential ROS-generating enzyme-inhibitors under the same experimental conditions. Cyclooxygenase enzymes are closely associated with both oxidative stress and inflammation but the scenario is quite complex. Cyclooxygenase enzymes can be activated by ROS and pro-inflammatory cytokines (Kanie and Kamata, 2000, Vanhoutte et al., 2005, Bagi et al., 2006); meanwhile, COX can generate ROS and propagate further inflammatory stimuli. Thus, COX can cause vascular hypercontractility by increasing the synthesis of constrictor prostanoid(s) (Pannirselvam et al., 2005, Erdei et al., 2007, Matsumoto et al., 2007), and by excessive formation of ROS (Kontos, 1987, Finkel and Holbrook, 2000, Finkel and Holbrook, 2000, Tang et al., 2007). In turn, ROS can directly depolarize cell membranes (Kourie, 1998), inhibit potassium channels (Gutterman et al., 2005), facilitate the mobilization of calcium from intracellular stores or extracellular fluids (Lounsbury et al., 2000, Forman et al., 2002), and/or enhance the formation of isoprostanes from membrane lipids which exhibit potent vasoconstrictor activity (Cracowski et al., 2002). To confirm the role of prostanoids, I examined the responses to the thromboxane A$_2$ analogue U-46619 and found an increased sensitivity to U-46619 in the aortae of $db/db$.

Therefore, the augmented contractions in the aortae of $db/db$ mice may be attributed to a dysfunction in cytosolic calcium regulation and increased sensitivity to constrictor prostanoid(s). The role of prostanoids in intracellular calcium regulation, particularly the sensitization of the contractile apparatus, awaits further investigation.
There were no significant differences in the internal diameters of the aortae between \( db/db \) and control mice at the age of 14-20 weeks old, when the vascular dysfunction was most obvious, suggesting that the dysfunction was mainly due to biochemical and not structural changes. Other studies (Xie et al., 2006, Guo et al., 2005) reported similar findings in the aortae in \( db/db \) mice and their controls.

### 4-2-2 Unaltered contractile responses of the carotid arteries of \( db/db \) mice

#### 4-2-2-1 Vascular heterogeneity

Contractile responses to both KCl and PE were similar in the carotid arteries from \( db/db \) and control mice at 10-14 weeks of age. The differential adaptation of the carotid arteries and the aortae to the diabetic milieu is another demonstration of vascular heterogeneity. Previous studies have reported differences in the alterations of the contractile function between distinct vascular beds or arteries under pathological conditions. Renal but not superior mesenteric artery from streptozotocin-induced diabetic rats exhibited an enhanced contractile response to U46619 (Arikawa et al., 2006). The vasopressin-induced contractions were reduced in basilar and coronary arteries, increased in renal arteries, and not modified in tail arteries in streptozotocin-induced diabetic rats (Garcia-Villalon et al., 2003). The responses to NE and 5-HT were enhanced in the aorta, while unchanged in the basilar artery from diabetic rabbits (Abiru et al., 1991). The sensitivity to norepinephrine was decreased in the femoral arteries but increased in the cerebral small arteries from cholesterol-fed rabbits (Simonsen et al., 1991). However, the mechanisms underlying the vascular heterogeneity in these studies were not reported.

The ability of the carotid arteries of \( db/db \) mice to resist the noxious milieu associated with diabetes and to maintain unaltered contractile responses may be explained by our finding that the carotid arteries relax rather than constrict in response to exogenously-generated ROS, in contrast to the responses in the aortae.
4-2-3 Diminished contractile responses in the femoral arteries of db/db mice

In contrast to the aortae, the femoral arteries of \( db/db \) mice showed diminished contractile responses to PE and KCl compared to control mice. Although the majority of studies have associated diabetes with enhanced contractile responses, some studies did report reduced contractile responses in experimental and clinical diabetes: in the aortae (Majithiya and Balaraman, 2005, Kawasaki, 1997, Takahashi et al., 2003, Hattori et al., 1996, Head et al., 1987), femoral (Shi et al., 2007, Shi and Vanhoutte, 2008), tail (Wang et al., 1998), basilar and coronary arteries (Garcia-Villalon et al., 2003), and mesenteric vasculature (Carvalho Leone and Coelho, 2004, Makino and Kamata, 1998) of streptozotocin-induced diabetic rats. Similarly, reduced contractions were demonstrated in the aorta (Lee et al., 2008) and caudal artery (Mita et al., 2010) of Goto-Kakizaki rats. Additionally, attenuated contractions were also reported in intact circulation in conscious streptozotocin-induced diabetic rats (Cheng et al., 2003, Cheng and Pang, 2004) and diabetic patients (van Gurp et al., 2006, Nugent et al., 1996, McAuley et al., 2000).

After normalizing PE-induced contractions to the maximum KCl contraction, the differences between \( db/db \) and control mice were eliminated, suggesting that the dysfunction occurred at a common step in both agents’ pathways and was not confined to adrenergic stimulation. The attenuated contractions in diabetic models were frequently attributed to upregulation of iNOS (Lee et al., 2008, Cheng and Pang, 2004), or dysregulation of COX (Shi and Vanhoutte, 2008, Carvalho Leone and Coelho, 2004, Makino and Kamata, 1998, Marrachelli et al., 2006); however, in my study the attenuated PE-induced contractions in the femoral arteries of \( db/db \) mice were not ameliorated after 30 minutes incubation with either L-NAME (\( 10^{-4} \) M) or indomethacin (\( 10^{-5} \) M).
4-2-3-1 Oxidative stress and vascular hypocontractility

Our results showed that KCl- and PE-induced contractions in the femoral arteries of control mice were suppressed by xanthine/xanthine oxidase preincubation, suggesting a role for oxidative stress in the attenuated contractions in the femoral arteries from db/db mice. Oxidative stress has been frequently associated with vascular hypercontractility (Shi et al., 2007, Feletou and Vanhoutte, 2006, Kanie and Kamata, 2000, Pannirselvam et al., 2005, Erdei et al., 2007, Shi et al., 2007, Ozcelikay et al., 2000, Matsumoto et al., 2007). Reactive oxygen species can cause/enhance either vasoconstriction or vasorelaxation depending on the type of ROS involved, their concentrations in the local environments and their target(s) in the vascular cells (Lounsbury et al., 2000). H$_2$O$_2$ is not only a relaxing hyperpolarizing factor but can also cause depolarization of the VSMs and vasoconstriction (Feletou and Vanhoutte, 2006). Similarly, O$_2^-$ can cause either vasoconstriction (Kanie and Kamata, 2000, Katusic and Vanhoutte, 1989) or vasorelaxation (Hong et al., 1989, Wei et al., 1996).

4-2-3-2 Phenylephrine signalling cascade

The contrasting responses of the femoral arteries and the aortae to the diabetic milieu raised questions about the signalling cascade mediating the contractions in these arteries and the possibility that ROS interact with different targets in the aortae and femoral arteries. Indeed, nifedipine abolished PE contractions in both the aortae and femoral arteries, while cyclopiazonic acid reduced the contractions only in the aortae indicating that PE contraction in the femoral artery was mainly dependent on extracellular calcium influx via VGCC, in contrast to the aorta that required both extracellular calcium influx and calcium release from the sarcoplasmic reticulum. Similarly, Weber et al. (1995) showed that the relative contributions of the signaling mechanisms activated by norepinephrine differ between different arteries.

Previous studies reported that ROS suppressed L-type calcium currents via modification of the sulphydryl groups in the channel protein (Kourie, 1998, Chiamvimonvat et al., 1995). In this
study, xanthine/xanthine oxidase suppressed KCl-induced contractions, which depend mainly on extracellular calcium influx via VGCC. Therefore, it is reasonable to speculate that ROS, targeting the VGCC, the main source for cytoplasmic calcium in the femoral arteries, are likely responsible for the deterioration in the contractile function. The femoral artery may be particularly vulnerable to oxidative stress compared to the aorta or the carotid artery as it strikingly lacks the expression of extracellular SOD, the key enzyme responsible for scavenging \( \text{O}_2^- \) in the space between the endothelium and VSMs.

Conversely, PE contractions in the aorta required both calcium influx from the extracellular space and release from intracellular stores. The interaction of ROS with the sarcoplasmic reticulum in the aorta could lead to a different functional outcome i.e. enhanced contractions (Lounsbury et al., 2000), particularly given that the expression and function of Rho kinase and CPI-17 were reported to be enhanced in the aortae of \( \text{db/db} \) mice (Nuno et al., 2009, Xie et al., 2006). Therefore, different signalling pathways could explain the divergent responses of distinct arteries to the same challenge such as oxidative stress.

4-2-3-3 Potassium channels and diabetes

Under resting conditions, 4-AP caused significant contractions in the femoral arteries of both \( \text{db/db} \) and control mice, indicating that \( \text{K}_v \) regulates basal tone. This finding is in agreement with previous studies highlighting the role of \( \text{K}_v \) in regulating the vasomotor tone (Gutterman et al., 2005, Li et al., 2004a, Tammaro et al., 2004, Sobey, 2001). Although an enhanced function of \( \text{K}_v \) was observed in the femoral arteries of \( \text{db/db} \) mice under resting conditions, it could not explain the attenuated PE contractions. Reduced function of \( \text{K}_v \) has been reported previously in diabetic animal models and was suggested as accounting for impaired vasodilatation (Chai et al., 2005, Bubolz et al., 2005, Chai et al., 2007). However, enhanced function of other subtypes of K channels (\( \text{K}_{\text{ATP}} \) and \( \text{K}_{\text{Ca}} \)) was reported in diabetic models (Sobey, 2001, Pataricza et al., 2008, Ye
et al., 2004) and was explained as an adaptive response to buffer enhanced vasoconstriction stimuli and/or to compensate for reduced NO release. It is also possible that diabetes eventually reduces the ability of the contractile proteins of the vascular smooth muscle to develop tension (Shi and Vanhoutte, 2008, Sanchez et al., 2005, Stephenson et al., 1994). Similarly, high levels of ROS have been shown to cause contractile dysfunction, resulting in skeletal muscle weakness and fatigue (Powers and Jackson, 2008).

Altogether, the femoral arteries of *db/db* mice showed attenuated contractile responses that could not be attributed to increased function of NOS, COX or K_v, but are possibly due to VGCC dysfunction or to the failure of the contractile apparatus. Also, the activity of K_v was increased under resting conditions.

In summary, our study revealed a marked heterogeneity in the adaptation of three arteries to the diabetic milieu. The aortae showed augmented contractile responses, likely due to increased sensitivity to constrictor prostanoid(s), and a dysfunction in cytosolic calcium regulation. The carotid arteries maintained unaltered contractions. On the other hand, the femoral arteries showed attenuated contractile responses, coinciding with the lack of expression of SOD-3 in this artery, possibly leading to excessive ROS accumulation and suppression of VGCC function. These findings suggest that although oxidative stress is a common denominator underlying vascular dysfunction, its final functional outcome can vary depending on the targeted cellular components and the integrity of the antioxidant defence mechanisms. Illustration (4-2) presents the mechanisms proposed to underlie the differential responses of the aorta and femoral artery to PE and high KCl under physiological and diabetic conditions.
Illustration 4-2: Schematic diagram illustrating the proposed mechanisms underlying the differential responses of the aorta and femoral artery to phenylephrine and high KCl under physiological and diabetic conditions

DAG= diacyl glycerol, IP$_3$= inositol triphosphate, MLCK= myosin light chain kinase, MLCP= myosin light chain phosphatase, PE= phenylephrine, PIP$_2$= phosphatidylinositol bisphosphate, PLC= phospholipase C, SOD= superoxide dismutase, VGCC= voltage gated calcium channel
4-3 Effect of exercise training on vascular reactivity in the aortae and femoral arteries of db/db mice

Our study investigated several aspects of vascular reactivity: endothelium-dependent (NO- and EDHF-mediated) and independent vasodilatation, as well as receptor-dependent and independent contractile responses in two different arteries—the aortae and femoral arteries of db/db mice and their controls. We examined changes in vascular reactivity in relation to alterations in cardiovascular risk factors and adaptations of eNOS/Akt, SOD and COX enzyme systems. The functional experiments were conducted on aortae and femoral arteries isolated from the same animal and compared simultaneously under identical conditions.

The present study shows that short-term exercise enhanced NO-mediated vasorelaxation in the aortae and femoral arteries of db/db mice in parallel with increased SOD-2 and SOD-3 protein expressions, reduced plasma oxidative stress and triglycerides, but independent of weight loss, glycemia, or inflammation. However, short-term exercise could not restore EDHF-mediated vasodilatation in the femoral arteries or mitigate the hypercontractility in the aortae of db/db mice. Long-term exercise reduced the body weights of db/db mice, which was associated with improved glucose tolerance, reduced systemic inflammation and increased aortic Akt and eNOS expressions. The correlation analysis suggests that the beneficial effect of exercise is attributed to the collective and not individual action of exercise.

4-3-1 Exercise and vasodilatation

My previous study delineated body weight as the major determinant of ACh-induced vasodilatation in the aortae of sedentary db/db mice and their controls (Sallam et al., 2011). It was expected that weight loss is required for improving ACh-induced relaxation. Surprisingly, the current findings show that short-term exercise restored ACh-induced relaxation in the aortae
of db/db mice without concurrent changes in body weight, but in association with reduced plasma triglycerides and 8-isoprostane levels in parallel with increased aortic expression of SOD-2 and SOD-3. It is possible that obesity plays the major role in the impaired vasodilatation in db/db mice; however, by suppressing the sequelae of obesity i.e. oxidative stress and dyslipidemia, exercise could improve the vasodilatation in the aortae of db/db mice. Other studies have also reported that exercise improved vasodilatation without weight loss in diabetic subjects (Green et al., 2003). Likewise, rosiglitazone and metformin ameliorated vascular dysfunction in rodent models of diabetes without changing body weight or glycemic control (Bagi et al., 2004, Matsumoto et al., 2008).

Exercise induces transient increase in oxidative stress in skeletal muscles, thereby upregulating the antioxidant defence system (Ji et al., 2006, Hollander et al., 2001). Similar responses probably occur in tissues other than skeletal muscles as indicated by exercise-induced increases in the protein expression of SOD isoforms in several arteries (de Moraes et al., 2008, Rush et al., 2003, Lee et al., 2011), heart (Moien-Afshari et al., 2008), and liver (Chang et al., 2004) of various animal models. We observed a reduction in plasma 8-isoprostane level, a marker of systemic oxidative stress, which occurred in parallel with increased aortic SOD-2 and SOD-3 expressions in db/db mice following 4 weeks of exercise, suggesting that the effect of exercise was generalized and not limited to the skeletal muscles or the aorta. Reducing oxidative stress could explain, at least in part, how short-term exercise improved vasodilatation in the aortae of db/db mice since ROS can interfere with NO signalling at multiple steps: receptor binding, eNOS activation, NO degradation, soluble guanylyl cyclase activation and/or the contractile apparatus functioning (De Vriese et al., 2000, Feletou and Vanhoutte, 2006). However, antioxidants have not shown significant therapeutic benefits for diabetic patients (Darko et al., 2002, Heart Protection Study Collaborative Group, 2002, Kataja-Tuomola et al., 2010),
suggesting that reducing oxidative stress may not be the sole mechanism by which exercise improved vasodilatation. Indeed, short-term exercise reduced both oxidative stress and plasma triglycerides in \textit{db/db} mice, while long-term exercise reduced body weight, plasma glucose, oxidative stress, inflammation and triglycerides. Additionally, long-term exercise increased the aortic expression of SOD-1, eNOS and Akt in \textit{db/db} mice; therefore, exercise increases NO bioavailability by dual mechanisms– increasing its synthesis and decreasing its breakdown.

We did not find a significant correlation between ACh-induced vasodilatation and any of the metabolic variables examined (body weight, plasma glucose, \(8\)-isoprostane, CRP or triglycerides) in the exercise groups, suggesting that no individual action is the major influence for the vascular benefits of exercise but rather that all these actions contribute. The multiple mechanisms by which exercise affects the body could explain the greater efficacy of exercise over other interventions such as food restriction (Sakamoto \textit{et al.}, 1998), insulin treatment (Aas \textit{et al.}, 2006), acarbose (Wagner \textit{et al.}, 2006), rosiglitazone (Kadoglou \textit{et al.}, 2010), metformin (Knowler \textit{et al.}, 2002) and antioxidant supplements (Darko \textit{et al.}, 2002, Heart Protection Study Collaborative Group, 2002, Kataja-Tuomola \textit{et al.}, 2010). Hence, a comprehensive pharmacological intervention that addresses the complete spectrum of diabetes pathophysiology, targeting inflammation, oxidative stress and dyslipidemia could be the optimal strategy for the management of diabetes.

4-3-2 \textbf{Exercise and EDHF}

Exercise restored the NOS-mediated component but not the EDHF-mediated component in ACh-induced vasodilatation in the femoral arteries of \textit{db/db} mice. Several studies reported that exercise improved EDHF-mediated vasodilatation in hyperlipidemic pigs (Woodman \textit{et al.}, 2003) and Otsuka Long-Evans Tokushima fatty rats, a model of type 2 diabetes (Minami \textit{et al.}, 2002) and hypertensive rats (Yen \textit{et al.}, 1995). However, a direct comparison with these results is hampered by
differences in the animal models used and the nature of EDHF in the different blood vessels studied.

4-3-3 Exercise and vasoconstriction

Diabetic vasculopathy usually involves not only impaired vasodilatation but also augmented vasoconstriction. Our results show that although short-term exercise restored vasodilatation in the aortae of \textit{db/db} mice, it exacerbated the augmented contractile responses. Several studies reported that exercise enhanced vasoconstriction under physiological (Korzick \textit{et al.}, 2004, Robles \textit{et al.}, 2011, Merkus \textit{et al.}, 2003) and diabetic conditions (Setty \textit{et al.}, 2004), while other studies reported that exercise had no effect (Moien-Afshari \textit{et al.}, 2008, Claudino \textit{et al.}, 2011, Khazaei \textit{et al.}, 2008), or even reduced (Esser \textit{et al.}, 2007, Mokelke \textit{et al.}, 2003) the contractile responses in diabetes animal models. Such discrepancies could be due to differences in the gender of the animals, type of blood vessels studied, signalling pathways of the contractile agents, and/or the structure of the exercise program used.

The present study shows that indomethacin suppressed the augmented aortic contractions in \textit{db/db} exercise mice, suggesting that COX mediate exercise-induced vascular hypercontractility. Exercise causes some damage to skeletal muscle fibres, thereby triggering a transient inflammatory response and increasing oxidative stress; such changes could upregulate redox-sensitive enzymes such as SOD-2 and COX (Ji, 2007). Indeed, short-term exercise increased the aortic protein expressions of COX-2, SOD-2 and SOD-3 simultaneously. Increased COX expression and/or activity is/are associated with enhanced vasoconstriction in diabetes (Bagi \textit{et al.}, 2005, Shi and Vanhoutte, 2008, Guo \textit{et al.}, 2005). It is also possible that exercise enhanced vasoconstriction by altering cytosolic calcium regulation (Bowles, 2000, Witczak \textit{et al.}, 2006, Witczak and Sturek, 2004) or upregulating protein kinase C signalling (Korzick \textit{et al.}, 2004).
Similarly, short-term exercise potentiated KCl and PE contractions in the femoral arteries of db/db mice. It is likely that similar mechanisms underlie exercise-enhanced contractions in the aortae and femoral arteries; however, this effect of exercise was transient and was not observed after 8 weeks of exercise, in parallel with the transient upregulation of COX-2.

**4-3-4 Exercise and regional vascular heterogeneity**

It is well accepted that individual vascular beds, exposed to varying local and neurohumoral influences, exhibit differences in vascular reactivity. Whether the aorta and femoral artery from db/db mice respond differently to exercise is unknown. Our findings show that exercise improved NOS-mediated vasodilatation and caused transient increases in the contractile responses simultaneously in both the aortae and femoral arteries of db/db mice. Therefore, our findings suggest that the effects of exercise on the vasomotor functions of the aortae and the femoral arteries are comparable and are probably triggered by systemic and not local changes.

**4-3-5 Short-term vs. long-term exercise**

Long-term exercise triggered different effects from short-term exercise at biochemical, molecular and functional levels. Long-term exercise reduced the body weights of both db/db and control mice. The weight reduction in db/db mice was associated with improved glycemic control and reduced systemic inflammation. Our results add to several previous reports demonstrating the capacity of exercise to reduce blood glucose in diabetic subjects with concurrent weight loss (Sakamoto et al., 1998, Xiang et al., 2005, Mikus et al., 2010, Haram et al., 2009), or independent of weight loss (Sigal et al., 2006, Lee et al., 2011, Sennott et al., 2008, Sennott et al., 2008, Loimaala et al., 2009, Kadoglou et al., 2007, de Lemos et al., 2007).

The ability of exercise to reduce inflammation was reported in experimental animals (Xiang et al., 2005, de Lemos et al., 2007) and diabetic patients (Kadoglou et al., 2007). Whether diminished inflammation occurred secondary to weight loss, or was triggered by exercise *per se*
is still open to investigation, but there is compelling evidence that obesity promotes inflammation (Aldhahi and Hamdy, 2003, Balistreri et al., 2010, Brooks et al., 2010) and that weight reduction per se can suppress inflammation (Esposito et al., 2003, Ziccardi et al., 2002).

Long-term exercise reduced systemic oxidative stress, but unlike short-term exercise, it increased the protein expression of SOD-1 but not SOD-2 or SOD-3 in the aortae of db/db mice. These results confirm earlier findings that exercise modulates the three SOD isoforms differently (Hollander et al., 2001, Rush et al., 2003, Chang et al., 2004, Higuchi et al., 1985); this may be attributed to the fact that the expression of SOD-1 and SOD-2 is regulated differently (Ji, 2008) as the promoter region of SOD-2 contains more ROS-sensitive binding sites (Ho et al., 1991). Also, the aortic eNOS and Akt protein expressions were increased in control mice 4 weeks post exercise, but were increased in db/db mice after 8 weeks of exercise. Several studies showed that the functional and molecular adaptations to exercise varied according to the duration of exercise. One week but not 16 weeks of exercise enhanced endothelium-dependent relaxation in the pulmonary arteries of miniature swine (Johnson and Laughlin, 2000, Johnson et al., 2001). The protein expression of SOD-1 in rat skeletal muscles was increased 48 hours post exercise, whereas SOD-2 protein content was increased after 10 and 24 hours, but not 48 hours (Hollander et al., 2001). Similarly, 12 and 24 weeks of exercise showed different effects on blood glucose, and heart rate in diabetic rats (Chakraphan et al., 2005). However, the mechanisms underlying these duration-dependent adaptations were not explained.

In summary, the exercise protocol used in the present study enhanced NO-mediated vasodilatation in db/db mice, but did not restore all the vascular functions. Further research is required for optimizing the structure of the exercise program and elucidating the upstream trigger for the multiple actions of exercise, which could represent the optimal drug target for effective management of type 2 diabetes.
5-Conclusions

5-1 Putting the data together

My findings demonstrate substantial heterogeneity between the aortae, carotid and femoral arteries in their vasodilatory and contractile responses under physiological and diabetic conditions. The aortae of \textit{db/db} mice exhibited progressive impairments in endothelial and VSMs functions possibly due to cytosolic calcium dysregulation; however, further investigation is needed to confirm that. The carotid artery was the most resilient and maintained unaltered vasodilatory and contractile responses despite a marked increase in systemic oxidative stress in \textit{db/db} mice probably because the carotid artery, in contrast to the aorta, relaxes in response to superoxide anion or peroxynitrite. The response of the carotid artery may reflect a physiological strategy to maintain the blood supply to the brain even under stressful conditions. The femoral artery was characterized by a larger contribution of EDHF to endothelium dependent ACh vasodilatation which was significantly reduced at an early age in \textit{db/db} mice. The contractile response to PE in the femoral artery, mainly dependent on the influx of calcium via VGCC, was attenuated in \textit{db/db} mice probably due to the lack of the expression of SOD-3 in the femoral artery leading to marked oxidative stress. The benefits of exercise training were confined to NO mediated vasodilatation in the aortae and femoral arteries of \textit{db/db} mice, and were associated with increased eNOS/Akt and SOD protein expressions and reduced cardiovascular risk factors.

\begin{tikzpicture}

\node at (0,0) {	extbf{Illustration 5-1: Summary of the vascular responses in the aortae, carotid and femoral arteries of \textit{db/db} mice}};

\node at (-5,0) {	extbf{Carotid artery:} ACh \leftrightarrow, PE \leftrightarrow};

\node at (5,0) {	extbf{Aorta:} ACh \downarrow, SNP \downarrow, KCl \uparrow, PE \uparrow};

\node at (10,0) {	extbf{Femoral artery:} ACh \downarrow, KCl \downarrow, PE \downarrow};

\node at (0,-2) {	extbf{Oxidative stress}};
\node at (0,-3) {	extbf{Inflammation}};
\node at (0,-4) {	extbf{Dyslipidemia}};
\node at (0,-5) {	extbf{Hyperglycemia}};

\node at (7,-1) {↑ increased};
\node at (7,-2) {↓ decreased};
\node at (7,-3) {↔ unchanged};
\node at (7,-4) {ACh= acetylcholine};
\node at (7,-5) {SNP= sodium nitroprusside};
\node at (7,-6) {PE= phenylephrine};

\end{tikzpicture}
5-2 Physiological relevance

Taking into consideration that the three studies included in my thesis are mainly *in vitro* studies that used isolated arteries from *db/db* mice, a genetic model of type 2 diabetes, and relied on using particular markers of oxidative stress and inflammation (study limitations are stated in the following section), the findings suggest the following:

1- Marked heterogeneity exists between regionally different arteries at functional and molecular levels under both physiological and pathological conditions. Therefore, my thesis calls for further detailed study of different arteries and vascular beds. However, and on a positive note, it is plausible that understanding regional differences in vasomotor control coupled with advanced drug delivery systems will open new avenues for developing selective therapies that target specific vascular beds without the complications of systemic side effects. Future therapies should go from personalized medicine to intra-personalized therapy.

2- ROS play both physiological and pathological roles in the vasculature contributing to regional vascular heterogeneity. Therefore, my study warrants against generalized use of antioxidants.

3- Oxidative stress plays a role in the vascular dysfunction in *db/db* mice; however, plasma 8-isoprostane, a marker of oxidative stress, did not show as the major determinant of vascular dysfunction in the aortae of *db/db* mice but was preceded by body weight and plasma CRP.

4- The exercise program implemented in the present study improved NO mediated vasodilatation by multiple mechanisms that included but was not limited to reducing oxidative stress.
5-3 Limitations of the study

1- The animals used in my thesis, \textit{db/db} mice, represent a genetic model of type 2 diabetes resulting from leptin receptor defects which are rare cases in diabetic patients. Therefore, \textit{db/db} mice cannot fully represent the aetiology or complexity of the human disease.

2- The present study examined the function of isolated arteries \textit{in vitro}; therefore, the findings cannot be extrapolated to whole vascular beds or \textit{in vivo} conditions. Comparable \textit{in vivo} studies will be useful to confirm my findings.

3- The functional experiments relied on the use of pharmacological agents, which frequently lose their reputation for specificity with time. The interpretation of the results is limited to the currently available information about the scope of actions of those pharmacological agents.

4- I used plasma 8-isoprostan e and CRP as markers of systemic oxidative stress and inflammation, respectively. While both are validated biomarkers in humans and mice; there are no published studies comparing them to other potential markers of oxidative stress or inflammation in \textit{db/db} mice.

5- The findings of my third study are limited to the structure and duration of the exercise program implemented in this study. Current evidence suggests that any change in the details of the exercise protocol can affect the outcome.
5-4 Strength of the study

1- Although the concept of vascular heterogeneity is well accepted and fairly documented in the literature; few studies have attempted to elucidate the mechanisms underlying such heterogeneity, particularly under pathological conditions. The present thesis is the first to address this issue specifically in db/db mice.

2- The aorta, carotid and femoral arteries were isolated from the same animal, either a db/db or control mouse; their functional responses were examined simultaneously under similar experimental conditions e.g. solution composition, pH and temperature to minimize errors due to methodological variability.

3- Previous studies examined the effect of exercise training on certain aspects of vascular reactivity in diabetic patients or animal models. However, the present thesis encompasses a detailed study of both vasodilatory and contractile functions of two different arteries, coupled with examination of molecular and biochemical changes following short and long term exercise in order to attain a more comprehensive understanding of the effects of exercise on the vasculature.

5-5 Future directions

1. Elucidation of the nature and levels of ROS produced in the aorta vs. the carotid by fluorescence dyes staining studies.

2. Detection of the signalling pathways of peroxynitrite and its potential interaction with thiol compounds to serve as a storage pool for NO.

3. Study of the nature and outcomes of the interactions of ROS with potassium channels in the vasculature.
4. Comparable *in vivo* studies examining the adaptation of different vascular beds to the progression of diabetes in *db/db* mice as well as other models of diabetes and diabetic patients.

5. Elucidation of the potential role of COX in cytosolic calcium regulation, particularly with respect to Rho kinase function.

6. Discovering the upstream trigger for the multifaceted actions of exercise and its site of action, which probably occurs in the nucleus, will be a significant step towards developing effective therapies for type 2 diabetes.


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